The Effect of Carbon Tetrachloride Poisoning on Subcellular Metal Distribution in Rat Liver*

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The mechanism by which the ingestion of carbon tetrachloride results in distinct and reproducible histological alterations of the liver has been investigated intensively, both in vivo and in vitro (9-11). The mitochondria of liver cells are particularly vulnerable to this lipid solvent, as shown by their increased permeability (3), decreased coenzyme content (7, 8), and the loss of their ability to oxidize substrates (8) and to carry out esterifications of high energy phosphate coupled with oxidation (9).

In a series of investigations on the mechanism of carbon tetrachloride poisoning in the liver of the rat, a specific and characteristic chemical lesion involving alkali metals and alkaline earths has been detected. Large increases in calcium and concomitant decreases in potassium are found reproducibly in liver mitochondria obtained from rats to which this agent was administered. Both in vivo and in vitro, these compositional changes correlate with alterations of the oxidative function of mitochondria. The present paper reports on the time courses of these abnormalities in metal content after the administration of toxic quantities of carbon tetrachloride to rats.

METHODS

Healthy young male rats (Hisaw strain, Harvard Biological Laboratories), weighing between 150 and 250 g, were maintained on a diet of Purina chow and water ad libitum. All rats were fasted 16 hours before being killed. A single dose of 0.25 ml of carbon tetrachloride (analytical grade) per 100 g of body weight was administered in 0.25 ml of mineral oil per 100 g of body weight through a polyethylene stomach tube. A number of animals were killed at 0, 2, 4, 8, 12, 16, 24, 32, 40, 56, 72, 96, and 120 hours after administration of the agent. Controls were fed 0.25 ml of mineral oil per 100 g of body weight ad libitum. Within 14 days these animals lost 30 to 40% of their initial body weight.

For one experiment, reserves of body fat were depleted by placing rats on a diet of only 3 to 5 g of Purina chow daily with water ad libitum. Aliquots of the mitochondrial fractions obtained from each rat liver were analyzed for sodium, potassium, magnesium, and calcium by flame spectrometry (14). All subcellular fractions obtained from pooled livers of rats killed at 16 and 40 hours were also analyzed for magnesium, calcium, iron, zinc, and manganese by spark spectrosopy, and for sodium and potassium by flame spectrometry.

Residual potassium, calcium, and magnesium content of mitochondria was determined after three successive suspensions and centrifugations of samples of mitochondria in fresh aliquots of 0.25 M sucrose; aliquots of both the mitochondria and the supernatant fluid were analyzed for these three elements after each centrifugation.

To measure lipid-bound metals, suspensions of mitochondria in 0.25 M sucrose were extracted successively five times either with 3 volumes of carbon tetrachloride, 20 volumes of 2:1 chloroform-methanol, or 20 volumes of 1:1 ethanol-diethyl ether. Fresh aliquots were homogenized with one of these lipid solvents for 1 minute, in a motor-driven Potter-Elvehjem glass homogenizer; the suspension was centrifuged, and the lipid solvent phase was removed. Pooled lipid solvent phases were evaporated to dryness and analyzed for their metal content.

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Phosphate, which interferes with the determination of magnesium and calcium in the hydrogen-oxygen flame, was removed, and tissue samples were prepared for flame spectrophotometric analysis, by the following procedure. To a 2- to 3-ml sample of the mitochondrial suspension in sucrose (containing 1 to 2 mg of nitrogen per ml of suspension) is added 6 ml of a 2:1 mixture of reagent grade nitric acid and high purity perchloric acid (G. F. Smith Company). The samples are digested completely at 170 ± 20° and then evaporated to dryness. The residue, dissolved in a small volume of water, is transferred quantitatively to a graduated centrifuge tube and brought to a 4.0 ml volume by addition of metal-free water and 1.0 ml of 2% stannic chloride in acetone. The tube is stoppered immediately with a polyethylene cap and the contents are mixed. Stannic phosphate precipitates, removing phosphate quantitatively from the solution. Excess tin is precipitated as stannic acid by heating at 70° for 30 minutes, cooling, and centrifuging at 2900 r.p.m. for

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† Based on an unpublished method of M. Margoshes and B. L. Vallee.
10 minutes. Sodium, potassium, magnesium, and calcium are completely recovered in the water-acetone supernatant solution which is analyzed directly with a flame spectrometer (13).

The precision of the flame spectrometric procedure, expressed as a coefficient of variation of individual data is 0.03, and that of the spark spectrographic method varies from 0.08 to 0.20, depending upon the specific metal and its concentration (15).

Phosphate was determined by the method of Fiske and SubbaRow (16). Acid-soluble phosphate of mitochondria was isolated by the method of Schneider (17). Nitrogen was determined by a Kjeldahl microtechnique with a coefficient of variation of 0.02.

RESULTS

Metal Content of Mitochondria—The changes in the contents of sodium, potassium, magnesium and calcium of mitochondria obtained from rat livers are shown in Fig. 1 as a function of the time of sacrifice after the administration of carbon tetrachloride. The calcium content of mitochondria is doubled as early as 2 to 4 hours after the administration of the agent, increasing steadily during the next 10 hours and becoming 4-fold by 12 hours. Thereafter, it rises abruptly to more than 10-fold by 16 hours and is maximally increased to almost 20 times the normal value at 32 to 40 hours. Conversely, the mitochondrial potassium content is slightly decreased by 8 hours, and decreases steadily to one-fourth that of the control by 40 hours. Both the mitochondrial calcium and potassium contents return to normal values by 72 hours after the ingestion of the specified amount of carbon tetrachloride.

The excess calcium is not removed by repeated washing of the mitochondrial pellet with isotonic sucrose. Mitochondria from the liver of a rat, killed 16 hours after the administration of carbon tetrachloride, contained 230 μg of calcium per g of sedimented mitochondria, compared to 40 μg per g in the control animal. After three successive washings in fresh isotonic sucrose, calcium is recovered quantitatively in the mitochondrial pellet. By identical treatment, 42% of the potassium and 17% of the magnesium are removed.

Between 12 and 56 hours, concomitant with the maximal alterations in calcium and potassium contents, the sodium content of mitochondria increases up to 1.5 μg per mg N, while the magnesium content decreases by 2.5 μg per mg N. These alterations, however, are more variable than those of the calcium and potassium concentrations. The sum of the concentrations of these four major cations in mitochondria remains strikingly constant even while the extensive changes of calcium and potassium are maximal (Table I).

The nitrogen content of the mitochondria was employed as the base line for the calculation of concentrations of metals. Table I shows the variation of the mitochondrial nitrogen content, both from animal to animal (standard error of the mean) and as a function of time after administration of carbon tetrachloride. The nitrogen content does not vary markedly. The apparent decrease in the averages at 40 and 72 hours is not significant compared to the variation between animals as expressed by the standard error.

The increase in calcium does not appear to be secondary to an increase in phosphate. The acid-soluble and total phosphate contents of mitochondria are not altered before or during the early phase of these marked changes in the calcium content of mitochondria (Table I). Although the calcium content at 16 hours is over 10 times the initial value, the acid-soluble phosphate and total phosphate contents are not changed at that time. Only at and beyond 40 hours does an increase in phosphate take place, by which time the calcium content has reached a maximum value and is decreasing.
Mitochondria were extracted with carbon tetrachloride to test the hypothesis that the increased amount of calcium in mitochondria might be associated with lipids. In this manner, 11% of the percentage of calcium removed does not rise proportionately to the accumulation of total calcium in the mitochondria; in fact it decreases. Sodium, potassium, and magnesium are not extracted with this solvent. Extraction of mitochondrial pellets with chloroform and methanol, or ethanol and diethyl ether dissolves 24 and 17% of calcium, respectively, as well as substantial amounts of magnesium and potassium. The accumulation of calcium in mitochondria after carbon tetrachloride poisoning is unchanged by the depletion in rats of their peripheral fat by fasting before administration of the poison.

**Metal Content of Other Subcellular Fractions**—The metal contents of other subcellular fractions also change with time. Table III shows the metal content of whole liver and all subcellular fractions at the beginning (16 hours) and at the maximum point (40 hours) of the alterations in mitochondrial metal content.

The aggregation of calcium in the mitochondria and the residue fraction accounts for the total increase in the calcium concentration of the whole liver 16 hours after administration of carbon tetrachloride. At this time the calcium concentration in other subcellular fractions has not changed. By 40 hours, however, when the calcium concentration of the whole liver is increased further, the calcium content of all fractions is increased.

The loss of potassium from the mitochondria represents such a small proportion of the total potassium of rat liver that it is not reflected in its total content of potassium, either at 16 or 40 hours, even though there are small decreases in the residue and microsomes. The potassium content of the supernatant fraction, which contains 60% of the intracellular potassium (13), is virtually unchanged.

The sodium concentration is elevated 2-fold in all fractions obtained from the liver sample taken at 16 hours (Table III); normal values are observed in the sample taken at 40 hours. The magnesium concentration is increased in the supernatant fraction, but there is no other significant change.

The concentrations of zinc and manganese in whole liver and the subcellular fractions are virtually stable throughout the period of marked changes in calcium and potassium contents. Iron is altered, being elevated at 40 hours in both mitochondria and microsomes.

**DISCUSSION**

The administration of carbon tetrachloride results in striking increase of calcium and decrease of potassium contents of the mitochondria of rat liver as compared with normal mitochondria (13). Calcium, initially present in mitochondria in only minor amounts, increases 15-fold to become the dominant mitochondrial cation between 16 and 40 hours after the administration of carbon tetrachloride; concomitantly potassium, normally the most abundant cation, decreases to one-fourth of its initial concentration.

Earlier biochemical studies have emphasized that mitochondria are specific loci of attack in the liver cell injury resulting from carbon tetrachloride poisoning (3-11). The present findings demonstrate that the earliest changes in the intracellular cation concentration are localized to this organelle and are consistent with this view.

This chemical lesion is all the more unique since it appears to be relatively specific; it does not occur in conjunction with other types of experimental liver injuries such as those produced by diets deficient in cysteine and tocopherol, or magnesium. The magnitude and reproducibility of the observed alterations lie far outside of the range of possible analytical artifacts. The nitrogen content remains quite constant throughout the experimental period.

The increase in calcium precedes the loss of potassium, suggesting an interdependence. Further, the total cation content of mitochondria remains stable throughout the period during which changes in the concentrations of individual metals occur (Table I), suggesting either an exchange of calcium for potassium.

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**TABLE II**

<table>
<thead>
<tr>
<th>CCl₄ extraction* of mitochondria from livers of CCl₄ rats</th>
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<tbody>
<tr>
<td>Hours after CCl₄</td>
<td>Calcium in mitochondria</td>
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</tr>
<tr>
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<tr>
<td>40</td>
<td>13.6</td>
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<tr>
<td>96</td>
<td>1.7</td>
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</table>

* Extracted 5 times with 10 volumes of fresh CCl₄ per g of mitochondria each time at 4°C. Extracts pooled and analyzed.

**TABLE III**

<table>
<thead>
<tr>
<th>Effect of CCl₄ on metals in subcellular fractions of rat liver</th>
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<tr>
<td>Metals were determined by spark spectroscopy, except Na and K</td>
<td>by flame spectrometry.</td>
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<tr>
<td>Fraction</td>
<td>Time after CCl₄</td>
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<td>Mitochondria</td>
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<td>16</td>
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<td>40</td>
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<td>Microsomes</td>
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<td>40</td>
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<td>40</td>
<td>13.0</td>
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2 E. S. Reynolds, R. E. Thiers, and B. L. Vallee, unpublished work.
3 J. Vitale, R. E. Thiers, and B. L. Vallee, unpublished work.
at a fixed number of metal-binding sites on or in the mitochondrial membranes or an isosmolar exchange of potassium ion for calcium ion.

Calcium forms more stable compounds than does potassium with substrates, such as citrate and pyrophosphate. cofactors such as ATP, fatty acids, phosphatidic acid, and other compounds associated with the membranous solid portions of mitochondria (18). When mitochondria are washed with isotonic sucrose or even disrupted by repeated freezing and thawing, calcium remains entirely within the solid particles whereas most of the potassium is removed into the supernatant liquid.  

Mitochondria from normal rat livers are known to concentrate calcium (18) and maintain large gradients against the suspending medium. Although the anionic sites which bind calcium in the mitochondria are unknown it seems clear that under normal circumstances many of them are occupied by other cations, among which is possibly potassium. The binding of calcium may interfere with the normal function of these sites. However, no data have yet been obtained which can distinguish between cellular or mitochondrial functions which result from the alterations in cation concentrations and those which cause them.

The source of the calcium which accumulates in the liver mitochondria in carbon tetrachloride poisoning is not known. The serum may well be the immediate source since a transient drop in the serum calcium concentration of carbon tetrachloride poisoned rats was sometimes seen during this work, concurrent with the maximum rate of increase of mitochondrial calcium. It was observed early that, dependent upon their previous intake of calcium, dogs poisoned with carbon tetrachloride died of tetany typical of hypocalcemia, which could be relieved or prevented by administration of calcium salts (19, 20).

An increase in phosphate-containing compounds in mitochondria does not account for these changes (Table I) nor can the formation of calcium complexes with phosphatidic acid (Table II), or the formation of calcium soaps, be shown to be accountable. Actually, sufficient phosphate is already present in normal mitochondria to bind all the mitochondrial calcium even when this metal is increased maximally.

The ability of mitochondria to retain potassium is an essential concomitant of function and may be a prerequisite for performance of oxidative phosphorylation (21). On the other hand, excess calcium interferes with normal mitochondrial function. Mitochondrial swelling (22), loss of potassium (22, 23) and the inability to carry out respiration (22, 24) and oxidative phosphorylation (25) have all been observed when mitochondria are exposed to excess concentrations of calcium. It has been postulated that a solution of carbon tetrachloride, a nonpolar solvent, soluble in the monomolecular layers of lipid, phospholipid, and protein of the cristae mitochondrial membranes and mitochondria, exerts its effects by interfering directly with the physical and chemical integrity of these structures (3, 10).

The well known synergistic toxic effect of ethanol and carbon tetrachloride on the liver suggests alternatively that carbon tetrachloride may be converted metabolically into a more toxic compound. Thus the condensation of ethanol and carbon tetrachloride could result in the formation of the monoethanol ester of phosgene, ethyl chlorofomate. In our experimental trials with this agent, death of the animals always resulted before the establishment of histological alterations of the liver. Thus, this suggestion requires further study.

**Summary**

The administration of sublethal doses of carbon tetrachloride results in an increased content of calcium in rat liver mitochondria as early as 2 hours after its administration. Concomitantly mitochondrial potassium decreases, the concentration of calcium becoming maximal and that of potassium becoming minimal within 16 to 40 hours, both returning to normal values within 72 hours.

The increased calcium content of liver mitochondria after carbon tetrachloride poisoning is not associated with an increase in mitochondrial phosphorus, nor is it accounted for by proportional increase in calcium soaps or calcium salts of phosphatidic acid.

**References**

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