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HEPATOTOXICITY AND LIPID METABOLISM—III

CHANGES IN PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE DURING HEPATIC INJURY CAUSED BY CARBON TETRACHLORIDE

MICHIHIRO SUGANO, SHUJI CHO, KATSUMI IMAIZUMI and MASAFUTO WADA

The Laboratory of Nutrition Chemistry,
Department of Food Science and Technology,
Kyushu University School of Agriculture, Fukuoka, Japan

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Abstract—The concentration and composition of hepatic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were compared in rats treated with carbon tetrachloride (CCl_4) and in control rats. Administration of CCl_4 brought about a decrease in concentration of total phospholipids, PC and PE in the liver. However, the percentage concentration of PE was increased and that of PC decreased, resulting in an elevation of the PE:PC ratio after CCl_4 treatment. The fatty acid composition of PC and PE was altered by CCl_4; arachidonic and docosahexaenoic acids were decreased (the latter in PC only) and oleic and linoleic acids were increased. Stearic acid was also increased at the expense of palmitic acid. Similar changes were observed at the 1- and 2-positions as well as in four molecular species of these phospholipids. There were no significant changes in the percentage of molecular species of PC and PE, except for a considerable increase in the monoenoic fraction. These alterations were more prominent in the females than in males, and in the livers of rats killed 20 hr after the dose of CCl_4 than in the livers of those killed at 6 hr. The data further confirm that the metabolism of hepatic glycerophosphatides is altered by CCl_4 treatment. However, the fatty acid compositions of triglyceride and phospholipids were altered in different ways and there was a time difference between the two glycerides in response to CCl_4.

IN PREVIOUS studies,[1,2] we have demonstrated that liver triglycerides from rats sacrificed 6 hr after carbon tetrachloride (CCl_4) treatment contained, in terms of percentage composition, significantly more palmitic acid and less linoleic acid, than livers of control rats. This effect was attributed to a change in composition at the 1,3-positions of the glycerides. Since hepatic triglyceride and glycerophosphatides are synthesized mainly from a common precursor, 1,2-diacylglycerol, these alterations by CCl_4 in the positional composition of triglycerides indicated the possibility of similar changes in glycerophospholipids.

Horning *et al.*[3] have described changes in the composition of liver phosphatides after CCl_4 poisoning; CCl_4 treatment produced a decrease in arachidonic acid of the lecithin and cephalin fractions. More recently, Miller and Cornatzer[4] have shown that CCl_4 decreased the amount of arachidonic acid found in lecithin from liver mitochondria and microsomes. CCl_4 administration also resulted in a decrease in docosahexaenoic acid of mitochondrial lecithin. It is well known that the fatty acids of the 1- and 2-positions of hepatic phosphatidylcholine[5] and ethanolamine[6] are composed almost exclusively, but not entirely, of the saturates and unsaturates respectively. The

phospholipid changes caused by CCl_4, therefore, suggest that some alteration in structure of these lipids may possibly take place. The aim of this paper is to determine whether these changes do in fact occur.

EXPERIMENTAL

Experimental animals

Female and male Wistar rats, weighing 190–230 g and fed on a standard laboratory chow (Oriental Rat Chow NMF), were fasted for 15 hr. The animals were given intragastrically, under light ether anesthesia, either CCl_4 (0·25 ml/100 g body weight, diluted with an equal volume of liquid paraffin) or liquid paraffin (0·5 ml/100 g) and were exsanguinated 6 and 20 hr later.

Lipid analyses

The rats were killed by decapitation. The liver lipids were extracted and purified by the procedure of Folch *et al.*[7] and were finally dissolved in chloroform. Total lipids (by weighing) and total phospholipid phosphorus[8] were determined on an aliquot of this solution. Phosphorus determination of individual phospholipid fractions separated on thin-layer plates and revealed with iodine vapor was carried out according to the method of Rouser *et al.*[9] without the previous elution of the lipids from adsorbent.

Isolation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE)

A preliminary separation into neutral lipid and phospholipid was made on a silicic acid column by eluting first with chloroform and then with absolute methanol. Phospholipids were separated into their components by thin-layer chromatography on Silica gel G using a $CHCl_3$–CH_3OH–H_2O(70:30:4, v/v) mixture. The plates were sprayed with 0·1% bromthymol blue solution (containing ammonia) and the bands corresponding to PC and PE were scraped. The pigment was removed with several portions of an aqueous solution of 15% (1:1, v/v) methanol–acetone and the phospholipids were extracted first with a small amount of ethyl ether and then with chloroform–methanol–water(65:40:5, v/v).[10] By this procedure, the dye-free phospholipids were eluted quantitatively. The fatty acid composition of these samples was analyzed. In case of analyses of positional distribution of fatty acids in phospholipids and of the molecular species, these lipid fractions were further purified by repeating the thin-layer chromatography step. Identification of the phospholipid components was carried out by comparing with purified standards. In this way, chromatographically pure PC and PE were obtained.

Degradation of PC and PE with phospholipase A

PC and PE were hydrolyzed in an atmosphere of nitrogen with snake venom phospholipase A (*Crotalus adamanteus*, Sigma Chemical Company), according to the procedure of Long and Penny,[11] except that the amount of the enzyme for PE was ten times that described. The incubation periods were 2 and 5 hr for PC and PE respectively. After termination of the reaction with diluted HCl, the reaction mixture was separated by thin-layer chromatography into lyso-phospholipids, unesterified fatty acids and unreacted phospholipids using $CHCl_3$–CH_3OH–H_2O (60:40:4, v/v). During this incubation, the degradation of PC was almost complete (more than 95

per cent), while that of PE was 50–70 per cent complete, this being checked by phosphorus determination after thin-layer chromatography.

Subfractionation of PC and PE

PC and PE were subfractionated into four molecular species, according to degree of unsaturation, by the method of Arvidson.[12] Pentadecanoic acid was added as an internal calibration standard prior to extraction of the components from silica gel.[13]

Gas–liquid chromatography of fatty acids

Fatty acid methyl esters from the glycerophosphatides were analyzed as previously reported,[1] using a Nihon Electron Optics Laboratory gas chromatograph, model 750F, equipped with a hydrogen ionization detector. The column was 2 m × 4 mm (internal diameter) packed with 5% diethylene glycol succinate polyester on Chromosorb P(AW) DMCS, 80–100 mesh.

RESULTS

Fatty acid composition of hepatic PC and PE (experiment 1)

The composition of the major fatty acids of the PC and PE fractions in the liver from rats treated with CCl_4 and control rats is summarized in Figs. 1 and 2. At 20 hr after the dose of the haloalkane, the hepatic PC and PE fractions contained, in terms

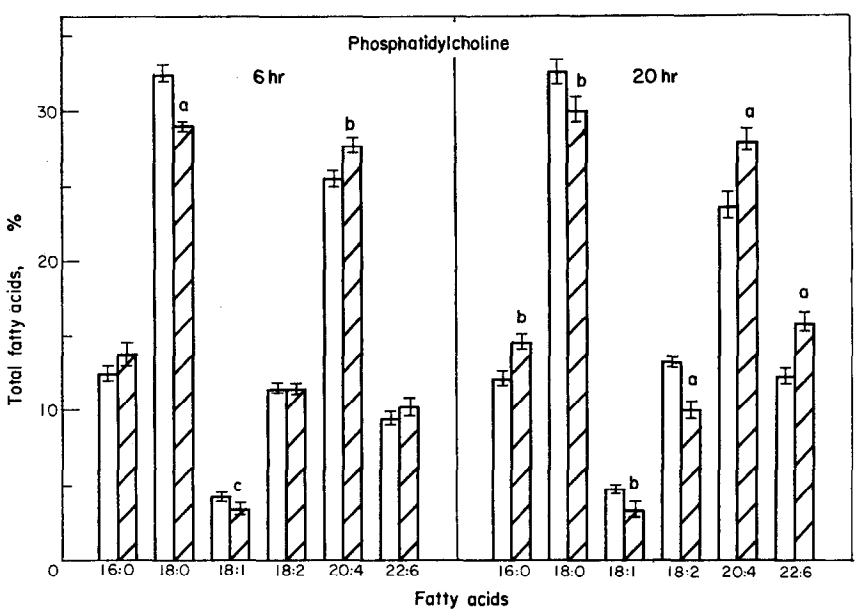

FIG. 1. Fatty acid composition of liver phosphatidylcholine in CCl_4-treated and control female rats. Fatty acids whose amounts were less than 2% of the total fatty acids detected are not shown in the figures. Rats were sacrificed at the indicated time after a single dose of CCl_4 or paraffin. Values are expressed as means of the per cent of total fatty acids ± S.E.; five to six female rats/group. Numbers before the colon represent carbon chain length; those after it represent the number of double bonds. Differences between treated and control rats are significant at: a) $P < 0{\cdot}01$; b) $0{\cdot}01 < P < 0{\cdot}02$; and c) $0{\cdot}02 < P < 0{\cdot}05$. □, CCl_4; ▨, paraffin.

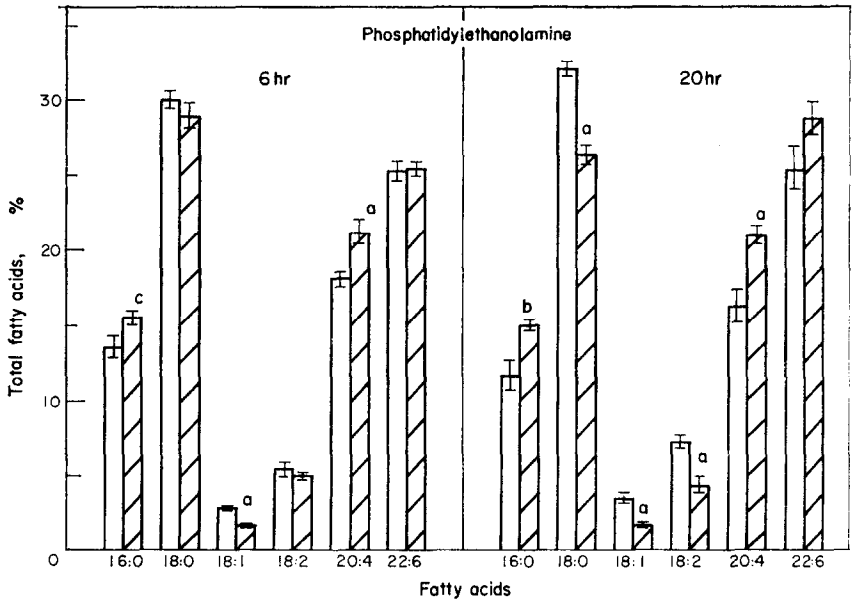

FIG. 2. Fatty acid composition of liver phosphatidylethanolamine in CCl_4-treated and control female rats. For details, see legend to Fig. 1.

of percentage composition, more stearic, oleic and linoleic acids and less palmitic and arachidonic acids in comparison to those from the paraffin controls. The PC fraction contained less hexadocosaenoic acid, but the PE fraction did not. These changes, due to CCl_4, in the fatty acid composition of the hepatic phospholipids were less in the 6-hr specimens. The data reported are in general agreement with those of Horning *et al.*,[3] Sgoutas[14] and Sgoutas and Kummerow.[15]

Analogous changes were observed in male rats treated similarly, but only arachidonic acid was decreased (26·2 to 17·7 per cent) and oleic acid was increased (4·7 to 6·2 per cent) at 20 hr after CCl_4 treatment. In male rats, there were no significant changes in the composition 6 hr after the treatment.

Concentration of liver phospholipids (experiment 2)

Administration of CCl_4 caused a significant decrease in the concentration of hepatic lipid phosphorus (Table 1). Male rats also responded similarly, but to a lesser extent. Accompanying the decrease, the concentration of the individual phospholipid components was also decreased. The percentage of PE-P was elevated, however, and that of PC-P was lowered by CCl_4, thus bringing about a fall in the PC-P:PE-P ratio [at 20 hr after the dose, CCl_4 1·33 $\pm$ 0·04, paraffin 1·73 $\pm$ 0·06($P < 0{\cdot}01$); at 6 hr after, CCl_4 1·57 $\pm$ 0·15, paraffin 1·76 $\pm$ 0·02] (Table 2).

Fatty acid configuration of PC (experiment 2)

Total PC. CCl_4 changed the fatty acid composition of hepatic PC as shown in Table 3. As in experiment 1, at 20 hr after a dose of CCl_4, the percentage of palmitic, arachidonic and docosahexaenoic acids decreased. This was balanced by an increase

TABLE 1. ANALYSES OF TOTAL LIVER LIPIDS AND PHOSPHOLIPIDS IN CCl_4-TREATED AND CONTROL FEMALE RATS*

Treatment	6 hr		20 hr	
	Total lipids (mg/g)	Phospholipids (mg/g)	Total lipids (mg/g)	Phospholipids (mg/g)
CCl_4	83·5 ± 2·21†	35·7 ± 0·48†	190·6 ± 4·07†	30·2 ± 0·67†
Paraffin	63·1 ± 3·40	41·6 ± 0·42	63·5 ± 1·47	43·2 ± 0·37

* Values are expressed as means ± S.E.; five to six rats per group.
† Difference from the control value is significant at $P < 0·01$.

TABLE 2. COMPOSITION OF LIVER PHOSPHOLIPIDS OF FEMALE RATS*

Fraction†	Per cent of total lipid-P		P (mg/g liver)	
	CCl_4	Paraffin	CCl_4	Paraffin
6 hr				
PE	29·0 ± 1·7	27·2 ± 0·5	0·43 ± 0·02	0·45 ± 0·01
PC	44·5 ± 1·8	47·7 ± 0·5	0·64 ± 0·03‡	0·79 ± 0·01
20 hr				
PE	32·6 ± 0·4‡	27·5 ± 0·7	0·40 ± 0·51	0·48 ± 0·02
PC	43·5 ± 0·9‡	47·3 ± 0·6	0·51 ± 0·02‡	0·82 ± 0·01

* Liver phospholipids were separated into the constituents by thin-layer chromatography and the content of lipid-P in each fraction was determined. Values are expressed as means ± S.E.; five to six rats per group.
† PE and PC represent phosphatidylethanolamine and phosphatidylcholine respectively.
‡ Difference from the control value is significant at $P < 0·01$.

TABLE 3. POSITIONAL DISTRIBUTION OF FATTY ACIDS IN LIVER PHOSPHATIDYLCHOLINE IN CCl_4-TREATED AND CONTROL FEMALE RATS*

Fatty acids	CCl_4			Paraffin		
	Total	1-Position	2-Position	Total	1-Position	2-Position
6 hr						
16:0	12·9 ± 0·6†	23·0 ± 1·5‡	3·0 ± 0·2§	16·0 ± 0·9	28·7 ± 1·2	1·4 ± 0·1
16:1	0·3 ± 0·0	0·4 ± 0·0	0·2 ± 0·0	0·3 ± 0·0	0·4 ± 0·0	0·2 ± 0·0
18:0	38·1 ± 0·8	70·9 ± 1·6	0·9 ± 0·1	35·6 ± 0·8	65·6 ± 1·4	0·5 ± 0·0
18:1	4·9 ± 0·3§	1·7 ± 0·1	7·8 ± 0·6§	3·4 ± 0·3	1·9 ± 0·1	5·0 ± 0·3
18:2	12·5 ± 0·5	1·4 ± 0·3	25·1 ± 1·1	11·8 ± 0·9	1·2 ± 0·1	25·1 ± 1·5
20:4	24·7 ± 1·0	1·6 ± 0·2	48·8 ± 1·8	26·3 ± 0·9	1·4 ± 0·3	52·8 ± 1·8
22:6	4·9 ± 0·2		11·5 ± 0·9	4·9 ± 0·2		12·4 ± 0·8
20 hr						
16:0	11·5 ± 0·3§	21·2 ± 0·6§	3·4 ± 0·1§	15·9 ± 0·3	29·4 ± 0·4	1·7 ± 0·2
16:1	0·3 ± 0·0	0·4 ± 0·0	0·3 ± 0·0	0·3 ± 0·0	0·3 ± 0·0	0·2 ± 0·0
18:0	41·1 ± 0·4§	73·1 ± 0·8§	1·0 ± 0·1§	35·1 ± 0·6	64·3 ± 0·6	0·3 ± 0·0
18:1	5·1 ± 0·1§	2·1 ± 0·6	7·7 ± 0·2§	3·6 ± 0·2	1·7 ± 0·2	5·0 ± 0·1
18:2	16·1 ± 0·5§	1·7 ± 0·1	32·3 ± 1·1§	11·9 ± 0·6	1·2 ± 0·1	24·1 ± 0·8
20:4	19·2 ± 1·0§	1·3 ± 0·1§	40·4 ± 1·9§	26·4 ± 0·2	2·3 ± 0·2	52·7 ± 1·7
22:6	4·3 ± 0·2‡		12·2 ± 0·7	5·5 ± 0·2		14·4 ± 0·8

* The fatty acid composition of the 1- and 2-positions of phosphatidylcholine was determined after hydrolyzing with snake venom phospholipase A. Values are expressed as percentage of total fatty acids detected. Means of five to six rats per group ± S.E. See also Figs. 1 and 2.
† Difference between treated and control rats is significant at $0·05 > P > 0·02$.
‡ Difference between treated and control rats is significant at $0·02 > P > 0·01$.
§ Difference between treated and control rats is significant at $P < 0·01$.

in stearic, oleic and linoleic acids. Thus, despite the changes in profile of fatty acid patterns, the total amounts of saturated and unsaturated fatty acids in the liver PC did not change. After CCl_4 treatment, however, the percentage of polyunsaturated acids in liver PC was decreased by one-third. The change in the stearate–palmitate ratio was also seen in the 6-hr specimens, but not in the arachidonate–linoleate ratio. The values obtained are given in Table 4.

TABLE 4. EFFECT OF CCl_4 ON RELATIVE CONCENTRATION OF STEARATE-PALMITATE AND ARACHIDONATE-LINOLEATE OF HEPATIC PHOSPHATIDYLCHOLINE*

Ratios	6 hr		20 hr	
	CCl_4	Paraffin	CCl_4	Paraffin
18:0/16:0	3·20 ± 0·24†	2·27 ± 0·13	3·59 ± 0·11†	2·22 ± 0·06
20:4/18:2	2·03 ± 0·15	2·29 ± 0·23	1·20 ± 0·09†	2·23 ± 0·11

* Values were calculated from the data shown in Table 3.
† Difference from the control value is significant at $P < 0·01$.

TABLE 5. FATTY ACID COMPOSITION OF MOLECULAR SPECIES OF LIVER PHOSPHATIDYLCHOLINE (LECITHIN)*

Molecular species	Percentage concn†	Fatty acids (% of total)						
		16:0	16:1	18:0	18:1	18:2	20:4	22:6
CCl_4 (20 hr)								
Monoenoic	11·6	36·4	2·1	25·8	31·1	3·8		
Dienoic	23·7	17·1	1·1	29·6	3·2	48·0	0·2	
Tetraenoic	40·0	7·2	0·2	41·7	0·8	0·3	48·6	
Hexaenoic	24·8	8·7	0·5	36·5	1·3	1·0	2·6	42·2
Unfractionated lecithin		12·5	0·4	36·5	4·9	12·2	21·9	9·0
Paraffin (20 hr)								
Monoenoic	6·4	39·0	2·2	20·4	33·2	4·0		
Dienoic	20·2	21·7	0·5	26·4	2·6	48·2		
Tetraenoic	48·7	11·0	0·2	37·4	1·0	0·2	49·7	
Hexaenoic	24·6	15·9	0·7	27·3	1·4	1·6	2·1	47·3
Unfractionated lecithin		15·0	0·3	31·4	3·2	11·0	26·4	11·0

* Each molecular species was isolated by $AgNO_3$-impregnated thin-layer chromatography.[12] Values are means of the duplicate analyses of pooled livers from six female rats per group. See also Figs. 1 and 2.
† The percentage concentration of each molecular species was determined by the internal standard (pentadecanoic acid) method.[13]

Positional distribution. PC from rats receiving CCl_4 20 hr previously contained more stearic acid and less palmitic acid at the 1-position of the molecule. The percentage of the total saturated acids, however, was equal in the treated and the control rats. Fatty acids associated with the 2-position of hepatic PC from CCl_4-intoxicated rats were composed of more palmitic, stearic, oleic and linoleic acids and less arachidonic acid. These positional changes due to CCl_4 in the fatty acid composition appeared to reflect an alteration in the composition of the total molecules.

The interpretation of positional composition data of the PE fraction was rather arbitrary because of the indefinite extent and incompleteness of the hydrolysis of this phospholipid. The data are, however, indicative of the alterations in the positional distribution of fatty acids essentially similar to those observed in the PC fraction. Thus, the fatty acids combined at the 1-position of PE from the treated rat livers contained, in terms of percentage composition, more stearic acid and less palmitic acid, and those at the 2-position contained more arachidonic acid and less linoleic acid, in comparison to control rat livers.

Molecular species of PC and PE (experiment 3)

The fatty acid composition of four molecular families of liver PC, separated according to degree of unsaturation, is given in Table 5. The modification by CCl_4 in the ratio of palmitic and stearic acids from the ratio observed in the intact PC was again demonstrated in each species and was most outstanding in the hexaenoic subfraction. The percentage of the individual subfractions remained apparently unchanged after the treatment, except for an apparent increase in the monoenoic fraction. Similar trends were also observed in the PE fraction (Table 6).

TABLE 6. FATTY ACID COMPOSITION OF MOLECULAR SPECIES OF LIVER PHOSPHATIDYLETHANOLAMINE (CEPHALIN)*

Molecular species	Percentage concn†	Fatty acids (% of total)						
		16:0	16:1	18:0	18:1	18:2	20:4	22:6
CCl_4 (20 hr)								
Monoenoic	8·6	20·0	2·0	32·4	35·1	8·3	0·2	
Dienoic	12·5	15·0	1·0	32·4	4·1	45·5		
Tetraenoic	26·9	6·2	0·7	40·1	1·3		49·8	
Hexaenoic	52·0	8·7	0·4	33·0	1·2		4·6	47·0
Unfractionated cephalin		9·1	0·2	33·7	3·6	7·0	16·8	25·9
Paraffin (20 hr)								
Monoenoic	2·2	24·7	6·3	25·3	26·5	10·2	0·7	
Dienoic	10·7	18·3	1·7	29·4	4·5	44·3	0·6	
Tetraenoic	34·0	8·9	0·5	37·9	1·4		49·4	0·5
Hexaenoic	53·1	17·4	0·6	24·5	0·9		3·9	49·0
Unfractionated cephalin		13·1	0·1	29·0	1·5	5·9	20·9	26·5

* Values are means of the duplicate analyses of pooled livers from six female rats per group. See also Table 5 and Figs. 1 and 2.

† The percentage concentration of each molecular species was determined by the internal standard (pentadecanoic acid) method.[13]

DISCUSSION

The present study indicated that CCl_4 induces quantitative and qualitative alterations in the hepatic phospholipids. The concentration of total phospholipids was significantly decreased 20 hr after a dose of CCl_4. CCl_4 treatment also caused a change in the pattern of the phospholipid components, characterized by a decrease in the percentage of PC and an increase in the percentage of PE. The modifications in the pattern of phospholipids did not correspond to those previously reported for whole

liver.[14,15] Although the reason for this discrepancy was not apparent, one possible explanation is based on the differences in the sex of the animals, in the analytical procedure, and in the periods of exposure to CCl_4. However, the data are in general agreement with those reported on mitochondria and microsomes.[4]

These alterations induced by CCl_4 can be interpreted simply as the results either of the decreased synthesis of PC and PE from the diglycerides or of the decreased conversion of PE to PC. The former possibility is compatible with the observation that CCl_4 brings about degeneration of the endoplasmic reticular membrane where most, if not all, synthesis of phospholipid occurs.[16] The latter possibility is supported by the observation tabulated in Table 3.

Other observed changes in the composition of the hepatic glycerophosphatides are concerned with the elongation of fatty acids. First, CCl_4 treatment appeared to inhibit the interconversion of linoleic to arachidonic acids and probably of linoleic to hexadocosaenoic acids. This phenomenon may be attributed to: (1) an inhibition of the enzyme systems participating in the elongation, since at 20 hr after the treatment the hepatic organelles involved in the interconversion of these polyunsaturated acids tended to degenerate;[4,16,17] and (2) the direct peroxidation of these polyunsaturated acids.[16,18] However, it is indeed difficult to ascribe the decrease in arachidonic or hexadocosaenoic acids simply to peroxidative degradation, since the PE fraction showed no demonstrable change in the percentage of hexadocosaenoic acid and the percentage of linoleic acid still remained high.

Second, CCl_4 caused an alteration in the proportion of palmitic and stearic acids. Since hepatic mitochondria are undergoing functional degeneration at 20 hr after the dose of CCl_4,[16,17] the observation is difficult to interpret. It is probable that the pool of palmitic acid available for the synthesis of the glycerophosphatides is small, due to preferential utilization of this acid, but not of stearic acid, for triglyceride rather than for phospholipid synthesis.[1,2]

The alterations reported were much more distinct in the rats treated for 20 hr than in those treated for 6 hr. In contrast, the structural changes in hepatic triglycerides were remarkable in the 6-hr specimens.[1,2] This difference in the response of the different glycerides seems to be reflected in the extent of the modification in functioning of the enzymatic systems forming these two glycerides. This may, in turn, indicate that the metabolic pool(s) of diglycerides available for the synthesis of triglycerides and phospholipids is substantially disturbed by CCl_4. Moreover, it has been suggested that the tetraenoic subfraction of liver phosphatidylcholine and ethanolamine is synthesized mainly from 1-acyl lysoderivatives,[19–21] while for the formation of the mono- and dienoic subfractions, a *de novo* synthesis as established by Kennedy[22] would appear to be responsible. The hexaenoic molecular species of hepatic lecithin appeared to be derived mainly from cephalin by methylation.[23] Thus, the observed phospholipid alterations after CCl_4 treatment would be controlled by the complex responses of a number of enzymatic systems, the pool size and the metabolic turnover of the individual components.

Experimental evidence is inadequate at the present time to correlate the changes in hepatic phospholipids with the mechanism of fatty infiltration. The primary biochemical lesion responsible for the observed phospholipid changes seems to be attributable to a degenerating membrane system, primarily in the endoplasmic reticulum, possibly due to peroxidation.[16]

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