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EFFECTS OF CALCIUM CHANNEL BLOCKING AGENTS ON MEMBRANE MICROVISCOSITY AND CALCIUM IN THE LIVER OF THE CARBON TETRACHLORIDE TREATED RAT

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Abstract—Membrane microviscosity was determined from the polarized fluorescence of diphenylhexatriene in plasma membranes and microsomes prepared from the liver of carbon tetrachloride treated rats. It was greatly depressed between 12 and 24 hr after the administration of the carbon tetrachloride. Depression of microviscosity was also seen in the liposomes which were prepared from these membranes. There were decreases in phospholipid content and phospholipid methyltransferase activity, but these changes did not appear to explain the decreased microviscosity. A large accumulation of calcium occurred in the liver cells between 12 and 24 hr after the administration of carbon tetrachloride. Chlorpromazine, verapamil and nifedipine, when administered prior to the carbon tetrachloride, partially reduced the later accumulation of calcium and reduced the degree of histological damage observed. When these agents were administered 12 hr after the administration of carbon tetrachloride, they did not reduce the subsequent accumulation of calcium. When administered prior to and 7 hr after carbon tetrachloride, they had a small but potentially significant effect on the microviscosity change. It is suggested that at low levels of microviscosity a critical threshold may exist below which entry of calcium into the cell is poorly controlled and that calcium channel blocking agents may be ineffective if administered at a time when membrane microviscosity is very low. Tissue calcium accumulation was associated with visible cell damage.

Increase of intracellular calcium is a potential mechanism of tissue cell injury following contact with toxic agents [1–10] or tissue anoxia [11, 12]. The administration of the hepatotoxic agent carbon tetrachloride at selected doses leads to a massive and apparently reversible accumulation of calcium by the liver beginning about 12 hr after the administration of the toxic agent [13–15]. The carbon tetrachloride is quickly metabolized after its administration and the metabolic activation of the compound in the rat liver is associated with peroxidation of lipids in the endoplasmic reticulum membranes and with the formation in the endoplasmic reticulum of free radicals which interact with cell constituents [16–19]. Within 1 hr calcium sequestering activity of liver microsomes is severely depressed [20, 21] and the calcium content of liver microsomes is diminished [20]. Hours after this initial metabolic activation of the administered carbon tetrachloride, there is a large increase in liver calcium content that suggests a critical change has occurred in the effectiveness of the liver plasma membrane as a barrier maintaining calcium homeostasis.

The polarized fluorescence of diphenylhexatriene (DPH), when incubated with cell membranes, has been widely employed as an experimental measure of the apparent microviscosity of cell membranes [22–27]. DPH was employed in the present study to determine if the hepatotoxic agent carbon tetrachloride changes the apparent microviscosity of the liver plasma membrane and whether the calcium accumulation that is found in liver cells poisoned by carbon tetrachloride is associated with changes in membrane microviscosity.

There are several chemical changes involving membrane phospholipids which may affect the microviscosity of the membrane lipids. A decrease in the ratio of cholesterol to phospholipid decreases the microviscosity [22]. Methylation of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine may decrease the membrane microviscosity [25–27]. An increase in unsaturated fatty acids contained in the phospholipid decreases the membrane microviscosity [22]. An increase in the ratio of phosphatidylethanolamine to phosphatidylcholine increases microviscosity [28]. Removal of protein from the phospholipid bilayer decreases the microviscosity [27].

In the present study, the microviscosity of the liver membrane and liver membrane lipids was compared with the levels of calcium that had accumulated in the rat liver cells. Several membrane phospholipid parameters were analyzed in the present study and were compared with the changes occurring in membrane lipid microviscosity. Calcium channel blocking agents were employed to reduce the calcium influx into the liver cells and to determine if modification of the calcium accumulation in the liver cell changes the effects of carbon tetrachloride on membrane microviscosity.

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MATERIALS AND METHODS

Rats used in these experiments were male Sprague–Dawley (200–400 g) from Sasco, Inc. (St. Louis, MO). Carbon tetrachloride was obtained from Fisher Scientific (Fair Lawn, NJ). Chlorpromazine HCl was purchased from the Sigma Chemical Co. (St. Louis, MO). Nifedipine and verapamil HCl were gifts from Pfizer, Inc. (Brooklyn, NY) and the Knoll Pharmaceutical Corp. (Whippany, NJ) respectively.

Sucrose used in the fractionation procedure was ultrapure (ribonuclease free) grade from Schwarz/Mann (Spring Valley, NY). *S*-[*methyl*-^{3}H]-Adenosyl-L-methionine ([^{3}H]SAM) was obtained from the New England Nuclear Corp. (Boston, MA) at 55–85 Ci/mmole in sulfuric acid (pH 2.0)–ethanol, 9:1. Aquasol and all other radiochemicals used in the assays were also purchased from the New England Nuclear Corp.

Standard phospholipids were used to identify methylated phospholipids on TLC. Phosphatidylcholine, phosphatidyl-*N*,*N*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine and phosphatidylethanolamine were purchased from the Grand Island Biological Co. (Grand Island, NY). Silica gel G plates for TLC were purchased from Fisher Scientific (Pittsburgh, PA). The fluorescent probe diphenylhexatriene (DPH) was obtained through the Aldrich Chemical Co., Inc. (Milwaukee, WI).

Drug administration. Carbon tetrachloride was given by stomach tube at one dosage, 2.5 ml/kg rat weight. Animals had free access to food and water throughout the experiments. Chlorpromazine HCl, verapamil HCl and nifedipine were given as intraperitoneal injections at 25 mk/kg rat weight using a 25 mg/ml solution in deionized water or ethanol (nifedipine only). Usually these calcium antagonists were injected 1 hr prior to carbon tetrachloride administration, but at times other schedules were used as indicated.

Preparation of livers. At different intervals (3, 12, 18 and 24 hr) after carbon tetrachloride administration the rats were decapitated. The livers were perfused *in situ* with 20 ml of isotonic sucrose before removal to a dish of ice-cold isotonic sucrose. An 8.0-g aliquot of the liver was homogenized with 20 ml of isotonic sucrose in a size C glass homogenizing vessel with a Teflon pestle using 10–12 strokes at 1000 rpm (on ice). The homogenate was filtered through four layers of gauze and centrifuged at 800 rpm (100 *g*) for 10 min. The homogenate supernatant was then fractionated by differential centrifugation.

Fractionation procedure. All fractions and reagents were kept at 0–4°. Low speed spins were performed in a refrigerated Sorvall RC-2 centrifuge using a SS-34 rotor. The 105,000 *g* spin was performed in a Beckman L2-65 ultracentrifuge using a Ti-50 rotor.

The homogenate supernatant was centrifuged at 2500 rpm (755 *g*) for 10 min to produce P_1. The P_1 supernatant was centrifuged at 10,000 rpm (12,100 *g*) for 10 min. The 10,000 rpm supernatant was centrifuged at 40,000 rpm (105,000 *g*) to produce the microsomal fraction. Microsomal subfractions were made by centrifuging the 10,000 rpm supernatant at 15,000 rpm (27,000 *g*) for 10 min to produce a heavy microsomal subfraction. The 15,000 rpm supernatant was then centrifuged at 40,000 rpm (105,000 *g*) for 60 min to produce a light microsomal subfraction.

The P_1 was fractionated by the procedure of Fitzpatrick *et al.* [29] which was designed to obtain a plasma membrane rich fraction. P_1 was resuspended in 2 M sucrose (1 ml/g original tissue) and was centrifuged at 10,500 rpm (13,300 *g*) for 10 min. The supernatant was brought back to isotonicity by slowly adding 7 vol. of cold deionized water while swirling. The expanded supernatant was centrifuged at 17,000 rpm (35,000 *g*) for 10 min to produce a biphasic pellet. Isotonic sucrose was used to remove the top (pink) pellet from the lower (brown) pellet. The resuspended upper pellet was recentrifuged at 17,000 rpm and served as the plasma membrane enriched fraction.

Calcium content. Calcium content of rat liver homogenates and subcellular fractions was determined by the method of Moore *et al.* [13], Homogenates or fractions were mixed 1:1 with 16% trichloroacetic acid and were centrifuged at 27,000 *g* for 10 min. The supernatants were made basic with 1.25 N KOH. The alkaline supernatants were titrated for calcium with 0.1 mM EDTA using calcein (Fisher Scientific) as end point indicator.

Microviscosity procedure. Rat liver particulate fractions or liposomes were labeled with DPH as described by Jaiswal *et al.* [27]. A solution of the probe (2×10^{-3} M) in tetrahydrofuran was diluted 1000-fold by injection into a vigorously stirred solution of 50 mM Tris–HCl, pH 7.4. One milliliter of 50 mM Tris–HCl, pH 7.4, containing 100 μg of particulate fraction protein or 2 ml or the same buffer containing a suspension of liposomes was vortexed to mix with an equal volume of the diluted probe solution and incubated for 1 hr at 24°.

Fluorescence measurements were made using an Aminco SPF-500 corrected spectrofluorometer equipped with a polarizing attachment. Fluorescence intensities (emission wavelength 430 nm, slit width 8 nm) were measured with polarizers parallel and perpendicular to the vertically polarized excitation (360 nm, slit width 5 nm) beam. Microviscosity at 24° was determined as described by Shinitzky and Barenholz [22].

Preparation of liposomes. Lipids were extracted from rat liver fractions (2.0 mg protein) by shaking for 15 min with 2.0 ml of chloroform–methanol–HCl (2:1:0.02). The extracts were washed twice with 1.0 ml of 0.1 M KCl in 50% methanol. The aqueous layer was aspirated and discarded each time. The chloroform layer was evaporated under nitrogen, and 2.0 ml of 50 mM Tris–HCl, pH 7.4, was added to the residue. The resulting suspension was sonicated for 15 min at 20° as described by Jaiswal *et al.* [27]. For microviscosity measurements, the resulting liposome suspension was treated as described above.

Extraction of lipids for cholesterol and phospholipid. The rat liver fractions (1.0 mg protein) were extracted by shaking the fractions for 15 min with 3.0 ml of chloroform–methanol–HCl (2:1:0.02). The extract was centrifuged at 12,100 *g* and 2.0 ml of the

resulting supernatant was transferred to another tube. The supernatant was then washed twice by 1.0 ml of 0.1 M KCl in 50% methanol, aspirating and discarding the aqueous layer each time. The chloroform layer was then evaporated under nitrogen, and the residue was redissolved in 1.0 ml of chloroform.

Phospholipid phosphorous assay. An aliquot of the chloroform extract was evaporated at 80°. The residue was digested by 2.5 ml of 0.02 N sulfuric acid for 3.5 h at 150–160°. Two drops of phosphate-free hydrogen peroxide were then carefully added and digestion was continued for another 1.5 hr. After cooling, phosphorous was determined by a modification of the Fiske–Subbarow method as described by Gradwohl [30]. Optical densities were read at 630 nm.

Cholesterol assay. An aliquot of the chloroform extract was evaporated at 80°. To the residue was added 1.0 ml of glacial acetic acid, 2.0 ml of *o*-phthaldehyde (50 mg/100 ml in glacial acetic acid), and 1.0 ml of concentrated sulfuric acid. The resulting mixture was then vortexed until thoroughly mixed. After 20 min the optical densities were read at 550 nm and compared with those of standard cholesterol solutions in glacial acetic acid.

Phospholipid methylation. The assay of phospholipid methyltransferase activity was carried out as follows. The phospholipid methylation incubation mixture (50 μl total volume) was composed of two parts: 10 μl of [^{3}H]SAM diluted to 1 μM final concentration in 100 mM Tris–glycylglycine buffer, pH 8.0, (containing 5 mM $MgCl_2$ and 0.1 mM EDTA) and 40 μl of 50 mM Tris–glycylglycine buffer, pH 8.0, (also with 5 mM MgCl and 0.1 mM EDTA) containing 200 μg of rat liver subcellular fraction protein. The incubation was started by adding the first part to the second in a 37° shaking water bath. After 30 min the reation was stopped by adding 3.0 ml of chloroform–methanol–HCl (2:1:0.02) and shaking for 10 min. The extract was then washed twice with 2.0 ml of 0.1 M KCl in 50% methanol, aspirating and discarding the aqueous layer each time. An aliquot of the chloroform layer was evaporated to dryness and counted in 10 ml of Aquasol to determine total methyl group incorporation. An equal aliquot was analyzed by thin-layer chromatography on silica gel G plates described by Jaiswal *et al.* [27]. Standard phospholipids were chromatographed simultaneously. The spots were visualized by iodine vapor. The percentage of the total methyl group radioactivity on the thin-layer plate corresponding to each individual phospholipid was determined. Approximately 10% of the methyl group radioactivity on the plate was not associated with phospholipid. The radioactivity was expressed as fmoles of product formed/μg protein.

Other assays. Protein was estimated according to the method of Lowry *et al.* [31] using bovine serum albumin as a standard. Cholinephosphotransferase was assayed using the method of Wuytack *et al.* [32]. For histologic estimate of cell damage caused by carbon tetrachloride, liver lobes were fixed in formalin, sectioned and stained with hematoxylin and eosin. Statistical analysis of the data in this study employed Student's *t*-test and where applicable Duncan's new multiple range test [33].

RESULTS

The polarized fluorescence of DPH was measured in the liver plasma membrane and in two subfractions of liver microsomes that were prepared from male Sprague–Dawley rats receiving carbon tetrachloride. There was a progressive decline in microviscosity of these membranes between 0 and 18 hr after the administration of carbon tetrachloride (Fig. 1). The decrease in microviscosity at 18 hr was a constant

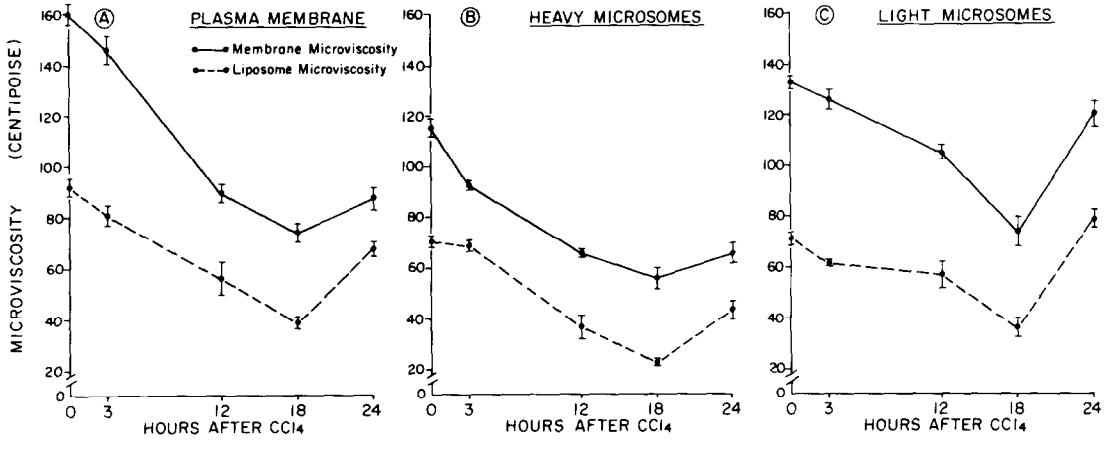

Fig. 1. Microviscosity of rat liver membrane fractions and extracted liposomes prepared at various intervals after CCl_4 administration (2.5 ml/kg by stomach tube). Microviscosity was evaluated after incubating the membranes or liposomes with the fluorescent probe diphenylhexatriene (1 μM final concentration) as described in Materials and Methods. The bars represent S.E. of twelve or more individual animals (0 and 24 hr) and five or more individual animals (3, 12 and 18 hr after CCl_4). The following differences in microviscosity with time were statistically significant ($P < 0.01$ with Student's *t*-test or $P < 0.05$ with Duncan's new multiple range test): plasma membrane and light microsomes 0 to 12 hr, 12 to 18 hr and 18 to 24 hr; heavy microsomes 0 to 12, 18 and 24 hr.

Table 1. Effects of carbon tetrachloride administration (2.5 ml/kg) on total phospholipid and cholesterol content of rat liver membrane fractions*

	Phospholipid (μmoles/mg protein)			Cholesterol (μmoles/mg protein)		
	Plasma membrane	Heavy microsomes	Light microsomes	Plasma membrane	Heavy microsomes	Light microsomes
Control	0.33 ± 0.016 (10)	0.27 ± 0.009 (22)	0.40 ± 0.004 (18)	0.186 ± 0.004 (7)	0.135 ± 0.006 (7)	0.138 ± 0.002 (6)
18 Hr post CCl_4	0.30 ± 0.009 (8)	0.28 ± 0.009 (3)	0.33 ± 0.01† (8)	0.178 ± 0.002 (3)		
24 Hr post CCl_4	0.26 ± 0.008† (12)	0.26 ± 0.017 (13)	0.29 ± 0.006† (12)	0.184 ± 0.008 (9)	0.138 ± 0.007 (4)	0.135 ± 0.002 (7)

* Values presented are means ± S.E. of (N) individual animals.
† Signifies $P < 0.01$ for experimental vs control.

fraction of the control when membrane microviscosity was measured over a temperature range between 10° and 40°. There appeared to be some gain in microviscosity between 18 and 24 hr after the carbon tetrachloride. The lipids were extracted from the isolated membrane fractions and liposomes were prepared. Liposomal microviscosity was about 60% of that of the intact membrane. A decline in microviscosity of the liposomes following administration of the carbon tetrachloride was very similar to that of the intact membrane (Fig. 1).

Phospholipid and cholesterol contents of the membranes are presented in Table 1. Phospholipid of plasma membrane was decreased significantly (21%) at 24 hr after the administration of carbon tetrachloride and the phospholipid of light microsomes was decreased significantly (18%) at 18 hr and (28%) at 24 hr after the administration of carbon tetrachloride. The cholesterol of the membrane preparations remained unchanged.

Synthesis of phosphatidylcholine in rat liver is principally via a pathway involving cholinephosphotransferase [34]. Choline phosphotransferase activity in rat liver homogenates was unchanged at 12 hr and was decreased 54 ± 7% of control (N = 11) at 24 hr after the administration of carbon tetrachloride.

Phospholipid methyltransferase activity contributes significantly to phosphatidylcholine formation in the liver by methylating phosphatidylethanolamine to phosphatidylcholine [35]. Phospholipid methyltransferase activity in isolated membrane fractions was diminished about 16% at 12 hr after carbon tetrachloride administration and was decreased between 30 and 40% at 24 hr after the administration of carbon tetrachloride (Table 2). The formation of all three methylated products was reduced.

Accumulation of calcium in the liver following the administration of carbon tetrachloride was measured for purposes of comparison with changes in the microviscosity of membranes (Fig. 2). As noted in an earlier study [13], a very large influx of calcium occurred between 12 and 24 hr. The largest fraction of this influx occurred between 12 and 18 hr. The initial calcium level was somewhat lower than in the previous study and this was found to be a consequence of perfusing the liver with isotonic sucrose prior to the homogenization.

Table 2. Effects of carbon tetrachloride administration (2.5 ml/kg) on phospholipid methylation in rat liver membrane fractions*

	N	*N*-Monomethyl-phosphatidyl-ethanolamine	*N*-Dimethyl-phosphatidyl-ethanolamine	Phosphatidyl-choline
		(fmoles product formed/μg protein/30 min)		
Plasma membrane				
Control	13	24 ± 0.5	28 ± 0.6	14.7 ± 0.3
3 Hr post CCl_4	5	21 ± 1.6	24 ± 0.8	16 ± 1.3
12 Hr post CCl_4	4	20 ± 0.5†	23 ± 0.6†	12 ± 0.3†
24 Hr post CCl_4	6	14 ± 0.5†	18 ± 0.6†	8.7 ± 0.3†
Microsomes				
Control	13	26 ± 0.5	26 ± 0.5	13 ± 0.2
3 Hr post CCl_4	7	24 ± 1.4	24 ± 1.4	12.3 ± 0.7
12 Hr post CCl_4	4	22 ± 0.8†	22 ± 0.9†	12 ± 0.45
24 Hr post CCl_4	6	18 ± 0.8†	20.5 ± 0.6†	9.7 ± 0.3†

* Membrane fractions were incubated at 37° in the presence of 1 μM SAM at pH 8.0. Values presented are means ± S.E. of N individual animals.
† $P < 0.01$ for experimental control.

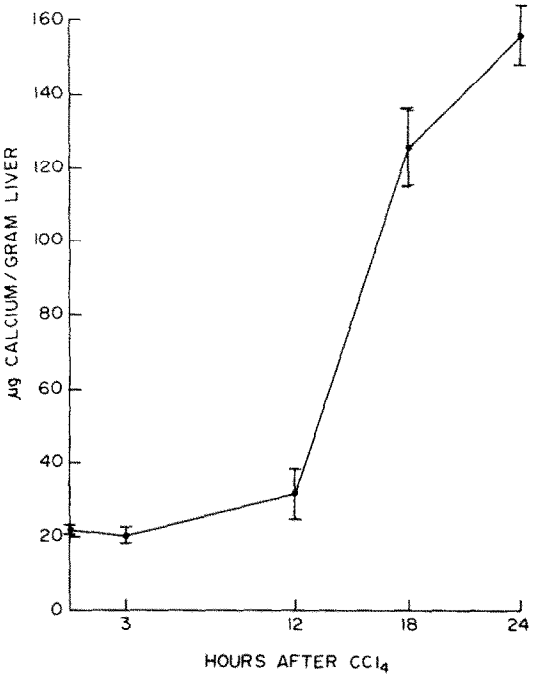

Fig. 2. Calcium content of rat liver homogenates (μg calcium/g liver) as a function of time after carbon tetrachloride administration. Carbon tetrachloride was given (2.5 ml/kg) by stomach tube and the rats were killed at the designated times after administration. Calcium was determined as described in Materials and Methods. Each point represents mean ± S.E of N individual animals (N = 37, 10, 7, 17 and 39 at 0, 3, 12, 18 and 24 hr). P is less than 0.01 for difference between calcium content at 12 and 18 hr. P is less than 0.05 for difference between calcium content at 18 and 24 hr. Analysis by Student's *t*-test.

Administration of the calcium channel blocking agents verapamil, nifedipine or chlorpromazine partially reduced the calcium accumulation when administered as a single dose 1 hr prior to the carbon tetrachloride or with an additional dose at 7 hr after the carbon tetrachloride (Table 3). When these agents were administered at a single dose 12 hr after the carbon tetrachloride, at a time when the microviscosity was much decreased and large scale calcium accumulation was beginning, there was no effect on the calcium accumulation (Table 3).

Administration of chlorpromazine 1 hr prior to the administration of carbon tetrachloride did not interfere with the fall in microviscosity of the membrane fraction at 18 hr or in microviscosity of liposomes prepared from the membrane fractions (Fig. 3). The single dose of chlorpromazine diminished the secondary rise in microviscosity between 18 and 24 hr.

Administration of chlorpromazine verapamil and nifedipine in two doses 1 hr prior to and 7 hr after the carbon tetrachloride reduced calcium accumulation considerably (Table 3). The large fall in plasma membrane microviscosity at 18 hr was reduced about 20% (Table 4). The mean microviscosity values after the two doses of these agents were similar to the microviscosity in the untreated control 12 hr after the carbon tetrachloride and prior to the onset of calcium accumulation. This change may be important if a critical microviscosity threshold exists for the control of calcium entry.

Histologic examination of the rat livers 24 hr after carbon tetrachloride revealed a moderate degree of centrilobular necrosis. Six livers of each treatment group were examined histologically. Approximately 13% of the cells were necrotic and 15% of the cells showed some swelling or fatty infiltrate. At 12 hr

Table 3. Effects of calcium channel blockers (25 mg/kg) on calcium content of rat liver 24 hr after carbon tetrachloride administration (2.5 ml/kg)*

	Calcium (μg/g liver)			
	No drug	Chlorpromazine	Verapamil	Nifedipine
0 Hr				
no drug, no CCl_4	21.4 ± 0.9 (37)			
24 Hr after CCl_4				
Drug administered 1 hr prior to CCl_4	156 ± 5.4† (39)	113 ± 6.8‡ (20)	101 ± 17‡ (10)	112 ± 12.6‡ (5)
Drug administered, CCl_4 omitted		21.5 ± 0.9 (11)	22 ± 1.4 (2)	21 ± 1.3 (2)
Drug administered 1 Hr prior to and 7 hr post CCl_4		86.8 ± 18.3‡ (10)	71 ± 3.6‡ (7)	39 ± 1.7‡ (12)
Drug administered, CCl_4 omitted		24.2 ± 1.3 (4)	23.7 ± 8 (4)	23.6 ± 2.1 (4)
Drug administered 12 hr post CCl_4		159 ± 14.1 (7)	167 ± 8 (8)	157 ± 17 (6)

* Values presented are mean ± S.E. of (N) individual animals. Statistical analysis employed both Student's *t*-test and Duncan's new multiple range test.

† $P < 0.01$ vs control at 0 hr.

‡ $P < 0.01$ vs value for no drug (156 ± 5.4) at 24 hr post CCl_4.

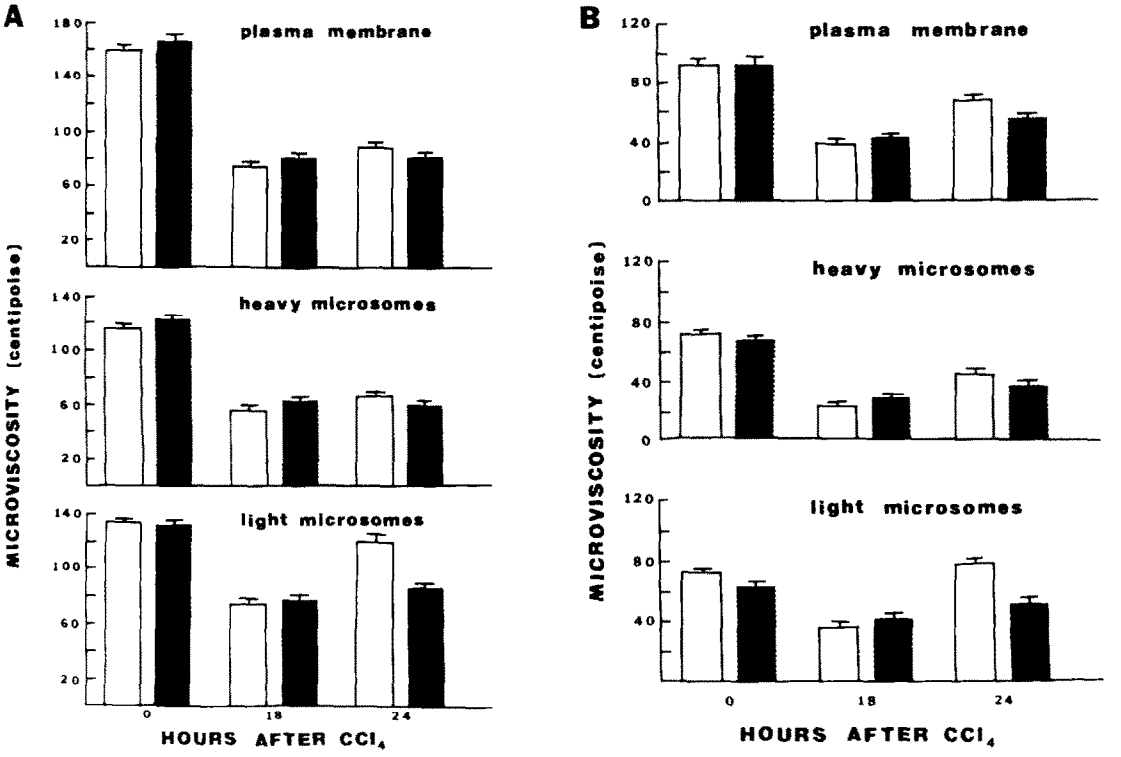

Fig. 3. Effects of chlorpromazine on microviscosity of rat liver membrane fractions (A) and extracted liposomes (B) prepared at various intervals after CCl_4 administration (2.5 ml/kg by stomach tube). Microviscosity was evaluated after incubating the membranes or liposomes with the fluorescent probe diphenylhexatriene (1 μM final concentration) as described in Materials and Methods. Open bars represent no pretreatment and shaded bars represent chlorpromazine pretreatment (25 mg/kg) 1 hr prior to CCl_4 administration. Values presented are means ± S.E. of nine or more individual animals (0 and 24 hr) and five or more individual animals 18 hr after CCl_4). The chlorpromazine diminished the secondary rise in microviscosity at 24 hr of the light microsomal membranes and liposomes and the plasma membrane liposomes. P was less than 0.01 with Student's *t*-test.

after carbon tetrachloride only 4% of the cells were necrotic. Chlorpromazine (two doses) and nifedipine (two doses) eliminated cell necrosis at 24 hr (less than 1% cell necrosis) and reduced the number of swollen cells at 24 hr. Verapamil (two doses) diminished the cell necrosis at 24 hr after carbon tetrachloride (2–4% cell necrosis).

DISCUSSION

A large fall in membrane microviscosity as measured by DPH polarized fluorescence was observed at 12–18 hr after the administration of carbon tetrachloride. This microviscosity change was also found in the liposomes, a mixture of neutral lipids and of phospholipids which were isolated from the membrane fractions. The microviscosity change was observed in several membrane fractions making it very unlikely that an undetected artifact in membrane fractionation was involved. The protein recovery in membrane fractions as percentage of total appeared consistent at all time periods. The initial microviscosity of the plasma membrane preparation was considerably higher than that of the microsomes and the subsequent decrease in microviscosity was

Table 4. Effects of calcium channel blockers (25 mg/kg) on microviscosity of rat liver plasma membrane 18 hr after carbon tetrachloride administration (25 mg/kg)*

	Microviscosity (centipoise) No CCl_4†	18 Hr post CCl_4
No drug	160.5 ± 4.1 (14)	73.2 ± 2.3 (8)
Chlorpromazine 1 hr pre CCl_4	168.0 ± 4.0 (7)	79.4 ± 4.2 (7)
Chlorpromazine 1 hr pre and 7 hr post CCl4	172.2 ± 8.0 (5)	91.3 ± 4.8‡ (8)
Verapamil 1 hr pre and 7 hr post CCl_4	173.1 ± 5.3 (8)	87 ± 6 (5)
Nifedipine 1 hr pre and 7 hr post CCl_4	160.3 ± 7.5 (8)	95 ± 5‡ (12)

* Values presented are mean ± S.E. of (N) individual animals. Statistical analysis employed both Student's *t*-test and Duncan's new multiple range test.

† Calcium channel blockers were given and animals were killed on the same schedule as animals given CCl_4.

‡ $P < 0.01$ vs value for no drug (73.2 ± 2.3) at 18 hr post CCl_4.

greater in that fraction. Phospholipid content was highest in the light subfraction of microsomes.

The effect of phospholipid changes on microviscosity is explained in the introduction. The phospholipid changes seen in the present study would favor an increase in microviscosity. The cholesterol to phospholipid ratio was increased at 24 hr. There was an apparent decrease in the capacity to form phospholipid and the methylated intermediate phosphatidyl-*N*-monomethylethanolamine when cholinephosphotransferase and phospholipid methyltransferase activities were measured *in vitro*. Exposure to carbon tetrachloride also decreases the relative unsaturated fatty acid content of the liver phospholipid [36] and increases the ratio of phosphatidylethanolamine to phosphatidylcholine [37].

Products of phospholipid breakdown which accumulate in the cytosol may be responsible for the change in microviscosity of the membranes. Carbon tetrachloride causes lipid peroxidation in microsomal membranes and the liberation of long chain aldehydes [38]. Carbon tetrachloride also activates phospholipase C leading to breakdown of phospholipid [39].

There is an additional consideration which does involve phospholipid methylation. It has been reported that addition of calcium (10 μM) and ATP (10 μM) to liver microsomes doubles the rate of methylation of phospholipids and this effect is mediated by calmodulin [40]. It is conceivable that after carbon tetrachloride administration early cytosol calcium increases associated with the loss of calcium from the endoplasmic reticulum [20] would activate phospholipid methylation and contribute to a decrease in microviscosity of membranes. This increase in phospholipid methylation activity in the intact cell would not be detected in the *in vitro* assay of isolated membranes.

The microviscosity change is probably not secondary to the large increase in calcium between 12 and 24 hr after the administration of carbon tetrachloride. Most of the microviscosity change occurred before the large calcium increase. The large accumulation of calcium coincided with the lowest microviscosity levels.

It is not clear whether the observed microviscosity change contributed to the large net influx of calcium taking place between 12 and 24 hr after the administration of carbon tetrachloride. The cells appear to tolerate a considerable fall in microviscosity before a large scale entry of calcium occurs. The microviscosity change may be an associated phenomenon perhaps related to lipid peroxidation and play a relatively minor role. Alternatively, a critical threshold level of microviscosity may exist which is essential for the adequate control of calcium entry. The microviscosity may pass below this critical level 12 hr after the administration of carbon tetrachloride and this may facilitate entry of calcium.

Two doses of chlorpromazine, verapamil and nifedipine appear to have diminished the fall in microviscosity at 18 hr. This relatively small effect may be functionally significant if it is postulated that there exists a critical threshold below which the membrane barrier against calcium fails.

Also unexplained is how calcium channel blocking agents, administered 1 hr before the carbon tetrachloride, reduced the calcium influx 12 hr later. Experimentally liver microsomes lower free calcium to a 0.2 μM level [41]. Intracellular calcium regulation by the endoplasmic reticulum of liver is impaired within 30 min after the administration of carbon tetrachloride and free cytosol calcium levels in the liver probably increase. An increase of free cytosol calcium may, in turn, trigger other intracellular events. The calcium channel blocking agents by reducing influx of external calcium would mitigate this *postulated* increase in cytosol calcium and the ultimate consequences of an initial perturbation of calcium regulation.

Calcium channel blocking agents given 12 hr after the carbon tetrachloride had no effect on the increase in calcium content of the liver cells that begins at that time. The membrane microviscosity is much reduced at 12 hr. This may imply that these pharmacological agents are ineffective if the membrane microviscosity is too low. Microviscosity may be a consideration of practical importance in the clinical use of calcium channel blocking agents. A low microviscosity may simply lead to the formation of channels in the membrane which are not amenable to control by calcium channel blocking agents.

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REFERENCES

1. F. A. Schanne, A. B. Kane, E. E. Young and J. L. Farber, *Science* **206**, 700 (1979).
2. A. B. Kane, E. E. Young, F. A. Schanne and J. L. Farber, *Proc. natn. Acad. Sci. U.S.A.* **77**, 1177 (1980).
3. J. L. Farber and S. K. El-Mofty, *Am. J. Path.* **81**, 237 (1975).
4. S. K. El-Mofty, M. C. Scrutton, A. Serroni, C. Nicolini and J. L. Farber, *Am. J. Path.* **79**, 579 (1975).
5. R. E. Thiers, E. S. Reynolds and B. L. Vallee, *J. biol. Chem.* **235**, 2130 (1960).
6. J. D. Judah, K. Ahmed and A. E. M. McLean, in *Ciba Foundation Symposium on Cellular Injury* (Eds. A. V. S. de Reuck and J. Knight), pp. 187–205. J. & A. Churchill, London (1964).
7. J. D. Judah, *Br. med. Bull.* **25**, 274 (1967).
8. A. E. M. McLean, E. McLean and J. D. Judah, *Int. Rev. expl. Path.* **4**, 127 (1965).
9. A. F. Casini and J. L. Farber, *Am. J. Path.* **105**, 138 (1981).
10. J. L. Farber, K. R. Chien and S. Mittnacht, Jr., *Am. J. Path.* **102**, 271 (1981).
11. K. R. Chien, J. Abrams, A. Serrani, J. Martin and J. L. Farber, *J. biol. Chem.* **253**, 4809 (1978).
12. K. R. Chien, R. G. Pfau and J. L. Farber, *Am. J. Path.* **97**, 505 (1979).
13. L. Moore, R. G. Davenport and E. J. Landon, *J. biol. Chem.* **251**, 1197 (1976).
14. D. M. Silverman and D. R. Tavis, *Toxic. appl. Pharmac.* **61**, 172 (1981).
15. E. S. Reynolds, *J. Cell Biol.* **19**, 139 (1963).
16. G. L. Plaa and H. Witschi, *A. Rev. Pharmac. Toxic.* **16**, 125 (1976).
17. E. S. Reynolds and H. J. Ree, *Lab. Invest.* **25**, 269 (1971).

18. E. A. Glende, Jr., A. M. Hruszkewycz and R. O. Recknagel, *Biochem. Pharmac.* **25**, 2163 (1976).
19. S. Z. Cagen and C. D. Klaasen, *Fedn. Proc.* **39**, 3124 (1980).
20. L. Moore, *Biochem. Pharmac.* **29**, 2505 (1980).
21. K. L. Lowry, E. A. Glende and R. O. Recknagel, *Biochem. Pharmac.* **30**, 135 (1981).
22. M. Shinitzky and Y. Barenholz, *Biochim. biophys. Acta* **515**, 367 (1978).
23. V. Chauhan, S. C. Sikka and V. K. Kalra, *Biochim. biophys. Acta* **688**, 357 (1982).
24. S. M. Johnson and R. Robinson, *Biochim. biophys. Acta* **558**, 282 (1979).
25. F. Hirata and J. Axelrod, *Nature, Lond.* **275**, 219 (1978).
26. B. V. R. Sastry, C. N. Statham, R. G. Meeks and J. Axelrod, *Pharmacology*, **23**, 211 (1981).
27. R. K. Jaiswal, B. V. R. Sastry and E. J. Landon, *Pharmacology*, **24**, 355 (1982).
28. J. D. Esko, J. R. Gilmore and M. Glaser, *Biochemistry* **16**, 1881 (1977).
29. D. F. Fitzpatrick, G. R. Davenport, L. Forte and E. J. Landon, *J. biol. Chem.* **244**, 3561 (1969).
30. *Gradwohl's Clinical Laboratory Methods and Diagnosis* (Eds. S. Frankel, S. Reitman and C. Sonnenwirth), 7th Edn, p. 185. The C. V. Mosby Co., Saint Louis (1970).
31. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
32. F. Wuytack, E. J. Landon, S. Fleischer and J. G. Hardman, *Biochem. biophys. Acta* **540**, 253 (1978).
33. H. L. Alder and E. R. Roessler, *Probability and Statistics*, pp. 301–3. W. H. Freeman, San Francisco (1972).
34. E. G. Kennedy and S. B. Weiss, *J. biol. Chem.* **222**, 193 (1965).
35. R. Sundler and B. Akesson, *J. biol. Chem.* **250**, 3359 (1975).
36. C. C. Weddle, R. R. Hornbrook and P. N. McCay, *J. biol. Chem.* **251**, 4973 (1976).
37. M. Sugano, S. Cho, K. Imaizumi and M. Wada, *Biochem. Pharmac.* **19**, 2325 (1970).
38. A. Benedetti, M. Comporti and H. Esterbauer, *Biochem. biophys. Acta* **620**, 281 (1980).
39. R. G. Lamb and D. W. Schwartz, *Toxic. appl. Pharmac.* **63**, 216 (1982).
40. S. Alemany, I. Varela, J. Harper and J. Mato, *J. biol. Chem.* **257**, 9249 (1982).
41. G. L. Becker, G. Fiskum and A. L. Lehninger, *J. biol. Chem.* **255**, 9009 (1980).