Studies of rat liver microsomal diglyceride acyltransferase and cholinephosphotransferase using microsomal-bound substrate: effects of high fructose intake

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Abstract Radiolabeled phosphatidate and diglyceride were prepared bound to rat liver microsomes. These compounds were used as substrates in studies of diglyceride acyltransferase, cholinephosphotransferase, and CTP:phosphatidic acid cytidylyltransferase. Optimum incubation conditions for these reactions in microsomes from normal male rats are described. High fructose diets were fed to rats for 11 days; this resulted in an increased rate of neutral lipid formation from *sn*-glycerol-3-phosphate by liver microsomal preparations. This was attributed, in part, to a previously reported increase in liver phosphatidate phosphatase activity. The significance of this increase is supported by the finding of a fall in microsomal phosphatidate content and a doubling in microsomal diglyceride. In addition, diglyceride acyltransferase measured with microsomal-bound diglyceride was increased twofold with no equivalent change in cholinephosphotransferase activity. Such a change should result in preferential triglyceride formation from the increased microsomal diglyceride pool. CTP:phosphatidic acid cytidylyltransferase activity was depressed by the high fructose diet. These combined alterations would lead to an accelerated hepatic triglyceride formation, a result found in vivo during high fructose feeding. The high fructose diet decreased slightly the total microsomal phospholipid content and markedly depressed phosphatidylethanolamine levels.

Previous studies have reported alterations in the pathways of hepatic glycerolipid biosynthesis in response to various environmental changes (1–7). In the case of fasting (1), partial hepatectomy (2), and high fructose intake (4, 5), a marked enhancement in microsomal phosphatidate phosphatase activity, as measured with microsomal-bound substrate, was found. The feeding of a high fat diet raises *sn*-glycerol-3-phosphate acyltransferase activity (3), and diabetic ketosis and triiodothyronine administration will increase diglyceride acyltransferase activity (8).

Recently, the rise in liver microsomal and supernatant phosphatidate phosphatase activity in rats fed high fructose diets has been correlated with an increase in microsomal diglyceride and triglyceride formation and total hepatic triglyceride synthesis as estimated in vivo and in vitro (4, 5). No corresponding increase in liver phosphatidylcholine formation was observed in these studies.

Preliminary experiments, using aqueous dispersions of substrate, did not demonstrate any change in microsomal cholinephosphotransferase or diglyceride acyltransferase activity during the high fructose feeding. This finding is surprising if we assume that a common microsomal diglyceride pool is utilized in the biosynthesis of both phosphatidylcholine and triglyceride. Although alternative explanations for the preferential formation of triglyceride from the diglyceride pool are possible, recent reports cast doubt on the physiological relevance of data obtained from experiments using aqueous dispersions of lipid substrates for determination of membrane-associated enzyme activities (1, 9–11).

Therefore, techniques have been developed to prepare microsomal-bound ^{14}C-labeled diglyceride to be used as a substrate for determination of diglyceride acyltransferase and cholinephosphotransferase. In principle, the methods resemble those reported by Johnston et al. (12) for preparation of membrane-bound [^{14}C]diglyceride in intestinal microsomes. The substrate proved satisfactory for measurements of these enzymes in liver. The effect of high fructose intake on both reactions was determined using the microsomal-bound ^{14}C-labeled diglyceride substrate. A substantial increase in diglyceride acyltransferase was observed, but there was no significant change in cholinephosphotransferase activity. Changes in microsomal neutral and phospholipid constituents were also observed during high fructose feeding. When taken together, the changes in liver enzyme activities and microsomal diglyc-

Abbreviations: TLC, thin-layer chromatography.

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eride level would favor the increased and preferential incorporation of *sn*-glycerol-3-phosphate into triglyceride, in accordance with the observed in vivo findings (4).

METHODS AND MATERIALS

Animal diets and preparation of microsomes

Male Wistar rats weighing 200–250 g were fed laboratory chow (Muracon I; Trouw & Co., Petten, Netherlands) until the onset of the study. The rats were then divided into two groups. One group continued to eat laboratory chow but was pair-fed with a group given a high fructose diet containing, per 1000 g: 665 g of fructose, 150 g of vitamin-poor casein (Koch-Light), 80 g of sucrose, 30 g of corn oil, 50 g of salt mixture (Trouw), 22 g of cellulose, and 3 g of vitamin mixture (Trouw). The two groups were fed for 11 days and then killed by exsanguination from the abdominal aorta. Weight gain in the two groups was approximately the same after the first day.

Livers were promptly removed and homogenized in 4 vol of 0.25 M sucrose containing 0.05 M Tris–HCl, pH 7.4, and 2.5 mM EDTA. The various cell fractions were sedimented as described previously (13). The final microsomal preparation was washed once in the same buffer, resuspended in 0.25 M sucrose, 0.05 M Tris–HCl, pH 7.4, and quickly frozen in dry ice–acetone before storage at −16°C. The same microsomal preparation was used for enzyme studies and quantitative lipid analyses. Microsomes used in the development of methods were prepared from nonfasting male Wistar rats (200 g) fed laboratory chow.

Preparation of microsomal-bound ^{14}C-labeled phosphatidate

Unless stated otherwise, the following incubation mixture was used in preparation of diacyl-*sn*-[1,3-^{14}C]glycerol-3-phosphate bound to microsomal particles. Approximately 2.5 mg of microsomal protein was incubated in a final volume of 1.725 ml containing 6.25 mg of fatty acid-poor bovine albumin (fraction V, Pentex Inc., Kankakee, Ill.), 0.15 mM dithiothreitol, 0.3 mM *rac*-glyceride-3-phosphate containing 0.7 μCi of *sn*-[1,3-^{14}C]glycerol-3-phosphate, 60 mM NaF, 35 mM Tris–HCl, pH 8.3, 50 μM palmitoyl CoA, and 50 μM oleoyl CoA. The mixture was incubated for 20 min at 37°C, and an additional 50 nmoles of palmitoyl CoA and 50 nmoles of oleoyl CoA were added in a volume of 0.125 ml. The incubation was continued for a further 20 min and stopped by placing the mixture in ice. The mixture was then placed over 5 ml of 0.3 M sucrose containing 0.1 mM dithiothreitol and sedimented in a 40 rotor at 80,000 *g* for 60 min in a Beckman ultracentrifuge. The pellet was washed with 0.3 M sucrose and carefully homogenized in 0.7 ml of 0.3 M sucrose containing 0.1 mM dithiothreitol. When indicated, an aliquot was removed for lipid extraction by a modification (14) of the Bligh and Dyer technique, using 0.2 M HCl instead of water. In a typical experiment, 9.3 nmoles of phosphatidate was formed per milligram of microsomal protein and contained 60,500 dpm. This incubation mixture is identified as preparation I in subsequent sections.

Preparation of microsomal-bound ^{14}C-labeled diglyceride

Microsomes, prepared containing ^{14}C-labeled phosphatidate as described above, were incubated with a fraction of rat liver cytosol precipitated by 40% NH_4SO_4 (5). This supernatant protein fraction contained phosphatidate phosphohydrolase activity. Approximately 5 mg of supernatant protein was added to 1.0–2.0 mg of microsomal protein in the presence of 0.15 M Tris–HCl, pH 8.3, and 8 mM $MgCl_2$ in a final volume of 2 ml. The mixture was incubated for 35 min at 37°C, cooled on ice, and placed over 5 ml of 0.3 M sucrose containing 0.1 mM dithiothreitol. The mixture was sedimented at 80,000 *g* for 60 min in a 40 rotor, and the pellet surface was washed twice with 0.3 M sucrose and suspended with gentle homogenization in 0.7 ml of 0.25 M sucrose containing 0.05 M Tris–HCl, pH 7.5. In a typical experiment, approximately 50% of the microsomal protein was lost during the sedimentation steps. 50–75% of the remaining radioactive phosphatidate was converted to diglyceride. A variable small amount (4–8%) was converted to triglyceride, and the remaining radioactivity was recovered as phosphatidate. This incubation system is identified as preparation II. Membrane-bound ^{3}H-labeled diglyceride was prepared from [2-^{3}H]glycerol in the same manner except for the addition of 2 units of glycerokinase (Boehringer) in the initial reaction mixture.

Assay of diglyceride acyltransferase

The isolated washed microsomal preparation (0.1–0.3 mg of protein) containing microsomal-bound ^{14}C-labeled diglyceride was incubated with 2 mM dithiothreitol, 2.5 mg of fatty acid-poor bovine albumin, 0.1 mM $MgCl_2$, 50 mM NaF, 35 nmoles of palmitoyl CoA, 35 nmoles of oleoyl CoA, and 0.05 M potassium phosphate buffer, pH 6.5, in a final volume of 0.45 ml. The mixture was incubated at 37°C for 2–4 min and the reaction was stopped by addition of 1.5 ml of methanol–chloroform 2:1. Lipids were extracted as described above (14), and radioactivity in neutral lipids was determined after separation by TLC (vide infra). The triglyceride and diglyceride were identified by comparison with known standards and scraped directly into scintillation fluid containing 3.125 g of 2,5-diphenyloxazole (PPO) and 0.1875 g of *p*-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) per liter in a mixture of toluene, Triton X-100, and water 1:0.5:0.1 (by vol). Radioactivity was determined in a Packard liquid scintilla-

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tion counter with correction for quenching when present. This assay system is identified as preparation III. Diglyceride acyltransferase was also determined using an aqueous dispersion of diglyceride as described by Young and Lynen (8).

Cholinephosphotransferase

Microsomes containing [^{14}C]diglycerides (0.4–0.6 mg of protein) were incubated in 0.1 M Tris–HCl buffer, pH 7.2, with 10 mM $MgCl_2$, 50 mM β-mercaptoethanol, and 0.5 mM CDPcholine in a final volume of 0.2 ml. The mixture was incubated at 37°C for 2–4 min and the lipids were extracted as described. Phosphatidylcholine synthesis was measured from the increase in radioactivity in the lecithin spot identified by TLC. Phosphatidylcholine synthesis from exogenous diglyceride was measured by methods previously described (15).

CTP:phosphatidic acid cytidylyltransferase

CDPdiglyceride formation from phosphatidate and CTP was measured with 40 mM $MgCl_2$ as described earlier (16).

Total glycerolipid formation

The incorporation of *sn*-[1,3-^{14}C]glycerol-3-phosphate into glycerolipids by microsomal preparations was measured in the presence of 0.015 M Tris–HCl, pH 8.3, 0.035 M ATP, 0.05 mM CoA, 0.75 mM dithiothreitol, 35 mM $MgCl_2$, 0.5 mM *sn*-glycerol-3-phosphate, 0.025 M sucrose, 0.1 μCi of *sn*-[1,3-^{14}C]glycerol-3-phosphate, 1.5 mM sodium palmitate, and 0.5 mg of fatty acid-poor albumin in a final volume of 0.4 ml. The reaction was stopped by addition of 1.5 ml of methanol–chloroform 2:1, and lipids were extracted as described above. Products were identified by TLC (13).

Identification techniques

Neutral lipids were identified after separation on standard TLC plates prepared with silica gel HR in a solvent containing petroleum ether (bp 40–60°C)–diethyl ether–formic acid 60:40:1.5. Tripalmitin, triolein, and monopalmitin used as standards were purchased from Applied Science Laboratories, Inc., State College, Pa. Diglycerides were prepared from rat liver lecithin by action of phospholipase C.

Phospholipids were identified by TLC on silica gel HR plates using solvent systems described previously (13, 14). Sodium phosphatidate was prepared from egg lecithin in the presence of phospholipase D as described by Davidson and Long (17). Lysolecithin was prepared from egg lecithin (18) and used to prepare sodium lysophosphatidate in the presence of phospholipase D (19).

Quantitative lipid analysis

Microsomal phosphatidylcholine, phosphatidylethanolamine, phosphatidate, and diglyceride were measured after separation of suitable lipid extracts by TLC (14). In some studies two-dimensional chromatography was used to provide better separations of phosphatidylethanolamine and phosphatidylcholine (20). A modification of the Bartlett determination of phosphate was used (21). Diglyceride was extracted from 6 mg of microsomal protein and separated by TLC. The diglyceride spot was scraped from the plate and subjected to methanolysis. Total diglyceride fatty acid was determined by gas–liquid chromatography using a C_{20} fatty acid standard. All lipid determinations were performed at least twice.

Materials

All reagents were reagent grade or redistilled before use. Acyl CoA derivatives were prepared and their purity assessed by described methods (13). *sn*-[1,3-^{14}C]Glycerol-3-phosphate, [2-^{3}H]glycerol, and [^{14}C]palmitoyl CoA were obtained from New England Nuclear Corp., CDP[^{14}C]choline from ICN, and CDPcholine from Sigma Chemical Co. Other materials were obtained from standard commercial sources.

RESULTS

Preparation and evaluation of membrane-bound substrate

Microsomal-bound [^{14}C]phosphatidate was prepared from *sn*-[1,3-^{14}C]glycerol-3-phosphate in the presence of various acyl CoA derivatives by methods similar to those reported earlier (5, 22). When specific [^{14}C]phosphatidates were not required, 2.5 mM ATP, 30 μM CoA, 25 mM $MgCl_2$, and 0.2 mM fatty acid could substitute for the acyl CoA substrates. Under these latter conditions, a mixed [^{14}C]phosphatidate was formed because of the presence of other fatty acids in the microsomal preparations and fatty acid-poor albumin. The incubation conditions described are not optimal for determination of the rate of phosphatidate formation because excessively high concentration of microsomal protein was necessary to obtain adequate microsomal pellets in subsequent sedimentation steps. Additional acyl CoA was added after 20 min because microsomal acyl CoA hydrolase resulted in substantial loss of this substrate during incubation. NaF was required during the synthesis of [^{14}C]phosphatidate to inhibit the microsomal phosphatidate phosphohydrolase activity that is present when microsomes are prepared by the methods described. The specific activity of phosphatidate formed could be varied over a 10-fold range by corresponding alterations in *sn*-glycerol-3-phosphate concentrations. Endogenous microsomal phosphatidate levels were determined independently, and dilution of the [^{14}C]phosphatidate in the total pool of endogenous and newly formed phosphatidate was presumed to be complete for specific activity determinations.

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TABLE 1. Incorporation of *sn*-[1,3-^{14}C]glycerol-3-phosphate into phosphatidate and lysophosphatidate in the presence of various acyl CoA substrates

Substrate	Phosphatidate	Lysophosphatidate
	cpm × 10^{-3}	*cpm × 10^{-2}*
None	1.7	9.8
Palmitoyl–oleoyl CoA	116	27.2
Oleoyl CoA	115	28.4
Palmitoyl–linoleoyl CoA	90	26.6
Palmitoyl CoA	89	22.1
Linoleoyl CoA	78	17.9

The incubation conditions are described in Methods (preparation I). The acyl CoA concentration was 90 μM in each assay, and acyl CoA mixtures contained equal quantities of each.

Several acyl CoA derivatives and mixtures were used to prepare microsomal-bound [^{14}C]phosphatidate and subsequently microsomal-bound diglyceride. The relative activities are compared in **Table 1.** Oleoyl CoA or a mixture of oleoyl CoA and palmitoyl CoA gave the highest yield of both lysophosphatidate and phosphatidate under these conditions.

Microsomal-bound phosphatidate was converted to microsomal-bound diglyceride by incubation with a rat liver supernatant fraction containing active phosphatidate phosphohydrolase. As noted in **Table 2,** approximately 80% of the available phosphatidate was converted to the corresponding diglyceride under the incubation conditions described. Approximately 50% of the initial microsomal protein used in the incubation with *sn*-[1,3-^{14}C]glycerol-3-phosphate was lost during the initial two incubations and resedimentation steps (I and II), accounting for the reduction in total lipid radioactivity recovered.

Diglyceride acyltransferase was determined in the presence of equal concentrations of palmitoyl and oleoyl CoA. The effects of various omissions from the reaction mixture are shown in **Table 3.** The microsomal-bound diglyceride used in most of these studies was formed from [^{14}C]phosphatidate, which was synthesized in the presence of both palmitoyl and oleoyl CoA. The formation of [^{14}C]triglyceride was linear over a 2–4-min interval under the incubation conditions described.

TABLE 2. Distribution of radioactive lipids formed during each incubation (I, II, III)

Preparation	PA	LPA	DG	TG	Total Lipids
			cpm		
I	94,115	755	10,602	1,673	107,145
II	6,580	1,315	35,315	665	43,875
III	7,100	1,132	28,210	5,600	42,042

A single microsomal fraction was used, and values were calculated as total radioactivity recovered in cpm in each lipid class. The initial reaction contained 2.5 mg of microsomal protein and 1.5 × 10^6 dpm of *sn*-[1,3-^{14}C]glycerol-3-phosphate. After incubation II, a total of 1.12 mg of microsomal protein was recovered, accounting for the loss of radioactive lipids. Only negligible amounts of radioactivity were recovered in monoglyceride or other phospholipids. PA, phosphatidate; LPA, lysophosphatidate; DG, diglyceride; TG, triglyceride.

TABLE 3. Effects of omissions from incubation mixture on diglyceride acyltransferase activity using microsomal-bound diglyceride as substrate

Omission	% Activity
None	100
Dithiothreitol	95
FAP albumin	35
FAP albumin, 50%	80
$MgCl_2$	100
Mcsm diglyceride	0

Details of assay conditions are described under Methods. FAP, fatty acid-poor; Mcsm, microsomal-bound.

The pH optimum for this reaction is indicated in **Fig. 1.** When Tris buffer replaced the phosphate buffer, a pH optimum of approximately 7.0 was observed. The effect of increasing acyl CoA concentrations on reaction rate is also noted in Fig. 1. The optimum albumin-bound acyl CoA concentration was approximately the same as that noted previously for *sn*-glycerol-3-phosphate acyltransferase (13). The amount of ^{14}C-labeled diglyceride formed was altered over the concentration range determined in microsomes from control and fructose-fed rats as shown in **Fig. 2.** There was no significant increase in reaction rate except at very high microsomal diglyceride levels.

The substrate preference of diglyceride acyltransferase was evaluated by preparing microsomal-bound diglyceride species from palmitoyl CoA, oleoyl CoA, and mixtures of

TABLE 4. Diglyceride species prepared from *sn*-glycerol-3-phosphate and the indicated acyl CoA substrates

Species	Palmitoyl CoA	Oleoyl CoA	Palmitoyl CoA–Oleoyl CoA	Palmitoyl CoA–Linoleoyl CoA
		% of total diglyceride		
Δ 0	84.5	17.3	12.7	11.4
Δ 1	4.4	26.5	43.5	7.9
Δ 1-1	0.4	32.2	15.0	6.1
Δ 2	10.0	12.8	19.1	62.0
Δ 1-2	0.0	10.0	8.4	2.9
Δ poly	0.4	1.0	1.2	9.6

Membrane-bound diglyceride was prepared as indicated under Methods. Microsomal lipids were extracted and the neutral lipids were separated on TLC. The diglyceride regions were scraped, eluted from the silica gel with chloroform–methanol 1:2 (v/v), and separated by argentation chromatography with chloroform–ethanol 97:3 (v/v) as the solvent system. Diglyceride standards were prepared by degradation of egg lecithin, rat liver lecithin, and synthetic dipalmitoyl, dioleoyl, and dilinoleoyl lecithins with phospholipase C. Δ 0 = disaturated; Δ 1 = monoenoic, one saturated and one monounsaturated fatty acid; Δ 1-1 = two monounsaturated fatty acids; Δ 2 = dienoic, one saturated and one diunsaturated fatty acid; Δ 1-2 = one monounsaturated and one diunsaturated fatty acid; Δ poly = species containing more than three double bonds in their total fatty acid constituents.

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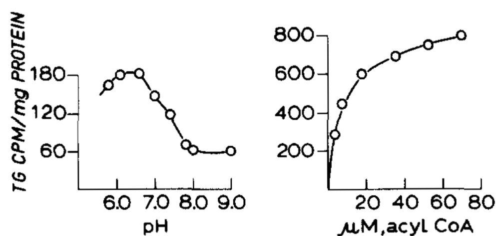

Fig. 1. Effect of pH (*left*) and acyl CoA substrate concentration (*right*) on diglyceride acyltransferase activity in rat liver microsomes. The pH curve was obtained using 0.05 M potassium phosphate buffers of the indicated pH at 30°C. The acyl CoA concentration curve was determined using palmitoyl CoA as substrate. The specific activity of the membrane-bound [^{14}C]diglyceride preparation was different in these two experiments.

palmitoyl and oleoyl CoA or palmitoyl and linoleoyl CoA. The diglyceride species formed under these incubation conditions are shown in **Table 4.** Each preparation was then incubated with each of the same acyl CoA derivatives or mixtures. Despite the marked diversity of diglyceride species, 33 to 39% of the membrane-bound diglyceride was converted to triglyceride during a 4-min period, indicating that no substantial specificity exists among the various diglyceride and acyl CoA substrates tested. These findings from experiments in which a microsomal membrane-bound diglyceride substrate was used substantiate previous observations reported from experiments in the intact animal (23, 24) and other in vitro systems (25–27).

Microsomal-bound ^{14}C-labeled diglyceride was studied as a substrate for the diglyceride acyltransferase activity present in fresh, unlabeled microsomal preparations. The bound ^{14}C-labeled diglyceride was prepared in the usual manner, and the intrinsic acyltransferase activity was then inactivated by heating the preparation at 90°C for 5 min or by letting it stand at room temperature for 24 hr. These preparations were then incubated under standard conditions with fresh microsomes known to contain active diglyceride acyltransferase activity. No formation of triglyceride could be demonstrated in several different experiments.

Synthesis of phosphatidylcholine was studied using membrane-bound [^{14}C]diglyceride samples obtained from incubation I prepared from either palmitoyl CoA, oleoyl CoA, or a mixture of palmitoyl and oleoyl CoA or palmitoyl and linoleoyl CoA. In all cases, phosphatidylcholine synthesis was linear for only 3–4 min, and no gross differences in the rate of synthesis were observed for the different molecular diglyceride species. In a 4-min period, about 20–30% of the available diglyceride was converted into phosphatidylcholine.

Dietary studies

The high fructose diet or control diet was fed to six rats for 11 days in two separate experiments. Microsomes were prepared independently from each rat, divided into several aliquots, and quickly frozen in dry içe–acetone. Microsomal-bound [^{14}C]diglyceride was prepared twice with each of the 12 microsomal fractions in both experiments.

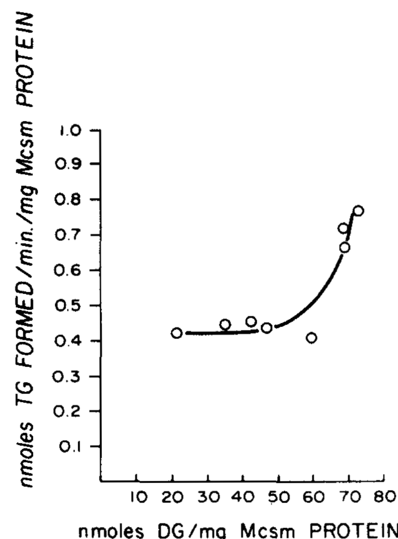

Fig. 2. Effect of increasing microsomal diglyceride content on diglyceride acyltransferase activity in microsomes from rats fed chow. The higher levels of diglyceride were achieved by prolonged initial incubations, raising the concentration of phosphatidate to 50 or more nmoles/mg of microsomal protein. Exposure of these microsomes to phosphohydrolase activity for 1 to 60 min produced the range of diglyceride levels recorded.

Total microsomal glycerolipid synthesis was measured by the incorporation of *sn*-[1,3-^{14}C]glycerol-3-phosphate into the individual lipids in the presence of palmitate, ATP, and CoA. The time course of this reaction in control experimental microsomes is shown in **Fig. 3.** Incorporation into phosphatidate was maximum at about 10 min in both groups. However, incorporation into diglyceride and triglyceride continued throughout the incubation. No incorporation into phosphatidylcholine, phosphatidylethanolamine, or CDPdiglyceride occurred in the absence of added CDP esters or CTP.

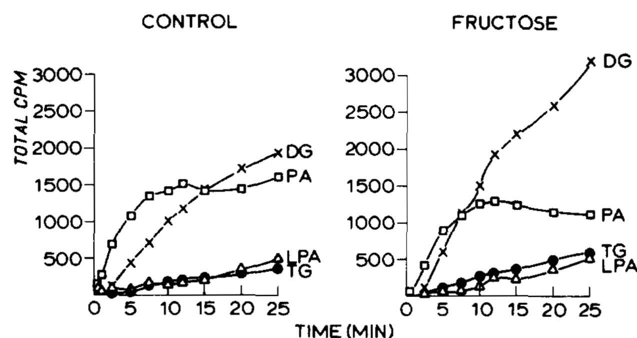

Fig. 3. Incorporation of *sn*-[1,3-^{14}C]glycerol-3-phosphate into glycerolipids by microsomal preparations in the presence of palmitate, ATP, and CoA as indicated in Methods. Microsomes were prepared from control rats (*left*) or rats fed the high fructose diet for 11 days (*right*). DG, diglyceride; PA, phosphatidate; TG, triglyceride; LPA, lysophosphatidate.

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TABLE 5. Diglyceride acyltransferase measured with membrane-bound diglyceride substrate

Diet Experiment	Microsomal Diglyceride Preparation	Diet	Diglyceride Acyltransferase	*P* Value
			nmoles triglyceride/min/mg protein ± SEM	
1	A	Control	0.43 ± 0.02	<0.001
		Fructose	0.79 ± 0.05	
	B	Control	0.44 ± 0.03	<0.01
		Fructose	0.74 ± 0.06	
	C	Control	0.56 ± 0.03	<0.001
		Fructose	1.01 ± 0.06	
2	A	Control	0.63 ± 0.04	<0.001
		Fructose	1.34 ± 0.07	

Results of four separate experiments are given. Differences in the control activity among the four experiments are attributable largely to variations in final membrane-bound diglyceride levels. In experiment 1-A, palmitoyl CoA alone was used as the acyl donor, and in experiments 1-B, 1-C, and 2-A an equal mixture of palmitoyl CoA and oleoyl CoA was used as described under Methods. Statistical differences between control and fructose values are indicated for each experiment.

In accordance with our previous findings, a greater incorporation of radioactivity into neutral lipids occurred when microsomes from rats fed fructose were used. Correspondingly, the maximum level of isotope incorporation into phosphatidate was lower, a finding that is consistent with the reported increase of phosphatidate phosphohydrolase activity in response to fructose feeding (5).

The same incubation was repeated in the presence of 0.4 mM and 2 mM CDPcholine. Incorporation of isotope into phosphatidylcholine occurred at both levels of CDPcholine. In contrast to neutral lipid formation, there was no increase in phosphatidylcholine radioactivity when microsomal preparations from rats given fructose were compared with controls.

There was no significant increase in diglyceride acyltransferase activity, as measured with an aqueous dispersion of diglyceride, when rats were fed fructose for 11 days. The values were 2.65 ± 0.62 nmoles of triglyceride/min/mg of protein in controls and 3.06 ± 0.41 in rats given fructose. However, determination of microsomal

TABLE 6. Total phosphatidate and diglyceride content of microsomes from the two diet experiments

Diet Experiment	Phosphatidate	Diglyceride
	nmoles/mg protein	
1. Control	5.2	17.5
Fructose	4.5	34.1
2. Control	4.6	22.4
Fructose	4.1	38.6

Phosphatidate recovery was monitored by addition of [^{14}C]-phosphatidate to lipid extracts before TLC separation. Details of techniques are described in Methods.

TABLE 7. Simultaneous calculation of diglyceride acyltransferase activity based on diglyceride and acyl CoA incorporation into triglyceride

Experiment	Substrate	Diglyceride Acyltransferase: Diglyceride	Diglyceride Acyltransferase: Palmitoyl CoA
		nmoles/min/mg mcsm protein	
1	[^{3}H]Diglyceride [^{14}C]Palmitoyl CoA	0.98 ± 0.05	1.08 ± 0.04
2	[^{14}C]Palmitoyl CoA		1.10 ± 0.05
3	[^{14}C]Diglyceride	0.85 ± 0.04	

The usual incubation conditions prevailed except for the use of [2-^{3}H]glycerol in the preparation of ^{3}H-labeled microsomal diglyceride in experiment 1 and ^{14}C-labeled palmitoyl CoA in determination of the reaction rate in experiments 1 and 2. The specific activity of the diglyceride was determined as described in Methods, and the usual concentration of palmitoyl CoA (0.133 mM) was used in all three experiments. The two different calculations for diglyceride acyltransferase activity were based on the specific activity of diglyceride or palmitoyl CoA as indicated. Reaction rates were similar for a dual-isotope experiment (no. 1) and for two individual experiments using the same microsomal preparation (nos. 2 and 3). Seven separate assays were conducted for each experiment and means ± SEM are recorded.

diglyceride acyltransferase using membrane-bound diglyceride showed a consistent rise in activity in the group fed fructose. The values shown in **Table 5** are the results of two separate microsomal-bound [^{14}C]diglyceride preparations from the initial (experiments 1-A, 1-B, and 1-C) and subsequent (experiment 2-A) fructose diet experiments. Calculation of the nanomoles of triglyceride formed is dependent upon the specific activity of diglyceride and required determination of the microsomal phosphatidate and diglyceride content in both control rats and those fed fructose. The results are shown in **Table 6.** There is no information to suggest a separation of microsomal diglyceride pools in liver, as has been reported for intestine (12). Therefore, it was assumed that isotope was completely mixed in the microsomal pool of these two lipids in the calculations of precursor specific activity.

To test this hypothesis, the rate of diglyceride acyltransferase was compared using ^{3}H-labeled membrane-bound diglyceride and ^{14}C-labeled palmitoyl CoA as substrates. The results for a dual-isotope experiment and two independent measures using ^{14}C-labeled palmitoyl CoA or diglyceride are shown in **Table 7.** The data show similar reaction rates when calculated from the specific activity of diglyceride, as estimated by the methods described, or from the determined specific activity of palmitoyl CoA. This finding supports the validity of the diglyceride specific activity calculations and would indicate that a single microsomal diglyceride pool exists under the conditions of these experiments.

The effect of the high fructose diet on cholinephosphotransferase activity as measured under several different incubation conditions is shown in **Table 8.** No decrease in the activity of this enzyme was observed, indicating that a

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TABLE 8. Enzymatic activities for cholinephosphotransferase and CTP:phosphatidic acid cytidylyltransferase in control and fructose-fed rats

Enzyme	Assay Method	Activity	
		Control	Fructose
		nmoles/min/mg ± SEM	
Cholinephos-photrans-ferase	Endogenous diglyceride	0.57 ± 0.04	0.69 ± 0.03
	Exogenous diglyceride	2.30 ± 0.16	2.31 ± 0.11
	Membrane-bound [14C]diglyceride	1.07 ± 0.09	1.16 ± 0.14
CTP:phos-phatidic acid cytidylyl-transferase	Endogenous phosphatidate	0.21 ± 0.01	0.16 ± 0.01
	Exogenous phosphatidate	2.08 ± 0.08	1.49 ± 0.22

All assays were conducted on microsomes prepared from diet experiment 1. Exogenous substrates were prepared by sonication (see Methods). None of the differences in cholinephosphotransferase activity was statistically significant. The fall in CTP:phosphatidic acid cytidylyltransferase in the fructose group was significant when either endogenous phosphatidate ($P < 0.02$) or exogenous phosphatidate ($P < 0.05$) was used.

TABLE 9. Total phospholipid content of microsomes from control and fructose-fed rats

Experiment	Total Phospholipid	PE	PC
	nmoles/mg protein		
1. Control	684	15.7	55
Fructose	630	11.0	56
2. Control	717	23.6	59
Fructose	660	9.5	62

The distribution of phospholipid phosphorus in PE (phosphatidylethanolamine) and PC (phosphatidylcholine) is indicated. The PE and PC were separated by both one- and two-dimensional TLC and corrected for total phospholipid phosphorus recovered from the plates. The experiment numbers refer to the two separate diet experiments.

reduced capacity for phosphatidylcholine synthesis does not contribute to the enhanced triglyceride formation of fructose feeding. In addition, this diet decreases slightly the rate of CDPdiglyceride synthesis from phosphatidate. This latter enzyme presumably competes for the same microsomal phosphatidate pool used in diglyceride formation.

The microsomal concentrations of phosphatidate, phosphatidylethanolamine, phosphatidylcholine, and diglyceride are shown for control and fructose-fed rats in Table 6 and **Table 9.** The microsomes from rats fed fructose had higher levels of diglyceride but lower concentrations of phosphatidate, phosphatidylcholine, and, especially, phosphatidylethanolamine. The total phospholipid-to-protein ratio was consistently lower in the microsomes of fructose-fed rats despite various attempts to remove attached or intracisternal protein by sonication or by washing microsomal pellets in 10 mM Tris–HCl, pH 8.6, prior to final sedimentation. Although both of these procedures increased the phospholipid–protein ratio by 15–20%, the difference between the fructose and control preparations remained.

DISCUSSION

These studies demonstrate the feasibility of preparing various species of ^{14}C-labeled microsomal-bound diglycerides by direct biosynthesis from [^{14}C]phosphatidate. The microsomal preparations remain active in the formation of triglyceride and phosphatidylcholine under proper incubation conditions. However, microsomal-bound [^{14}C]diglyceride apparently is not a substrate for these reactions unless it is formed by the microsomal preparations to be used in the assay. A microsomal fragment is unable to utilize membrane-bound diglyceride contained in another discrete particle with no enzymatic activity. This observation indicates the probable importance of substrate and enzyme arrangement within the microsomal membrane. They also cast some doubt on the physiological relevance of previous data on the activity and specificity of these reactions when aqueous dispersions of substrates are used. A similar conclusion has been reached in regard to phosphatidate phosphohydrolase (5, 9, 22).

Diglyceride acyltransferase was found to have a pH optimum of 6.5 in phosphate buffer and was more active when the acyl CoA substrates were added bound to fatty acid-poor albumin. At very high microsomal diglyceride concentrations an increase in the calculated reaction rate occurred. However, at the physiological levels as recorded in these experiments, the rate of diglyceride acyltransferase appeared to be independent of diglyceride concentration. No preference for various microsomal diglyceride species or acyl CoA derivatives could be demonstrated, confirming data obtained previously by several different in vivo and in vitro techniques (23, 25, 26).

Membrane-bound diglyceride was also a suitable substrate for measurement of cholinephosphotransferase. The reaction rate was somewhat lower than values obtained with aqueous dispersions of diglyceride prepared by sonication.

The increased formation of neutral lipids from *sn*-glycerol-3-phosphate by liver microsomal preparations from rats fed high fructose diets confirms previous results (4, 5). The lower phosphatidate and greater diglyceride accumulation in these particles also is compatible with the observation that microsomal phosphatidate phosphohydrolase activity is increased under these conditions. However, the previous results did not explain the observed increase in triglyceride formation in comparison with phosphatidylcholine as measured both in vitro (4, 5) and in vivo (4). Various possible explanations for this latter observation would include (*1*) a reduction in cholinephosphotrans-

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ferase activity, resulting in preferential synthesis of triglyceride; (*2*) higher diglyceride acyltransferase activity; (*3*) differences in the substrate kinetics of the two enzymes; or (*4*) compartmentalization of the diglyceride pool. The present work attempts to resolve several of these possibilities by measurement of both enzyme activities using membrane-bound diglyceride as substrate.

The results indicate that diglyceride acyltransferase activity, as measured with membrane-bound diglyceride, is increased in microsomes of rats fed fructose and that there is no fall in cholinephosphotransferase levels. However, the rise in diglyceride acyltransferase is not observed when an aqueous dispersion of diglyceride is used as substrate, indicating the probable importance of using more physiological substrate conditions in comparative studies of these membrane-bound enzymes.

Apparently, the rise in microsomal diglyceride acyltransferase activity does not fully compensate for the accelerated rate of diglyceride formation, as indicated by the twofold rise in microsomal diglyceride content in rats fed fructose. This result conforms to previous findings of a quantitatively greater effect of high fructose feeding on the activity of microsomal and supernatant phosphohydrolase (5).

The mechanism for the increased diglyceride acyltransferase activity cannot be resolved with assurance at present. The increase in microsomal diglyceride content does not account for the greater activity, as shown by the substrate concentration curve. Other explanations such as activation of diglyceride acyltransferase activity by structural changes related to the microsomal lipid alterations, removal of substances that inhibit diglyceride acylation by microsomal preparations, or a direct increase in the diglyceride acyltransferase content of the microsomes may account for the increased activity. Regardless of mechanism, increased diglyceride acylation should lead to an acceleration of triglyceride formation from *sn*-glycerol-3-phosphate without equivalent changes in phosphatidylcholine formation. Precisely, this effect has been observed in studies conducted in vivo and in liver homogenate preparations (4). The various changes in microsomal glycerolipid biosynthesis, as measured in vitro, may account for the many observations in man and experimental animals indicating that diets of high sugar content accelerate the hepatic formation and release of triglyceride into the blood (3, 4).

The values for phospholipid content of control microsomes are similar to those reported previously (28, 29). The effect of fructose on microsomal phospholipid content is most marked in the phosphatidylethanolamine fraction. Park, Marai, and Mookerjea (30) have reported a similar marked decrease in phosphatidylethanolamine content and a lesser decrease in phosphatidylcholine in both the rough and the smooth endoplasmic reticulum when fasted rats are fed a high sucrose diet for 48 hr. They have correlated this change with a profound fall in the linoleic and arachidonic acid content of the liver phospholipids. The present results suggest that such alterations may occur with fructose feeding as well, are persistent for at least 11 days, and do not require prior fasting. The implications of these variations in microsomal membrane lipids for the various metabolic functions of the organelle remain to be investigated.

APPENDIX

Calculation of specific activities

nmoles PA formed = dpm PA/sp act GP

sp act PA = dpm PA/nmoles PA (formed + microsomal pool)

nmoles DG formed = dpm DG/sp act PA

sp act DG = dpm DG/nmoles DG (Formed + microsomal pool)

nmoles TG formed = dpm TG/sp act DG

Activity DG acyltransferase = nmoles TG formed/min/mg microsomal protein

Abbreviations: PA, phosphatidate; GP, *sn*-glycerol-3-phosphate; DG, diglyceride; TG, triglyceride.

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