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TOPOGRAPHY OF PHOSPHATIDYLCHOLINE, PHOSPHATIDYLETHANOLAMINE AND TRIACYLGYCEROL BIOSYNTHETIC ENZYMES IN RAT LIVER MICROSOMES

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Summary

The topography of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol biosynthetic enzymes within the transverse plane of rat liver microsomes was investigated using two impermeant inhibitors, mercury-dextran and dextran-maleimide. Between 70 and 98% of the activities of fatty acid : CoA ligase (EC 6.2.1.3), *sn*-glycerol-3-phosphate acyltransferase (EC 2.3.1.15), phosphatidic acid phosphatase (EC 3.1.3.4), diacylglycerol acyltransferase (EC 2.3.1.20), diacylglycerol cholinephosphotransferase (EC 2.7.8.2) and diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) were inactivated by mercury-dextran. Dextran-maleimide caused 52% inactivation of the *sn*-glycerol-3-phosphate acyltransferase. Inactivation of each of these activities except fatty acid : CoA ligase occurred in microsomal vesicles which remained intact as evidenced by the maintenance of highly latent mannose-6-phosphatase activity (EC 3.1.3.9). These glycerolipid biosynthetic activities were not latent, indicating that substrates have free access to the active sites. Moreover, ATP, CDP-choline and CMP appeared unable to penetrate the microsome membrane. These data indicate that the active sites of these enzymes are located on the external surface of microsomal vesicles.

It is concluded that the biosynthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol occurs asymmetrically on the cytoplasmic surface of the endoplasmic reticulum.

Introduction

The principal site of phospholipid and triacylglycerol biosynthesis in liver is the endoplasmic reticulum. While the pathways of phospholipid and triacylglycerol synthesis have been established for some time [1,2] and the individual enzyme activities investigated [2,3], only recently has the orientation of these enzymes within the transverse plane of microsomal vesicles been investigated [4,5]. Homogenization of liver results in extensive fragmentation of the endoplasmic reticulum. These fragments seal to form closed vesicles called microsomes which maintain proper sidedness [6,7]. Since the enzymes of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol biosynthesis were inactivated by proteases in intact microsomal vesicles, it was suggested that these enzymes were located on the cytoplasmic surface of the membrane [4]. However, as pointed out by these workers [4], it is possible that proteolysis of a cytoplasmic domain causes inactivation of a lumenal active site. Additionally, Moonen and van den Bosch [8] suggested that residual protease could inactivate a lumenal enzyme if the microsomal vesicles were disrupted under assay conditions.

The enzymes of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol synthesis were also suggested to be externally oriented because none of the activities appeared latent [4]. The latency argument rested on the presumption that cytoplasmically derived precursors/products such as glycerol phosphate, CMP, ATP, CDP-choline and CDP-ethanolamine do not freely penetrate the microsomal membrane [4].

In this paper, we report new investigations on the topography of glycerolipid enzymes in microsomal vesicles which utilize impermeant inhibitors. * Additionally, measurements of the permeation of water-soluble substrates of three biosynthetic enzymes into microsomal vesicles are reported. From these two new lines of investigation and previous studies [4], it is concluded that phosphatidylcholine, phosphatidylethanolamine and triacylglycerol synthesis occurs asymmetrically on the cytoplasmic surface of microsomal vesicles.

Materials and Methods

Materials. Adenosine 5'-[γ-^{32}P]triphosphate, [2-^{14}C]glycerol, [*carboxyl*-^{14}C]-dextran ($M_{r,av}$ = 20 000), [U-^{14}C]adenosine 5'-monophosphate, [*methyl*-^{14}C]-cytidine diphosphocholine, [G-^{3}H]coenzyme A and $^{32}P_i$ were obtained from New England Nuclear Corp. (U.S.A.); [5-^{3}H]cytidine 5'-monophosphate and cytidine 5'-diphospho[2-^{14}C]ethan-1-ol-2-amine were products of Amersham (U.S.A.); palmitoyl-coenzyme A and coenzyme A were products of P-L Biochemicals; diolein and dioleoyl phosphatidic acid were supplied by Serdary (Canada); silicone fluids were a gift of the Silicone Products Department of General Electric (U.S.A.). All other materials were obtained from commercial sources.

Preparation of microsomes. Charles River CD female rats (200—250 g), which were fasted for 18 h before killing, were used. Microsomes were isolated

* A preliminary report of this work has been published [9].

as previously described [4,10] and were stored at −20°C. Detergent-disrupted microsomes were prepared by supplementing 8.5 vols. of intact microsomes (approx. 1 mg/ml) with 1.5 vols. of 4% sodium taurocholate, pH 7.4. The mixture was incubated on ice for 20 min and assayed immediately. In some experiments, microsomes were disrupted in a similar manner with 1% Triton X-100. Calcium-aggregated microsomes were prepared as described previously [11].

Synthesis of impermeant inhibitors. Mercury-dextran was prepared according to the method of Pitha [12] except that dextran of $M_{r,av}$ 18 400 was used. The mercury-dextran contained 28.8% mercury by weight or approx. 0.33 mercury atoms/glucose residue. Mercury-dextran is quite stable in aqueous solutions near neutrality for extended periods of time [12]. The mercury-dextran solutions employed did not contain any free mercury as measured electrochemically by differential pulse polarography [13].

Dextran-maleimide was synthesized essentially by using the method of Abbott and Schachter [14]. The starting material, dextran-O-CH_2-CHOH-CH_2-S-SO_3-Na (M_r 75 000), was a gift of Dr. J. Pitha. It was reduced, dialyzed against water at 4°C and lyophilized. The dextran-O-CH_2-CHOH-CH_2-SH (100 mg) was dissolved in 15 ml water and added dropwise to a solution of 20 mg bismaleimidomethyl ether (a gift of Dr. W. Lennarz) at 70° C. The solution was stirred constantly and allowed to cool at room temperature for 1 h. After lyophilization, the material was dissolved in 2 ml water and subjected to chromatography on a Sephadex G-25 column (60 × 2.0 cm). The maleimide content was estimated by determining sulfhydryl depletion after reaction with excess 2-mercaptoethanol using 5,5'-dithiobis(2-nitrobenzoic acid) [15]. The purified dextran-maleimide was lyophilized, resuspended in water and small samples were stored at −20°C.

Synthesis of radioactively labeled substrates. Mannose 6-[^{32}P]phosphate was synthesized by a modification of the method of Slein [16] as described previously [4]. *sn*-Glycerol 3-[^{32}P]phosphate [17], *sn*-[2-^{3}H]glycerol 3-phosphate [17] and [^{3}H]palmitoyl-CoA [18] were synthesized as previously described. [^{32}P]Phosphatidic acid was synthesized enzymatically from [γ-^{32}P]ATP and 1,2-dioleoyl-*sn*-glycerol with *Escherichia coli* diacylglycerol kinase. * [γ-^{32}P]-ATP was generated in situ by using the method of Glynn and Chappell [20]. The [^{32}P]phosphatidic acid was purified by thin-layer chromatography on Silica gel H plates with a chloroform/methanol/acetone/acetic acid/water (10:20:40:20:10, v/v) solvent system [21], and stored at −20° C in chloroform.

Enzyme assays. Mannose-6-phosphatase activity was determined at 23°C with 1 mM mannose 6-[^{32}P]phosphate essentially as described previously [22]. The volume of the assay was reduced to 200 μl and the amount of protein employed was similar to that employed for the assay of the glycerolipid synthetic activities. *sn*-Glycerol-3-phosphate acyltransferase activity was assayed with 0.3 mM *sn*-[^{3}H]glycerol 3-phosphate and 50 μM palmitoyl-CoA as previously described [23] except that 50 mM Tris-cacodylate buffer, pH 6.5, was used and dithiothreitol was omitted. Diacylglycerol acyltransferase activity was deter-

* Membranes were prepared from the hybrid plasmid-bearing strain, BB26-VL1A/pVL1, as previously described [19]. This strain bears the structural gene for the diglyceride kinase *dgk* and overproduces the kinase more than 10-fold (Bell, R.M., Lightner, V. and Raetz, C.H.R., unpublished observations).

mined with 30 μM [^{3}H]palmitoyl-CoA, 150 μM diolein essentially as described previously [24] except that 50 mM Tris-cacodylate buffer, pH 7.0, was employed; the diolein was added in 10 μl of absolute ethanol and diisopropylfluorophosphate (2.5 mM) was added [25]. Diacylglycerol cholinephosphotransferase activity [26] and diacylglycerol ethanolaminephosphotransferase activity [26] were estimated as previously described except that 50 mM Tris-cacodylate buffer, pH 7.0, 150 μM diolein added in 10 μl absolute ethanol and 100 μM CDP-[^{14}C]choline or CDP-[^{14}C]ethanolamine, were used, respectively. Fatty acid : CoA ligase activity was assayed in the reverse direction. The reaction mixture (200 μl) contained 50 mM Tris-cacodylate, pH 7.0, 8 mM $MgCl_2$, 50 μM [^{3}H]palmitoyl-CoA, 10 mM AMP, 10 mM PP_i and 2 mg/ml albumin. After 10 min at 23°C, the reaction was terminated with a solution of 1.5 ml isopropanol/heptane/water (80:20:2, v/v/v). This was followed by 0.5 ml water and 1 ml heptane. A sample of the heptane phase was counted. Greater than 90% of the heptane-extractable label comigrated with authentic palmitic acid on Silica gel G plates developed with petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v). Phosphatidic acid phosphatase activity was assayed at 23°C for 5 min using 10 μM [^{32}P]phosphatidic acid (added in ethanol), 0.5 mg/ml bovine serum albumin and 50 mM Tris-cacodylate buffer, pH 7.0, in a final volume of 200 μl. The reaction was stopped with 3 ml chloroform/methanol (2:1, v/v) and 0.8 ml H_2O was added to separate phases. A portion of the aqueous layer was counted. All enzyme activities were assayed under conditions where activity was proportional with time and the amount of protein (10—40 μg) employed. Radioactivity was determined in an Intertechnique SL-30 scintillation counter at 4°C. Quench corrections were made by the external standard method [27]. Protein was determined by using the method of Lowry et al. [28] with bovine serum albumin as standard.

Substrate permeation measurements. Substrate permeation into microsomal vesicles was investigated by a modification of the microcentrifugation method previously described [11]. The incubation temperature was 23°C and 50 mM Tris-cacodylate buffer, pH 7, was used. The bottom quenching layer contained 0.1 ml 30% glycerol in 1% perchloric acid; the silicone fluid density and viscosity were determined empirically and consisted of a mixture of versilube F-50/SF-96(50) (4:1, w/w); the composition of the permeation mixtures was kept constant in terms of the concentrations of glycerol, dextran and glycerolipid enzyme substrates by using labeled or unlabeled solutes as required. This was essential because the measured volumes were influenced by added substrates. When other solutes were added in attempts to decrease non-specific binding, the same concentration of solute was added to each of the permeation mixtures. Substrate stability was investigated by analysis of the quenched permeation mixtures by either thin-layer chromatography or extraction of the particular solute.

Results

Inhibition of glycerolipid biosynthetic enzymes by impermeant inhibitors

Since high molecular weight dextrans do not permeate the microsomal membrane [11,29], the location of active sites of enzymes in the transverse plane of

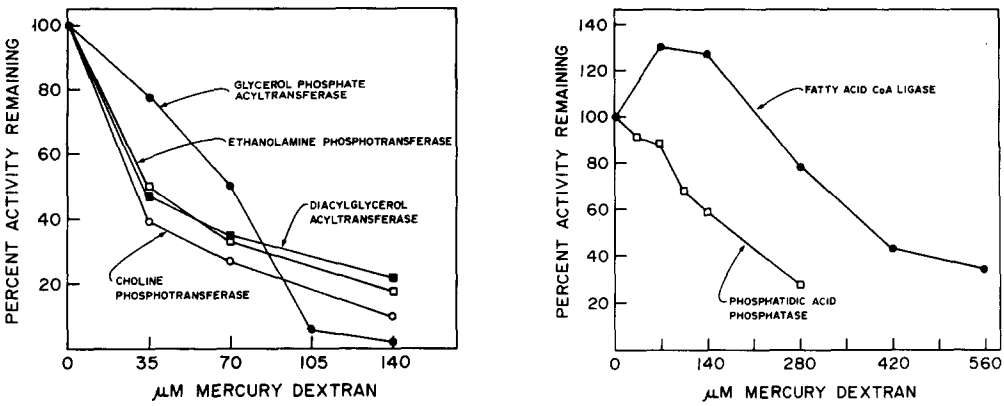

Fig. 1. Inhibition by mercury-dextran. The effect of mercury-dextran on glycerophosphate acyltransferase (●——●) (1.2); diacylglycerol acyltransferase (■——■) (1.7), diacylglycerol cholinephosphotransferase (○——○) (5.6) and diacylglycerol ethanolaminephosphotransferase (□——□) (1.3) is shown. The specific activities (nmol/min per mg) in the absence of inhibitor are shown in parentheses. Assays were performed in the presence of the indicated concentrations of inhibitor. Glycerophosphate acyltransferase and diacylglycerol acyltransferase were preincubated for 3 min with inhibitor in the assay. The reactions were initiated with palmitoyl-CoA/glycerol phosphate for glycerophosphate acyltransferase and palmitoyl-CoA/diolein for diacylglycerol acyltransferase. Other details are described in Materials and Methods. The concentration of inhibitor is reported as the concentration of mercury present as mercury-dextran.

Fig. 2. Inhibition of fatty acid : CoA ligase (●——●) (6.6) and phosphatidic acid phosphatase (○——○) (3.4) by mercury-dextran. Specific activities (nmol/min per mg) in the absence of inhibitor are shown in parentheses. Fatty acid : CoA ligase was preincubated for 3 min with inhibitor in the assay. The reaction was initiated with PP_i and palmitoyl-CoA. Other details are as described in Materials and Methods and Fig. 1.

microsomal vesicles could be probed with dextrans containing maleimide and mercury functional groups in covalent linkage. Mercury-dextran proved to be an effective inhibitor for each of the biosynthetic enzyme activities investigated. The concentration dependences of mercury-dextran inhibition of glycerophosphate acyltransferase, diacylglycerol acyltransferase, diacylglycerol cholinephosphotransferase and diacylglycerol ethanolaminephosphotransferase activities are shown in Fig. 1. These enzyme activities were inhibited 80—98% at 140 μM impermeant inhibitor. The inhibition of phosphatidic acid phosphatase activity was 70% at 280 μM mercury-dextran (Fig. 2). Since mercury-dextran would bind the CoASH required for fatty acid activation, fatty acid : CoA ligase activity was assayed by determining the AMP- and PP_i-dependent release of labeled palmitic acid from palmitoyl-CoA. Fatty acid : CoA ligase activity was inhibited 65% by 560 μM mercury-dextran (Fig. 2). The increased ligase activity noted in the presence of 70 and 140 μM mercury-dextran most likely occurs by release of product inhibition. Similar modest increases in ligase activity were noted in the presence of 5,5′-dithiobis(2-nitrobenzoic acid). Dextran alone did not affect any of the enzyme activities. All enzyme activities except fatty acid : CoA ligase were inhibited by mercury-dextran in intact vesicles (see below). The effect of mercury-dextran on enzyme activities in detergent-disrupted microsomes was essentially identical to that observed in intact microsomes.

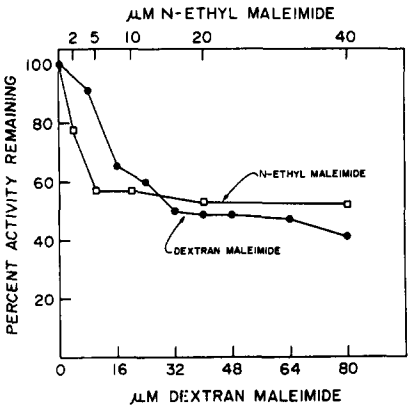

Fig. 3. Glycerophosphate acyltransferase inhibition by dextran-maleimide (●———●) and *N*-ethylmaleimide (□———□). Microsomes (1 mg/ml) were preincubated 10 min at 4°C with the indicated concentration of dextran-maleimide or *N*-ethylmaleimide and then assayed. Other details are as described in Materials and Methods. The specific activity of the uninhibited reactions were 1.4 and 1.1 nmol/min per mg, respectively. The concentration of impermeant inhibitor is reported as the concentration of reactive maleimide present as dextran-maleimide.

Dextran-maleimide proved to be a less useful probe of the location of the active sites of glycerolipid biosynthetic enzymes in the transverse plane of microsomal vesicles than mercury-dextran. *N*-Ethylmaleimide inhibited glycerophosphate acyltransferase activity about 50% (Fig. 3 and Ref. 23). None of the other biosynthetic enzyme activities investigated was inhibited by *N*-ethylmaleimide. Dextran-maleimide inhibited the glycerophosphate acyltransferase activity about 50% at concentrations greater than 10 μM (Fig. 3). Complete alkylation of an enzyme need not cause complete loss of enzyme activity. Similar inhibition was noted in detergent-disrupted microsomes. These data are consistent with an external location for the active site of the glycerophosphate acyltransferase.

Microsomal vesicles remain intact in the presence of inhibitors under assay conditions

The inhibition of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol biosynthetic activities by mercury-dextran suggests that the active sites of these enzymes are exposed on the cytoplasmic surface of microsomal vesicles. This conclusion would be valid only if microsomal vesicles remained intact in the presence and absence of inhibitors under assay conditions (i.e., in the presence of the glycerolipid enzyme assay components). This was established by employing mannose-6-phosphatase latency [10] as a quantitative index of microsomal integrity. In order to establish a valid index of microsomal integrity, it was necessary to define a common set of assay conditions for mannose-6-phosphatase and the glycerolipid biosynthetic activities. This necessitated compromises in the assay conditions used. The pH of the mannose-6-phosphatase assay was raised to 7.0, the pH of the assays of glycerolipid enzyme activities was changed to 7.0, and a common buffer was used (see Materials and Methods). Mannose-6-phosphatase latency was determined at the same protein concentration as that employed to determine glycerolipid syn-

TABLE I

EFFECT OF ASSAY COMPONENTS AND IMPERMEANT INHIBITORS ON MANNOSE-6-PHOSPHATASE ACTIVITY

Mannose-6-phosphatase was assayed in the presence of glycerolipid enzyme assay components and impermeant inhibitors. Other details are described in Materials and Methods. Latency is defined as (1—intact activity/disrupted activity) × 100.

Enzyme assay components added	Additions: impermeant inhibitors (μM)	Mannose-6-phosphatase activity (nmol/min per mg)		
		Intact	Disrupted	Latency
None	none pH 6.5	26	543	95
	pH 7.0	28	395	93
Glycerolphosphate acyltransferase *	none	12	253	95
	dextran-maleimide (80)	3	300	99
	mercury-dextran (140)	21	390	95
Diacylglycerol acyltransferase **	none	1	97	99
	mercury-dextran (140)	8	88	91
Diacylglycerol cholinephosphotransferase **	none	15	173	89
	mercury-dextran (140)	45	296	85
Diacylglycerol ethanolaminephosphotransferase **	none	39	327	88
	mercury-dextran (140)	66	325	80
Fatty acid : CoA ligase **	none	9	135	93
	mercury-dextran (560)	43	103	58
Phosphatidic acid phosphatase **	none	14	318	95
	mercury-dextran (280)	45	284	84

* Latency of mannose-6-phosphatase was determined at pH 6.5 (Tris-cacodylate buffer) with the assay components for glycerolphosphate acyltransferase, except NaF was omitted due to its inhibitory effect on the activity of mannose-6-phosphatase in disrupted microsomes.

** Assay pH = 7.0 Tris-cacodylate buffer.

thetic enzyme activities. The latency of mannose-6-phosphatase remained high at the higher pH (Table I) indicating that the microsomal permeability barrier remained intact (see also Ref. 30). The activity of the glycerolipid biosynthetic enzymes was maintained, albeit, at somewhat lower activity than that observed at other pH values. Mannose-6-phosphatase latency generally remained high in the presence or absence of impermeant inhibitors when glycerolipid assay components were added (Table I). The single exception, fatty acid : CoA ligase, in Table I should be noted. Apparently, the higher levels of mercury-dextran employed in these experiments caused significant disruption. These findings strongly suggest that the impermeant inhibitors are affecting the biosynthetic enzyme activities except fatty acid : CoA ligase in intact microsomal vesicles.

Since Hg^{2+} is an inhibitor of mannose-6-phosphatase [31], inhibition by mercury-dextran (Hg^{+}) might have prevented measurement of mannose-6-phosphatase latency. However, the mercury-dextran did not affect the activity of mannose-6-phosphatase (see Table I and compare disrupted activities with and without inhibitor in the presence of glycerolipid components). The decrease in mannose-6-phosphatase activities in disrupted microsomes was due to the presence of substrates for the glycerolipid enzymes.

Mercury-dextran inhibition of glycerolipid synthetic enzymes under other assay conditions

Although mercury-Dextran was a good inhibitor of the glycerolipid enzymes (see Figs. 1 and 2), the possibility remained that interaction between inhibitor and substrates occurred without a direct effect on the enzymes. In order to eliminate the possibility that the inhibitor removed substrate from the reaction, the degree of inhibition at two levels of mercury-dextran was determined at several concentrations of substrates. The results are shown in Table II. The inhibition of enzyme activities observed at either concentration of mercury-dextran remained essentially unchanged with increasing amounts of substrate. These data also agree with the data shown in Figs. 1 and 2 where different sub-

TABLE II

INHIBITION OF GLYCEROLIPID SYNTHETIC ENZYMES BY MERCURY-DEXTRAN AT VARIOUS SUBSTRATE LEVELS

Assays were performed at the indicated concentrations of substrates in the absence or presence of two concentrations of mercury-dextran. Assays were performed as described in Materials and Methods. Activity is expressed as nmol/min per mg.

Glycerolphosphate acyltransferase

Glycerol phosphate (μM)	Palmitoyl-CoA (μM)	Activity	Mercury-dextran (μM)	
			70	140
			Activity remaining(%)	
100	50	1.81	33	1
600	50	2.72	28	2
300	12.5	0.77	51	1
300	150	0.71	50	2

Diacylglycerol acyltransferase

Palmitoyl-CoA (μM)	Diolein (μM)	Activity	Mercury-dextran (μM)	
			35	70
			Activity remaining(%)	
10	150	0.44	65	50
20	150	0.86	68	42
50	150	0.61	79	52
30	50	1.17	53	34
30	200	1.71	42	29

Diacylglycerol ethanolaminephosphotransferase

CDP-ethanolamine (μM)	Diolein (μM)	Activity	Mercury-dextran (μM)	
			35	70
			Activity remaining(%)	
25	150	0.24	69	58
200	150	0.26	68	50
100	50	0.20	78	61
100	450	0.26	70	66

Fatty acid : CoA ligase

Palmitoyl-CoA (μM)	AMP * (mM)	Activity	Mercury-dextran (μM)	
			140	280
			Activity remaining(%)	
20	10	4.41	116	64
35	10	7.62	112	56
50	2.5	6.61	110	57
50	5	7.22	115	61

Diacylglycerol cholinephosphotransferase

CDP-choline (μM)	Diolein (μM)	Activity	Mercury-dextran (μM)	
			35	70
			Activity remaining (%)	
50	150	1.48	46	32
200	150	1.60	46	28
100	50	1.24	42	36
100	450	1.81	49	28

* PP_i fixed at 10 mM

strate concentrations were employed. The integrity of the microsomal vesicles remained intact under similar conditions (see Table I). It should be pointed out that up to 140 μM mercury-dextran did not inhibit all microsomal enzyme activities investigated. * Thus, the inhibition by mercury-dextran appeared to be a direct effect on the biosynthetic enzymes.

Non-latency of biosynthetic enzyme activities and non-penetration of water-soluble substrates

Investigations of latency for any membrane-bound enzyme require that the integrity of the microsomal membrane be known. Conditions were established under which the microsomal membrane remained intact and glycerolipid synthetic enzyme activities could readily be measured (Table I). Under these conditions, glycerolipid synthetic activities were not latent (Table III). The conditions are different from those reported earlier [4]. Non-latency was observed for some of these activities previously at different pH values using different buffers [4]. The non-latency of fatty acid : CoA ligase activity is reported for the first time. The non-latency of these synthetic activities indicates that the substrates have free access to the active sites of these enzymes. An external location for the active sites of these enzymes in the membrane is suggested if free permeation of substrates through the microsomal membrane does not occur.

The permeation of water-soluble substrates of the glycerolipid biosynthetic enzymes (Table IV) was investigated using calcium-aggregated microsomes and

* Mercury-dextran did not inhibit mannose-6-phosphatase, ethanol acyltransferase, acyldihydroxyacetone-phosphate oxidoreductase, lysophosphatidylcholine acyltransferase, the serine base exchange enzyme, or phosphatidic acid : CTP cytidyltransferase activities under similar conditions to those employed for Table II.

TABLE III

ACTIVITIES OF GLYCEROLIPID BIOSYNTHETIC ENZYMES IN INTACT AND DETERGENT-DISRUPTED MICROSOMES

Enzyme activities were determined at pH 7.0 with Tris-cacodylate buffer. Glycerolphosphate acyltransferase activity determined at pH 6.5 with Tris-cacodylate buffer. Other details are stated in Materials and Methods. Activities are expressed as nmol/min per mg.

Enzyme	Intact	Disrupted *
Fatty acid : CoA ligase	12.6	12.8
Glycerophosphate acyltransferase	1.5	1.5
Phosphatidic acid phosphatase	0.8	1.3
Diacylglycerol acyltransferase	2.1	1.6
Diacylglycerol cholinephosphotransferase	2.8	2.2
Diacylglycerol ethanolaminephosphotransferase	2.2	1.9

* Microsomes were disrupted with Triton X-100 or taurocholate (see Materials and Methods).

TABLE IV

PERMEATION OF SUBSTRATES OF THE GLYCEROLIPID BIOSYNTHETIC ENZYMES INTO MICROSOMAL VESICLES

The incubation mixture contained 50 mM Tris-cacodylate buffer (pH 7.0) and approx. 1 mg/ml of calcium-aggregated microsomes (final volume 1.0 ml). The microsomes were incubated for 3 min in the presence of the various solutes and then centrifuged. Values are reported as the means (±S.E.). Other details are given in Materials and Methods and in Ref. 11. Dextran-accessible volume = extramicrosomal volume. Glycerol-accessible volume = total volume.

Substrate (1 mM)	Substrate-accessible volume (μl/ml)	Dextran-accessible volume (μl/mg)	Glycerol-accessible volume (μl/mg)
ATP *	3.5 ± 0.2	3.5 ± 0.4	5.6 ± 0.3
CMP **	15.3 ± 0.7	15.3 ± 0.8	18.6 ± 0.9
CDP-choline ***	11.7 ± 0.9	11.4 ± 0.6	15.8 ± 1.3

* Differences between substrate volume and total volume are statistically highly significant ($P < 0.0005$).

** Statistically highly significant ($P < 0.005$).

*** Statistically highly significant ($P < 0.025$).

the microcentrifugation technique [11]. In these studies, the volume accessible to high molecular weight dextrans is used to measure the extraluminal volume, while the volume accessible to glycerol is used to measure the total volume, since glycerol readily permeates the membrane [11]. A comparison of volumes accessible to substrates, dextran and glycerol provides an index of whether substrates permeate the membrane [11].

The spaces occupied by ATP, CDP-choline and CMP were essentially identical to the extraluminal space, the dextran-accessible space. This suggests that these substrates do not permeate the membrane. Since these molecules are substrates of the fatty acid : CoA ligase, and the diacylglycerol choline- and ethanolamine-phosphotransferases, their impermeability of the microsomal membrane is consistent with the active sites of these enzymes being located on the external surface of microsomal vesicles. When similar studies were performed on other substrates, glycerol 3-phosphate, CoASH and CDP-ethanolamine, the

substrate-accessible volumes were greater than the dextran-accessible volumes, even when additional solutes were added in an attempt to decrease non-specific adsorption (data not shown); hence, these substrates may permeate the membrane. Proof that such permeation occurs would require the demonstration of a metabolically active lumenal pool [11].

Discussion

Two new lines of evidence are reported which strongly suggest that the active sites of the enzymes of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol synthesis are located on the cytoplasmic surface of rat liver microsomes. These are (1) inactivation by impermeant inhibitors, and (2) substrate impermeability and non-latency. Interpretation of both of these new lines of evidence is critically dependent on microsomal integrity. The latency of mannose-6-phosphatase activity generally proved useful as a quantitative index of microsomal integrity. Since inactivation by impermeant inhibitors occurred in microsomal vesicles which generally remained intact under assay conditions, critical residues of these biosynthetic enzymes are located on the cytoplasmic surface.

The inability of ATP, CDP-choline and CMP to readily permeate the microsomal membrane (Table IV) coupled with the non-latency of these biosynthetic enzymes (Table III and Ref. 4) constitutes additional evidence that the active sites of the fatty acid : CoA ligase, and the diacylglycerol ethanolamine- and cholinephosphotransferases are located on the cytoplasmic surface. Additionally, palmitoyl-CoA does not appear to cross the microsomal membrane [25]. This observation and the non-latency of fatty acid : CoA ligase, glycerophosphate acyltransferase and diacylglycerol acyltransferase (Table III) suggest a cytoplasmic location for the active sites of these enzymes. These observations suggest that transport systems like those described by Arion and coworkers [11,32] for glucose 6-phosphate and P_i (Arion, W.J., Walls, H.E., Lange, A.J. and Ballas, L.M., unpublished observations) do not exist to facilitate movement of substrates of these biosynthetic enzymes into the lumen of microsomal vesicles.

Additional evidence for the site of glycerolipid synthesis in microsomal vesicles might be obtained if a newly synthesized lipid could be localized to the cytoplasmic or lumenal surface. Recent studies of Higgins [33] employing phospholipase C to localize phosphatidylcholine synthesized in vivo and in vitro in microsomal vesicles are consistent with synthesis occurring on the cytoplasmic surface [4]. Attempts to localize phosphatidylethanolamine synthesized in vitro to the cytoplasmic surface of microsomes by trinitrobenzenesulfonate-labeling procedures as employed by Rothman and Kennedy [34] have not been successful (Coleman, R.A. and Bell, R.M., unpublished observations). Bulk and newly synthesized microsomal phosphatidylethanolamine was labeled at one rate with trinitrobenzenesulfonate, suggesting that either phosphatidylethanolamine is present exclusively on the cytoplasmic surface or that phosphatidylethanolamine moves rapidly between the cytoplasmic and lumenal surfaces. *

* While mannose-6-phosphatase activity remained highly latent under the labeling conditions used, the possibility that trinitrobenzenesulfonate permeates the microsomal membrane has not been excluded.

The rapid movement of phospholipids between the two halves of the microsomal bilayer has been implied from studies using phospholipid exchange proteins [35,36].

The two new lines of evidence reported in this paper, and the finding that most of these biosynthetic activities are inactivated by proteases in intact microsomal vesicles [4,5] provide three lines of evidence which strongly imply that phosphatidylcholine, phosphatidylethanolamine and triacylglycerol synthesis occur asymmetrically on the cytoplasmic surface of rat liver microsomal vesicles. All three approaches are in agreement and no reliable contradictory evidence exists. The cytochemical studies of Higgins [37] suggested a lumenal location for the glycerophosphate acyltransferase. These results are questionable because controls demonstrating microsomal integrity were not performed. The amounts of palmitoyl-CoA employed in these studies have been demonstrated to disrupt microsomal vesicles (cf. Ref. 25). Additionally, palmitoyl-CoA does not appear to permeate the microsomal membrane [25]. The intestinal diacylglycerol cholinephosphotransferase was suggested to be located on the lumenal surface because it was not inactivated by nagarse [38]. Data employing other proteases [4,39] suggest that this interpretation may be incorrect. More than one protease should have been employed and inactivation in disrupted vesicles should have been demonstrated [4,39].

Assuming that microsomal vesicles mirror the properties of the endoplasmic reticulum, the data indicate that the biosynthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol occurs asymmetrically on the cytoplasmic surface of the endoplasmic reticulum. Since the active sites of these enzymes face the cytoplasm, the enzymes would have ready access to fatty acids, CDP-choline and CDP-ethanolamine which are synthesized in the cytoplasm and to substrates like ATP, CoASH and glycerol phosphate. The endoplasmic reticulum is the major site of phosphatidylcholine and of phosphatidylethanolamine biosynthesis and these are the most abundant membrane phospholipids. The assembly of phospholipid bilayers in the endoplasmic reticulum membrane occurs by asymmetric assembly of phospholipids on the cytoplasmic surface. The origin of asymmetric bilayers might reflect selective transmembrane movement of phospholipids [4]. Asymmetric synthesis of glycerolipids also has important implications in terms of the biogenesis of the serum lipoproteins.

Studies of the topography of enzymes of glycerolipid metabolism within the transverse plane of microsomal vesicles are limited. Recently, Rock et al. [40] reported a highly latent dihydroxyacetone-phosphate acyltransferase activity present in rabbit harderian gland microsomes. Moonen and van de Bosch [8,41] concluded that the active center of the bovine lysophospholipase II is located on the lumenal side of the microsomal membrane. It would be interesting to know whether other enzymes of glycerolipid synthesis are located on the cytoplasmic surface of microsomes from other tissues. The data of Moonen and van de Bosch [8,41] in conjunction with the data supporting asymmetric biosynthesis of glycerolipids on the cytoplasmic surface bring up the intriguing possibility that synthetic and degradative events may occur on opposite sides of the microsomal membrane.

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