Calcium Uptake of a Rat Liver Microsomal Subcellular Fraction in Response to \textit{in Vivo} Administration of Carbon Tetrachloride*

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ATP-dependent calcium uptake of rat liver microsomes is examined following ingestion of CCl₄ (2.5 ml/kg). Within 30 min there is an abrupt drop in calcium uptake activity of the liver microsomes. This activity remains down for 48 hours before slowly returning to normal levels. The effect is specific for CCI₄, as contrasted with CHCl₃ and CH₂Cl₂. The CCI₄ does not affect similar calcium uptake activity of kidney microsomes. Calcium uptake activity of liver mitochondria is unaffected. The first 12 hours after CCl₄ ingestion there is a relatively slow rise in the calcium content of the liver tissue and mitochondria. After 12 hours a much larger influx of calcium into the tissue and the mitochondria takes place. Forty-eight hours after CCl₄ ingestion the process begins to slowly reverse.

The following postulated sequence may relate to the CCl₄ hepatotoxicity. CCl₄ is activated to free radicals by the liver endoplasmic reticulum. The free radicals inactivate calcium pump activity of the liver endoplasmic reticulum. Calcium levels of the cytoplasm increase and significantly modify ion permeability of the plasma membrane. High levels of external calcium enter the cytoplasm and are sequestered in the mitochondria. The high level of mitochondrial calcium uptake inhibits mitochondrial oxidative phosphorylation.

The specific sensitivity of the calcium pump activity of liver microsomes to CCl₄ further establishes the identity of a system separate from the mitochondrial system. The above postulated sequence of events would suggest a critical role in liver metabolism for calcium pump activity of the endoplasmic reticulum.

The hepatotoxin carbon tetrachloride has been extensively studied as a model compound producing hepatocellular necrosis. The onset of pathological change in the centrolobular region of the liver is rapid after oral administration of the compound. In the rat, metabolism of lipid material by the liver is destroyed within 30 min after orally administered carbon tetrachloride (1). Within 1 hour there are alterations in the appearance of the endoplasmic reticulum (2), changes in microsomal drug-metabolizing enzyme activity (3), and depression of hepatic protein synthesis. Within 1 hour the calcium content of the liver has doubled (9, 4) and at later time periods calcium sequestered by the mitochondria will reach 15 to 20 times normal (5, 6).

The endoplasmic reticulum of the liver cell is the first cellular organelle disrupted by carbon tetrachloride poisoning. Indeed the hepatotoxic response to the agent depends upon metabolic activation by the enzymes of the endoplasmic reticulum (7, 8). A free radical derived from carbon tetrachloride appears to be responsible for the hepatotoxicity of the compound. Studies with compounds that induce the microsomal drug-metabolizing enzymes suggest that this enzyme complex is responsible for activation of carbon tetrachloride. Carbon tetrachloride toxicity in the rat can be potentiated by pretreatment of animals with agents which induce the mixed function oxidase complex of the liver endoplasmic reticulum. This has been demonstrated with phenobarbital (9, 10), 3,4-benzpyrene (11), and with 1,1,1,-trichloro-2,2-bis(p-chlorophenyl)ethane (12).

Alteration of calcium metabolism has been suggested as a potential mediator of organ-specific cell death produced by a number of compounds. These include carbon tetrachloride and galactosamine in the liver (13-17), isoproterenol in the heart (18), and adriamycin in the heart (19).

In this study we have determined the effect of \textit{in vivo} carbon tetrachloride administration on the calcium pump activity of the liver endoplasmic reticulum. The effect of this administration of carbon tetrachloride on whole liver and liver mitochondrial calcium levels had also been studied. Destruction of calcium pump activity in liver endoplasmic reticulum is an early manifestation of carbon tetrachloride toxicity.

\textbf{MATERIALS AND METHODS}

The animals employed in this study were male rats of the Sprague-Dawley strain weighing approximately 250 g. The animals...
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were obtained from the Holzman Co., Madison, Wis. *CaCl₂ (8.0 mCi/mg of calcium) was obtained from the New England Nuclear Corp.

Carbon tetrachloride was administered by stomach tube to rats (2.5 mg/kg). Rat liver microsomes and mitochondria were isolated according to procedures previously described (20). Protein was determined by the method of Sutherland et al. (21).

Calcium uptake was measured in the following medium: 100 mM KCl, 30 mM imidazole histidine buffer (pH 6.8), 5 mM sodium azide, 5 mM MgCl₂, 5 mM ATP (pH adjusted with imidazole to 6.8), 20 mM CaCl₂, and 0.1 nCi/ml of *¹⁵CaCl₂ in a total volume of 3 ml. The assay was initiated at 37° by the addition of the subcellular fraction to the medium.

The fixed preparation was then dehydrated and embedded in English araldite. Thin sections were stained with uranyl acetate followed by lead citrate and were examined in a Hitachi HT-11B electron microscope.

**RESULTS**

Fig. 1 demonstrates the effect of CCl₄, chloroform, and methylene dichloride on ATP-dependent calcium uptake by liver microsomes. Animals were treated with doses equimolar to 2.5 ml of CCl₄/kg, all given by stomach tube. The animals were killed 1 hour after the dose. Carbon tetrachloride inhibits calcium uptake activity more than 80% while the other two compounds have no effect on this activity. Chloroform, when given in this manner, has been reported to inhibit drug metabolism (3). In the current study both CHCl₃ and CH₂Cl₂ produced gross changes in the gastrointestinal tract. Animals treated with these compounds exhibited marked flatulence and grossly hemorrhagic areas in the gastrointestinal tract. Hemorrhagic areas were noted only after CCl₄. Inadequate absorption from the gastrointestinal tract has not been excluded as a reason for the lack of an effect of CHCl₃ on the liver microsomal calcium pump.

The time course of CCl₄ inhibition of calcium uptake activity of liver microsomes is presented in Fig. 2. In this experiment calcium uptake activity is only 37% of control 30 min after the dose of CCl₄. Between 4 and 8 hours the maximal inhibition is reached at 10% of control activity.

The toxicity of CCl₄ is currently thought to involve a free radical produced by the mixed function oxidase system of the liver endoplasmic reticulum (7, 8). The data presented in Fig. 3 suggests that inhibition of the liver microsomal calcium pump is not simply a physical effect of CCl₄. Renal microsomes from animals treated for 1 hour with CCl₄ demonstrate only 15% inhibition of ATP-dependent calcium uptake while microsomes from the liver of these animals demonstrate a 75% inhibition of the activity. This observation may suggest that
the liver microsomes activate \( \text{CCl}_4 \) to a reactive species responsible for inhibition of the liver microsomal calcium pump.

Experiments were carried out to determine the effect of \( \text{CCl}_4 \) on calcium content of whole liver and of liver mitochondria. Animals were treated with 2.5 ml of \( \text{CCl}_4 \)/kg by stomach tube and were killed after various time periods. The results are presented in Fig. 4. By 3 hours after administration of carbon tetrachloride calcium levels increase in both the whole liver and mitochondria. By 12 hours the increase is 3-fold in the whole liver and 40% in the mitochondria. A more precipitous change is seen after 12 hours. By 24 hours the calcium level in the whole liver is 15 times that of the control and by 48 hours the level in mitochondria has increased 8-fold. This data clearly indicate that after \( \text{CCl}_4 \) ingestion calcium content of the liver markedly increases. The mitochondria appear to sequester a major portion of the excess cellular calcium.

In the rat liver the effect of \( \text{CCl}_4 \) appears to be specific for the microsomal calcium pump. When rats are treated with \( \text{CCl}_4 \), and are killed either 1 or 24 hours after the dose, the mitochondrial calcium uptake is not affected, in contrast to the inhibition observed in the microsomes (Fig. 5). This experiment was conducted in an assay medium identical with that used for the microsomal studies, except that sodium azide was omitted. When 100 \( \mu \text{M} \) calcium and 5 \( \mu \text{M} \) phosphate were substituted for 20 \( \mu \text{M} \) calcium to test calcium uptake under relatively higher calcium loading conditions that may occur in the \( \text{CCl}_4 \)-damaged liver, the uptake capacity of the mitochondria was again unaffected 1 hour after the \( \text{CCl}_4 \) dose (data not shown).

After \( \text{CCl}_4 \) treatment the measured protein content of mitochondria and microsomes per g of tissue is eventually decreased. Microsomal protein is maximally decreased (about 40%) at 24 hours and then gradually recovers. Mitochondrial protein content per g of tissue is only slightly decreased at that time (about 15%). At 48 hours after the \( \text{CCl}_4 \) is administered the mitochondrial protein content per g of tissue is decreased 40% and remains decreased at 96 hours.

Mitochondrial and microsomal fractions of the rat liver were examined with an electron microscope as described under "Materials and Methods." The microsomes were comprised of smooth and rough endoplasmic reticulum membrane vesicles. The majority of the mitochondria were in the condensed form associated with high ATP content. There was detectable a very small amount of endoplasmic reticulum in this fraction. In contrast to the biochemical changes reported in this study, 24 hours after administration of carbon tetrachloride the isolated microsomes and mitochondria showed no obvious changes in physical structure. It should be emphasized that this study deals with a dose of carbon tetrachloride producing a reversible nonlethal liver toxicity.

**DISCUSSION**

The previous study demonstrates the occurrence of an energy-dependent calcium uptake activity in the microsomal subcellular fraction of the rat liver (20). The present report establishes inhibition of the calcium pump of the liver microsomal fraction as another of the early events after administration of \( \text{CCl}_4 \), to the rat. Inhibition of the calcium pump by the hepatotoxin is prompt. Within 30 min (the earliest time period examined) the activity in animals treated orally with \( \text{CCl}_4 \) is only 37% of control. Four hours after administration of \( \text{CCl}_4 \), the calcium pump activity is 10% of control activity. Recovery of a functional calcium pump is relatively slow; at 4 days after treatment calcium uptake activity increases to 48% of control. The recovery of mitochondria from massive calcium accumulation after 48 hours appears to parallel the apparently significant recovery of microsomal calcium pump activity after 48 hours.

The effect of \( \text{CCl}_4 \) is specific for the endoplasmic reticulum of the hepatocyte. After the standard dose of \( \text{CCl}_4 \), the calcium pump in renal microsomes is only slightly inhibited and liver mitochondrial calcium uptake is not inhibited 1 or 24 hours after treatment. A number of other liver endoplasmic reticulum enzymes are inhibited early in the course of carbon

![Fig. 4. Cellular calcium in the liver of animals treated with carbon tetrachloride. Rats were treated with \( \text{CCl}_4 \) (2.5 ml/kg, by stomach tube) and killed after the indicated time periods. Total calcium content of the whole liver homogenate of mitochondrial subcellular fraction was determined as described under "Materials and Methods." Early changes in cellular calcium have been implicated in the cause of cellular necrosis in several tissues and for this reason the early portion of the chart has been expanded. Each point represents the mean ± S.E. for the determination in three or four preparations.](http://www.jbc.org/)

![Fig. 5. Calcium uptake by rat liver mitochondria in vitro after \( \text{CCl}_4 \) treatment of rats in vivo. After a \( \text{CCl}_4 \) dosage of 2.5 ml/kg by stomach tube, the animals were killed at 1 or 24 hours. Calcium uptake was determined as described under "Materials and Methods." The initial calcium level was 20 \( \mu \text{M} \). Each point represents the mean ± S.E. for the determination in six mitochondrial preparations.](http://www.jbc.org/)
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tetrachloride toxicity. These include the microsomal mixed function oxidase system (3) and glucose-6-phosphatase (2). Morphological evidence suggests that the endoplasmic reticulum is the first cellular organelle to be damaged by CCl₄ (2, 15).

Total liver calcium rises slowly during the first 12 hours after CCl₄ is administered. Calcium pump inhibition in the liver cells begins after 24 hours. Calcium accumulation in the whole liver parallels calcium accumulation by liver mitochondria. What role inhibition of the endoplasmic reticulum calcium pump by CCl₄ has in the mitochondrial accumulation of calcium is a matter of speculation. The endoplasmic reticulum calcium pump may represent part of a triad responsible for the control of ionic calcium levels in the cytoplasm. One can envision the calcium pumps in liver plasma membrane (22), endoplasmic reticulum (20), and mitochondria (23) acting in concert to control cytoplasmic calcium levels. Disruption of one portion of this control system could result in an increase in intracellular ionic calcium levels and the accumulation of calcium by another element of the system, or extrusion from the cell. After injury to the calcium pump activity of the endoplasmic reticulum, the mitochondria may begin to sequester excessive amounts of calcium from the cytoplasm in an attempt to maintain cytoplasmic calcium at a normal level.

We have demonstrated, as have several others (2, 5, 6, 14), accumulation of calcium in the liver after CCl₄ intoxication. During the later stages of hepatic damage (after 12 hours) much of this calcium is localized in the mitochondria (Fig. 4 of this study). The accumulation of calcium in necrotic tissue is well known. Several workers, however, have implicated calcium in the early events of organ-specific cellular necrosis produced by toxic compounds. These include carbon tetrachloride (2, 4, 14), thioacetamide (24), and galactosamine (16, 17) in the liver. In the heart, calcium has been suggested to be important in the production of necrosis by isoproterenol (18). In summary, the present observations are compatible with the interpretation of Judah et al. (14) concerning cytoplasmic calcium. The following postulated sequence may describe the pertinent events of CCl₄ hepatotoxicity. CCl₄ is activated to free radicals by the liver endoplasmic reticulum. Calcium levels of the cytoplasm increase and significantly modify the ion permeability of the plasma membrane. High levels of external calcium enter the cytoplasm and are sequestered in the mitochondria. The high levels of mitochondrial calcium uptake inhibit oxidative phosphorylation in the liver mitochondria (6, 34, 35) and this may represent a major toxicity in the CCl₄-treated animal.

In several tissues a store of calcium has been identified with the endoplasmic reticulum. These tissues include the liver (25, 26), fat cells (27), and acinar cells of the pancreas (28). One may speculate that the rapid inhibition of calcium uptake by the liver endoplasmic reticulum could result in a substantial redistribution of cellular calcium. Calcium is an important regulator of membrane permeability (30-32). An increase in cytosolic calcium alters plasma membrane function (29, 33) and may result in the massive calcium permeability and other changes seen later in the hepatotoxic cascade.

Inhibition of the liver endoplasmic reticulum calcium pump by CCl₄ provides a plausible site from which an early redistribution of cellular calcium may occur. Unfortunately, measurements of total liver calcium (Fig. 4) (2, 4) give no information about changes of ionized calcium in the cytosol or redistribution of calcium stores within the cell. Damage to the liver endoplasmic reticulum calcium pump and the membrane of this organelle could result in a redistribution of as much as 20 to 40% of cellular calcium (25, 26) and yet this would be undetected with the measurements of total cell calcium. The later changes in mitochondrial calcium may be a response to increases in cytosol calcium. These organelles may sequester the excess calcium in an attempt to normalize calcium levels in the cytosol.

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In the present study the recovery of the microsomal calcium pump after 48 hours is associated with the reversal of other changes described. The microsomal or endoplasmic reticulum calcium pump activity appears to play a critical role in liver cell metabolism. The findings of this study also support the identity of a separate calcium control system in the liver endoplasmic reticulum.

The assumption in the postulated sequence is that the principal membrane site directly attacked by CCl₄ is the endoplasmic reticulum. The data directly demonstrate calcium pump damage at this site by the CCl₄. The data directly indicate that the mitochondrial calcium pump activity is intact for 24 hours. Plasma membrane calcium regulation needs further direct study. The data on cell calcium content do suggest that during the first hours after CCl₄, the plasma membrane role in cell calcium regulation is relatively intact when compared with changes that are manifest at 24 hours after CCl₄ administration.

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