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The phospholipids of the hepatic endoplasmic reticulum

Structural change in liver injury

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Endoplasmic-reticulum phospholipids were measured during the first hour after carbon tetrachloride administration to male Sprague–Dawley rats and compared with carbon tetrachloride challenge of microsomes from control animals *in vitro*. The extracted lipids were separated by high-pressure liquid chromatography. No significant differences in the abundance of phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol or phosphatidylcholine were found after either treatment when compared with untreated controls. Diene conjugate formation in each separated phospholipid was determined by measuring A_{232} and expressed on the basis of lipid phosphorus. Phosphatidylserine was peroxidized 6-fold greater than in controls after challenge *in vivo*, reaching maximal change after 15 min, whereas the other phospholipids showed little or no alteration. Fatty acid composition analysis was performed by g.l.c. after transesterification of individual phospholipids. Phosphatidylserine revealed two types of response: an abrupt decrease in relative abundance of oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) without further loss and a slower, linear decrease in arachidonic acid ($C_{20:4}$) over the first hour. Similar changes were not seen in other phospholipids. In the *'in vitro'* model, the relative amounts of the phospholipids do not change. The extent of peroxidation was greater in all the phospholipids than found *in vivo*, with phosphatidylserine peroxidized to the greatest extent. These data suggest that carbon tetrachloride injury *in vivo* produces an early peroxidative event and that a specific phospholipid (phosphatidylserine) is selectively modified, although maintaining its relative concentration in the membrane. Dissection of this process *in vitro* will require refinement of existing systems to reduce the non-specific changes associated with the model system.

There is increasing evidence that the lipids of membranes modify their functions, and that this lipid composition is subject to dynamic variation. Dietary means have been used to change phospholipid and/or fatty acid components and then specific functions assayed (Marshall & McLean, 1971; Lowe & Wills, 1976). Cell poisons may also operate, at least in part, by modification of lipid composition. Carbon tetrachloride poisoning affects the structure and function of the endoplasmic reticulum early in the evolution of liver cell injury. Changes in both the lipid component (Lombardi, 1965; Rao & Recknagel, 1968, 1969) as well as protein functions (Smuckler *et al.*, 1961, 1962; Reynolds & Yee, 1967) have been described. Lipid peroxidation is considered by some authors to be a key factor in the genesis of this injury (Recknagel & Ghoshal, 1966; Recknagel & Glende, 1973; Slater, 1978). Peroxidation (measured as diene conjugation) is half-maximal 5 min after oral carbon tetrachloride administration (Rao & Recknagel, 1968), peaks by 15 min (the present paper) and seems predominantly to affect a single phospholipid, PtdSer (the present paper), or PtdEtn, as reported by Benedetti *et al.* (1976). These changes occur long in advance of maximal carbon tetrachloride liver concentration (Recknagel & Litteria, 1960), and point to early rapid chemical interaction suggested by Slater (1976). The arachidonic acid component of the endoplasmic-reticulum phospholipids decreases when carbon tetrachloride is administered *in vivo* (Horning *et al.*, 1962; Comporti *et al.*, 1969, 1971)

Abbreviations used: PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

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and it is the preferred substrate for lipid peroxidation when microsomes are incubated in air in the presence of NADPH (Villarruel *et al.*, 1976). In fact, arachidonic acid loss seems to be a common change with cytochrome *P*-450 loss after alkyl halide exposure (Moody *et al.*, 1980, 1981), but notably at longer time periods than the initial peroxidation. This observation suggested other fatty acids might be modified at earlier time periods (see below). The carbon tetrachloride-induced lipid peroxidative process was shown to affect the several phospholipids differently (Benedetti *et al.*, 1976). Also, only restricted forms of the cytochrome *P*-450 complex were more susceptible to carbon tetrachloride poisoning (Head *et al.*, 1981). These findings are consistent with a focal nature of the initial injury, and point to a potential interaction between the formation of the proximate toxin (the active metabolic product of carbon tetrachloride), the phospholipid and the cytochrome.

We sought to confirm and extend information concerning the potential focal response of the membrane alteration. We used a technique to isolate and identify lipid components that reduced manipulation (and artefactual peroxidation), and gave a good yield of polyunsaturated fatty acids (James *et al.*, 1981). We found that an intimate relationship of PtdSer to injury exists. Also, a dynamic response in fatty acid composition of this phospholipid, involving several of the unsaturated species, occurs during the first hour of poisoning.

Experimental

Male Sprague–Dawley rats (Charles River Breeding Laboratory, Wilmington, MA, U.S.A.), weighing 180–220 g, were housed separately in cages with steel-screened bottoms and maintained on Purina Rodent Chow and water *ad libitum* for 4 days before experimentation. Food was removed 14–16 h before the experiments were begun.

Carbon tetrachloride-induced lipid peroxidation in vivo

Spectral-grade carbon tetrachloride (Mallinkrodt, St. Louis, MO, U.S.A.) was administered at a dosage of 2.5 ml/kg body wt. by gastric intubation in an equal volume of mineral oil. The rats were killed at intervals (specified in the Tables) up to 60 min. Control rats were given mineral oil alone. The animals were killed by exsanguination under light diethyl ether anaesthesia.

Livers were perfused *in situ* with 0.9% NaCl via the thoracic aorta, removed, minced and weighed in tared beakers containing ice-cold 0.1 M-potassium phosphate buffer (pH 7.4). Homogenization was carried out using a homogenizer with a Teflon pestle driven at 1000 rev./min, in 3.4 vol. of the buffer. All further manipulations were carried out at 4°C unless otherwise specified. The resulting brei was centrifuged at 10000 $g_{av.}$ for 10 min in an SS34 rotor in a Sorvall RC5 centrifuge; microsomes were harvested from this supernatant fluid by centrifugation at 143000 *g* for 60 min in a Beckman 60 Ti rotor. The resultant sediment was resuspended in 15 vol. of 50 mM-Tris/HCl buffer (pH 7.4) containing 175 mM-KCl, and re-centrifuged at 143000 *g* for 60 min. The final pellet was suspended in 0.1 M-potassium phosphate buffer (pH 7.4) and its protein content was measured by the Lowry technique with crystalline bovine serum albumin as standard (Lowry *et al.*, 1951).

Carbon tetrachloride-induced lipid peroxidation employing purified microsomal suspensions in vitro

Microsomal suspensions in 0.1 M-potassium phosphate buffer (pH 7.5) were incubated as follows. Some controls were incubated in phosphate buffer alone, others were incubated in phosphate buffer to which NADPH was added. Carbon tetrachloride reactions were incubated with 100 mM-carbon tetrachloride and 570 μM-NADPH in phosphate buffer. Ferroxidant mixtures were incubated with Fe^{3+}/ADP and NADPH by additions of 286 μM-ADP, 857 μM-$FeCl_3$ and 570 μM-NADPH (all quantities are final concentrations). All additions were made with freshly prepared components. The protein concentrations of the microsomal suspensions were 1.02 mg/ml in a total volume of 45 ml.

The reaction was started by the addition of the NADPH. The flasks were then placed in a shaking water bath at 37°C and incubated for 20 min in the dark. At intervals, portions were removed and the thiobarbituric acid assay for peroxidation was carried out (Sidwell *et al.*, 1954). After 20 min (this time was selected on the basis of pre-experiments suggesting the reaction was completed) the suspensions were diluted with ice-cold buffer and centrifuged at 143000 *g* for 1 h. The pellets were scraped into glass tubes for lipid extraction.

Lipid extraction and purification

Microsomal pellets from experiments *in vivo* and *in vitro* were extracted by dispersion with a Polytron homogenizer (Brinkman, Westbury, NY, U.S.A.) in 20 vol of hexane/propan-2-ol (3:2, v/v) (Burdick and Jackson Laboratories, Muskegon, MI, U.S.A.) as previously described (James *et al.*, 1981). This method involved centrifuging the resulting homogenate at 770 *g* for 10 min at 4°C. The organic phase was removed and a portion of the extract, generally containing 2–4 mg of phospholipid, was reduced in volume by evaporation under N_2 to 100 μl; 100 μl of this concentrated fraction was injected into a Hewlett–Packard 1084A high-pressure liquid chromatograph through a Rheodyne injection port

(Rheodyne, Berkeley, CA, U.S.A.). A Hewlett–Packard Si100 10-μm column was used after saturation with the mobile-phase solvent.

Phospholipid elution was accomplished with a continuous gradient from 100% solvent A [hexane/propanol/water (24:32:3, by vol.)] at zero time to 100% solvent B [hexane/propanol/water (12:16:3, by vol.)] at 5 min. The flow rate was 1.5 ml/min. Elution was monitored with a Varian spectrophotometer at 206 nm. Identification of specific lipids was made by comparison with the elution times of standards and by t.l.c. comparison with standards. Additional t.l.c. analysis was carried out as previously described (James *et al.*, 1981) and identification was made with ninhydrin, phosphorus and rhodamine stains (Dittmer & Wells, 1969). Phospholipids were quantified by u.v. absorption and by phosphorus determinations (Bartlett, 1959). Diene conjugate concentrations were determined with a Cary 118 spectrophotometer, by measuring A_{232}, and the amount of phospholipid present (expressed in mg) was used to yield specific peroxidation activity.

After high-pressure-liquid-chromatographic separation, the phospholipid fractions were dried to a small volume under dry N_2; fatty acid methyl esters were obtained by reacting the phospholipid residue with methanolic base reagent (Supelco, Bellefonte, PA, U.S.A.) at 80°C for 15 min in a Teflon-lined screw-cap 15 ml bottle. The esters were then analysed on a Hewlett–Packard 5830A gas chromatograph using an SP-2330, 100/120 Chromosorb WAW column (Supelco). The column temperature at the beginning of the run was 170°C and after 1 min raised to 200°C at a rate of 2°C/min and maintained at this temperature for the remainder of the run (10 min). The helium flow rate was 20 ml/min. Peaks were identified by comparison with fatty acid methyl esters of palmitate, stearate, oleate, linoleate, eicosatrienoate, arachidonate and docosahexaenoate standards (Supelco). Selected analysis were further checked by g.l.c.–mass spectrometry.

Statistical analysis

Each experiment was repeated a minimum of three times. The values obtained from these different experiments were used to calculate means and standard deviations. Assay for significance of difference between means was done by the Student's *t* test. Tests for linearity of arachidonate loss were carried out by using a statistical program for linear regression designed for a Hewlett–Packard model 97 programmable calculator.

Results

Phospholipids isolated from microsomes and analysed as described had compositional values similar to previous findings using either column or thin-layer techniques (Glaumann & Dallner, 1968; Davison & Wills, 1974; DePierre & Ernster, 1977). Our separation technique permitted a recovery of $91.7 \pm 1.3\%$ of the phospholipids applied to the column. Exposure of male rats to carbon tetrachloride for up to 1 h resulted in no significant differences in total phospholipid content, and only small shifts in the relative amount of the individual phospholipid species (Table 1). This suggested that we could examine the isolated component for the effect of injury during the first hour without artefactual selection or enrichment.

The individual phospholipids were analysed for diene conjugation as indicated (Fig. 1). We found, in contrast with a previous report that used a thin-layer procedure (Benedetti *et al.*, 1976), that PtdSer was most peroxidized (Fig. 1) and that it and PtdIns were modified earliest after oral carbon tetrachloride

Table 1. *Phospholipid changes after carbon tetrachloride poisoning in vivo*

Animals were given either carbon tetrachloride in mineral oil or mineral oil alone. After 1 h, the rats were killed and the microsomes were isolated. The lipids were extracted and phospholipids purified by high-pressure liquid chromatography and phospholipid phosphorus quantified (for details see the Experimental section). Results are expressed as means ± 1 S.D. of three or more experiments.

Phospholipid	Treatment	Phospholipid phosphorus (mg/100 g body wt.)
PtdEtn	Control	21.1 ± 2.1
	Carbon tetrachloride	23.9 ± 0.7
PtdIns	Control	9.7 ± 3.2
	Carbon tetrachloride	8.0 ± 2.0
PtdSer	Control	2.7 ± 0.4
	Carbon tetrachloride	3.2 ± 0.7
PtdCho	Control	50.7 ± 1.6
	Carbon tetrachloride	46.8 ± 1.7

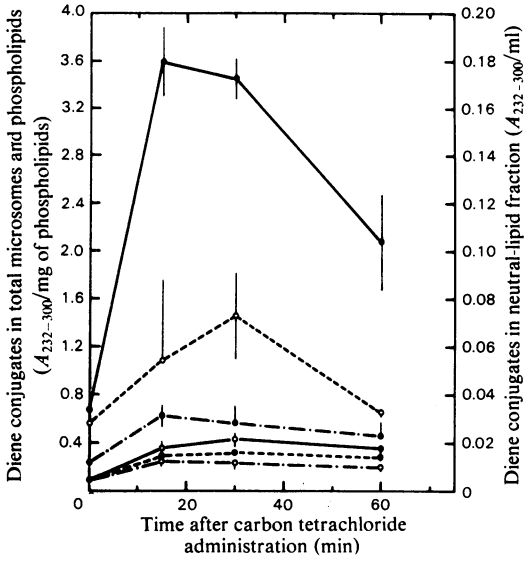

Fig. 1. *Lipid peroxidation of isolated hepatic microsomal phospholipids from rats treated with carbon tetrachloride*

Male rats were treated with 2.5 ml of carbon tetrachloride/kg body wt. for the times indicated. Hepatic microsomes were isolated and a lipid extract was prepared. Phospholipids were separated by high-pressure liquid chromatography as described in the Experimental section and diene conjugates and organic phosphorus content were determined in fractions of PtdIns (●-·-●), PtdEtn (○-·-○), PtdSer (○——○), PtdCho (●----●), neutral lipids (○----○) and total lipids (○——○). Values are means ± S.D. (represented by the vertical lines through the points) for three or more samples.

administration. The changes in PtdCho and PtdEtn were not significant; even the PtdIns alteration was much less than PtdSer (2- compared with 6-fold). The specific peroxidation of PtdSer was not static, but decreased during the latter part of the first hour. Surprisingly, neutral lipids also showed an early change in peroxidation, but after the modification in PtdSer.

PtdSer may have been preferentially peroxidized because of a unique content of unsaturated fatty acid, or because it occupies a select topological position in the membrane adjacent to the site of metabolism of carbon tetrachloride. The transformation of this haloalkane to a chemically reactive form, the proximate toxin, interacts with the PtdSer to result in the peroxidation. To test these possibilities transmethylation and fatty acid analysis of phospholipid was undertaken. These analyses revealed preservation of significant quantities of $C_{22:6}$ fatty acid in control preparations. Furthermore, PtdSer was not richer in unsaturation, especially $C_{20:4}$ fatty acid (Table 2), than other phospholipid species. Lipid analysis of PtdSer at different times after carbon tetrachloride showed that $C_{18:1}$ and $C_{18:2}$ fatty acids decrease in relative abundance during the first 15 min to 66.2% of the control value for $C_{18:1}$ and 58.6% for $C_{18:2}$ fatty acid, whereas $C_{20:4}$ fatty acid was 92.8% of the control value. $C_{22:6}$ fatty acid was not changed during 15 min (Fig. 2). Similar changes were not seen in other phospholipids.

During the remainder of the first hour of poisoning, there was little further alteration in $C_{18:1}$

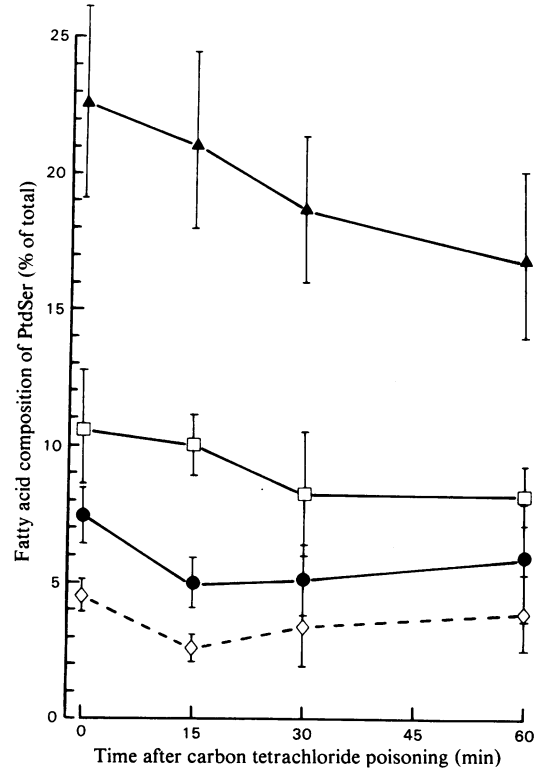

Fig. 2. *Fatty acid content of PtdSer isolated from hepatic microsomes of rats treated with carbon tetrachloride*

PtdSer fractions were prepared from lipid extracts of hepatic microsomes as described in the legend to Fig. 1. Fatty acid methyl esters were prepared and separated by g.l.c. as described in the Experimental section. The relative contents of arachidonate ($C_{20:4}$; ▲), docosahexanoate ($C_{22:6}$; □), oleate ($C_{18:1}$; ●) and linoleate ($C_{18:2}$; ◊) are shown. Values are means ± S.D. (represented by the bars) for three or more samples. Changes in $C_{18:1}$ and $C_{18:2}$ fatty acids are statistically different from controls at 15 and 20 min at $P < 0.05$. Loss of $C_{20:4}$ fatty acid is linear from 1 h.

or $C_{18:2}$, but $C_{20:4}$ fatty acid showed a linear decay with a correlation coefficient of $r = 0.969$. The greatest modification at 1 h was in $C_{20:4}$ fatty acid. There was also an increase in $C_{14:0}$ fatty acid in relative quantity (results not shown). The $C_{20:4}$ component of other phospholipids was not statistically altered (Table 2). The loss of diene conjugation and the shifts in fatty acids suggest the possibility of repair and/or relocation resynthesis early in the injury. It also suggests the potential of two different processes, a rapid one modifying $C_{18:1}$ and $C_{18:2}$, and a second changing $C_{20:4}$ fatty acid. These may or may not be directly related. A critical analysis of potential fatty acid relocation and phospholipid position, as well as a survey of potentially related processes, would be more readily obtained by using an isolated microsomal peroxidation system. We compared the results of such a system with the carbon tetrachloride response *in vitro*.

Table 2. *Fatty acid changes in phospholipid fractions after carbon tetrachloride poisoning in vivo (% of total)*
Animals were given either carbon tetrachloride in mineral oil or mineral oil alone. After 1 h, the rats were killed, the microsomes isolated and the lipids extracted. Phospholipids were purified by high-pressure liquid chromatography, methylated and fatty acid analysis was performed by g.l.c. Fatty acid components are expressed as the percentage of the total fatty acids in the sample.

		Fatty acid content (%)	
Phospholipid	Treatment	$C_{20:4}$ fatty acid	$C_{22:6}$ fatty acid
PtdEtn	Control	27.3 ± 1.6	8.60 ± 0.7
	Carbon tetrachloride	24.9 ± 0.4	9.50 ± 0.6
PtdIns	Control	33.3 ± 2.2	3.60 ± 0.4
	Carbon tetrachloride	32.6 ± 0.9	4.20 ± 0.3
PtdSer	Control	22.8 ± 1.4	10.70 ± 2.2
	Carbon tetrachloride	16.9 ± 3.6*	8.20 ± 1.1
PtdCho	Control	29.2 ± 0.5	3.93 ± 0.7
	Carbon tetrachloride	25.2 ± 1.5	4.50 ± 0.1

* $P < 0.05$.

Table 3. *Comparison of phospholipid changes after incubations in vitro and the corresponding specific activities (diene conjugation) in each species*
Microsomes were prepared from control rats are incubated at 37°C with each addition as outlined in the text. After incubation the microsomes were pelleted, lipids extracted and phospholipids were purified by high-pressure liquid chromatography and quantified as outlined in the Experimental section.

Phospholipid	Conditions	Phospholipid phosphorus (mg/100 g body wt.)	Specific activity (A_{232}/mg of phospholipid phosphorus)
PtdEtn	Buffer	21.70 ± 0.9	0.32 ± 0.10
	Carbon tetrachloride/NADPH	21.60 ± 0.7	1.87 ± 0.25*
	NADPH	21.70 ± 0.7	1.33 ± 0.29*
	Fe^{3+}–ADP/NADPH	21.60 ± 0.8	1.25 ± 0.22*
PtdIns	Buffer	8.70 ± 2.6	0.44 ± 0.15
	Carbon tetrachloride/NADPH	8.30 ± 1.7	2.08 ± 0.24*
	NADPH	8.40 ± 1.4	1.64 ± 0.46*
	Fe^{3+}–ADP/NADPH	8.50 ± 1.5	1.76 ± 0.54*
PtdSer	Buffer	2.80 ± 0.4	1.25 ± 0.40
	Carbon tetrachloride/NADPH	3.14 ± 0.7	8.02 ± 3.60*
	NADPH	3.10 ± 0.6	5.50 ± 2.10*
	Fe^{3+}–ADP/NADPH	2.90 ± 0.8	6.06 ± 2.60*
PtdCho	Buffer	50.50 ± 1.6	0.22 ± 0.10
	Carbon tetrachloride/NADPH	52.50 ± 1.6	1.72 ± 0.65*
	NADPH	51.30 ± 1.6	1.22 ± 0.37*
	Fe^{3+}–ADP/NADPH	51.70 ± 1.7	1.12 ± 0.38*

* $P < 0.05$.

Table 4. *Comparison of fatty acid composition in phospholipid fractions from microsomes after exposure to buffer, carbon tetrachloride, NADPH or Fe^{3+}–ADP/NADPH in vitro*

Microsomes were prepared from control rats and incubated with appropriate additions. After incubation, microsomes were pelleted, the lipids were extracted and phospholipids purified by high-pressure liquid chromatography. Fatty acid methyl esters were analysed by g.l.c. Fatty acid components are expressed as fractions of the total fatty acids in the sample. Changes are given as the percentage of the control (buffer) quantity.

		Fatty acids and associated decreases			
Phospholipid	Conditions	$C_{20:4}$ fatty acid (%)	Change (%)	$C_{22:6}$ fatty acid (%)	Change (%)
PtdEtn	Buffer	26.1 ± 3.2		8.4 ± 1.5	
	Carbon tetrachloride/NADPH	20.9 ± 4.3	−20	5.9 ± 2.2	−30
	NADPH	22.3 ± 3.8	−15	6.4 ± 1.9	−25
	Fe^{3+}–ADP/NADPH	22.1 ± 4.0	−15	6.3 ± 2.1	−25
PtdIns	Buffer	30.4 ± 3.1		2.78 ± 1.2	
	Carbon tetrachloride/NADPH	26.4 ± 4.3	−13	2.24 ± 1.8	−20
	NADPH	28.3 ± 4.0	−7	2.36 ± 1.6	−15
	Fe^{3+}–ADP/NADPH	27.7 ± 4.5	−9	2.30 ± 2.0	−16
PtdSer	Buffer	22.4 ± 3.8		9.52 ± 4.0	
	Carbon tetrachloride/NADPH	17.9 ± 4.4	−20	6.57 ± 4.3	−32
	NADPH	19.6 ± 4.1	−10	9.36 ± 3.8	—
	Fe^{3+}–ADP/NADPH	19.3 ± 4.3	−10	8.37 ± 4.6	−12
PtdCho	Buffer	27.5 ± 1.2		3.90 ± 1.2	
	Carbon tetrachloride/NADPH	23.4 ± 2.6	−15	3.12 ± 1.2	−20
	NADPH	24.8 ± 2.7	−12	3.32 ± 1.4	−15
	Fe^{3+}–ADP/NADPH	24.0 ± 2.7	−13	3.28 ± 1.4	−15

Freshly isolated microsomes and microsomes incubated with buffer alone had phospholipid compositions that were identical. Addition of carbon tetrachloride and NADPH *in vitro* did not modify this composition (consistent with results *in vivo*). When phospholipids were isolated and assayed for diene conjugation all species were peroxidized, but PtdSer more so than any of the phospholipids (Table 3). Addition of NADPH alone, and in conjunction with Fe^{3+}/ADP, were done to assay for the specificity of the change. This resulted in more peroxidation of PtdSer but with a greater background effect. From the fatty acid analyses (Table 4) it is obvious that a widespread attack of the polyunsaturated fatty acids in each phospholipid species occurred, in marked contrast with the circumstances *in vivo*. Also, carbon tetrachloride with NADPH produces more diene conjugates and fatty acid loss than either NADPH alone or with Fe^{3+}/ADP, suggesting the possibility of a more reactive species. When the results of the system *in vivo* are compared with those of the one *in vitro*, significantly different phospholipid species are modified. This suggests that the system *in vitro* only partially mimics the changes *in vivo*. Further analysis of these differences is required.

Discussion

Available data point to an early alteration in structure and function of the endoplasmic reticulum after administration of a wide variety of hepatotoxins (Clifford & Rees, 1966; Svoboda & Higginson, 1968; Comporti *et al.*, 1971; Reynolds, 1972; Villarruel *et al.*, 1976). Specific attention has been directed toward lipid modification after carbon tetrachloride poisoning, and especially to a role for oxidative and/or free-radical-initiated changes. The early and extensive peroxidation found in the PtdSer fraction in the present study and the binding of a ^{14}C metabolite derived from carbon tetrachloride reported by Reynolds & Moslen (1974) and Benedetti *et al.* (1976) point to a focus of injury in this phospholipid. ^{14}C metabolites were preferentially localized to the PtdSer fraction with four times the frequency found in other phospholipid fractions (Reynolds & Moslen, 1974). The binding frequency corresponds with our findings on diene conjugation; PtdSer showed a 6-fold increase in A_{232} after carbon tetrachloride poisoning. Furthermore, the correlation of changes in lipid peroxidation and endoplasmic-reticulum binding of Ca^{2+} (Moore *et al.*, 1971), and the role for PtdSer in Ca^{2+} transport (Jacobson & Papahadjopoulos, 1978; Brownig & Seelig, 1980) suggests a relationship of the PtdSer change and functional defect. These results contrast with the conclusions drawn by others (Benedetti *et al.*, 1976). We believe that the concordance of ^{14}C binding to PtdSer and the techniques employed in the present paper indicate that the interpretations of Benedetti *et al.* (1976) were based on systems that masked the PtdSer change and resulted in the apparent PtdEtn change they reported.

The changes associated with the high specific activity observed for diene conjugation and the binding data are also reflected in fatty acid analysis of PtdSer. There is a marked early loss of $C_{18:1}$ and $C_{18:2}$ fatty acids and a small loss in the $C_{20:4}$ fatty acid during the first 15 min. Subsequently little further change in the $C_{18:1}$ and $C_{18:2}$ fatty acids occurred, but a linear reduction of $C_{20:4}$ fatty acid takes place during the remainder of the first hour. Other phospholipids showed insignificant changes in $C_{20:4}$ and $C_{22:6}$ fatty acids. Availability of double bonds for peroxidation cannot explain the focus of attack on the fatty acids in PtdSer, but rather suggests a functional and/or topological placement of the PtdSer and emphasizes the injury evolution concept of Slater (1976).

The temporal sequence of both the specific-activity changes in diene conjugation and the modification of fatty acids of the phospholipids point to a further complexity. First, the most significant increase in peroxidation (reflected as diene conjugation) seems to occur within 15 min of oral administration, and during this period changes in $C_{18:1}$ and $C_{18:2}$ fatty acids and, to a much smaller extent, $C_{20:4}$ fatty acid occur. Within the next 15 min the specific activity of peroxidation decreases, and concomitantly $C_{18:1}$ and $C_{18:2}$ fatty acids are no longer modified (and in fact may be healed), but $C_{20:4}$ fatty acid is lost. These changes are most pronounced in PtdSer, and seem restricted to this phospholipid. This points to a sequence of injury and potential partial repair. The initial change in $C_{18:1}$ and $C_{18:2}$ fatty acids appears to be connected with an early response potentially related to the 'proximate' toxin. The latter evolution of the $C_{20:4}$ fatty acid loss over a longer period of time seems to be a secondary phenomenon, but the relationship of this change to the similar fatty acid loss and to peroxidation is not clear. Finally the relationship between the $C_{20:4}$ fatty acid loss, PtdSer peroxidation and a specific cytochrome *P*-450 change suggests a topological juxtaposition between $C_{20:4}$ fatty acid, cytochrome *P*-450 and PtdSer. Indeed, $C_{20:4}$ fatty acid is lost when agents known to inactivate cytochrome *P*-450 without stimulating peroxidation are administered to rats (Comporti *et al.*, 1971; Villarruel *et al.*, 1976; Moody *et al.*, 1981).

When the data *in vivo* are compared with those obtained from an *'in vitro'* model, the relative amounts of the phospholipids do not change, but a higher level of peroxidation is noted in all the phospholipids. Although PtdSer is peroxidized to a higher specific activity *in vitro*, a background effect was evident in the other phospholipids. Peroxidizing systems *in vitro* using NADPH with or without carbon tetrachloride contain more or less Fe^{3+}, added as an ADP–Fe^{3+} complex or present inadvertently as an impurity in various buffer preparations or other additives. Recent evidence (Kornbrust & Mavis, 1980) indicates that Fe^{2+}, maintained in the reduced form by NADPH, initiates peroxidation of lipids along the microsomal membrane. When rat liver microsomes were prepared in the presence of EDTA to chelate ions (i.e., Fe^{2+}, Fe^{3+}, Cu^{2+}), then incubated *in vitro* with NADPH, there was no lipid peroxidation (Mason *et al.*, 1979; Kornbrust & Mavis, 1980). Our results *in vitro* seem to indicate that there is a more diffuse attack on the $C_{20:4}$ and $C_{22:6}$ fatty acids and this may reflect the presence of Fe^{2+}. When we added Fe^{3+}–ADP to our microsomal preparation, there was a slight increase in the extent of peroxidation; however, the pattern followed the same generalized background observed in the non-Fe^{3+}-supplemented system, which may indicate that Fe^{3+} was already present in these microsomes. It has been proposed that the NADPH/haloalkane system free of Fe^{2+} probably mirrors the situation *in vivo* more faithfully than do systems containing Fe^{2+} and it was suggested (Willis, 1980) that studies on NADPH/Fe^{2+} microsome systems should be compared with equivalent studies employing NADPH/haloalkane/microsome systems free of Fe^{2+}. This seems to be supported by our data.

These data, taken together, suggest that due to injury, carbon tetrachloride does produce an early peroxidative event, that a specific phospholipid (PtdSer) is involved and that a complexity of fatty acid targets exists. It follows that a structural topology of the microsomal membrane is present in which PtdSer, cytochrome *P*-450 and the site of carbon tetrachloride metabolism are in proximity. Further changes in $C_{18:1}$ and $C_{18:2}$ fatty acids, and in fact their partial restoration, may imply a healing of the injury with reconstitution of the membrane occurring during cell injury. Careful dissection of this process will require refinement of existing systems *in vitro* to reduce the field effect, and may provide a scheme for the topology of the hepatic endoplasmic reticulum.

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