EVIDENCE THAT BIOSYNTHESIS OF PHOSPHATIDYLETHANOLAMINE, PHOSPHATIDYLCHOLINE, AND TRIACYLGLYCEROL OCCURS ON THE CYTOPLASMIC SIDE OF MICROSOMAL VESICLES
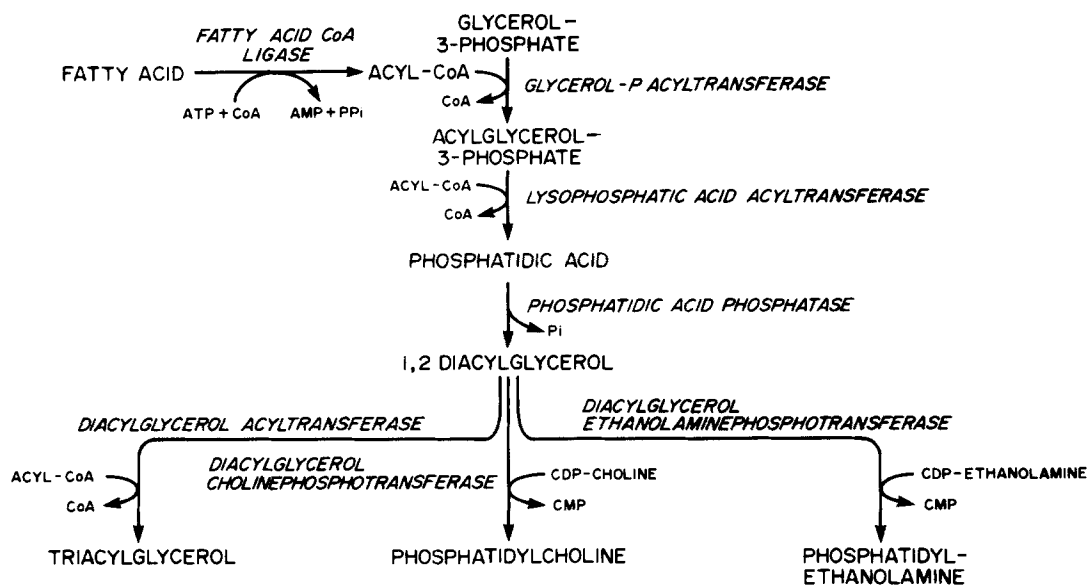
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Experiments were performed to localize the hepatic microsomal enzymes of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol biosynthesis to the cytoplasmic or lumenal surface of microsomal vesicles. Greater than 90% of the activities of fatty acid-CoA ligase (EC 6.2.1.3), sn-glycerol 3-phosphate acyltransferase (EC 2.3.1.15), lysophosphatidic acid acyltransferase, diacylglycerol acyltransferase (EC 2.3.1.20), diacylglycerol cholinephosphotransferase (EC 2.7.8.2), and diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) was inactivated by proteolysis of intact microsomal vesicles. The phosphatidic acid phosphatase (EC 3.1.3.4) was not inactivated by any of the proteases tested. Under the conditions employed, <5% of the lumenal mannose-6-phosphatase (EC 3.1.3.9) activity was lost. After microsomal integrity was disrupted with detergents, protease treatment resulted in a loss of >74% of the mannose-6-phosphatase activity. The latency of the mannose-6-phosphatase activity was not affected by protease treatment. Mannose-6-phosphatase latency was not decreased by the presence of the assay components of several of the lipid biosynthetic activities, indicating that those components did not disrupt the microsomal vesicles. None of the lipid biosynthetic activities appeared latent. The presence of a protease-sensitive component of these biosynthetic activities on the cytoplasmic surface of microsomal vesicles, and the absence of latency for any of these biosynthetic activities suggest that the biosynthesis of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol occurs asymmetrically on the cytoplasmic surface of the endoplasmic reticulum.

KEY WORDS lipid biosynthesis · membrane asymmetry · asymmetric biosynthesis · glucose-6-phosphatase · lipoprotein biogenesis

The location of biosynthetic activities within the transverse plane of the endoplasmic reticulum is of particular interest for enzymes whose products may be either secreted or retained within the cell. Phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol account for the vast majority of hepatic glycerolipid biosynthesis. The phospholipids are utilized for hepatic membrane biogenesis and for the formation of lipoproteins, and the triacylglycerols are incorporated into lipoproteins or accumulate within the hepatocyte in certain disease states (14). The enzymes responsible for the biosynthesis of these glycerolipids (Scheme I) from fatty acids and glycerol-3-P have all been localized to the microsomal subcellular fraction (12, 16, 29, 30). Microsomes are derived from the endoplasmic reticulum and are sealed vesicles which maintain proper sidedness (11, 22). The external surface of these vesicles corresponds to the cytoplasmic surface of the endoplasmic reticulum. Macromolecules destined for secretion must pass into the lumen of the endoplasmic reticulum (5, 23). Uncharged molecules of up to ~600 daltons are able to enter the lumen of rat liver microsomes, but macromolecules and charged molecules of low molecular weight do not cross the vesicle membrane (10, 11).

Because proteases neither cross the microsomal membrane nor destroy the permeability barrier of the microsomal vesicles, only the enzymes and proteins located on the cytoplasmic surface of microsomal vesicles are susceptible to proteolysis unless membrane integrity is disrupted (10, 11). By use of this approach, several enzymes and proteins have been localized in the transverse plane of microsomal membranes (11). With the possible exception of cytochrome P 450, all of the enzymes and proteins investigated were localized asymmetrically by the proteolysis technique (11). By studies of this type, as well as by product

FATTY ACID CoA LIGASE

GLYCEROL-3-PHOSPHATE

FATTY ACID ——————→ ACYL-CoA

ATP + CoA   AMP + PPi

CoA

GLYCEROL-P ACYLTRANSFERASE

ACYLGLYCEROL-3-PHOSPHATE

ACYL-CoA

CoA

LYSOPHOSPHATIC ACID ACYLTRANSFERASE

PHOSPHATIDIC ACID

PHOSPHATIDIC ACID PHOSPHATASE

Pi

1,2 DIACYLGLYCEROL

DIACYLGLYCEROL ACYLTRANSFERASE

DIACYLGLYCEROL ETHANOLAMINEPHOSPHOTRANSFERASE

ACYL-CoA

CoA

DIACYLGLYCEROL CHOLINEPHOSPHOTRANSFERASE

CDP-CHOLINE

CMP

CDP-ETHANOLAMINE

CMP

TRIACYLGLYCEROL   PHOSPHATIDYLCHOLINE   PHOSPHATIDYL-ETHANOLAMINE

SCHEME 1 Microsomal enzymes of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol biosynthesis.

localization, glucose-6-phosphatase (EC 3.1.3.9) has been localized to the lumenal surface of microsomal vesicles (11) and of the endoplasmic reticulum (18, 19). All microsomal vesicles contain glucose-6-phosphatase (18, 19) which can effectively utilize mannose-6-P as a substrate, provided the permeability barrier of the vesicles has been disrupted to allow the substrate access to the active site located on the lumenal surface (4). An exact correspondence between mannose-6-phosphatase activity and membrane permeability to EDTA has been established (4). The latency of mannose-6-phosphatase activity provides a quantitative index of microsomal integrity (4).

Few of the microsomal enzymes in the synthesis of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol have been solubilized and/or purified, and little is known about the topography of these enzymes in the transverse or lateral planes of the endoplasmic reticulum. An asymmetric location of these biosynthetic enzymes on the cytoplasmic or lumenal surface of microsomal vesicles may provide a mechanism for regulation of the glycerolipids to be retained or secreted by the cell, and for the biogenesis of asymmetric phospholipid bilayers.

In this paper, we report investigations on the localization of all seven microsomal enzymes (Scheme I) in the biosynthesis of triacylglycerol, phosphatidylcholine, and phosphatidylethanola-

mine, using the protease technique with mannose-6-phosphatase serving as lumenal control activity. The latency of these lipid biosynthetic enzymes was also investigated, using the latency of mannose-6-phosphatase as an index of microsomal integrity.

MATERIALS AND METHODS
Materials

Hexokinase, mannose, mannose-6-phosphate, D,L-α-glycerolphosphate, sodium taurocholic acid, egg lecithin phosphatidic acid, bovine serum albumin (essentially fatty acid free), 5,5'-dithiobis-(2-nitrobenzoic acid), and Tween 20 were purchased from Sigma Chemical Co., St. Louis, Mo. Elastase, α-chymotrypsin, micro-protease, and trypsin-L-(tosylamido 2-phenyl)ethyl chloromethyl ketone (TPCK) were obtained from Worthington Biochemical Corp., Freehold, N. J. Papain, thermolysin, and pronase were obtained from Calbiochem, San Diego, Calif. Subtilisin was purchased from Nutritional Biochemicals Corp., and Proteinase K from Beckman. 1-oleoylglycerol 3-P and 1,2-sn-dioleoylglycerol were obtained from Serdary Research Laboratories, Inc., London, Ontario, Can., and palmitoyl-CoA, oleoyl-CoA, adenosine triphosphate (ATP), and CoA from P-L Biochemicals Inc., Milwaukee, Wis. Triton WR 1339 was purchased from Ruger Chemical Co., Irvington-on-Hudson, N. Y. Silica gel HR plates (250 μm) were obtained from Analtech, Inc., Newark, Del., sodium dodecyl sulfate from BDH Chemicals Ltd., Poole, England, and sodium deoxycholate from Schwartz/Mann

Div., Becton, Dickinson & Co., Orangeburg, N. Y. CDP-[14C]choline, 32P$_i$, [3H]palmitic acid, and Aquasol were obtained from New England Nuclear, Boston, Mass., and CDP-[14C]-ethanolamine from ICN Pharmaceuticals Inc., Cleveland, Ohio. [3H]palmitoyl-CoA (1), [3H]glycerol 3-P (6), and γ-[32P]ATP (13) were synthesized by previously reported methods.

Isolation of Liver Microsomes

Livers from 200–250 g Charles River CD strain female rats were homogenized by 10 rapid up-and-down strokes in a motor-driven, Teflon-glass homogenizer in 3 vol of cold Medium I (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-Cl, pH 7.4). The homogenate was centrifuged at 22,000 g for 15 min. The resulting supernate was centrifuged at 100,000 g for 1 h. The pellet was suspended in Medium I and centrifuged at 100,000 g for 1 h. The resulting pellet was suspended in Medium I without EDTA. Protein was determined by the method of Lowry et al. (20), using bovine serum albumin as the standard.

Protease Treatment of Microsomes

Intact and disrupted (by 10-min exposure at 4°C to 0.05% deoxycholate and 50 mM KCl in Medium I without EDTA) microsomes (30 mg microsomal protein) were treated for 25 min at 30°C with either chymotrypsin, 100 μg/mg microsomal protein, or pronase, 50 μg/mg microsomal protein, as described by Nilson and Dallner (21). Control intact and disrupted microsomes were treated similarly except that the protease was omitted. At the end of the incubation, the samples were cooled in ice water and centrifuged immediately at 100,000 g for 2 h. The recovered microsomes were suspended in Medium I without EDTA, and protein was determined according to the method of Lowry et al. (20). Enzyme assays were performed immediately or within 3 days on samples stored at −15°C. No activity was lost by freezing and thawing one time.

Protease Treatment of Nitrogen-Cavitated Microsomes

Microsomes (10 mg/ml in Medium I without EDTA) were disrupted under nitrogen by passage through a French Pressure Cell at 20,000 pound per square inch and immediately treated for 25 min at 30°C with chymotrypsin, 100 μg/mg microsomal protein. Disrupted and nondisrupted control microsomes were treated similarly except that chymotrypsin was omitted. After the incubation, the microsomes were collected by centrifugation and resuspended as described above.

Synthesis of [32P]Mannose-6-P

[32P]mannose-6-P was synthesized by a modification of the method of Slein (28). The reaction mixture (2.5

ml) contained 50 mM Tris HCl, pH 8.1, 8 mM MgCl$_2$, 20 mM mannose, 16.8 mM [32P]ATP (24 μCi/μmol), and ~0.5 mg of hexokinase. After 1 h at 23°C, the mannose-6-P was purified from the reaction mixture by chromatography on Whatman no. 1 paper in Solvent I (methanol/formic acid per H$_2$O, 120/19.5 per 10.5, vol/vol per vol). The [32P]mannose-6-P was located by autoradiography and eluted from the paper with water. Typically, 20–40% of the labeled ATP was converted to [32P]mannose-6-P. Greater than 94% of the labeled mannose-6-P chromatographed with authentic mannose-6-P in Solvent I. The [32P]mannose-6-P was employed at a specific activity of 1.36 μCi/μmol.

Enzyme Assays

The activities of mannose-6-phosphatase (assayed at 2 mM mannose-6-P) (3), glycerol-3-P acyltransferase (EC 2.3.1.15) (27), and fatty acid-CoA ligase (EC 6.2.1.3) (24) were determined by previously described radiochemical methods. The activities of diacylglycerol acyltransferase (EC 2.3.1.20) (7), diacylglycerol cholinephosphotransferase (EC 2.7.8.2) (8), and diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) (8) were determined by previously described radiochemical methods except that the 1,2-sn-dioleoylglycerol was added in 0.5% sodium taurocholate so that the final incubation mixture contained 400 μM dioleoylglycerol and 0.005% sodium taurocholate. Lysophosphatidic acid acyltransferase was determined spectrophotometrically (17), using 50 μM oleoylglycerol-P and 50 μM oleoyl-CoA. Phosphatidic acid phosphatase (EC 3.1.3.4) activity was determined by monitoring orthophosphate release (9) as modified by R. Mavis,[1] using 400 μM phosphatidic acid and 50 mM Tris-maleate, pH 7.0. Assays were performed at 23°C except that the phosphatidic acid phosphatase activity was determined at 37°C. All assays were linear with the time and protein employed, using either intact or disrupted microsomes.

RESULTS

Effect of Proteases on Enzymes of Phosphatidylcholine, Phosphatidylethanolamine, and Triacylglycerol Biosynthesis

Treatment of microsomal vesicles with pronase had no effect on mannose-6-phosphatase activity or latency (Table I). Because mannose-6-phosphatase activity is a lumenal enzyme activity (4), these data demonstrate that the nondetergent-treated microsomal preparation employed remained intact during the pronase digestion. Under

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[1] R. Mavis, University of Rochester, personal communication.

TABLE I

Effect of Pronase on Microsomal Activities

	Intact microsomes*			Disrupted‡ microsomes§		
	Control	+Pronase‖	Pronase/control	Control	+Pronase‖	Pronase/control
	nmol/min		%	*nmol/min*		%
Mannose-6-phosphatase¶ **	2,260	2,140	*94.7*	1,880	492	*26.1*
Fatty acid-CoA ligase	1,300	46.7	*3.6*	917	18.5	2.0
Glycerolphosphate acyltransferase¶	12.9	0.59	*4.6*	9.7	0.15	1.5
Lysophosphatidic acid acyltransferase	1,152	150	*13.0*	122	18.5	15.0
Phosphatidic acid phosphatase	131	130	*99.2*	101	105	104.0
Diacylglycerol acyltransferase¶	49.6	2.92	*5.9*	38.3	1.48	3.8
Diacylglycerol cholinephosphotransferase¶	438	147	*33.6*	190	151	79.4
Diacylglycerol ethanolaminephosphotransferase¶	36.4	30.4	*83.5*	17.3	16.1	93.1

* Protein recoveries from intact control and pronase-treated microsomes were 23.5 and 19.5 mg, respectively.

‡ Treated with 0.05% deoxycholate, 50 mM KCl.

§ Protein recoveries from disrupted control and pronase-treated microsomes were 15.2 and 12.4 mg, respectively.

‖ Exposed to 50 μg pronase/mg microsomal protein for 25 min at 30°C.

¶ Assayed after treatment with 0.5% taurocholate. Final assay mixtures contained <0.05% taurocholate.

** Mannose-6-phosphatase latency was 90% in the initial microsomal preparation. After incubation and collection of the microsomes, latencies were 80 and 77% for intact control and pronase-treated microsomes, respectively.

these conditions, the activities of fatty acid-CoA ligase, glycerolphosphate acyltransferase, and diacylglycerol acyltransferase were almost completely inactivated (Table I). Lysophosphatidic acid acyltransferase and diacylglycerol cholinephosphotransferase underwent substantial inactivation. In contrast, the phosphatidic acid phosphatase and the diacylglycerol ethanolaminephosphotransferase were hardly affected by pronase treatment. When the pronase treatment was performed in the presence of deoxycholate to disrupt the microsomal vesicles, 74% of the mannose-6-phosphatase activity was lost. The extent of inactivation of the lipid biosynthetic activities by pronase was not significantly enhanced by addition of deoxycholate (Table I).

When a similar experiment was performed with chymotrypsin, more than 98% of the mannose-6-phosphatase activity was recovered in the nondetergent-treated microsomal vesicles, and over 84% of the activity was inactivated in the microsomal vesicles disrupted with deoxycholate (Table II).[2] The preservation of microsomal integrity was also demonstrated by the mannose-6-phosphatase latency of 85%. Greater than 90% of the activities of fatty acid-CoA ligase, glycerolphosphate acyltransferase, diacylglycerol acyltransferase, diacylglycerol cholinephosphotransferase, and diacylglycerol ethanolaminephosphotransferase was lost by chymotrypsin proteolysis of the intact microsomal vesicles (Table II). Little additional inactivation was seen when deoxycholate was added to disrupt the integrity of the vesicles (Table II). The phosphatidic acid phosphatase was not inactivated by chymotrypsin in either intact or disrupted microsomes (Table II). Attempts to inactivate phosphatidic acid phosphatase by incubation of intact or disrupted microsomes with 100 μg protease/mg microsomal protein were unsuccessful with the use of trypsin, subtilisin, papain, thermolysin, elastase, collagenase, microprotease, or proteinase K. Because the microsomes employed in the latter experiments were not reisolated by centrifugation, solubilized, but active, phosphatidic acid phosphatase would not be distinguished from the microsomal-bound activity.

[2] The possibility that chymotrypsin inactivation of mannose-6-phosphatase activity was dependent on the presence of deoxycholate was eliminated by investigations employing microsomes disrupted by nitrogen cavitation. Mannose-6-phosphatase latency was decreased from 89 to 49% by nitrogen cavitation. Treatment of the nitrogen-cavitated microsomes with chymotrypsin led to the inactivation of 55% of the mannose-6-phosphatase activity.

TABLE II

Effect of Chymotrypsin on Microsomal Activities

	Intact microsomes*			Disrupted‡ microsomes§		
	Control	+Chymotryp- sin‖	Chymotrypsin/ Control	Control	+Chymotryp- sin‖	Chymotrypsin/ Control
	nmol/min		%	*nmol/min*		%
Mannose-6-phosphatase¶ **	1,453	1,437	*98.9*	2,098	324	*15.4*
Fatty acid-CoA ligase	1,257	36.0	*2.9*	1,263	19.8	1.6
Glycerolphosphate acyltransferase¶	9.46	0.85	*9.0*	6.97	0.36	5.2
Phosphatidic acid phosphatase	103	119	*119.0*	130	115	88.8
Diacylglycerol acyltransferase¶	32.1	1.90	*5.9*	36.5	1.40	3.8
Diacylglycerol cholinephosphotrans- ferase¶	186	1.04	*0.6*	134	0.12	0.1
Diacylglycerol ethanolaminephos- photransferase¶	24.1	0.49	*2.0*	21.5	0.30	1.4

* Protein recoveries from intact control microsomes and chymotrypsin-treated microsomes were 20.0 and 19.0 mg, respectively.

‡ Treated with 0.05% deoxycholate, 50 mM KCl.

§ Protein recoveries from disrupted control microsomes and disrupted, chymotrypsin-treated microsomes were 20.0 and 11.6 mg, respectively.

‖ Exposed to 100 μg of chymotrypsin/mg microsomal protein for 25 min at 30°C.

¶ Assayed after treatment with 0.5% taurocholate. Final assay mixtures contained <0.05% taurocholate.

** Mannose-6-phosphatase latency was 90% in the initial microsomal preparation. After incubation and collection of the microsomes, latencies were 87 and 85% for the intact control and chymotrypsin microsomes, respectively.

Latency of Mannose-6-Phosphatase and Enzymes of Phosphatidylcholine, Phosphatidylethanolamine, and Triacylglycerol Biosynthesis

The latency of mannose-6-phosphatase activity varied between 88 and 93% for the microsomal preparations employed, in substantial agreement with the work of others (4). Optimum detergent concentrations required for maximal mannose-6-phosphatase activity were 0.5-0.75% taurocholate, or 0.1% deoxycholate. With either detergent, full expression of activity required slightly higher concentrations than previously reported (2, 4). In the presence of 50 mM KCl, maximal activity was seen at 0.05% deoxycholate.

The manifestation of latent mannose-6-phosphatase activity was employed to determine whether microsomal integrity was altered by assay components for the enzymes of phosphatidyl-choline, phosphatidylethanolamine, and triacyl-glycerol biosynthesis (Table III). Mannose-6-phosphatase latency was 91% for the microsomal preparation used. The components employed in assaying glycerolphosphate acyltransferase, phos-

phatidic acid phosphatase, diacylglycerol acyl-transferase, diacylglycerol cholinephosphotrans-ferase, and diacylglycerol ethanolaminephospho-transferase did not cause the latent mannose-6-phosphate activity to be manifested (Table III), nor did they significantly inhibit the latent activity (data not shown). The fatty acid-CoA ligase reaction components, which contained Triton WR 1339, caused a 3.4 fold increase in mannose-6-phosphatase activity (Table III) and did not inhibit the remaining latent activity. The reaction components for the lysophosphatidic acid acyltransferase inhibited both the nonlatent (Table III) and latent mannose-6-phosphatase activity (data not shown). Thus, assay of the activities of glycerolphosphate acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, diacylglycerol cho-linephosphotransferase, and diacylglycerol etha-nolaminephosphotransferase was possible without disrupting microsomal vesicles.

The latency of those biosynthetic enzyme activities which could be assayed in intact microsomal vesicles was investigated as the transmembrane movement of their substrates to an active site located on the lumenal surface might limit activity, in a manner analogous to that of the mannose-6-phosphatase activity (4). The addition of various

levels of taurocholate, deoxycholate, or Tween 20 to microsomes failed to reveal any increase in the activities of glycerolphosphate acyltransferase, phosphatidic acid phosphatase, or diacylglycerol ethanolaminephosphotransferase. The activities of diacylglycerol acyltransferase and diacylglycerol cholinephosphotransferase increased slightly at detergent concentrations fivefold lower than required for the expression of mannose-6-phosphatase latency (data not shown). Microsomes exposed to 0.5% taurocholate, where maximal mannose-6-phosphatase activity was expressed, showed no significant increase in activities of glycerolphos-

phate acyltransferase, phosphatidic acid phosphatase, or diacylglycerol ethanolaminephosphotransferase (Table IV). The activities of diacylglycerol acyltransferase and diacylglycerol cholinephosphotransferase increased 60 and 40%, respectively. Thus, none of the enzymes in biosynthesis of the phosphatidylcholine, phosphatidylethanolamine, or triacylglycerol appeared to be latent by comparison with the mannose-6-phosphatase activity which increased 1,130% in disrupted microsomal vesicles.

Consideration was given to the possibility that the lipid biosynthetic enzymes were located on

TABLE III

Effect of Assay Components of Enzymes in Biosynthesis of Phosphatidylcholine, Phosphatidylethanolamine, and Triacylglycerol on Mannose-6-Phosphatase Latency

Enzyme	Assay components added	Nonlatent mannose-6-phosphatase activity
		%
Mannose-6-phosphatase*	None	100
Fatty acid-CoA ligase	50 μM CoA, 10 mM ATP 30 μM palmitic acid‡, 1 mg/ml Triton WR 1339	337.2
Glycerolphosphate acyltransferase	0.6 mM D-L glycerol-P, 1.0 mM dithiothreitol, 50 μM palmitoyl-CoA	92.6
Lysophosphatidic acid acyltransferase	50 μM oleoyl-CoA, 50 μM 1-oleoylglycerol 3-P, 1 mM DTNB§, 100 mM Tris, pH 7.4	46.5
Phosphatidic acid phosphatase	0.4 mM phosphatidic acid	91.8
Diacylglycerol acyltransferase	400 μM dioleoylglycerol‖, 0.05% taurocholate, 30 μM palmitoyl-CoA	
Diacylglycerol cholinephosphotransferase	400 μM dioleoylglycerol‖, 0.05% taurocholate, 0.5 mM EGTA, 100 μM CDP-choline	98.6
Diacylglycerol ethanolaminephosphotransferase	400 μM dioleoylglycerol‖, 0.05% taurocholate, 0.5 mM EGTA, 100 μM CDP-ethanolamine	93.8

* Assayed as described under Experimental Procedures. The latency of mannose-6-phosphatase activity was 91%.
‡ Palmitic acid added in Triton WR 1339.
§ 5,5'-dithiobis(2-nitrobenzoic acid).
‖ dioleoylglycerol added in taurocholate.

TABLE IV

Latency of Mannose-6-Phosphatase and Enzymes in Biosynthesis of Phosphatidylcholine, Phosphatidylethanolamine, and Triacylglycerol

	Specific activity, nmol/min/mg		
Enzyme	Intact microsomes	Disrupted*	Disrupted/intact
Mannose-6-phosphatase	9.0	102	11.3
Glycerol-3-P acyltransferase	0.46	0.46	1.0
Phosphatidic acid phosphohydrolase	4.9	4.8	1.0
Diacylglycerol acyltransferase	0.97	1.50	1.6
Diacylglycerol cholinephosphotransferase	16.2	22.2	1.4
Diacylglycerol ethanolaminephosphotransferase	9.8	10.3	1.11

* Disrupted microsomes were exposed to 0.5% taurocholate before assay.

the lumenal surface and required transmembrane movement of their substrate(s) by a membrane protein component. In this case, protease-treatment of intact microsomes would appear to inactivate the lipid biosynthetic enzymes due to proteolysis of the protein component responsible for the transmembrane movement of substrate(s). Subsequent disruption of microsomes would then allow full expression of enzyme activities. This possibility was eliminated by the finding that protease-treated microsomal vesicles did not contain latent biosynthetic activities.[3]

DISCUSSION

Interpretation of Protease and Latency Experiments in Relation to the Localization of Phosphatidylcholine, Phosphatidylethanolamine, and Triacylglycerol Biosynthetic Enzymes in the Transverse Plane of Microsomal Vesicles

The treatment of microsomal vesicles with proteases (Tables I and II) resulted in ~90% inactivation of the activities of fatty acid-CoA ligase, glycerolphosphate acyltransferase, lysophosphatidic acid acyltransferase, diacylglycerol acyltransferase, diacylglycerol cholinephosphotransferase, and diacylglycerol ethanolaminephosphotransferase. Because the protease treatment did not seriously affect the latency of mannose-6-phosphatase activity, the integrity of the microsomal vesicles was maintained. The resistance of >95% of the mannose-6-phosphatase activity to proteolysis, unless the integrity of the microsomal membrane was disrupted by detergents or by nitrogen cavitation, further demonstrates that the microsomal vesicles remained intact during the proteolytic treatment. Thus, the six lipid biosynthetic enzymes listed above are inactivated by proteolysis of polypeptide component(s) exposed on the cytoplasmic surface of microsomal vesicles. The protease data do not exclude the possibility that these enzymes span the membrane and that proteolysis of the cytoplasmic domain results in loss

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[3] Lipid biosynthetic activities which had been inactivated in intact microsomes by proteolysis with pronase or chymotrypsin were also assayed after microsomal disruption by 0.5% taurocholate. The intact and disrupted microsomes showed comparable activities.

of activity at the lumenal surface. This possibility seems unlikely, as these activities do not appear latent.[4] Evidence either for or against a spanning orientation for these enzymes awaits the development of methods which would allow proteases independent access to the microsomal lumenal surface and/or the raising of specific antibodies to the purified enzyme. Additional evidence regarding the location of the active sites might be obtained by localization of the reaction products.

The sensitivity of the glycerolphosphate acyltransferase[5] to proteolysis is in agreement with Schlossman and Bell who demonstrated inactivation by trypsin (27), and with the data of Hüllsmann and Kurpershoek-Davidov who employed subtilisin (15). The latter workers suggested that the glycerolphosphate acyltransferase and fatty acid-CoA ligase were located on the cytoplasmic surface of intestinal epithelial microsomes, and that the diacylglycerol cholinephosphotransferase was located on the lumenal surface as it was resistant to subtilisin (15). Our data obtained on pronase and chymotrypsin inactivation of this activity clearly demonstrate that resistance to a single protease can result from the absence of an accessible proteolytic site. This point was recently emphasized by DePierre and Ernster in a review on enzyme topology of intracellular membranes (11).

The ability to assay several of these lipid biosynthetic activities in intact microsomes under conditions where mannose-6-phosphatase latency was not manifested (Table III) suggests that the substrates have access to the active sites of the enzymes in intact microsomes (Table IV). Because transmembrane movement of many of the

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[4] It is possible that the apparent absence of latency noted in Table II might have resulted from inhibition of the activity (latent and nonlatent) by the detergent employed to disrupt the microsomal vesicles; however, no changes in biosynthetic enzyme activities were observed when a variety of detergent concentrations was tested. Under these conditions, the expression of latent activity and the detergent inhibition of the activity would not be expected to exactly balance one another.

[5] Rat liver microsomal dihydroxyacetone phosphate acyltransferase and glycerolphosphate acyltransferase activities appear to be dual catalytic functions of a single microsomal enzyme (27). Both activities are equally susceptible to proteolysis by chymotrypsin in intact microsomal vesicles. D. M. Schlossman and R. M. Bell, unpublished data.

substrates employed is presumed to be slow (11),[6] the enzymes' active sites as well as their protease-sensitive sites would most likely be present on the cytoplasmic surface of the microsomal vesicles. The failure to demonstrate latency for any of the lipid biosynthetic activities in control or protease-treated microsomal vesicles is consistent with the active sites being located on the cytoplasmic surface and with the absence of (or need for) facilitated transmembrane movement of substrates (intermediates). Only the latency argument can be made for the localization of the phosphatidic acid phosphatase, as it was resistant to all the proteases tested in both intact and disrupted microsomes. However, a lumenal location of this single enzyme in the biosynthesis of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol (Scheme I) seems unlikely. It appears reasonable that all seven enzymes are located asymmetrically on the cytoplasmic surface of the endoplasmic reticulum.

Consequences of Asymmetric Biosynthesis of Phosphatidylcholine, Phosphatidylethanolamine, and Triacylglycerol

The data suggest that biosynthesis of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol occurs asymmetrically on the cytoplasmic surface of the endoplasmic reticulum.[7] This orientation of enzyme activities would allow ready access to fatty acids, CDP-choline, and CDP-ethanolamine which are synthesized by soluble enzymes in the cell cytoplasm, and to substrates such as ATP and CoA, that are required for the activation of fatty acids (26). The location of these enzymes on the cytoplasmic surface might allow the amounts of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol synthesized to be regulated by cytoplasmic metabolites. In addition, the data suggest that membrane phospholipid asymmetry does not result from the syn-

thesis of a given species of lipid on each face of the bilayer by oppositely oriented enzymes.[8] Transmembrane movement of phosphatidylcholine and phosphatidylethanolamine will be necessary for the biogenesis of the phospholipid bilayer. Evidence for the rapid translocation of phospholipids in intact microsomal vesicles has been reported recently by Zilversmit and Hughes (31) who demonstrated extensive exchange of rat liver microsomal phospholipids. The biogenesis of lipoproteins in the lumen of the endoplasmic reticulum will probably require transmembrane movement of triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine.

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[6] It is not known whether substrates such as glycerol-phosphate, CoA, ATP, CDP-choline, and CDP-ethanolamine can pass into the lumen of the microsomal vesicles. Further, data are presently not available to indicate whether substrates such as fatty acyl-CoA are present on the cytoplasmic surface, lumenal surface, or both.

[7] Asymmetric biosynthesis of phosphatidylethanolamine in *Bacillus megaterium* has recently been demonstrated by Rothman and Kennedy (25).

[8] For a discussion of the origin of phospholipid asymmetry, see references 10 and 26.

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