Biochemical Pharmacology, Vol. 30, pp. 135–140.

0006-2952/81/0115-935 $02.00/0

DESTRUCTION OF LIVER MICROSOMAL CALCIUM PUMP ACTIVITY BY CARBON TETRACHLORIDE AND BROMOTRICHLOROMETHANE*

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(*Received* 1 *May* 1980; *accepted* 15 *July* 1980)

Abstract—Disturbed cellular calcium homeostatis has been observed during carbon tetrachloride (CCl_4) poisoning, with large alterations in calcium content occurring 8 hr after administration. Moore *et al.* [10] have shown that the hepatic smooth endoplasmic reticulum can sequester calcium and that this ability is decreased severely within 30 min after CCl_4 administration to rats. It was suggested that disturbed endoplasmic reticulum calcium pump activity may have a critical role in the expression of CCl_4 hepatotoxicity. We examined the effect of bromotrichloromethane ($BrCCl_3$) and CCl_4 metabolism on the calcium pump of Fe^{2+}-free rat liver microsomes. It was determined that severe deficits in calcium uptake can be correlated with minimal lipid peroxidation induced by these agents. At a given level of lipid peroxidation, calcium uptake was affected more severely than were the activities of the microsomal enzymes glucose-6-phosphatase and aminopyrine demethylase. Calcium uptake was increased 7-fold by the presence of 5 mM ATP in incubations prior to assay of calcium sequestration. Lipid peroxidation induced by $BrCCl_3$-NADPH was accompanied by leakage of calcium from calcium-loaded microsomes. These results strengthen the possibility that disturbances in intracellular calcium homeostasis may be a key event in liver injury induced by $BrCCl_3$ and CCl_4.

Perturbation of cellular calcium homeostasis has been documented in a number of diverse pathological phenomena including ischemic cell injury [1], adriamycin-induced cardiomyopathy [2], acute renal uranium intoxication [3], and thioacetamide intoxication in the liver [4]. Pathological excess of intracellular calcium has been implicated as a final common pathway of cell death produced by a wide range of toxins including the hepatotoxin carbon tetrachloride (CCl_4) [5, 6].

For the case of CCl_4 poisoning, the pattern of alterations in whole liver calcium content has been documented. Typically, there is a modest increase in calcium content during the first 8–12 hr after administration. Some workers [7–9] have observed a transient 2- to 3-fold elevation of calcium content during the first and second hours, while others [10] have observed a linear increase during this period. After 8–12 hr, a rapid increase in calcium content is noted, peaking between 24 and 36 hr, and reaching seven to twenty times normal levels [7–10]. These large alterations in cellular calcium content during CCl_4 poisoning occur well after the initial events critical to manifestation of toxicity [8]. Recently, attention has focused on hepatic calcium homeostasis in the early hours of intoxication. Moore *et al.* [11] have reported that the hepatic endoplasmic reticulum has the ability to sequester calcium and that a deficit in this function occurs as early as 30 min after CCl_4 administration [10]. Although the precise role of the endoplasmic reticulum in calcium regulation is unknown, it was suggested that this early alteration in a calcium homeostatic function might be an important intermediate step in pathological processes induced by CCl_4.

The present study shows that microsomal calcium sequestration is extremely sensitive to the metabolism of bromotrichloromethane ($BrCCl_3$) and CCl_4 *in vitro*. The incubation conditions employed promoted minimal but significant levels of lipid peroxidation. The results support the possibility that disturbed calcium homeostasis may be a significant intermediate event standing between $BrCCl_3$ or CCl_4 metabolism and the overt manifestations of toxigenic haloalkane liver cell injury.

METHODS

Materials. Animals used in this study were male, Sprague–Dawley rats between 200 and 450 g, supplied by Zivic–Miller Laboratories Inc. (Allison Park, PA). NADP, ATP, isocitrate, and isocitric dehydrogenase (EC 1.1.1.42) were supplied by the Sigma Chemical Co. (St. Louis, MO). $^{45}Ca^{2+}$ was obtained as aqueous $^{45}CaCl_2$ from the New England Nuclear Corp. (Boston, MA).

Microsome preparation. To prepare microsomes, liver samples from decapitated rats were homogenized with ice-cold 0.154 M KCl containing 3 mM EDTA at pH 7.4, using a Teflon-glass Potter–Elvehjem homogenizer. The whole homogenate was centrifuged at 2700 *g* for 10 min, in a Sorvall RC2-B centrifuge at 4°. The supernatant fraction was centrifuged for 30 min at 80,000 *g* and 4° in a Spinco

* This work was supported by Grant 9-R01-ES01821 from the National Institute of Environmental Health Sciences, NIH, USPHS. K.L. was supported by a NSF graduate fellowship.

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ultracentrifuge. The microsomal pellet was resuspended in an appropriate volume of cold Tris–maleate buffer (0.05 M Tris, 0.05 M maleate, 0.1 M KCl, pH 7.4).

Experimental procedures. Experiments concerned with the actions of $BrCCl_3$ or CCl_4 on the calcium uptake system of microsomes employed two sequential incubations. For the first, fresh microsomes were incubated with a range of haloalkane concentrations in the presence of NADPH. Incubation mixtures for the first step contained microsomes at a concentration of 1.0 to 1.2 mg protein/ml, and reagents for NADPH generation [100 μM NADP, 0.07 units (Sigma) isocitric dehydrogenase/ml, 2.5 mM nicotinamide, 5 mM $MgCl_2$, and 3 mM D,L-isocitrate] dissolved in buffer. $BrCCl_3$ was added to the incubation medium dissolved in heptane. The total volume of heptane added with $BrCCl_3$ was 0.05 to 0.5 μl/ml of incubation medium, and the final concentration of $BrCCl_3$ in the medium was 0.5 to 50 nl/ml. In experiments performed to compare the action of $BrCCl_3$ to CCl_4, CCl_4 was added undiluted to a final concentration of 10–100 nl/ml incubate. Addition of 5 mM ATP in some experiments (see below) was an important feature of the work. Incubation was initiated by addition of $BrCCl_3$ or CCl_4 to ice-cold microsomal suspensions. Flasks were sealed and incubated at 37° for 30 min. Samples for determination of malonic dialdehyde (MDA), an index of lipid peroxidation, were assayed by the thiobarbituric acid method [12].

After the primary incubation with $BrCCl_3$ or CCl_4, microsomes were recovered by centrifugation at 4° and 80,000 *g* for 30 min. Very little further MDA formation occurred during recovery of the microsomes. Microsomal pellets were resuspended in imidazole–histidine buffer (30 mM imidazole, 30 mM histidine, 100 mM KCl, pH 6.8) and assayed for calcium uptake in the system of Moore *et al.* [11]. Briefly, microsomes at a protein concentration of 0.1 to 0.15 mg/ml were incubated with 5 mM NaN_3, 5 mM $MgCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 20 μM $CaCl_2$ and $^{45}CaCl_2$ (0.1 to 0.15 μCi/ml) in imidazole–histidine buffer at 37° for 60 min. Microsomes were filtered under house vacuum, on 0.2 μm cellulose triacetate filters (Metricel) wetted with buffer, and rinsed immediately with 10 ml of buffer. Filters were air dried and immersed in scintillant (Scintisol, Isolab Corp., Akron, OH). Radioactivity was monitored by a Packard 2002 liquid scintillation counter at room temperature. Counts were corrected for quenching (3–10 per cent) by the channels-ratio method.

In other experiments, the order of haloalkane exposure and calcium loading was reversed. Microsomes at a concentration of 0.2 mg protein/ml were loaded with labeled calcium by incubation with 100 μM $CaCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 10 mM $MgCl_2$, 5 mM NaN_3 and $^{45}CaCl_2$ (0.6 μCi/ml) for 60 min at 37°. Microsomes were recovered by centrifugation at 80,000 *g* for 30 min at 4°, and resuspended in imidazole–histidine buffer. The calcium-loaded microsomes were then incubated with NADPH and either $BrCCl_3$ or $FeSO_4$; both of the latter are potent pro-oxidants. Samples were incubated at a concentration of 0.3 to 0.4 mg protein/ml for 50 min with $BrCCl_3$ (0.07 μl/ml of incubation medium) and the reagents for NADPH generation [0.14 mM NADP, 2.5 mM nicotinamide, 3 mM (D,L)-isocitrate, 0.07 units (Sigma) isocitric dehydrogenase/ml, 5 mM $MgCl_2$]. For this $BrCCl_3$–NADPH system, 0.1 mM EDTA was also included to exclude any possible peroxidation induced by iron contaminants. In parallel experiments, lipid peroxidation was initiated by 40 μM $FeSO_4$ in the presence of an NADPH-generating system as given immediately above. Samples for the determination of ^{45}Ca content and malonic dialdehyde production were taken in the manner described above.

For some experiments, microsomes sequestering calcium were exposed to NADPH plus $BrCCl_3$. Microsomes (0.2 mg protein/ml) were incubated with the reagents for calcium uptake [100 μM $CaCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 10 mM $MgCl_2$, 5 mM NaN_3, and $^{45}CaCl_2$ (0.8 μCi/ml] and for NADPH generation [0.14 mM NADP, 2.5 mM nicotinamide, 3 mM (D,L)-isocitrate, 0.07 units (Sigma) isocitric dehydrogenase/ml]. Samples were taken for malonic dialdehyde and ^{45}Ca determination during the incubation as described above. At 12 min, either $BrCCl_3$ or $FeSO_4$ was added (0.06 μl $BrCCl_3$/ml or $FeSO_4$ at a final concentration of 22 μM) and incubation continued for 75 min.

Enzyme assays. Microsomes exposed to $BrCCl_3$–NADPH were also assayed for glucose-6-phosphatase (EC 3.1.3.9) and aminopyrine demethylase activity. Glucose-6-phosphatase activity was measured by incubating microsomes (0.15 mg protein/ml) with glucose-6-phosphate (35 mM) in imidazole–histidine buffer for 30 min at 37°. Inorganic phosphate was measured by the method of Fiske and Subbarow [13]. To measure aminopyrine demethylase activity, microsomes (0.5 to 0.6 mg protein/ml) were incubated in Tris–maleate buffer with aminopyrine (9 mM) and an NADPH-generating system as outlined above for 30 min at 37°. One-ml aliquots were placed in 0.5 ml of 15% trichloroacetic acid and centrifuged at 1000 *g* for 10 min. The supernatant fractions were assayed for formaldehyde by the method given by Nash [14]. Protein was assayed after the method of Lowry *et al.* [15] using bovine serum albumin as standard.

RESULTS

Rat liver microsomes prepared in a medium containing EDTA to remove iron contaminants did not peroxidize in the presence of either NADPH or $BrCCl_3$, but when both NADPH and $BrCCl_3$ were present peroxidation was observed (Fig. 1). As can be seen, the degree of lipid peroxidation was directly related to the amount of $BrCCl_3$ added; hence, the extent of lipid peroxidation could be controlled by the amount of $BrCCl_3$ used. This experiment was repeated using CCl_4 as the pro-oxidant. Results followed the same trend as for $BrCCl_3$ except that CCl_4 was about one-third as potent as $BrCCl_3$ per unit volume (data not shown). It should be emphasized that the MDA yield in the $BrCCl_3$–NADPH system (Fig. 1) was much lower than for Fe^{2+}-NADPH-dependent lipid peroxidation. For example, in experiments with 40 μM $FeSO_4$ (final concentration)

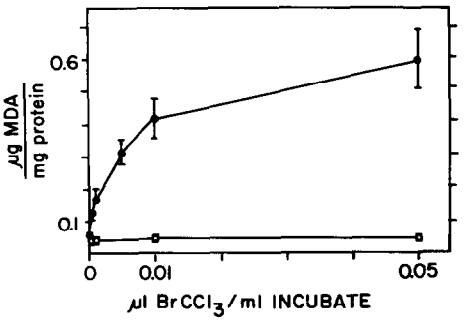

Fig. 1. Bromotrichloromethane–NADPH-dependent lipid peroxidation in liver microsomes prepared in the presence of EDTA. Liver microsomes were incubated at 37° for 30 min in 0.10 M KCl, pH 7.4, with 0.05 M Tris–maleate. The final volume of 10.0 ml contained 1.0 to 1.2 mg microsomal protein/ml, 5 mM ATP, and the following additions: (●)$BrCCl_3$ and NADPH-generating system as given in Methods (N = 9–13 for each point); or (○) $BrCCl_3$ (N = 3–7). Malonic dialdehyde (MDA) was measured after 30 min. $BrCCl_3$ was added in less than 5 μl heptane/ml incubate. Addition of heptane alone (5 μl/ml incubate) to microsomes produced only 0.052 μg MDA/mg protein in 30 min. Data are means ± S.E.M.

and NADPH present, yields were 5.11 ± 0.11 μg MDA/mg protein (N = 5), about 10-fold greater than for the $BrCCl_3$-NADPH system.

Bromotrichloromethane-induced lipid peroxidation was associated with severely compromised microsomal calcium uptake capacity (Fig. 2). Cal-

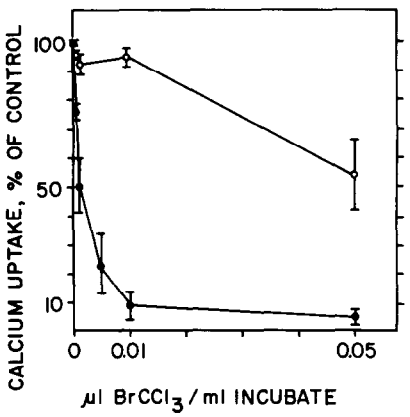

Fig. 2. Calcium uptake in microsomes previously incubated with various amounts of $BrCCl_3$, with and without NADPH. Procedures for the initial incubation are given in the legend of Fig. 1. Recovered microsomes, at a concentration of 0.10–0.15 mg protein/ml, were incubated for 60 min at 37° in 30 mM imidazole–histidine buffer at pH 6.8 with 5 mM NaN_3, 5 mM $MgCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 20 μM $CaCl_2$ and $^{45}CaCl_2$ (0.1 to 0.15 μCi/ml). Data are expressed as percent of unperoxidized microsomal calcium uptake at 60 min (control $\bar{x}$ ± S.E.M. for twelve experiments was 137.5 ± 6.0 nmoles Ca^{2+}/mg protein). Data for microsomes preincubated with $BrCCl_3$ and the NADPH-generating system (N = 4) are given as solid circles (●). Data for microsomes preincubated with $BrCCl_3$ alone (N = 3) are given as open circles (○). $BrCCl_3$ was added in a heptane carrier; 5 μl heptane/ml incubate caused no loss of uptake activity in 60 min at 37°. Data are means ± S.E.M.

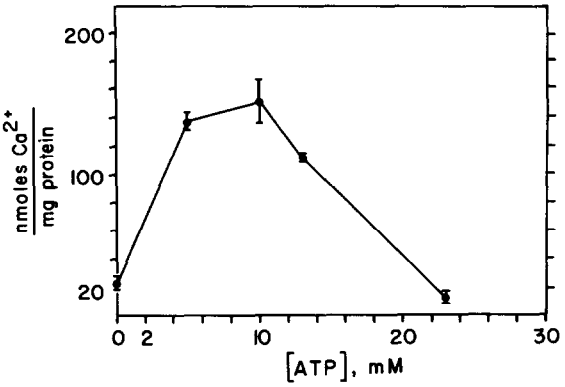

Fig. 3. Effect of ATP in initial microsomal incubations on subsequent microsomal calcium uptake. Microsomes at a concentration of 1.0 to 1.2 mg microsomal protein/ml were incubated in Tris–maleate buffer, pH 7.4, for 30 min at 37° with various concentrations of ATP. Microsomes were recovered by centrifugation and assayed for calcium uptake according to procedures given in Methods; see also Fig. 2. Data are means ± S.E.M. (N = 3).

cium taken up by unperoxidized microsomes during a 60-min incubation was 137.5 ± 6.0 nmoles/mg protein (N = 12). Lipid peroxidation induced by 0.01 μl $BrCCl_3$/ml of incubation mixture coincided with a 90 per cent decrease in calcium sequestration. At the same concentration, $BrCCl_3$ alone produced only an insignificant decrease.

The presence of ATP during an initial incubation of microsomes had a profound effect on subsequently determined calcium uptake. It was found that addition of ATP both elevated subsequent calcium uptake and reduced inter-experimental variability. For example, a maximal 7-fold elevation of calcium uptake by non-peroxidized microsomes was seen when 5–10 mM ATP was present during the initial incubation (Fig. 3); the concentration of 5 mM ATP was selected as optimal for further work. $BrCCl_3$-induced lipid peroxidation was not affected by 5 mM ATP. The correlation between depression of microsomal calcium sequestration and $BrCCl_3$-induced lipid peroxidation is given in Fig. 4. Ninety per cent of residual calcium uptake activity was lost at lipid peroxidation levels represented by the production of 0.25 to 0.30 μg MDA/mg protein. Significant losses of calcium sequestration were seen with as little as 0.10 to 0.15 μg MDA/mg protein. At comparable levels of MDA production, no difference was noted between $BrCCl_3$- and CCl_4-induced lipid peroxidation with respect to subsequent calcium uptake (data not shown).

The relationship between low levels of lipid peroxidation and depression of microsomal aminopyrine demethylase or glucose-6-phosphatase was measured (Fig. 4). Aminopyrine demethylase activity was decreased 90 per cent at MDA levels of 0.5 μg/mg protein, whereas glucose-6-phosphatase was decreased by only 45 per cent. A comparison of calcium uptake and enzyme activities at a peroxidation level of 0.3 μg MDA/mg protein shows a 90 per cent loss of calcium uptake activity versus a 60 per cent loss of aminopyrine demethylase activity and a 25 per cent loss of glucose-6-phosphatase activity. At lower ranges of MDA production, cal-

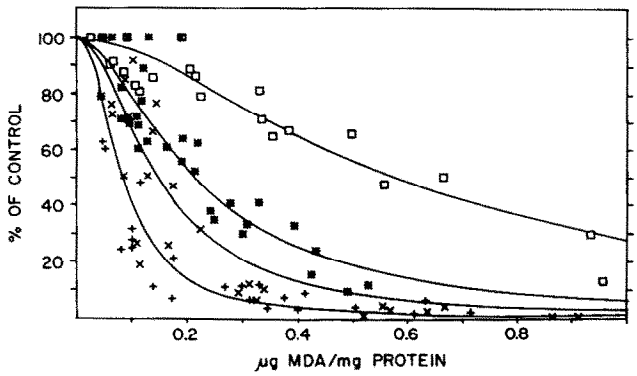

Fig. 4. Microsomal calcium uptake and enzyme activity as a function of lipid peroxidation. Microsomes incubated by the procedures given in Fig. 1 were recovered and assayed for calcium uptake (procedure given in Fig. 2) and for glucose-6-phosphatase and aminopyrine demethylase activities (procedure given in Methods). Data are plotted as amount of MDA ($BrCCl_3$–NADPH-dependent) produced in the initial incubation versus per cent of unperoxidized microsomal values as determined in subsequent assays. Key: (+) calcium uptake of microsomes initially incubated without 5 mM ATP (control $\bar{x} \pm$ S.E.M. was 25.5 ± 4.5 nmoles Ca^{2+}/mg protein per 60 min; N = 10); (×) calcium uptake of microsomes initially incubated with 5 mM ATP (control $\bar{x} \pm$ S.E.M. was 137.5 ± 6.0 nmoles Ca^{2+}/mg protein per 60 min; N = 11); (*) aminopyrine demethylase activity (control $\bar{x} \pm$ S.E.M. was 4.2 ± 0.8 μg HCHO formed/mg protein per 30 min; N = 3); and (□) glucose-6-phosphatase activity (control $\bar{x}$ + S.E.M. was 0.42 ± 0.09 mg inorganic phosphate split/mg protein per 30 min; N = 3). Lines are fitted to the Hill equation ($\%\ \text{inhibition} = \frac{(\text{MDA})^x}{y + (\text{MDA})^x}$). Respective values of x and y, ± S.E., for each group are: (+) 2.18 ± 0.44, 0.004 ± 0.005; (×) 1.93 ± 0.45, 0.025 ± 0.023; (*) 1.75 ± 0.19, 0.067 ± 0.021; and (□) 1.73 ± 0.21, 0.383 ± 0.077. Values for x and y were determined with a Hewlett–Packard 9825A computer using a program developed by Dr. B. Lindley, employing a non-linear least-squares fit.

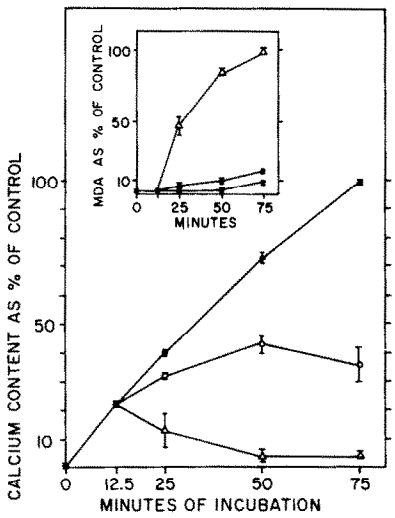

Fig. 5. Calcium uptake in microsomes undergoing peroxidation. Microsomes at a concentration of 0.2 mg protein/ml were incubated for 75 min at 37° with 100 μM $CaCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 10 mM $MgCl_2$, 5 mM NaN_3, $^{45}CaCl_2$ (0.8 μCi/ml), 0.14 mM NADP, 2.5 mM nicotinamide, 3 mM (D, L)-isocitrate, and 0.07 units (Sigma) isocitric dehydrogenase/ml. At 12 min either $FeSO_4$ at a final concentration of 22 μM (△) or $BrCCl_3$ in heptane at a final concentration of 0.06 μl/ml (○) was added. Solid circles (●) indicate no addition at 12 min. Data are expressed as percentage of calcium sequestered by unperoxidized microsomes at 75 min (control $\bar{x} \pm$ S.E.M. for five experiments was 313.1 ± 45.0 nmoles Ca^{2+}/mg protein). Inset shows MDA production with time as percentage of that produced at 75 min by $FeSO_4$–NADPH containing incubates (control $\bar{x} \pm$ S.E.M. for five experiments is 9.1 ± 2.4 μg MDA/mg protein). Data are means ± S.E.M.

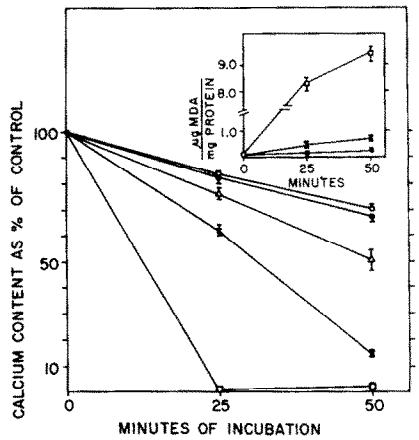

Fig. 6. Calcium content of preloaded microsomes during subsequent metabolism of $BrCCl_3$ or $FeSO_4$–NADPH-induced lipid peroxidation. Microsomes were initially incubated at a concentration of 0.2 mg protein/ml with 100 μM $CaCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 10 mM $MgCl_2$, 5 mM NaN_3, and $^{45}CaCl_2$ (0.6 μCi/ml) for 60 min at 37°. Microsomes were recovered and incubated at a concentration of 0.3 to 0.4 mg protein/ml for 50 min with reagents for NADPH generation (given in Methods) and 0.1 mM EDTA without further additions (○) or with 5 μl heptane/ml (●), or with 0.07 μl $BrCCl_3$/ml (×). Some incubates contained EDTA and $BrCCl_3$ but no NADPH-generating system (△), or the NADPH-generating system and 40 μM $FeSO_4$ but no EDTA (□). Data are expressed as percentage of the zero-time calcium content (control $\bar{x} \pm$ S.E.M. for five experiments was 330.6 ± 69.9 nmoles/mg protein). Inset shows MDA production with time. Key: (□) represents MDA production for the $FeSO_4$–NADPH system; and (×) represents MDA production for the $BrCCl_3$–NADPH system. MDA values for the controls NADPH alone, heptane + NADPH, and $BrCCl_3$ alone were essentially the same and represented by the solid circle (●). All data are means ± S.E.M. (N = 4–5).

cium uptake was the most severely affected of these three indicators of microsomal function.

In some experiments, lipid peroxidation was initiated in microsomal suspensions actively accumulating calcium. MDA production and calcium sequestration were measured concurrently. Pro-oxidants, either $BrCCl_3$ or $FeSO_4$, in the presence of NADPH, were added 12 min after the start of calcium accumulation. Calcium content of unperoxidized microsomes increased in a linear fashion for the 75 min of incubation (Fig. 5). After addition of $BrCCl_3$ in the presence of NADPH, microsomes displayed a lower rate of calcium accumulation compared to control microsomes. After 50 min no further calcium accumulation occurred. Subsequent to the addition of $FeSO_4$, microsomes lost accumulated calcium, and by 50 min the level of microsomal calcium approached basal, zero-time values.

In another series of experiments, microsomes were allowed to sequester calcium for 60 min. These calcium-loaded microsomes were recovered by centrifugation and resuspended; lipid peroxidation was induced by either $BrCCl_3$–NADPH or $FeSO_4$–NADPH. Calcium content and MDA production were monitored as peroxidation progressed. The calcium content of unperoxidized microsomes at 25 min varied between 75 and 85 per cent of the zero-time value (Fig. 6). At this time, microsomes that were recovered from the $BrCCl_3$–NADPH system retained 60 per cent of their initial calcium content, whereas microsomes that were recovered from the $FeSO_4$–NADPH system retained 2 per cent of zero-time calcium content.

DISCUSSION

The initial events of liver injury induced by CCl_4 or $BrCCl_3$ are thought to stem directly from carbon–halogen bond cleavage by the cytochrome P-450-mixed function oxidase system in the liver smooth endoplasmic reticulum. The free radical product of the interaction leads to the initiation of peroxidation of adjacent membrane lipids [16]. Widespread pathological phenomena [7], including disruption of the endoplasmic reticulum, loss of associated enzyme functions, triglyceride accumulation, and breakdown of the cell membrane, result from these initial events via unknown secondary mechanisms.

Recently, evidence has been presented that these secondary toxigenic mechanisms resulting in cellular dysfunction are accompanied by an extremely limited and focal process of lipid peroxidation. Masuda and Murano [17] showed, and Kornbrust and Mavis [18, 19] confirmed, that quantitatively minimal lipid peroxidation will occur in EDTA-prepared, Fe^{2+}-free rat liver microsomes incubated with NADPH and either CCl_4 or $BrCCl_3$. The latter authors [18, 19] also showed for the typical Fe^{2+}-NADPH microsomal-peroxidizing system that Fe^{2+} is the active pro-oxidant species, maintained in the reduced form by NADPH. Although the lipid peroxidation observed in NADPH–haloalkane systems is much less than that observed in the highly peroxidative NADPH–Fe^{2+} system, nevertheless it is potent in reducing the amount of cytochrome P-450 and glucose-6-phosphatase activity. This suggests that the haloalkane-initiated lipid peroxidation observed with EDTA microsomes is restricted to membrane sites at or near cytochrome P-450 and glucose-6-phosphatase. In our opinion, NADPH–haloalkane systems, free of Fe^{2+}, probably reflect much more faithfully the conditions in animals poisoned with $BrCCl_3$ or CCl_4 than do isolated microsomes in which lipid peroxidation is forced with NADPH and Fe^{2+}.

Secondary mechanisms of toxigenic cell injury arising from focal peroxidation sites along the endoplasmic reticulum may take several forms. First, it is possible that, in the process of lipid peroxidation, either diffusible or membrane-bound toxic products are generated which are directly responsible for spreading cellular dysfunction. Benedetti *et al.* [20] recently reported the isolation of diffusible, highly toxic carbonyl compounds from peroxidized microsomes. The major component of these toxic products, capable of inhibiting glucose-6-phosphatase as well as causing the hemolysis of erythrocytes, is 4-hydroxynonenal [21]. Hruszkewycz *et al.* [22] have shown that altered membrane lipids from peroxidized microsomes are also capable of inhibiting cytochrome P-450 and glucose-6-phosphatase; Willis and Recknagel [23] have reported on the isolation of these altered lipids with reference to hemolytic activity. Although these results have documented the toxic nature of peroxidative metabolites, the role of the metabolites *in vivo* during CCl_4 or $BrCCl_3$ poisoning has not been ascertained.

A second possibility for a toxigenic mechanism arising from CCl_4 and $BrCCl_3$ metabolism is that disturbance of the endoplasmic reticulum in itself may have immediate, direct consequences on cell functions. Moore *et al.* [10, 11] reported that Mg^{2+}–ATP-dependent calcium sequestration is reduced to 37 per cent of control activity in microsomes isolated 30 min after CCl_4 administration *in vivo*. Our results have demonstrated the sensitivity of calcium sequestration to $BrCCl_3$ and CCl_4 metabolism in Fe^{2+}-free systems *in vitro*. The decline in function is associated with exceedingly minimal levels of lipid peroxidation (Figs. 2 and 4). Possible effects on the calcium pump of covalent binding of cleavage products resulting from $BrCCl_3$ and CCl_4 metabolism are not known at this time. At a given level of lipid peroxidation, calcium uptake is more severely decreased than either glucose-6-phosphatase or aminopyrine demethylase (Fig. 4). Although the role of the endoplasmic reticulum in intracellular calcium homeostasis is by no means fully understood, destruction of microsomal calcium pump activity could result in disturbances in calcium concentration which, in turn, may affect a variety of cellular processes. Whole liver and subcellular calcium content increase in early phases of CCl_4 poisoning [7–10, 24–26]; an uncontrollable rise in calcium concentration is thought to be a final common path in toxic cell death [5, 6].

The calcium uptake system studied in Fe^{2+}-free microsomes was sensitive to the presence of ATP in a prior incubation. Calcium uptake was increased approximately 7-fold by the presence of 5–10 mM ATP during a preincubation (Fig. 3). Moore [27] reported that the presence of ATP during microsomal preparation increased calcium uptake 2-fold

and also resulted in the appearance of proteins identified by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis in the 105,000 *g* supernatant fraction. Further study of the liver microsomal calcium pump and its regulation is needed to evaluate these results.

The disturbance of microsomal calcium accumulation during peroxidation induced by $BrCCl_3$–NADPH was compared to that induced by $FeSO_4$–NADPH. Extensive lipid peroxidation induced in the Fe^{2+}–NADPH system resulted in a severe decline in calcium content of calcium-loaded microsomes within 25 min of the initiation of peroxidation (Fig. 5). The lesser peroxidation observed in the $BrCCl_3$–NADPH system was associated with a decline in rate of ^{45}Ca accumulation, and the amount of ^{45}Ca retained by the microsomes did not decline significantly in 25 min subsequent to initiation of peroxidation. The decline in accumulation seen in both types of peroxidized microsomes may be the result of an effect on permeability of microsomes to calcium, or to direct damaging effects on the pump itself. Concerning possible effects on permeability, we have shown that microsomes preloaded with calcium and subsequently peroxidized with either $FeSO_4$–NADPH or $BrCCl_3$–NADPH retain significantly less calcium than do control microsomes within 25 min after initiation of peroxidation (Fig. 6). It appears that in some manner lipid peroxidation decreases the capacity of microsomes to retain calcium; as would be expected, Fe^{2+}–NADPH-induced peroxidation is much more destructive than is the peroxidation that is dependent on $BrCCl_3$ metabolism. Alternatively, an inhibition of the calcium pump also seems likely. Martonosi *et al.* [28] reported that the dephosphorylation step of Ca^{2+}-ATPase in sarcoplasmic reticulum is retarded 80 per cent after vesicle treatment with phospholipase, suggesting that this calcium pump is sensitive to changes in the phospholipid environment. Chien *et al.* [29] also reported decreased calcium sequestration in ischemic myocardial microsomes associated with altered phospholipid metabolism. In the case of $BrCCl_3$ poisoning, alteration of membrane phospholipids may affect the calcium pump in a similar, direct manner.

This report has shown that depression of microsomal calcium sequestration correlates closely with minimal $BrCCl_3$-dependent lipid peroxidation. A disturbance in intracellular calcium homeostasis during toxigenic haloalkane poisoning *in vivo* may be a key event in the resulting cascade of cellular dysfunction. The area of intracellular calcium homeostasis must be further considered in the study of mechanisms of hepatotoxicity induced by $BrCCl_3$ and CCl_4.

Acknowledgements—We are grateful to Dr. Roger Willis for his generous assistance in the preparation of this manuscript. We also wish to thank Dr. Barry Lindley for his help in data presentation, and Dr. Robert Waller for his helpful comments on the manuscript.

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