review

Lipid topogenesis

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Abstract Investigations of the topography of glycerolipid synthetic enzymes within the transverse plane of microsomal vesicles indicated an exclusive cytoplasmic surface location of active sites. Evidence was derived from studies employing proteases and other impermeant inhibitors, from investigations of latency and substrate permeation, and from localization of products. These studies strongly suggest a total asymmetric synthesis of glycerolipids on the cytoplasmic surface of the endoplasmic reticulum. The data are critically reviewed, emphasizing the importance of appropriate controls for topographical studies of microsomal enzymes. The limited data on the location and topography of other enzymes of complex lipid metabolism in microsomes, peroxisomes, mitochondria, and other membranes are also reviewed. These new findings have important implications for the processes of "lipid topogenesis" which encompass complex lipid synthesis, the integration of lipids into membranes, and lipid translocation across membranes. Later events of lipid topogenesis involve lipid movement to other membranes and structures, the sorting of complex lipids from each other to assemble structures of distinct lipid composition, and the formation and maintenance of lipid asymmetry.—**Bell, R. M., L. M. Ballas, and R. A. Coleman.** Lipid topogenesis. *J. Lipid Res.* 1980. **22:** 391–403.

Understanding the mechanisms of complex lipid synthesis and the processes of intracellular lipid translocation promises valuable insights into the biogenesis and assembly of structures that contain complex lipids. These structures include plasma membranes, membranes of the intracellular organelles, serum lipoproteins, bile, cholesteryl ester droplets, and triacylglycerol stores. The recognition that proteins are oriented asymmetrically within the transverse plane of membranes prompted investigations into how membrane proteins acquire their characteristic orientation. These studies indicated that membrane proteins are unidirectionally translocated across or asymmetrically integrated into distinct intracellular membranes either during or shortly after their synthesis (1–7). Proteins synthesized on the rough endoplasmic reticulum are sorted from each other and selectively transported to specific intracellular membranes or compartments (2, 6). During these selective transport processes (which may occur via coated vesicles (8)), some membrane or secretory proteins undergo post-translational processing involving carbohydrate (2, 6, 8) and/or fatty acid (9, 10) additions. Studies of these processes termed "intracellular protein topogenesis" (6) are more abundant than studies of analogous processes involving lipids.

"Lipid topogenesis" is used here as a categorical term for those intracellular processes occurring simultaneously with or shortly after the synthesis of complex lipids. The early events of lipid topogenesis include lipid synthesis, the integration of lipids into membranes, and lipid translocation across membranes. Later events involve lipid movement to other membranes and structures, the sorting of different complex lipids from each other to assemble structures with distinct lipid compositions, and the formation and maintenance of lipid asymmetry within biological membranes and other structures.

This review will focus on early events of lipid topogenesis. The topography of glycerolipid synthetic enzymes within microsomal vesicles and other organelles will be reviewed. The implications of these findings in regard to the sites of synthesis of other complex lipids and to the assembly of membranes, lipoproteins, triacylglycerol droplets, and bile will be discussed.

Diversity of complex lipids and of physiological functions

Glycerolipids display great diversity in structure and function. **Fig. 1** shows a biosynthetic pathway for glycerolipids. Glycerophospholipids constitute the

Abbreviation: DHAP, dihydroxyacetone phosphate.

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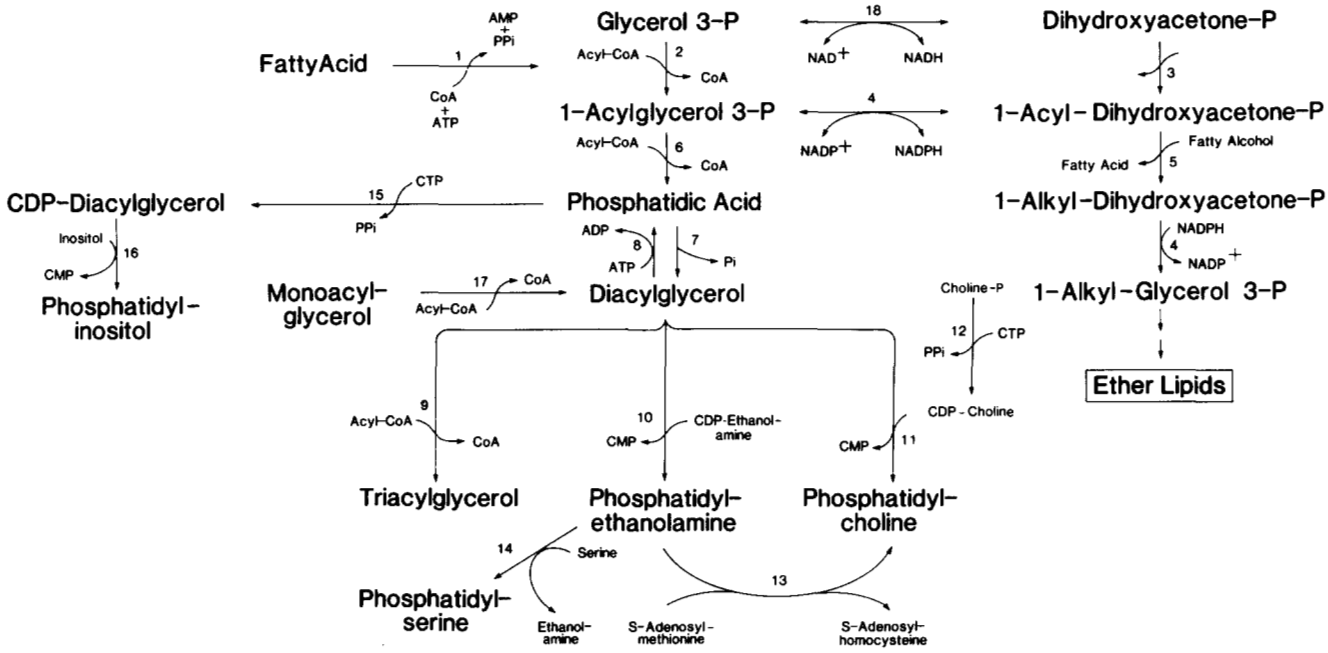

Fig. 1. Enzymes of glycerolipid biosynthesis. 1) Fatty acid CoA ligase (AMP) (EC 6.2.1.3); 2) *sn*-glycerol 3-P acyltransferase (EC 2.3.1.15); 3) dihydroxyacetone-P acyltransferase (EC 2.3.1.42); 4) acyl(alkyl)dihydroxyacetone-P oxidoreductase (EC 1.1.1.10); 5) alkyldihydroxyacetone-P synthase; 6) lysophosphatidic acid acyltransferase (EC 2.3.1.-); 7) phosphatidic acid phosphatase (EC 3.1.3.4); 8) diacylglycerol kinase; 9) diacylglycerol acyltransferase (EC 2.3.1.20); 10) diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1); 11) diacylglycerol cholinephosphotransferase (EC 2.7.8.2); 12) choline-P cytidyltransferase (EC 2.7.7.15); 13) phosphatidylethanolamine *N*-methyltransferase (EC 2.1.1.17); 14) phosphatidylethanolamine serinetransferase; 15) phosphatidic acid cytidyltransferase (CDP-diacylglycerol synthase) (EC 2.7.7.41); 16) phosphatidylinositol synthase; 17) monoacylglycerol acyltransferase; 18) glycerol-3-P dehydrogenase (EC 1.1.1.8).

major lipid component of membranes and triacylglycerols are the most important mammalian storage fuel. The ether glycerolipids are major components of membranes from brain cells and other tissues (11, 12). Although less abundant than glycerolipids, sphingolipids are the prominent glycolipids on cell surfaces and sphingomyelin, a phospholipid, is a component of cellular membranes (13, 14). Cholesterol undergoes a complex metabolism within cells and between cells (15). It is an important component of the plasma membrane and the serum lipoproteins, and is a precursor for steroid hormones and bile acids. Bile, which facilitates the excretion of bilirubin and other waste products, contains bile salts which aid in the digestion of lipid in the intestine.

The physical properties of complex lipids necessitate specialized metabolic routes of synthesis and transport. These occur within cells during growth and cell division, and between cells that produce and consume the plasma lipoproteins. Progress on elucidating the mechanisms by which the amounts and types of complex lipids are regulated in mammals has been hampered by the physical properties of substrates and the membrane association of the enzymes involved.

Lipid movement in and between membranes

Little complex lipid is present in free form in plasma or cell cytosol. Most complex lipids are associated with protein-containing structures, notably membranes and lipoproteins. Although transmembrane movement of phospholipids is extremely slow in model membranes, red cell membranes, and enveloped viruses (16–24), rapid transmembrane movement appears to occur in bacterial cytoplasmic membranes (25, 26) and in the endoplasmic reticulum (27–30). The rapid lateral movement of phospholipids within membranes occurs at rates near 1 μm/sec (31, 32). Within cells, phospholipids move rapidly from their sites of synthesis to their final cellular locations. Mechanisms available for the movement of complex lipids between membranes include transfer through regions of continuity or zones of adhesion, transport via intracellular vesicles including those of the coated vesicle/coated pit system (8, 33, 34), and exchange and net transport by phospholipid exchange proteins (27, 35). Fatty acids move extremely rapidly across and between membranes (36). Although phospholipid asymmetry is well established for the bilayers of the red cell membrane and for enveloped viruses (17, 37), lipid asymmetry remains unsubstantiated for

intracellular membranes such as the endoplasmic reticulum (38–41).

Sites of synthesis of complex lipid precursors

The building blocks of complex lipids are synthesized in the cytosol by soluble enzymes. Fatty acid synthase, acetyl-CoA carboxylase, and the enzymes that generate NADPH reside in the cytosol. Other precursors, including glycerophosphate, CDP-choline, CDP-ethanolamine, inositol, serine, β-hydroxy β-methylglutaryl CoA, and the sugar nucleotides are also thought to be synthesized in the cytosol. Palmitic acid, the principal de novo fatty acid product, undergoes activation, elongation, and desaturation within the endoplasmic reticulum membrane.

Subcellular location of glycerolipid synthetic enzymes

In rat liver the enzymes of glycerolipid synthesis are largely, but not exclusively, recovered in the microsomal subcellular fraction (42, 43). These include the enzymes responsible for the synthesis of the quantitatively most important glycerolipids, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and triacylglycerol (42, 43). Mitochondria contain significant amounts of fatty acid CoA ligase, glycerophosphate acyltransferase, and lysophosphatidic acid acyltransferase activities as well as the enzymes of phosphatidylglycerol and cardiolipin synthesis (43). The Golgi apparatus may possess the capacity for synthesis of phospholipids (42). Peroxisomes contain several enzymes of ether lipid biosynthesis, DHAP acyltransferase, acyl-DHAP oxido-reductase, alkyl-DHAP synthase (44–46), and fatty acid CoA ligase (47). Peroxisomes also participate in the β-oxidation of long chain fatty acids (48). Molecular characterizations of the glycerolipid biosynthetic enzymes are limited (43) since none, except for fatty acid CoA ligase (49), have been extensively purified. The existence of isoenzymes has not been addressed critically. For example, it is conceivable that separate diacylglycerol cholinephosphotransferases synthesize phosphatidylcholine destined for bilayers, lipoproteins, and bile. Such isoenzymes could have different topographical locations within the endoplasmic reticulum membrane.

Membrane protein and lipid asymmetry

The asymmetric orientation of membrane proteins within the transverse plane confers a functional asymmetry to membranes (3, 4). Each of the proteins associated with membranes has a fixed orientation with domains present on either the inner surface, the outer surface, or on both surfaces. All copies of each specific transmembrane protein span the membrane with a fixed orientation (3, 4). Reviews of membrane protein asymmetry (3, 4), intracellular protein topogenesis and the evolution of intracellular membranes (5, 6), topography of intracellular membranes (50), and endoplasmic reticulum structure (51) will provide important background for those unfamiliar with these areas.

Phospholipids, which comprise the structural matrix and the permeability barrier of membranes, are also asymmetrically distributed (3, 4, 37). Unlike membrane proteins, lipid asymmetry is only partial; each type of lipid is generally present on both sides of the membrane. The reader is referred to a review on lipid asymmetry (37) for a summary of the techniques employed to define lipid sidedness and for a discussion of the merits and weaknesses of these approaches. The phospholipid asymmetry of the erythrocyte membrane is well established (37). Whether lipid asymmetry is characteristic of other membranes is currently under investigation. The question of lipid asymmetry in microsomal vesicles is of particular interest, but existing studies conflict (38–41). The presence of non-lammelar lipid structures in biological membranes (52) may aid the rapid transmembrane movement of phospholipids in microsomal vesicles (27–29).

Properties of microsomal vesicles

Homogenization extensively fragments hepatic endoplasmic reticulum. These fragments seal, right side out, to form closed vesicles which are recovered in the microsomal subcellular fraction (50, 51, 53). The external surfaces of all microsomal vesicles correspond to the cytoplasmic surface of the endoplasmic reticulum (50, 51). Preparations of wrong-side-out microsomal vesicles are not available. Microsomal vesicles have been useful for studies of the transverse asymmetry of proteins, because they appear to mirror the properties of the endoplasmic reticulum (50, 51). Uncharged molecules of low molecular weight (less than 600–1000) (54, 55), readily penetrate microsomal vesicles, but most charged molecules, and high molecular weight dextrans and proteins (54, 55) do not cross the membrane.

Topography of glycerolipid synthesis in microsomal vesicles

Where are the active sites of the glycerolipid biosynthetic enzymes located in the transverse plane of the microsomal membrane? Three possible orientations are illustrated in **Fig. 2.** The domains of en-

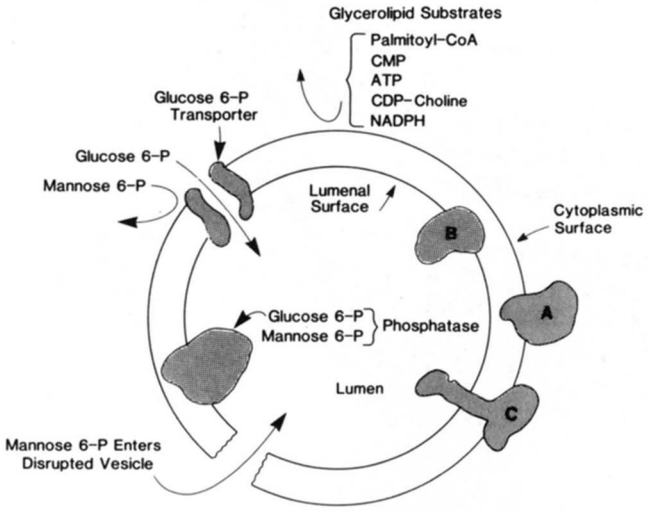

Fig. 2. Pertinent features of microsomal vesicles. The topography of proteins within the transverse plane of the membrane is illustrated. A. Protein facing the cytoplasm. B. Protein facing the lumen. C. A transmembrane protein. The lumenal glucose 6-phosphatase acts on either glucose 6-P or mannose 6-P. The entrance of glucose 6-P into the lumen is facilitated by a glucose 6-P transporter. Mannose 6-P and the glycerolipid substrates indicated do not enter the lumen of intact microsomal vesicles. Disruption of vesicles allows mannose 6-P access to the active site of glucose 6-phosphatase.

zymes could be located exclusively on the cytoplasmic surface (A) or on the lumenal surface (B), or the enzyme could span the membrane and have its active site on either side of the membrane (C). The location of the active sites of the glycerolipid biosynthetic enzymes should indicate whether synthesis occurs on one or both surfaces and help answer questions about later events of lipid topogenesis. Are bilayers assembled asymmetrically or by bilateral synthesis?[2] Where is triacylglycerol synthesized? Do isoenzymes with specialized functions exist on opposite sides of the membrane? Do specialized transport proteins facilitate the entrance of substrates of these enzymes of complex lipid synthesis into the lumen of microsomal vesicles? Does lipid asymmetry in membrane bilayers result from asymmetric synthesis or selective translocation?[2]

Experimental approaches and controls

Since most of the enzymes of glycerolipid synthesis have not been purified to homogeneity, studies on their topography in microsomal vesicles have, necessarily, employed indirect methods. The location of the active sites of these enzymes has been investigated using three different methods: *1*) inactivation by proteases and other impermeant inhibitors; *2*) demonstrations of latency and of substrate permeation; and *3*) localization of products by chemical, histochemical, or enzymatic methods. The need to perform appropriate controls that demonstrate vesicular integrity under each set of experimental conditions cannot be overemphasized. Although microsomal proteins do not undergo rapid transmembrane movement in microsomal vesicles, the lipids appear to do so (28, 29). Therefore, product localization studies demand knowledge not only of vesicular integrity but also of the rates of transmembrane movement of the lipid products.

Mannose 6-phosphatase latency provides a quantitative index of microsomal integrity

In liver, glucose 6-phosphatase has been localized to the lumenal surface of endoplasmic reticulum and microsomal vesicles (55, 58–60) (see Fig. 2). A membrane protein facilitates the entry of glucose 6-P into the lumen (55, 60), however, mannose 6-P is not transported by the glucose 6-P transporter (55). Mannose 6-P is hydrolyzed effectively by glucose 6-phosphatase when the permeability barrier of the vesicles has been disrupted to allow this alternate substrate access to the lumenal active site (55, 61). Thus, intact microsomes possess low mannose 6-phosphatase activity whereas activity increases 10- to 16-fold in disrupted vesicles (61). Latency of mannose-6-phosphatase activity corresponds exactly to membrane permeability to EDTA (61). A determination of mannose 6-phosphate latency, therefore, provides a quantitative index of microsomal integrity.

Studies with proteases

Rationale. Proteases do not cross the microsomal membrane or destroy the permeability barrier of microsomal vesicles under controlled conditions (40, 50, 51, 62). Therefore, inactivation by proteases can indicate whether essential domains of microsomal enzymes are exposed on the cytoplasmic surface. If inactivation by a protease occurs only after the vesicle is disrupted, essential domains may lie within the microsomal lumen. Coleman and Bell (63) were the first to combine protease treatment with the quantitative assessment of microsomal integrity afforded by determining mannose 6-phosphatase latency.

Results. Treatment of microsomal vesicles with proteases under conditions in which the microsomal vesicles remained intact caused inactivation of the enzymes of glycerolipid biosynthesis listed in **Table 1.** Coleman and Bell found that either chymotrypsin

[2] Consideration of erythrocyte membrane structure led Bretscher (56, 57) to recognize astutely the problem of assembly of asymmetric phospholipid bilayers. Bretscher (56, 57) was the first to propose that lipid biosynthesis takes place on one side of the membrane and to predict rapid transmembrane movement of phospholipids.

TABLE 1. Effect of proteases on glycerolipid synthetic activities in intact microsomal vesicles

	Percent Inactivation			
	Pronase	Chymotrypsin	Trypsin	References
Fatty acid CoA ligase	96	97		(63)
Glycerol-P acyltransferase	95	91		(63)
Lysophosphatidic acid acyltransferase	87	17		(63)
Phosphatidic acid phosphatase	1	0		(63)
Diacylglycerol acyltransferase	94	94		(63)
Diacylglycerol cholinephosphotransferase	66	99	>97[a]	(63, 65)
Diacylglycerol ethanolaminephosphotransferase	16	98	>97[a]	(63, 65)
Dihydroxyacetone-P acyltransferase		63	89	[b]
Acyl-DHAP oxidoreductase		100	100	[b]
Phosphatidic acid: CTP cytidyltransferase		0	96	[b]
CDP-diacylglycerol inositol phosphatidyltransferase	89	23	71	[b]
Monoacylglycerol acyltransferase		50	57	[b]
Diacylglycerol kinase		61	87	[b]
Serine base exchange		68	49	[b]
Cholinephosphate cytidyltransferase			>97	(65)
Phosphatidylethanolamine *N*-methyltransferase			>97	(65)

[a] This value is from reference 65.
[b] Ballas, L. M., and R. M. Bell. Submitted for publication.

or pronase inactivated fatty acid CoA ligase, glycerol-P acyltransferase, lysophosphatidic acyltransferase, diacylglycerol acyltransferase, diacylglycerol ethanolaminephosphotransferase, and diacylglycerol cholinephosphotransferase activities in intact microsomal vesicles (63).[3] Vance and coworkers (65) showed that trypsin inactivated choline- and ethanolaminephosphotransferases and the phosphatidylethanolamine *N*-methyltransferase activities in intact microsomes. In studies in which the maintenance of microsomal vesicular integrity was not investigated, trypsin or chymotrypsin impaired phosphatidylinositol synthesis (66). Ballas and Bell,[4] using intact microsomal vesicles, demonstrated that trypsin or chymotrypsin substantially inactivated phosphatidic acid: CTP cytidyltransferase and CDP-diacylglycerol inositol phosphatidyltransferase. DHAP acyltransferase and acyl-DHAP oxidoreductase, which are enzymes of ether lipid bisynthesis, monoacylglycerol acyltransferase, diacylglycerol kinase, and the serine base exchange enzyme were also inactivated in intact microsomal vesicles (Table 1).

These studies demonstrate that critical domains of the synthetic enzymes are exposed on the cytoplasmic surface and they rule out the possibility that domains of these enzymes are exposed exclusively on the lumenal surface (B in Fig. 2). These studies suggest, but do not prove, that the active sites face the cytoplasmic surface. It is possible, although we are not aware of any precedents, that proteolysis of a cytoplasmic domain could inactivate a lumenal active site.

Pitfalls. The use of proteases to investigate the location of enzymes within the transverse plane of microsomal vesicles is subject to several pitfalls. First, resistance to inactivation by proteases does not prove that an enzyme is located within the lumen (50). For example, note the differences in inactivation by pronase and chymotrypsin for lysophosphatidic acid acyltransferase and diacylglycerol ethanolamine phosphotransferase (Table 1). Diacylglycerol cholinephosphotransferase in intestinal microsomes was resistant to Nagarase treatment (67). If Nagarase treatment of disrupted microsomes had then inactivated the enzyme, a lumenal location would have been suggested. It is possible, however, that the detergent employed to disrupt membrane integrity could expose a cryptic proteolytic cleavage site on the cytoplasmic surface. The work of Coleman and Bell (68) on lysophosphatidic acid acyltransferase provides an example of a false suggestion of a lumenal location. Thus, the localization of lumenal microsomal enzymes should include studies using several proteases and employ more than one method of microsomal disruption. Moonen and van den Bosch (69) demonstrated that residual protease remains after reisolation of microsomes by centrifugation. Thus, if assay conditions disrupt the recovered microsomal vesicles, the residual protease will have access to lumenal sites. Protease treatment does not always afford useful topographical information. Phosphatidic acid phosphatase activity, for example, was not inactivated by

[3] Using microsomes turned inside-out on polylysine-coated glass beads, Dawidowitz and Sawyer (64) showed that the diacylglycerol choline- and ethanolamine-phosphotransferase activities were resistant to proteases while glucose 6-phosphatase was inactivated. This is consistent with a cytoplasmic location of active sites for the enzymes of glycerolipid synthesis.
[4] Ballas, L. M., and R. M. Bell. Unpublished data.

TABLE 2. Inhibition of glycerolipid biosynthetic enzymes by mercury-dextran

Enzyme	Percent Inhibition
Glycerol-P acyltransferase[a]	99
Diacylglycerol acyltransferase[a]	78
Diacylglycerol cholinephosphotransferase[a]	90
Diacylglycerol ethanolaminephosphotransferase[a]	82
Fatty acid CoA ligase (reverse reaction)[b]	65
Phosphatidic acid phosphatase[c]	72
Dihydroxyacetone-P acyltransferase[a]	70
CDP-diacylglycerol inositol phosphatidyltransferase[c]	99
Monoacylglycerol acyltransferase[c]	60
Diacylglycerol kinase[c]	75

[a] 140 μM mercury-dextran.
[b] 560 μM mercury-dextran.
[c] 280 μM mercury-dextran.
Data taken from reference 70 and Ballas, L. M., and R. M. Bell; submitted for publication.

exposure to several proteases in either intact or detergent-disrupted rat liver microsomes (63).

Studies using impermeant inhibitors

Since high molecular weight dextrans do not permeate the microsomal membrane (54, 55), the location of enzyme active sites can be probed with dextrans that have been covalently linked to maleimide or mercury functional groups. Mercury-dextran, the more effective inhibitor, inactivated the enzymes listed in **Table 2** (70). Dextran-maleimide inhibited glycerol-P acyltransferase about 50%. Dextran alone did not affect enzyme activities. The extent of inhibition by mercury-dextran was essentially identical in both intact and disrupted microsomes. Microsomal integrity in the presence and absence of mercury-dextran under assay conditions was established by determining mannose 6-phosphatase latency (70).

Assay of enzymes in intact vesicles

The protease and mercury-dextran data suggest that the active sites of the glycerolipid synthetic enzymes are located on the cytoplasmic surface of microsomal vesicles. A cytoplasmic location implies that substrates have free access to active sites and, thus, total enzyme activities should be readily detectable in intact microsomes. This appears to be true for these enzymes since mannose 6-phosphatase latency was maintained under assay conditions[4] (63, 70) and disruption by detergents did not greatly enhance glycerolipid enzyme activity. Several glycerolipid synthetic activities increased slightly with detergent concentrations that were too low to allow expression of latent mannose 6-phosphatase activity[4] (63). It is likely that the mechanism by which detergents act to enhance enzyme activity is unrelated to delivery of substrates to the lumenal compartment. The lack of latency is consistent with a cytoplasmic location of the active sites of the glycerolipid synthetic enzymes.

Do substrates of glycerolipid synthetic enzymes cross the microsomal membrane?

Charged molecules as small as acetate do not cross the microsomal membrane (54), unless specific transport proteins are present. Of the known microsomal transport systems, those for glucose-6-P and Pi are the best characterized (55, 71). The lengthy ultracentrifugation process required to assess solute penetration into microsomal vesicles (54) was refined by Ballas and Arion (55) who employed calcium-aggregated microsomes that could be rapidly sedimented in the microcentrifuge. The volumes occupied by substrates are compared to the volumes occupied by solutes like glycerol and glucose which freely penetrate into the microsomal lumen and to the volumes occupied by non-penetrating solutes like dextran, inulin, or acetate (54, 55, 70, 72). Direct measurements of this type suggest that ATP, CMP, and CDP-choline, which are substrates of glycerolipid synthetic enzymes, do not enter the microsomal lumen. Although the demonstration of metabolically active lumenal pools provides substantial evidence that molecules enter the lumen (55, 72), conclusive evidence that certain solutes are excluded from the lumenal compartment is more difficult to collect because adsorption and nonspecific binding to microsomes may occur. NADPH probably does not permeate (73), whereas fatty acids (36) and diacylglycerols (74) would be expected to readily undergo transmembrane movement.

Studies of a microsomal ethanol acyltransferase activity provided evidence that palmitoyl-CoA cannot readily penetrate the microsomal membrane (75). The data revealed a lag in the palmitoyl-CoA dependence that correlated exactly with the disruption of the permeability barrier of microsomal vesicles by palmitoyl-CoA. This lag in the palmitoyl-CoA dependence was abolished after disruption of microsomal vesicles by detergents. The inhibition of ethanol acyltransferase activity by added albumin occurred because albumin prevented microsomal disruption by palmitoyl-CoA. Latent ethanol acyltransferase and mannose 6-phosphatase activities were similarly expressed quantitatively. Ethanol acyltransferase was resistant to protease inactivation in intact microsomes and susceptible in disrupted microsomes. Since ethanol is known to permeate microsomal vesicles, the latency of ethanol acyltransferase must result from the limited penetration of palmitoyl-CoA to the lumenal surface of intact microsomal vesicles (75). This important conclusion suggests that all micro-

somal glycerolipid synthetic activities that use fatty acyl-CoA thioesters have active sites located on the cytoplasmic surface.

The lack of latency and the inability of substrates of several glycerolipid synthetic enzymes to penetrate into the lumen strongly suggest that the active sites are present on the cytoplasmic surface of microsomal vesicles. The possibility that proteolysis of transporter proteins resulted in an apparent loss of glycerolipid synthetic activities was eliminated by showing that protease-treated microsomes did not contain latent synthetic activities (63).

Product localization by chemical, histochemical, or enzymatic methods

Although Rothman and Kennedy (76) were able to locate newly synthesized phosphatidylethanolamine in *Bacillus megaterium* by labeling with trinitrobenzenesulfonate, this method did not prove useful in studying phosphatidylethanolamine synthesis in rat liver microsomes.[5] Since bulk and newly synthesized phosphatidylethanolamine were labeled at identical rates in intact microsomes, either all of the phosphatidylethanolamine is on the cytoplasmic surface, or the phosphatidylethanolamine moves rapidly between the two sides of the microsomal membrane. The latter suggestion is consistent with the rate of phospholipid translocation that was implied from studies using phospholipid exchange proteins (28, 29). Another possibility is that trinitrobenzenesulfonate readily crosses the microsomal membrane.

Higgins (77) employed phospholipase C to localize phosphatidylcholine synthesized in vitro in rat liver microsomes. Almost 90% of the phosphatidylcholine labeled in vitro for 2 min was hydrolyzed, whereas only 50% of the microsomal phosphatidylcholine labeled *in vivo* was hydrolyzed. These data were interpreted to suggest that most of the label is incorporated into the outer microsomal leaflet in vitro. It was not possible to chase the in vitro labeled phosphatidylcholine into a pool protected from phospholipase C hydrolysis (i.e., to the inner leaflet). This interpretation appears inconsistent with the extensive exchangeability of phosphatidylcholine present in rat liver microsomes (28, 29). These studies employing phospholipase C also revealed differences in specific activities of inner and outer leaflet phosphatidylcholine (77). These observations prompted Higgins to suggest that phosphatidylcholine synthesized by the CDP-choline pathway remains on the outer leaflet and that some alternate pathway gives rise to the inner leaflet phosphatidylcholine. Regrettably, the apparent location of phospholipids within the transverse plane of microsomes appears to be dependent on the methods employed (38–41, 77). Conclusive proof will require the development of several independent methods to localize the lipid present and the measurement of rates of lipid transmembrane movement.

Few studies have been reported localizing glycerolipid synthetic enzymes by histochemical methods. The exclusively lumenal location of the glycerol-P acyltransferase based on the histochemical detection of released thiol groups from acyl-CoA (78) is totally inconsistent with the results of experiments demonstrating protease inactivation (63), inactivation by impermeant inhibitors (70), lack of latency (63, 70), and impermeability of microsomes to palmitoyl-CoA (75). In the histochemical studies, maintenance of microsomal integrity was not demonstrated (78). Experiments in our laboratory show that the amounts of palmitoyl-CoA employed in these studies would have completely disrupted the microsomal vesicles (75).

Location of enzymes of glycerolipid degradation in microsomal vesicles

At present, little information exists on the topography of enzymes of glycerolipid degradation. Microsomal lysophospholipase II of bovine liver was resistant to chymotrypsin in intact microsomal vesicles (69). After the vesicles were disrupted with lysophosphatidylcholine, chymotrypsin inactivated greater than 95% of the lysophospholipase activity. Sonication released the microsomal lysophospholipase II free of lipids. These careful studies of Moonen and van den Bosch (69) suggest that the active site of lysophospholipase II is located within the lumen. The properties of the ethanol acyltransferase activity of rat liver microsomes suggested that it might be a lumenal esterase (75), perhaps the latent palmitoyl-CoA hydrolase activity described by Jambdar (79).[6] The orientation of other microsomal enzymes that degrade glycerolipids deserves further investigation. The cytoplasmic orientation of several glycerolipid synthetic enzymes and the implied lumenal location of two degradative enzymes raise the intriguing possibility that synthetic and degradative events may occur on opposite sides of the microsomal membrane.

Topography of lipid synthetic enzymes in organelles other than endoplasmic reticulum

Although the endoplasmic reticulum plays the dominant role in complex lipid synthesis, mito-

[5] Coleman, R. A., and R. M. Bell. Unpublished data.

[6] Since palmitoyl-CoA does not freely permeate microsomal vesicles, the physiological substrates are unknown for both these activities.

chondria, Golgi, and peroxisomes contain significant quantities of synthetic activities. Lysosomes contain several lipid-degrading enzymes (80), as well as the enzymes that synthesize *bis*-monoacylphosphatidylglycerol (81). Mitochondria contain glycerol-P acyltransferase, fatty acid CoA ligase, and other enzymes involved in phosphatidylglycerol and cardiolipin biosynthesis (43). Little data is available on the localization of glycerolipid synthetic enzymes within the transverse plane of membranes other than endoplasmic reticulum. Experiments localizing glycerol-P acyltransferase and fatty acid CoA ligase to the inner surface of the outer mitochondrial membrane must be considered tentative in view of the indirect assays that were performed and the absence of appropriate controls (82).

Hirata and Axelrod (83) have presented data suggesting that the active site of the phosphatidylethanolamine *N*-methyltransferase is located on the cytoplasmic side of the erythrocyte membrane, and that the subsequent *N*-methyltransferase reactions are catalyzed by a second enzyme located on the outer surface. The purity of the erythrocyte membranes was not established, however, and, in view of the extremely low specific activities reported, the activities may have been located in contaminating microsomes derived from leukocytes.

The lipid synthetic capacity of plasma membranes requires continued investigation. A recent report suggesting that rat adipocyte plasma membranes contain glycerol-P acyltransferase activity located on the outer surface must be regarded as unsubstantiated since the activity measured may have been in broken cells present in the population (84).

Fatty acids are incorporated into the inner leaflet of the erythrocyte membrane where the acylation of lysophospholipids occurs (21, 22). Diacylglycerol kinase is also located on the inner surface of the erythrocyte membrane (74). The renewal of phospholipids in red blood cells either by exchange with serum lipoproteins or by reacylation reactions has received considerable attention because mitochondria, endoplasmic reticulum, and other intracellular organelles are absent in erythrocytes (23, 24). These studies may reflect an unrecognized role of the plasma membrane of other cell types and represent a fruitful area for further investigation.

The topography of the peroxisomal enzymes of complex lipid metabolism is uncertain. Enzymes of lipid metabolism present in peroxisomes are just now being identified. Lipid synthetic activities associated with peroxisomes include fatty acid CoA ligase (47), DHAP acyltransferase, acyl-DHAP oxidoreductase, and alkyl-DHAP synthase (44–46). A latent DHAP acyltransferase activity was found in rat liver peroxisomes (46) and in a preparation of rabbit harderian gland microsomes which probably contained peroxisomes (85, 86). The amount of palmitoyl-CoA present in the assay mix proved important in estimating the degree of latency, probably reflecting the disruption of peroxisomal (microsomal) vesicles with palmitoyl-CoA (86). This latency, and the resistance of DHAP acyltransferase to trypsin inactivation in the absence of detergent, suggests a lumenal location for the active site (46, 85, 86). Similar data were reported for alkyl-DHAP synthase (85, 87). Since protease treatment of intact membranes abolished DHAP acyltransferase activity when a palmitoyl-CoA generating system was employed, it was suggested that fatty acid CoA ligase was on the cytoplasmic surface (86).

Peroxisomes synthesize the key intermediates of ether lipid synthesis. A lumenal site of synthesis is consistent with a peroxisomal DHAP pool since peroxisomes contain a glycerol-P dehydrogenase (88, 89). Since peroxisomes do not contain significant quantities of lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol cholinephosphotransferase, and diacylglycerol ethanolamine phosphotransferase,[7] it appears likely that the early intermediates of ether lipids are transferred to the endoplasmic reticulum for completion of ether lipid synthesis.[8]

Topography of other enzymes of complex lipid metabolism

Most other enzymes of complex lipid metabolism have not been rigorously localized. The production of unsaturated fatty acids must occur on the cytoplasmic surface of the endoplasmic reticulum since cytochrome b_5 and cytochrome b_5 reductase are located there (50, 90, 91). Recently, studies employing proteases and antibodies showed that Δ^9 terminal desaturase is exposed on the cytoplasmic surface of chicken liver microsomal vesicles (92).

It is likely that all enzymes of the endoplasmic reticulum that require NADPH or palmitoyl-CoA are located facing the cytoplasmic surface, since neither substrate penetrates readily into the lumen of microsomal vesicles. Thus, the synthesis of cholesterol, dolichol, cholesteryl esters, sphingolipids, and bile acids probably takes place on the cytoplasmic surface.

Inhibition of β-hydroxy-β-methylglutaryl-CoA reductase by antibodies suggested that critical domains were located on the cytoplasmic surface (93);

[7] Ballas, L. M., P. B. Lazarow, and R. M. Bell. Unpublished data.

[8] The existence of peroxisomal enzymes specific for alkylglycerolipid substrates has not been investigated.

however, controls showing maintenance of vesicular integrity were not performed. A cytoplasmic location for the active site was also suggested because NADPH is a substrate. The synthesis of the isopentyl pyrophosphate and of dimethylallyl pyrophosphate, precursors of cholesterol and dolichol, occurs by a series of enzymes located in the cytoplasm (15, 94), Microsomal enzymes catalyze the condensation of two molecules of farnesyl pyrophosphate (94). Since the conversion of lanosterol to cholesterol requires NADPH, this process probably occurs on the cytoplasmic surface. The microsomal acyl-CoA cholesterol acyltransferase which functions to produce cholesterol esters is located on the cytoplasmic surface of microsomal vesicles (95). In addition, oleoyl-CoA, a substrate for this enzyme, does not appear to permeate the microsomal membrane as indicated by a highly latent ethanol acyltransferase activity[4] (75). The biosynthesis of steroids from cholesterol involves the microsomal P-450 system which is accessible from the cytoplasmic surface (50). The synthesis of bile acids and bile salts involves several steps in the endoplasmic reticulum which are NADPH dependent (96). The hydroxylated intermediates are then transferred to mitochondria for side chain cleavage (96). Since microsomal cholic acid CoA ligase activity was inactivated by proteases and was not activated by disruption of the permeability barrier (97), a cytoplasmic location was suggested. The cholyl-CoA glycine acyltransferase is present in the cytosol or loosely associated with the microsomal membrane (98).

The biosynthesis of ceramide, the basic building block of the sphingolipids, is accomplished by a number of enzymes presumably located in microsomes (13). Since the palmitoyl-CoA (75) and NADPH (73) substrates cannot penetrate microsomes, it is likely that the active sites of these enzymes face the cytoplasmic surface. We are not aware of any topographical investigations of enzymes which add the carbohydrate or phosphocholine moieties of the final ceramide derivatives.

Asymmetric assembly of complex lipids in the endoplasmic reticulum

The data generated from studies of enzyme inactivation by proteases and other impermeant inhibitors, latency and substrate permeation, and product localization indicate that the active sites of microsomal enzymes that synthesize complex lipids are located on the cytoplasmic surface of the endoplasmic reticulum. The data are incompatible with domains exclusively located on the lumenal surface, but do not rule out the possibility that one or more of these enzymes spans the membrane and has domains exposed on both surfaces. As pointed out earlier, the studies suggesting a lumenal location for some of the glycerolipid synthetic enzymes are faulty in that either controls were not performed to demonstrate vesicular integrity or the experimental design was inadequate.

Assuming that the topography of microsomal vesicles mirrors that of the endoplasmic reticulum, most, if not all, complex lipids are synthesized on the cytoplasmic surface of the endoplasmic reticulum (**Fig. 3**). Fatty acids are synthesized in the cytosol by fatty acid synthase which uses cytoplasmic NADPH, acetyl-CoA, and malonyl-CoA. Fatty acids synthesized de novo and those derived from the diet, are activated on the cytoplasmic surface of the endoplasmic reticulum. Fatty acyl-CoA thioesters are elongated and desaturated on the cytoplasmic surface. The pool of acyl-CoA thioesters is then used for the esterification reactions. The other precursors, substrates, and enzymes of complex lipid synthesis are also present in or facing the cytosolic compartment. These substrates include glycerol-P, dihydroxyacetone-P, CDP-choline, CDP-ethanolamine, inositol, ATP, serine, and β-hydroxy-β-methylglutaryl-CoA. The subsequent steps of glycerolipid synthesis occur on the cytoplasmic surface (see Fig. 3). Sequential esterification of glycerol-P (or DHAP) produces phosphatidic acid from which diacylglycerol and CDP-diacylglycerol are derived. Phosphatidylcholine and phosphatidylethanolamine, the most abundant glycerolphosphatides, are synthesized by addition of the appropriate phosphoryl bases to diacylglycerol. Phosphatidylinositol is derived via a phosphatidyl transfer from CDP-diacylglycerol to inositol. Phosphatidylserine is derived by base exchange of serine for the ethanolamine moiety of phosphatidylethanolamine, and triacylglycerol is synthesized by a third esterification. Additionally, cholesterol, cholesteryl esters, and ceramide are probably synthesized on the cytoplasmic surface.

Lipid topogenesis

Asymmetric synthesis of all the complex lipids appears likely, although the sites of many enzymatic reactions have not been rigorously established. Specific membrane proteins would not be necessary to transport the charged substrates into the endoplasmic reticulum lumen. The phospholipid bilayer would grow by synthesis and insertion of new molecules on only the cytoplasmic surface of the endoplasmic reticulum. Following synthesis, phospholipids would be translocated to the lumenal surface during membrane biogenesis. Clearly, phospholipid asymmetry does not result from synthesis of different phospholipid classes on opposite surfaces. Rapid trans-

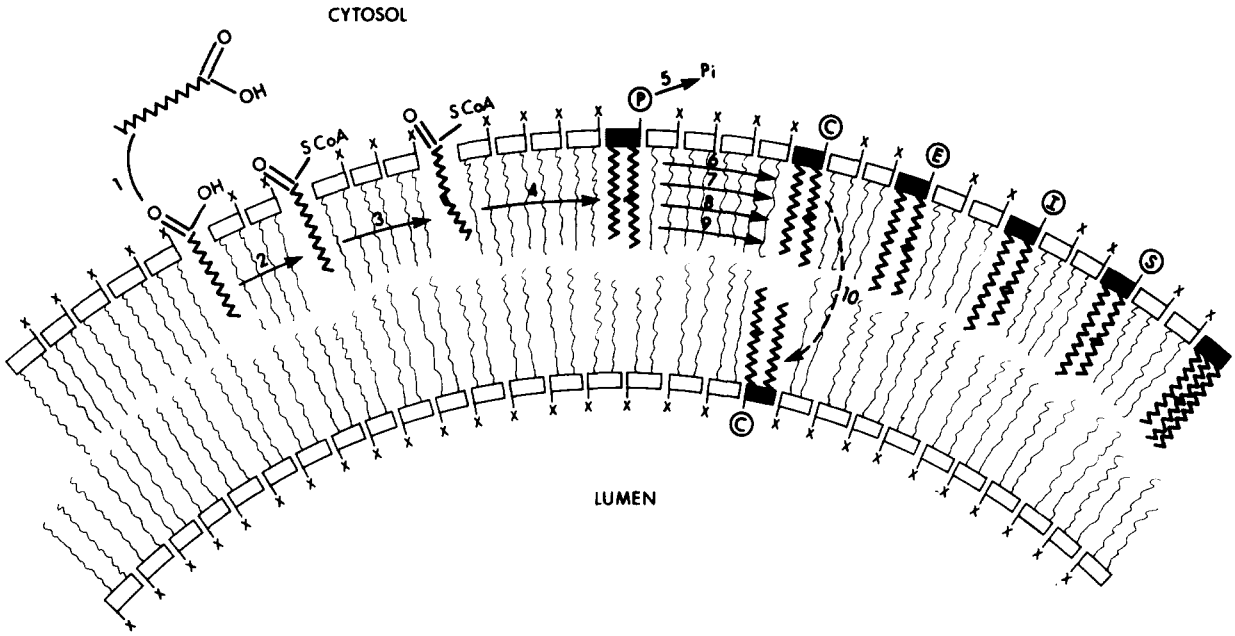

Fig. 3. Early events of lipid topogenesis. Fatty acids are inserted into the cytoplasmic surface of the endoplasmic reticulum (1) and activated to form acyl-CoA thioesters (2). The acyl chains may be elongated and/or desaturated (3). Glycerol-P undergoes acyl-CoA dependent esterification to form phosphatidic acid (4). The action of phosphatidic acid phosphatase (5) forms diacylglycerols that are converted to phosphatidylcholine and phosphatidylethanolamine by acquisition of phosphocholine and phosphoethanolamine polar head groups (6). Phosphatidyl serine synthesis occurs by base exchange (7). Triacylglycerol synthesis occurs by esterification of diacylglycerol (8). CDP-diacylglycerol is an intermediate in the synthesis of phosphatidylinositol (9). Once formed, the glycerolipids may move to the lumenal surface of the endoplasmic reticulum (10). C = choline; E = ethanolamine; I = inositol; S = serine; X = polar head group C, E, I, or S; P = PO_4.

membrane movement of phospholipids within the endoplasmic reticulum is implied by the extensive exchangeability of the phospholipids present. Membrane lipids organized in non-lamellar phases may provide a mechanism for rapid transmembrane movement of phospholipids. Other complex lipids such as cholesterol and its derivatives, triacylglycerol, sphingolipids, and bile acids must be transported through membrane bilayers during bile secretion and lipoprotein assembly.

Little data exist on the topography of complex lipid synthesis in organelles such as Golgi, mitochondria, and peroxisomes. The interactions between the intracellular organelles and the regulation of these interactions remains an intriguing area of investigation. The emerging role of vesicles as intracellular carriers of protein and lipids deserves further attention. Much remains to be learned about the transbilayer movement of complex lipids in endoplasmic reticulum and other organelles, and the mechanisms of sorting, targeting, and assembling of these lipids into their resulting structures. The possibility that protein topogenesis dictates and drives lipid topogenesis seems likely and may explain, in part, the characteristic complex lipid compositions of the organelles, the lipoproteins, and bile. The asymmetric distribution of membrane proteins may cause the observed partial lipid asymmetry either by specific lipid-protein interactions or by the generation of asymmetric ionic environments.

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