Effect of carbon tetrachloride administration on the synthesis of triglycerides and phospholipids in rat liver

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ABSTRACT In vivo incorporation of choline-methyl-^{14}C into liver lecithin and its biosynthetic precursors was studied in CCl_4-treated rats. Radioactivity in cytidine diphosphoryl (CDP-)choline and lecithin was reduced to one-third of control levels, whereas that of phosphorylcholine was increased to 4.7 times control levels. Incorporation of phosphorylcholine-^{32}P into lecithin by homogenates prepared from livers of CCl_4-treated animals was reduced, but conversion of CDP-choline-^{32}P to lecithin by the isolated microsomal fraction did not show any significant depression. A block in the synthesis of CDP-choline is indicated. The in vivo utilization of methionine for lecithin synthesis was not affected.

After intravenous injection of palmitic acid-1-^{14}C, radioactivity of triglycerides from microsomal and mitochondrial fractions was markedly lower than the controls, whereas radioactivity of triglycerides in the soluble fraction was greatly increased. Radioactivity of diglycerides changed from 0.5% of total lipids in the control to 10% of total lipids in CCl_4-treated animals. Incorporation of palmitic acid into phospholipids was also suppressed.

The results demonstrate that synthesis of both phospholipids and triglycerides is inhibited in rats 4–5 hr after CCl_4 administration.

SUPPLEMENTARY KEY WORDS reduced incorporation · choline-methyl-^{14}C · phosphorylcholine-^{32}P · CDP-choline · lecithin · palmitic acid-1-^{14}C · microsomes · diglyceride

ELECTRON-MICROSCOPIC investigations (1, 2) have disclosed that the primary site of cellular injury after CCl_4 intoxication is in the rough endoplasmic reticulum. Decreased incorporation of amino acids into liver and plasma proteins has been correlated with changes in the ribosomes (1). However, it is questioned that a reduction in the secretion of triglycerides (TG) from hepatic cells is solely the result of an inhibition in the synthesis of the protein moieties of very low density lipoprotein (3), and whether the disturbance of protein synthesis is the ultimate cause of cell death is still controversial (3, 4).

Microsomes are the site not only of the synthesis of protein, but also of the final step in the synthesis of lecithin—one of the main phospholipids (PL) of the liver—and also of the final step in triglyceride synthesis (5). Thus, injury of the endoplasmic reticulum is quite likely to influence the metabolism of these lipids.

It has been shown (6) that combination of PL with apoprotein is the primary step in the formation of high density lipoprotein and the neutral lipids are assumed to be bound to the phospholipid–protein matrix later (7). If this is also the case in low and very low density lipoprotein, and if synthesis of PL is impaired in CCl_4-poisoned liver, the accumulation of TG in the liver will partly be accounted for: newly synthesized lipids will fail to conjugate with protein (3).

It is now established that membranes of subcellular organelles are constructed of lipids and proteins (8). The prominent role of PL in maintaining mitochondrial function is known (9). These facts, together with the reported relatively short half-life of PL of hepatic microsomes and mitochondria (10, 11), suggest that the membrane systems of subcellular organelles are vulnerable to impairment of lipid synthesis.

Abbreviations: CTP, CDP, CMP–cytidine tri-, di-, and monophosphates, respectively; PL, phospholipids; TG, triglycerides; DG, diglycerides; FFA, free fatty acids.

Since TG and phosphoglycerides, the latter constituting more than 90% of hepatic PL (12), have diglycerides (DG) as a common precursor, and since the liver is an active site for production of both of these lipid classes, disturbed metabolic relationships between these lipids would be expected in pathological conditions.

In this paper we report marked alterations in synthesis of the lipid components of hepatic cells during the middle (4–5 hr) period of acute CCl_4 poisoning.

MATERIALS AND METHODS

Animals

Wistar rats of both sexes weighing 230–300 g were used. In the experiments in which incorporation of choline-methyl-^{14}C and L-methionine-methyl-^{14}C was studied, male animals were used. They were fed a standard rat chow (Nihon Clea Co., Tokyo, Japan). Food was removed 20 hr before the animals were killed.

Radioactive Materials

Palmitic acid-1-^{14}C (20 mc/mmole), phosphoric acid-^{32}P, and L-methionine-methyl-^{14}C (6.1 mc/mmole) were purchased from the Dai-ichi Chemical Co., Tokyo, Japan. Choline-methyl-^{14}C (51.8 mc/mmole) was obtained from The Radiochemical Centre, Amersham, England.

Palmitoyl-^{14}C CoA was synthesized according to the method of Vignais and Zabin (13) after palmitic acid-1-^{14}C had been converted to the anhydride by heating with acetic anhydride to 150°C. Phosphorylcholine-^{32}P was prepared by heating phosphoric acid-^{32}P and choline chloride (14). Cytidine diphosphoryl choline (CDP-choline)-^{32}P was synthesized from phosphorylcholine-^{32}P and CMP (15). The concentration of labeled CDP-choline used for the experiment was determined by absorption at 280 nm.

Incorporation of Choline-Methyl-^{14}C

CCl_4 (0.25 ml/100 g of body wt) was fed orally 1 or 4 hr prior to the isotope injection. 5 μc/100 g of body wt of choline-methyl-^{14}C was injected into the control and into the poisoned rats via the femoral vein under ether anesthesia.

After 1 hr animals were exsanguinated and livers were removed and washed with cold 0.15 M NaCl solution.

Incorporation of L-Methionine-Methyl-^{14}C

1 hr after injection of 5 μc/100 g of body wt via the femoral vein, livers were removed and lipids were extracted with chloroform–methanol 2:1.

Incorporation of Palmitic Acid-1-^{14}C

Palmitic acid-1-^{14}C was converted to its sodium salt and complexed with crystalline bovine serum albumin (Armour). 5 μc/100 g of body wt of the isotope was injected via the femoral vein. After 20 min the animals were bled from the abdominal aorta. The livers were irrigated with 0.15 M NaCl solution through the portal vein, then removed and chilled in ice. Livers were homogenized in 4 volumes of 0.25 M sucrose solution with the glass–Teflon homogenizer at 0°C. The homogenates were centrifuged at 800 *g* for 10 min to sediment nuclei and cell debris. The supernates were centrifuged at 13,000 *g* for 15 min. The precipitates of the second centrifugation were designated "mitochondrial fraction." The second supernates were centrifuged again at 105,000 *g* for 120 min to give "soluble fraction" and "microsomal fraction" (16). Lipid particles that adhered to the wall of the centrifuge tubes were scraped off with a glass rod and mixed with the supernate. The sediments after each centrifugation were not washed. The temperature was kept below 5°C throughout the procedure.

Extraction of Lipids and Lipid Analysis

Livers or liver cell fractions were extracted with chloroform–methanol 2:1 (17). Lipid extracts were placed on silicic acid columns and eluted with *n*-hexane, *n*-hexane–ethyl ether 9:1, chloroform, chloroform–methanol 1:1, and methanol, successively. Cholesteryl esters, TG, free fatty acids (FFA), cholesterol, and DG were eluted with the first three solvents, and if necessary they were separated by TLC on Silica Gel H in *n*-hexane–ethyl acetic acid 75:23:2. Cholesterol was not separated from DG. The PL, being eluted from the column with the last two solvents, were further separated by thin-layer chromatography on Silica Gel H in chloroform–methanol–water 65:25:4. Phosphatidyl ethanolamine and lecithin gave distinct spots, but other PL were difficult to identify precisely. TG (18), FFA (19), and phospholipid phosphorus (20) were determined colorimetrically, after they had been separated by silicic acid column or thin-layer chromatography as described above.

Analysis of Biosynthetic Precursors of Lecithin

PL and related compounds were extracted by a slight modification of the method used by Bjørnstad and Bremer (21). Livers were homogenized with 67% ethanol and the homogenates were centrifuged. The precipitates were extracted once with 67% ethanol and once with chloroform–methanol 3:1. The two ethanol extracts were combined in a separatory funnel, and two layers were obtained by the addition of chloroform and water. The lower phase was mixed with the chloroform–methanol extract of the precipitate. This mixture was washed with water, evaporated under nitrogen, and

further analyzed on column and thin-layer chromatography for PL. The water-soluble extracts, corresponding to 2 g of liver, were put on columns of Dowex-2 (formate form) and eluted by gradient chromatography with water–0.04 N formic acid. Nonradioactive CDP-choline was added to the extracts before chromatography as indicator for absorption at 280 nm. Fractions of 5 ml each were collected. Optical density at 280 nm was recorded continuously while the effluent passed through a UV detector. An aliquot of each fraction was plated and dried, and its radioactivity was determined. Radioactivity peaks appeared in the first water washing of the column, and in tubes 11–15 and 20–24. The last peak, which was very small compared to the first two, coincided with that of absorption at 280 nm, and was considered to be attributable to CDP-choline.

Determination of Fatty Acyl CoA

Long-chain acyl CoA was extracted by a modification of the method of Tubbs and Garland (22). To 4 ml of 13,000 *g*-15 min supernatant fraction of the liver homogenate, 12 ml of cold $HClO_4$ (8% w/v) was added and centrifuged at 5°C. The precipitate was washed with cold $HClO_4$ (5% w/v), dissolved in 0.4 N KOH containing 10 mM mercaptoethanol, and kept at 20°C for 20 min. It was then acidified with 1.2 N HCl, chilled in ice, and centrifuged at 0°C. The supernate was neutralized with Tris buffer to pH 7.0. To 0.2 ml of the extract, 0.15 μmole of cytochrome *c*, 15 μmoles of phenazine methosulfate, 0.2 ml of acyl CoA dehydrogenase prepared from beef liver (22), and 0.2 mmole of phosphate buffer of pH 7.0 were added. A blank without acyl CoA was also taken through the operation, and the reaction was followed at 366 nm (at 24°C) until the difference in extinction between the experimental and control samples was constant. The concentration of acyl CoA was calculated from $\Delta E_{366\ nm}$ (oxidized–reduced) for cytochrome *c*, which is 14.4 $cm^{-1}\ \mu M^{-1}$.

Design of Experiments In Vitro

Rats were fed CCl_4 4 hr before sacrifice. The livers were removed, washed in ice-cold saline solution, and homogenized in a glass–Teflon homogenizer in 0.25 M sucrose solution. The homogenates were fractionated by centrifugation as described above. The 13,000 *g* supernates were either used for experiments as "microsomes plus soluble fraction" or centrifuged again at 105,000 *g* for 120 min to obtain "microsomal fraction."

sn-1,2-Diacylglycerols were prepared from egg lecithin by treatment of the lecithin with phospholipase C (Sigma Chemical Co.) (23). They were purified by chromatography on silicic acid.

Radioactivity Measurement

Radioactivities of ^{14}C-labeled lipids were determined in a Packard Tri-Carb liquid scintillation spectrometer. The lipid spots on thin-layer chromatograms were scraped into counting vials and mixed with 1 ml of methanol for PL and 1 ml of *n*-hexane for other nonpolar lipids. The counting efficiency was checked by an internal standardization method. Radioactivities of ^{14}C-labeled choline, phosphorylcholine, and CDP-choline and of ^{32}P-labeled compounds were determined in a proportional gas-flow counter (type SC5, The Nihon Musen Co.).

RESULTS

Incorporation of Choline-Methyl-^{14}C Into Phospholipids and Phospholipid Precursors in the Liver In Vitro

1 hr after injection of choline-methyl-^{14}C, about 60% of the administered radioactivity was recovered in the livers of both control and CCl_4-treated (4 hr) rats; less than 0.15% was recovered in the lipids of blood plasma in a normal rat. The radioactivity in liver PL and specific activity of liver lecithin of the CCl_4-treated rats were 32 and 37% of the control, respectively (Table 1). CDP-choline, the direct precursor of lecithin, had very low radioactivity (0.16% of total counts in the livers of the control group). This fraction, too, showed a decrease to one-third of the control level.

In contrast to the other fractions, the radioactivity in phosphorylcholine was more than four times as high in CCl_4-treated (4 hr) livers as in controls, indicating in-

TABLE 1 INCORPORATION OF CHOLINE-METHYL-^{14}C INTO PHOSPHORYLCHOLINE, CDP-CHOLINE, AND PHOSPHOLIPIDS OF LIVER IN VIVO

Rats	Choline	Phosphorylcholine	CDP-Choline	Phospholipids	Specific Activity of Lecithin
		cpm/mg protein			*cpm/mg*
Control	3,850 ± 230	500 ± 155	12.9 ± 2.3	3,330 ± 420	26,100 ± 3,270
CCl_4-treated (4 hr)	3,330 ± 700	2,420 ± 890	4.4 ± 0.9	1,070 ± 67	9,560 ± 1,950
P	NS	<0.001	<0.001	<0.001	<0.001

5 μc/100 g of choline-methyl-^{14}C was injected (i.v.) 1 hr before the rats were killed. Means ± SD (n = 5) are given.

hibition at the step in which CDP-choline is synthesized. About half of the radioactivity of the liver was in free choline.

There was no significant difference in the specific activity of lecithin between the control and CCl_4-treated (1 hr) rats (23,100 ± 5,170 cpm/mg, n = 4).

Incorporation of CDP-Choline-^{32}P Into Phospholipids In Vitro

CDP-choline:1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2), which is found mainly in the microsomal fraction, catalyzes reaction I.

$$\text{CDP-choline} + \text{1,2-diglyceride} \rightleftharpoons \text{lecithin} + \text{CMP} \quad \text{(I)}$$

For the conditions described in Table 2, more than half the phosphorylcholine-^{32}P of CDP-choline was transferred to DG. The activity of this enzyme was unchanged by CCl_4 administration (Table 2).

Incorporation of Phosphorylcholine-^{32}P Into Lecithin In Vitro

CDP-choline synthesis occurs according to reaction II.

$$\text{phosphorylcholine} + \text{CTP} \rightarrow \text{CDP-choline} + \text{PPi} \quad \text{(II)}$$

(CTP:cholinephosphate cytidyltransferase) (EC 2.7.7.15)

The system we used involved both reaction II and reaction I. At first we tried to measure the activity of reaction II above, isolating CDP-choline from a deproteinized supernate of the reaction mixtures either by adsorption on Norit A or by column chromatography on Dowex-2. However, the incorporation of ^{32}P into CDP-choline was very low in comparison to the incorporation of ^{32}P into lecithin in the presence of sufficient diglyceride. Hence, we decided to measure the incorporation of phosphorylcholine into lecithin. The difficulty of making a quantitative assay of reaction II was also discussed by Wilgram and Kennedy (5).

The incorporation of ^{32}P into lecithin by the homogenate of CCl_4-treated rat liver was only 62% of the control (Table 2). Since reaction I proceeds equally well in the control and CCl_4-treated groups (Table 2), the decrease in the incorporation was considered to be due to the suppression of reaction II.

Incorporation of L-Methionine-Methyl-^{14}C Into Lecithin In Vitro

$$\text{3 S-adenosylmethionine} + \text{phosphatidyl ethanolamine} \rightarrow \text{lecithin} + \text{3 adenosylhomocysteine} \quad \text{(III)}$$

Although the quantitative significance of reaction III has not been established, a pathway for the formation of lecithin via methylation of phosphatidyl ethanolamine is well known. The reaction was reported to be quite active in the liver (24).

TABLE 2 INCORPORATION OF PHOSPHORYLCHOLINE-^{32}P INTO LECITHIN BY LIVER HOMOGENATE (A), AND TRANSFER OF RADIOACTIVITY OF CDP-CHOLINE-^{32}P INTO LECITHIN BY MICROSOMES PLUS SOLUBLE FRACTION (B)

Rats	A Phosphorylcholine-^{32}P	B CDP-Choline-^{32}P
	cpm/mg protein of incubation medium	
Control	10,800 ± 410*	1,110 ± 95*
CCl_4 (4 hr)	6,730 ± 980*	1,280 ± 94*
P	<0.01	NS

A: Each tube contained phosphorylcholine-^{32}P (1.04×10^6 cpm), 1 μmole of *sn*-1,2-diacylglycerols, 10 μmoles of $MgCl_2$, 10 μmoles of freshly neutralized cysteine, 0.5 μmole of CTP, 5 μmoles of ATP, 100 μmoles of Tris buffer of pH 7.4, 0.02 mg of Tween 20, and 0.2 ml of 800 *g*–10-min supernate of 20% liver homogenate in a final volume of 0.8 ml. The tubes were incubated at 37°C for 1 hr.

B: Each tube contained 0.6 μmole of CDP-choline-^{32}P (10,000 cpm), 1 μmole of *sn*-1,2-diacylglycerols, 10 μmoles of $MgCl_2$, 10 μmoles of freshly neutralized cysteine, 50 μmoles of Tris buffer of pH 7.4, 0.02 mg of Tween 20, and 0.2-ml of 13,000 *g*–15-min supernate of 20% liver homogenate (microsomes plus soluble fraction) in a final volume of 1.3 ml. The tubes were incubated at 37°C for 1 hr.

* Means ± SD (n = 4).

The specific activity of lecithin after injection of L-methionine-methyl-^{14}C showed no significant difference between the control and CCl_4-treated (4 hr) groups (Table 3).

Incorporation of Palmitic Acid-1-^{14}C Into Lipids of the Liver In Vivo

20 min after palmitic acid-1-^{14}C injection to normal rats, 44 and 53% of radioactivity of the liver lipids were found in TG and PL, respectively. When CCl_4 was fed 1 hr prior to the isotope injection, the radioactivity of TG in liver homogenate fell to 64% of the control, whereas that of PL did not change significantly (Table 4). 4 hr after CCl_4 feeding, the figures were quite different. The radioactivity in TG of the total liver homogenate was almost the same as in the control, but that in PL dropped to 65% of the control. Examination of the lipids of each cellular fraction at this period revealed (Table 4) marked suppression of the incorporation into

TABLE 3 INCORPORATION OF L-METHIONINE-METHYL-^{14}C INTO PHOSPHOLIPIDS OF LIVER IV VIVO

Rats	Phospholipids	Specific Activity of Lecithin
	cpm/mg protein	*cpm/mg*
Control	2,010 ± 250*	17,600 ± 530
CCl_4 (4 hr)	2,200 ± 270*	16,350 ± 640
P	NS	NS

5 μc/100 g of L-methionine-methyl-^{14}C was injected (i.v.) 1 hr before the rats were killed.

* Means ± SD (n = 4).

TABLE 4 INCORPORATION OF PALMITIC ACID-1-^{14}C INTO LIPIDS OF LIVER AND PLASMA IN VIVO

Lipids	Rats	Microsomes	Mitochondria	Soluble Fraction	Homogenate	Plasma	Specific Activity in Microsomes	Specific Activity in Mitochondria
		cpm/g liver				*cpm/ml*	*cpm/mg*	
TG	Control	273,000	121,000	337,000	853,000	8,100	67,800	30,600
	SD	±45,600	±48,000	±71,900	±90,400	±1,500	±17,300	±3,700
	CCl_4 (1 hr)	—	—	—	549,000	310	—	—
	SD				±109,000	±180		
	P				<0.01	*		
	CCl_4 (4 hr)	101,900	35,700	428,000	722,000	290	25,200	19,200
	SD	±28,000	±7,300	±4,400	±76,100	±160	±7,100	±4,600
	P	<0.001	<0.01	<0.05	NS	*	<0.005	<0.005
PL	Control	396,000	160,000	27,900	703,000	—	25,000	15,500
	SD	±65,800	±25,200	±7,500	±105,000		±5,000	±3,500
	CCl_4 (1 hr)	—	—	—	605,300	—	—	—
	SD				±76,000			
	P				NS			
	CCl_4 (4 hr)	262,000	76,500	39,700	460,000	—	18,800	10,100
	SD	±29.100	±11,700	±18,200	±54,700		±1,100	±700
	P	<0.01	<0.001	NS	<0.01		<0.05	<0.025
DG	Control	4,600	1,000	1,300	8,200	—	—	—
	SD	±2,600	±620	±870	±730			
	CCl_4 (1 hr)	—	—	—	222,000	—	—	—
	SD				±91,000			
	P				*			
	CCl_4 (4 hr)	53,400	15,500	53,000	144,000	—	—	—
	SD	±22,700	±6,000	±33,000	±61,000			
	P	*	*	*	*			
FFA	Control	22,300	16,100	14,700	60,700	19,500	—	—
	SD	±3,900	±8,700	±5,600	±18,400	±6,100		
	CCl_4 (1 hr)	—	—	—	139,000	17,800	—	—
	SD				±15,300	±4,600		
	P				<0.001	NS		
	CCl_4 (4 hr)	40,500	25,800	27,600	105,000	19,100	—	—
	SD	±12,800	±5,600	±10,000	±18,500	±4,700		
	P	<0.05	NS	NS	<0.01	NS		

5 μc/100 g of palmitic acid-1-^{14}C–bovine albumin complex was injected (i.v.) 20 min before the rats were killed. Five rats were used for each group. Mean liver weights were 6.31 ± 0.75 g for the control, 6.0 ± 0.74 g for CCl_4 (1 hr), and 6.5 ± 0.28 g for CCl_4 (4 hr) rats.

* The difference of the variances of two groups was too great to make Student's "*t*" test.

microsomal and mitochondrial TG. The accumulation of radioactivity in TG of the soluble fraction counterbalanced its reduction in the other two fractions.

The labeling of plasma TG fell drastically in both of the CCl_4-treated groups (Table 4, column 7).

The radioactivities in PL of microsomes and mitochondria, as calculated per 1 g of liver, were strikingly low in CCl_4-treated (4 hr) rats. But the changes in their specific activities were less statistically significant, because of the slightly lower PL contents in both fractions. No difference was observed in the distribution of radioactive palmitic acid among the major PL fractions between these two groups (Table 5).

DG, being a common precursor of both TG and PL biosynthesis, had a very low radioactivity in normal control rats (Table 4). After the feeding of CCl_4, radioactivity in the DG fraction increased to the extent that it contributed 15 and 10% of the total lipid radioactivity in the CCl_4 (1 hr) and CCl_4 (4 hr) groups, respectively.

The intrahepatic accumulation of labeled free fatty acid was also noticeable. Since plasma FFA levels remained unchanged until 5 hr after CCl_4 administration [610 ± 122 μM, n = 7 in the control, and 640 ± 160 μM, n = 8 in CCl_4 (5 hr)], the labeled fatty acid was considered to be diluted equally in both groups before it was trapped by the liver.

Incorporation of Palmitoyl-^{14}C CoA and Palmitic Acid-1-^{14}C Into Triglyceride In Vitro

fatty acid + ATP + CoA → acyl CoA + AMP + PPi (IV)

[acid:CoA ligase (AMP)](EC 6.2.1.3)

acyl CoA + D-1,2-diglyceride → triglyceride + CoA (V)

(acyl CoA:1,2-diglyceride *O*-acyltransferase)

(EC 2.3.1.20)

TABLE 5 DISTRIBUTION OF LABELED PALMITIC ACID AMONG THE PHOSPHOLIPIDS OF MICROSOMES AND MITOCHONDRIA

Rats	Microsomes		Mitochondria	
	Phosphatidyl Ethanolamine	Lecithin	Phosphatidyl Ethanolamine	Lecithin
	% of total phospholipids			
Control	27 ± 2	65 ± 2	27 ± 2	62 ± 2
CCl_4 (4 hr)	28 ± 2	60 ± 2	26 ± 2	62 ± 2

Experimental conditions were the same as in Table 4. The subfractionation of phospholipids was made by TLC. Means ± SD (n = 5).

To undergo esterification or oxidation, fatty acids must be activated through reaction IV. The acyl CoA thus formed combines with glycerophosphate to form diglyceride and then triglyceride (reaction V).

As the results obtained in living animals showed the striking increase in the incorporation of labeled fatty acids into the DG fraction, we examined whether reaction V was suppressed by CCl_4. The experiments, in which microsomes plus soluble fraction of the liver were used, revealed that the incorporation of both palmitoyl-^{14}C CoA and palmitic acid-1-^{14}C were greater in the preparation from CCl_4-treated (4 hr) rats (Table 6).

To estimate the dilution effect of labeled substrates, the concentrations of acyl CoA and FFA in the cell fractions used for the above experiments were measured. The concentration of fatty acyl CoA in microsomes plus soluble fraction was 92 ± 3.7 mμmoles (n = 4) in the control and 76 ± 6.6 mμmoles (n = 4) per gram of liver in CCl_4-treated rats (4 hr). This difference is too small

TABLE 6 INCORPORATION OF PALMITIC ACID-1-^{14}C AND PALMITOYL-^{14}C CoA INTO TRIGLYCERIDES BY MICROSOMES PLUS SOLUBLE FRACTION

Rats	A Palmitic acid-1-^{14}C	B Palmitoyl-^{14}C CoA
	cpm/mg protein of incubation medium	
Control	10,430 ± 1,260*	340 ± 71†
CCl_4 (4 hr)	21,600 ± 2,970*	862 ± 150†
P	<0.001	<0.001

A: Each tube contained 0.1 μc (0.005 μmole) of palmitic acid-1-^{14}C, 1 μmole of *sn*-1,2-diacylglycerols, 0.1 μmole of CoA, 10 μmoles of ATP, 10 μmoles of $MgCl_2$, 25 μmoles of NaF, 5 μmoles of freshly neutralized cysteine, 400 μmoles of Tris buffer of pH 7.0, 0.05 mg of Tween 20, and 0.2 ml of 13,000 *g*–15-min supernate (microsomes plus soluble fraction) of 20% liver homogenate in a final volume of 2.0 ml. The tubes were incubated at 37°C for 30 min.

B: Each tube contained 3 μmoles of palmitoyl-^{14}C CoA (53,000 cpm), 2 μmoles of *sn*-1,2-diacylglycerols, 8 μmoles of freshly neutralized cysteine, 50 μmoles of Tris buffer of pH 7.4, 0.02 mg of Tween 20, and 0.2 ml of 13,000 *g*–15-min supernate in a final volume of 1.0 ml. The tubes were incubated at 37°C for 30 min.

* Means ± SD (n = 4).

† Means ± SD (n = 5).

TABLE 7 EFFECT OF CONCENTRATION OF ADDED ATP ON FATTY ACID ESTERIFICATION BY MICROSOMES PLUS SOLUBLE FRACTION OF NORMAL RAT LIVER

	ATP (mM)			
	0	0.66	1.3	2.6
	% of total radioactivity of lipids			
Triglycerides	0.31	2.0	2.7	2.3
Diglycerides	0.18	2.1	2.9	3.8
Phospholipids	1.2	48.2	73.8	79.2
Lecithin + phosphatidyl ethanolamine		19.4	19.8	27.7
Unidentified fraction		24.1	47.2	47.5
Cholesteryl esters	0.05	1.5	2.0	3.1
Free fatty acids	98.22	46.2	18.6	11.6

Each tube contained 0.05 μc (0.0025 μmole) of palmitic acid-1-^{14}C, 10 μmoles of glycerol 3-phosphate, 0.1 μmole of CoA, 10 μmoles of $MgCl_2$, 25 μmoles of NaF, 5 μmoles of freshly neutralized cysteine, 400 μmoles of Tris buffer of pH 7.0, and 0.2 ml of 13,000 *g*–15-min supernate (microsomes plus soluble fraction) of 20% liver homogenate in a final volume of 3 ml. The tubes were incubated at 37°C for 30 min.

to account for the enhanced incorporation of palmitoyl-^{14}C CoA after CCl_4 administration. The FFA content of this cellular fraction did not exhibit any change (3.8 ± 0.4 μmoles in the control, and 4.3 ± 1.0 μmoles per gram of liver in the CCl_4-treated).

As ATP is required for acyl CoA formation, we also tested the effect of ATP concentration on the fatty acid incorporation into lipids in microsomes plus soluble fraction. Elevation of the ATP level in incubation medium promoted fatty acid esterification mainly into the fraction that had TLC characteristics similar to those of lysophosphatidic acid, but not into TG or PL (Table 7).

DISCUSSION

The results of our experiments in living animals demonstrated that 4–5 hr after CCl_4 administration the incorporation of both choline-methyl-^{14}C (Table 1) and palmitic acid-1-^{14}C (Table 4) into hepatic PL was strikingly depressed.

The accumulation of radioactivity in the DG fraction after injection of palmitic acid-1-^{14}C (Table 4) suggests that CCl_4 feeding suppresses either the transfer of phosphorylcholine and phosphoryl ethanolamine from their CDP-complexes to DG or the synthesis of these CDP-complexes. It is also conceivable that the transfer of additional fatty acids to DG to form TG is disturbed.

According to Wilgram and Kennedy (5), CDP-choline:1,2-diglyceride cholinephosphotransferase is located principally in the microsomal fraction. Judging from the reported very early effect of CCl_4 on microsomes (25), we expected the activity of this enzyme to fall. It did not; CCl_4 seemed to block instead, the formation of

CDP-complex from phosphorylcholine (Table 1). The effect was confirmed in the experiment in which liver homogenate was incubated with phosphorylcholine-^{32}P, CTP, and diglyceride (Table 2). Carbon tetrachloride therefore probably inhibits the enzyme CTP:choline-phosphate cytidyltransferase. In addition to this, an inadequate supply of CTP at the reaction site may possibly contribute to decreased CDP-choline formation.

As there were no alterations in the distribution of labeled palmitic acid among the major PL fractions in the poisoned liver (Table 5), we consider that the synthesis of phosphatidyl ethanolamine was inhibited to the same extent as lecithin.

The biosynthesis of lecithin from phosphatidyl ethanolamine and methionine, the participating enzymes being also located in the microsomal fraction, did not change 4 hr after CCl_4 (Table 3). This indicates that CCl_4 does not act indiscriminately on the biochemical processes taking place in microsomes.

There have been discrepancies in the results obtained by various laboratories concerning the effects of CCl_4 on PL synthesis (26, 27). In isolated perfused liver taken 3.5 hr after CCl_4 feeding, incorporation of palmitic acid-1-^{14}C into PL during 3 hr of perfusion was reported to be 56% of the control (reference 26; cf. also our Table 4). On the other hand, in an experiment in which linoleic acid-1-^{14}C was injected 4 hr after CCl_4 administration and livers were examined after 20 min, there was no difference in its incorporation into *total* PL (although radioactivity in phosphatidyl ethanolamine fell and that in cardiolipin and lecithin rose) (27). This discrepancy may be due partly to the different radioactive fatty acids used in the two sets of experiments. It has been shown that unsaturated acyl CoA esters are transferred to the 2-position of monoacyl glycerophosphorylcholine more rapidly than saturated ones (28).

The lower production of PL in the hepatic cells could interfere with the coupling of TG and protein to form plasma lipoproteins (29) and cause the intracellular TG deposition. However, our observation did not prove that this occurs in the very early period (1 hr) of CCl_4 poisoning, when a block in hepatic TG secretion was already apparent (Table 4). The significance of the decrease in hepatic PL formation 4–5 hr after CCl_4 feeding may lie, rather, in the deterioration of cellular functions in the late period of CCl_4 poisoning.

The TG of total liver homogenate became labeled to an almost equal extent in the control and the CCl_4 (4 hr) rats after injection of palmitic acid-1-^{14}C. There was, however, a striking change in the distribution of the radioactivity among cell fractions (Table 4).

TG are synthesized from DG and fatty acyl CoA, the reaction being catalyzed by an acyltransferase located in the microsomal fraction. Localization of TG synthesis in the endoplasmic reticulum has also been demonstrated by electron-microscopic radioautography (30). Thus, the fall of the incorporation of labeled fatty acids into microsomal TG was considered to imply a depressed TG synthesis. In CCl_4-treated rats, newly formed TG might be more easily detached from microsomes to soluble fraction during homogenization and centrifugation; this would lead to a subcellular lipid distribution seemingly different from that in normal liver (31). Such an effect may possibly have contributed to the apparent lower incorporation into TG in our experiment; however, the fall in specific activity of the microsomal TG, together with the striking rise in the radioactivity in microsomal DG lends support to the idea that TG synthesis is lower. The increased radioactivity of TG in the soluble fraction can be accounted for by the suppression of its release from the hepatic cells, which is reflected by the drastic fall in the level of labeled TG in plasma (Table 4). As plasma FFA levels 5-hr after CCl_4 administration showed no significant elevation, it seems unlikely that labeled palmitic acid was more diluted in the poisoned rats. These results suggest that, although TG accumulate in the liver of CCl_4-treated rats, their synthesis is actually reduced.

The experiments carried out in vitro were discordant with this view. The incorporation of palmitoyl-^{14}C CoA into TG by the microsomes from CCl_4-treated (4 hr) rats (Table 6) exceeded that from the controls, which indicates that acyl CoA:1,2-diglyceride *O*-acyl-transferase remained intact in these animals.

Rossi and Zatti (31) reported that hydroxamic acid formation by liver homogenate decreased 4–5 hr after CCl_4 administration. We therefore replaced palmitoyl-^{14}C CoA by palmitic acid-1-^{14}C and tested its incorporation in the presence of diglyceride and cofactors. Again, however, the cell fraction from poisoned animals gave more incorporation (Table 6).

In the assessment of these discrepant results, we have to take into consideration the different conditions in vivo and in vitro, such as the concentration of magnesium, ATP, and protein, and disorganization of intracellular structure during the preparation of cellular fractions. Changes in the concentration of magnesium in the liver reported in CCl_4 poisoning (32) are small. Augmentation of ATP content in microsomal preparation enhanced the esterification of radioactive fatty acid with glycerol phosphate (Table 7); increased radioactivity was found neither in TG nor in major PL fractions but in an unidentified PL, presumably lysophosphatidic acid.

The rate of microsomal glyceride synthesis has been shown to depend on the amount of protein added to the reaction mixture (33). It has also been reported that formation of TG from 1,2-diglyceride by microsomes is

only slightly stimulated by the protein of soluble fraction (34). Thus, at present, the discrepancy between the results obtained in vivo and in vitro is not fully accounted for by the changes in the amount of ATP and protein at the site where TG are actually synthesized.

It should be borne in mind that the enzyme activities found in our microsomal fraction did not necessarily represent those of the endoplasmic reticulum membrane in situ. The activities of some microsomal enzymes are known to be modified by changes in membrane structure (35–36). For the synthesis of such hydrophobic molecules as TG and cholesterol, membranes must probably remain intact in order to bridge the gap between hydrophilic and hydrophobic groups. The importance of structural integrity in TG metabolism is supported by electron-microscopic radioautography (30) which showed that fatty acid esterification occurred on or near the endoplasmic reticulum membrane, and the lipid, mainly TG, was transported along the Golgi apparatus. Considering these facts, we presume that the disarrangement of the endoplasmic reticulum, which is already apparent 1 hr after CCl_4 administration and is probably caused by lipoperoxidation, is responsible for the fall in labeling of TG in vivo.

Although there is a good reason to believe that lipoperoxidation in the endoplasmic reticulum intiates cell injury in CCl_4 poisoning (37), it seems quite likely that disturbance of TG and PL synthesis as demonstrated in our experiments further aggravates the damage, particularly in the middle to late period.

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References

1. Smuckler, E. A., O. A. Iseri, and E. P. Benditt. 1962. *J. Exp. Med.* **116:** 55.
2. Reynolds, E. S. 1963. *J. Cell Biol.* **17:** 208.
3. Recknagel, R. O. 1967. *Pharmacol. Rev.* **19:** 145.
4. Shimizu, Y., and K. Kawai. 1966. *Ind. Health.* **4:** 76.
5. Wilgram, G. F., and E. P. Kennedy. 1963. *J. Biol. Chem.* **238:** 2615.
6. Sodhi, H. S., and R. G. Gould. 1967. *J. Biol. Chem.* **242:** 1205.
7. Fleischer, B., and S. Fleischer. 1967. *Biochim. Biophys. Acta* **147:** 566.
8. Dallner, G., P. Siekevitz, and G. E. Palade. 1966. *J. Cell Biol.* **30:** 73.
9. Green, D. E., and S. Fleischer. 1963. *Biochim. Biophys. Acta.* **70:** 554.
10. Wise, E. M., Jr., and D. Elwyn. 1965. *J. Biol. Chem.* **240** 1537.
11. Taylor, C. B., E. Bailey, and W. Bartley. 1967. *Biochem. J.* **105:** 605.
12. Ansell, G. G., and J. N. Hawthorne. 1964. *In* Phospholipids—Chemistry, Metabolism and Function. Elsevier Amsterdam. 412.
13. Vignais, P. V., and I. Zabin. 1958. *Biochim. Biophys. Acta* **29:** 263.
14. Riley, R. F. 1944. *J. Amer. Chem. Soc.* **66:** 512.
15. Kennedy, E. P. 1956. *J. Biol. Chem.* **222:** 185.
16. Hogeboom, G. H., and W. C. Schneider. 1955. *In* The Nucleic Acids. E. Chargaff and J. N. Davidson, editor Academic Press, Inc., New York. **2:** 105.
17. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
18. Marsh, J. B., and D. B. Weinstein. 1966. *J. Lipid Res.* **7** 574.
19. Duncombe, W. G. 1963. *Biochem. J.* **88:** 7.
20. Allen, R. J. L. 1940. *Biochem. J.* **34:** 858.
21. Bjørnstad, P., and J. Bremer. 1966. *J. Lipid Res.* **7:** 38.
22. Tubbs, P. K., and P. B. Garland. 1964. *Biochem. J.* **9** 550.
23. Hanahan, D. J., and R. Vercamer. 1954. *J. Amer. Chem Soc.* **76:** 1804.
24. Spitzer, H. L., K. Morrison, and J. R. Norman. 196 *Biochim. Biophys. Acta.* **152:** 552.
25. Recknagel, R. O., and B. Lombardi. 1961. *J. Biol. Chem* **236:** 564.
26. Weinstein, T., G. Dishmon, and M. Heimberg. 196 *Biochem. Pharmacol.* **15:** 851.
27. Sgoutas, D. S., and F. A. Kummerow. 1966. *Proc. Soc Exp. Biol. Med.* **123:** 279.
28. Hill, E. E., and W. E. M. Lands. 1968. *Biochim. Biophys Acta.* **152:** 645.
29. Lombardi, B., G. Ugazio, and A. N. Raick. 1966. *Amer. J. Physiol.* **210:** 31.
30. Stein, O., and Y. Stein. 1967. *J. Cell Biol.* **33:** 319.
31. Rossi, F., and M. Zatti. 1960. *Experientia (Basel).* **16:** 513.
32. Thiers, R. E., E. S. Reynolds, and B. L. Vallee. 196 *J. Biol. Chem.* **235:** 2130.
33. Tzur, R., and B. Shapiro. 1964. *J. Lipid Res.* **5:** 542.
34. Brindley, D. N., M. E. Smith, B. Sedgwick, and G. Hübscher. 1967. *Biochim. Biophys. Acta.* **144:** 285.
35. Ernster, L., and L. C. Jones. 1962. *J. Cell Biol.* **15:** 563.
36. Segal, H. L., and M. E. Washko. 1959. *J. Biol. Chem* **234:** 1937.
37. Recknagel, R. O., and A. K. Ghoshal. 1966. *Exp. Mol Pathol.* **5:** 413.