Studies of Biochemical Changes in Subcellular Particles of Rat Liver and Their Relationship to a New Hypothesis Regarding the Pathogenesis of Carbon Tetrachloride Fat Accumulation*

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Recent work in our laboratory, carried out in collaboration with Dr. M. C. Schotz, has led to the formulation of a simple hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation. This hypothesis consists essentially of an extension of the work of Byers and Friedman (1) to the carbon tetrachloride-poisoned liver. These workers have recently shown that the liver is constantly secreting large quantities of triglycerides into the plasma. According to our hypothesis, the formation of triglycerides by the liver is not interfered with in the carbon tetrachloride-poisoned animal, but the hepatic triglyceride secretory mechanism is inhibited or destroyed. As a result, triglycerides accumulate in the liver. Two preliminary aspects of the problem had previously been clarified. It was first observed that carbon tetrachloride reached its peak concentration in the liver of the rat within 1 to 2 hours after force feeding of the hepatotoxin (2). This observation established a basic frame of reference for all subsequent studies. It was next shown (3) that hepatic content of triglycerides was elevated 34% within 1 hour and 195% within 3 hours after carbon tetrachloride poisoning. This observation indicated that a serious derangement of hepatic lipid metabolism occurred coincident with the arrival of the toxic compound in the liver. After establishment of these two preliminary points, the hypothesis stated above was developed from the two main considerations. The first of these was a systematic study of changes in biochemical properties of subcellular fractions obtained from livers of rats poisoned with carbon tetrachloride. A series of investigations (4, 5) had previously led to the tentative conclusion that the hepatic parenchymal cell mitochondria were probably not the primary loci for the attack by carbon tetrachloride. An extension of these studies has eliminated the mitochondria from serious consideration as possible primary targets. Study of some microsomal enzymes, on the other hand, indicated that pathological changes occur in the endoplasmic reticulum as early as 2 hours after carbon tetrachloride poisoning. The observation that carbon tetrachloride feeding results in pathological changes in the enzymic properties of the microsome fraction at a time when the toxin is concentrated in the liver, and when rapid accumulation of triglycerides in the liver is taking place, suggested that a hitherto unknown hepatic mechanism, intimately associated with hepatic lipid metabolism and probably localized in the membranous component of the endoplasmic reticulum, was most probably the key locus involved. Consideration of the role of the liver in mechanisms of lipid transport, recently reviewed by Fredrickson and Gordon (6) and by Olson (7), led directly to the formulation of the above hypothesis. In a test of the hypothesis (8) it was found that 90 minutes after intravenous administration to rats of the non-ionic detergent Triton (a non-ionic detergent: Winthrop Laboratories, WR - 1339), plasma triglycerides were elevated 12-fold. If the rats had been poisoned with carbon tetrachloride 2 hours previously, this post-Triton hypertriglyceridemia was almost completely absent. Concomitantly there was a threefold increase in liver content of triglycerides. A depression to one-fourth of the normal level of plasma triglycerides was observed in carbon tetrachloride-poisoned rats that were not given Triton. This direct demonstration of a major derangement in the metabolism of triglyceride by the liver, coincident in time with the attainment of the peak concentration of carbon tetrachloride in the liver (2) and with the rapid early rise in hepatic triglyceride content (3), provides a new insight into the problem of the pathogenesis of carbon tetrachloride fat accumulation. The present communication presents our studies of the alterations in subcellular fractions of rat liver which occur during the course of pathological changes set into motion by carbon tetrachloride feeding, and which led to the formulation of the above hypothesis.

EXPERIMENTAL PROCEDURE

Animals used in this study were rats of the Sprague-Dawley strain (Holtzman Rat Company, Madison, Wisconsin). Female rats were used for experiments in which mitochondrial content of citrate was determined. In all other experiments, males were used. Carbon tetrachloride intoxication was produced by force feeding under light ether anaesthesia a 1:1 mixture of carbon tetrachloride and mineral oil at a dose of 0.5 ml of the mixture per 100 g of rat body weight. Control rats received 0.25 ml of mineral oil per 100 g of body weight. After force feeding, rats were kept in individual cages. In all the experi-
mental work reported in this communication, some control and some intoxicated animals were processed on the same day, under presumably identical conditions. Details pertinent to the various experiments were as follows.

**Citrate Experiments**—Except where indicated, rats were fed *ad libitum* until 6 hours before carbon tetrachloride feeding, whenupon all food was removed for the duration of the experiment. Water was provided *ad libitum*. Ten hours after carbon tetrachloride feeding, 19 hours of fasting (13 hours after carbon tetrachloride feeding; 19 hours of fasting) the rats were killed by cervical section, exsanguinated, and the livers removed and weighed. The homogenization medium, 0.30 M sucrose, containing 0.002 M EDTA, at pH 7.2. Five grams of liver were homogenized in 35 ml of medium and centrifuged at 600 × g for 12 minutes in the International table model centrifuge, head No. 216. The nuclear fraction was resuspended and recentrifuged once. Suitable aliquots of the combined nuclei-free homogenate were centrifuged at 7000 × g for 20 minutes (American Instrument Company, Inc., high speed angle centrifuge equipped with refrigeration, tube angle 32°). The supernatant fractions were removed without removing the fluffy layer. One centrifuged mitochondrial pellet was used for protein analysis, and one was extracted with 5 ml of 5% trichloroacetic acid. The trichloroacetic acid extract was used for protein analysis, and one was extracted with 5 ml of 5% trichloroacetic acid. The trichloroacetic acid extract was analyzed for citric acid essentially according to Schneider et al. (9).

**Mitochondrial ATPase Transformation Test**—In previous work (4), an ATPase transformation test was used to detect mitochondrial damage. In this test, ATPase activity of isolated mitochondria in presence of 2,4-dinitrophenol and EDTA is compared to ATPase activity with added magnesium ions. For normal mitochondria, 2,4-dinitrophenol-ATPase is considerably more active than Mg**2+**-ATPase. With mitochondrial damage, 2,4-dinitrophenol-ATPase declines, whereas Mg**2+**-ATPase is activated. For the 2,4-dinitrophenol ATPase, 1.5 ml of final reaction medium contained 6 μmoles of ATP (crystalline Na₂ATP·4H₂O, Sigma, neutralized to pH 7.4 with KOH); 30 μmoles of Tris pH 7.4, 325 μmoles of sucrose, 2 μmoles of EDTA pH 7.4, 3 × 10⁻⁵ M 2,4-dinitrophenol as activator. For the Mg**2+**-activated ATPase, the conditions were the same except that 2,4-dinitrophenol and EDTA were omitted and MgCl₂ was added to a final concentration of 0.002 M. For both assays, 12.5 μg of mitochondria were added as 0.5 ml of a 2.5% suspension of mitochondria in 0.3 M sucrose containing 0.002 M EDTA at pH 7.4. This medium was used for preparation of mitochondria.

**Mitochondrial Respiratory Control**—Existence of mitochondrial respiratory control was demonstrated by showing that the rate of oxidation of glutamate was increased on addition of 2,4-dinitrophenol to a respiring system deficient in ADP. Oxygen consumption was measured manometrically at 30°. In a final volume of 3.0 ml, the complete system contained 30 μmoles of inorganic phosphate (potassium salt, pH 7.4), 10 μmoles of MgCl₂, 30 μmoles of glutamate (potassium salt, pH 7.4), 530 μmoles of sucrose, 0.6 μmoles of EDTA. The EDTA and 90 μmoles of sucrose entered the system on addition of 0.3 ml of mitochondrial suspension in 0.30 M sucrose, 0.002 M EDTA medium. 2,4-Dinitrophenol was added to a final concentration of 3 × 10⁻⁵ M. Center wells contained 0.2 ml of alkali and filter paper strips.

**Octanoate Oxidation**—Octanoate oxidation was measured manometrically at 30° in the same Warburg bath in which mitochondrial respiratory control was measured. A final volume of 2.0 ml contained 40 μmoles of inorganic phosphate (potassium salt, pH 7.4), 4.5 μmoles of ATP, 10 μmoles of MgCl₂, 2 μmoles of octanoate, 1 μmole of fumarate, 550 μmoles of sucrose, 1 μmole of EDTA, and 2 mg of crystalline bovine serum albumin. The EDTA and 150 μmoles of sucrose entered the otherwise complete system on addition of 0.5 ml of mitochondria suspended in 0.30 M sucrose, 0.002 M EDTA medium.

**Glucose-6-Phosphatase**—Activity of this enzyme was determined either in whole rat liver homogenized in 0.30 M sucrose, 0.002 M EDTA medium, or in the microsome fraction sedimented from nuclei- and mitochondria-free homogenates at 100,000 × g in the Spinco preparative ultracentrifuge. Final conditions for the enzyme assay were as follows: 30 μmoles of Tris maleate buffer, pH 6.6; 20 μmoles of glucose-6-P, pH 7.0; either 6 mg of whole liver homogenate or 6 eq mg of microsome fraction; final volume 1.5 ml; 20 minutes of incubation at 30°. Preliminary studies indicated that substrate saturation was reached at 10 μmoles of glucose-6-P per 1.5 ml. Activity was proportional to enzyme concentration up to 8 mg of whole liver. Under the conditions given, only about 5% of added substrate was hydrolyzed.

**Microsomal DPNH Cytochrome c Reductase**—A total of 25 ml of 10% homogenate of rat liver, prepared in 0.30 M sucrose, 0.002 M EDTA, at pH 7.4, was centrifuged at 10,000 × g for 12 minutes in the SW 25.1 head of the Spinco preparative ultracentrifuge. The upper fatty layer was removed and discarded and the supernatant fraction set aside. The sediment was suspended in 20 ml of sucrose-EDTA medium and centrifuged. Supernatant fractions were combined and adjusted to 50 ml final volume. Just before assay, 0.4 ml of this nuclei- and mitochondria-free homogenate was diluted to 20 ml, of which 0.2 ml, corresponding to 0.2 mg wet weight of liver, was added to the final reaction medium. DPNH cytochrome c reductase activity was determined in a Beckman DU spectrophotometer as follows. To small test tubes were added, in a volume of 2.1 ml, 100 μmoles of potassium phosphate buffer pH 7.4, 1 μmole of KCN, 80 μmoles of nicotinamide. Then 0.3 ml of 3 × 10⁻⁴ M cytochrome c was added, the test tubes were placed in a 30° water bath, 0.2 ml of enzyme was next added, and finally 0.4 ml of 1.59 × 10⁻⁴ M DPNH in 1 × 10⁻⁴ M NaOH. Each enzyme extract was analyzed in triplicate against a blank cuvette which contained all additions except 0.4 ml of 1 × 10⁻⁴ M NaOH instead of the DPNH addition. Contents of the test tubes were transferred to suitable optical cuvettes and optical density was determined at 550 μm at 1, 2, and 3 minutes after addition of DPNH. The rate of reduction of cytochrome c was linear over the first 3 minutes. Reaction rate was linear with respect to...
the amount of nuclei- and mitochondria-free homogenate added, from 0.1 to 0.4 eq mg of liver, wet weight, per cuvette.

Chemical Procedures—Analysis for inorganic phosphate was carried out according to a method adapted after Berenblum and Chain (10) involving extraction of phosphomolybdic acid into isobutanol-benzene and reduction with SnCl₄. Protein was determined according to Lowry et al. (11), with bovine serum albumin as standard. Total liver fat was extracted essentially according to Folch et al. (12), dried down, and weighed. It was then redissolved in chloroform, and an aliquot passed over a silicic acid column. The chloroform eluate of the silicic acid column was analyzed for triglycerides according to Van Handel and Zilversmit (13).

RESULTS

Concentration of Citrate in Liver Mitochondria of Fluoroacetate-Poisoned Rats—In 1956, Schneider et al. (9) found that endogenous citrate of rat liver was localized in the mitochondrial fraction obtained by differential centrifugation of sucrose homogenates. Increased citrate in livers of female rats poisoned with fluoroacetate was also found in the mitochondrial fraction. Mitochondrial citrate was rapidly lost when mitochondria were lysed in water, incubated at 37° in presence of fluoroacetate, or disrupted by sonic vibrations. In our experiments, citrate was lost from liver mitochondria of normal rats or of rats previously given fluoroacetate when the mitochondria were suspended in an isotonic sucrose medium containing carbon tetrachloride (Table I). In previous work from this laboratory (14), it was shown that isolated mitochondria swell rapidly when suspended in isotonic sucrose solutions containing carbon tetrachloride. It was suggested that swelling was due to osmotic effects of the Donnan equilibrium brought into play by breakdown of mitochondrial membrane selective permeability. As in the case of the protolytic loss of potassium ions from red cells attacked by lytic agents (15), breakdown of mitochondrial membrane selective permeability would be expected to lead to loss of intramitochondrial citrate. Data of Table I and rapid loss of mitochondrial potassium ions under similar conditions (5), constitute objective evidence that carbon tetrachloride in vitro attacks the mitochondrial membrane, as was suggested by swelling experiments reported earlier (14). With regard to the hepatotoxic action of carbon tetrachloride, it has been shown that this toxic compound reaches its peak concentration in the liver within 1 to 2 hours after intubation into the stomach (2). Liver triglycerides are elevated three-fold within 3 hours (3). If the hepatotoxic action of carbon tetrachloride involves a breakdown of mitochondrial membrane selective permeability as a primary event, as has been suggested by others (16, 17), then during the onset and early course of the pathological disturbances, one might reasonably expect to find an impairment in the ability of isolated mitochondria to retain citrate. Such does not appear to be the case. Thirteen hours after carbon tetrachloride feeding, isolated liver mitochondria contained more citrate than liver mitochondria from corresponding controls not fed carbon tetrachloride (Table II). Furthermore, when carbon tetrachloride-fed rats were further treated with fluoroacetate, isolated liver mitochondria were found to contain 3½ times as much citrate as corresponding noncarbon tetrachloride-fed, fluoroacetate-poisoned controls. The finding that mitochondrial levels of citrate are higher in carbon tetrachloride-fed animals than in their corresponding controls was unexpected. However, the main point is unmistakably clear; viz in the period of 10 to 13 hours after carbon tetrachloride feeding, carbon tetrachloride-poisoned rats can respond to fluoroacetate by building up their levels of mitochondrial citrate. These increased levels of citrate are retained throughout subsequent homogenization and differential centrifugation procedures.

It is possible that the two substances, carbon tetrachloride and fluoroacetate, have the joint effect of producing even higher citrate levels than the 575 µg level found by analysis, and that the particular level found represents the remainder after possible substantial losses during homogenization and centrifugation. Since the main point of the carbon tetrachloride-fluoroacetate experiments was to determine whether the mitochondrial membrane is defective, mitochondrial storage experiments were carried out (Table III). The data clearly indicate that little citrate is lost from the mitochondria during 3 hours of additional storage. The over-all loss in 3 hours of storage in the carbon tetrachloride plus fluoroacetate-treated rats was only 7.5%, as compared to a 66.1% loss of citrate after 25 minutes of storage in sucrose-carbon tetrachloride in vitro (Experiment 3, Table I). During the period of storage from 1 to 3 hours, the loss of citrate from the liver mitochondria of the carbon tetrachloride-fluoro-
acetate-poisoned rats was 5.3%, as compared to 6.1% for the corresponding time period in the controls. The slight rise in citrate levels in the noncarbon tetrachloride-fed controls during the 1st hour of storage justifies comparison of citrate loss over the final 2 hours, rather than over the whole period of storage. Furthermore, it was found that when mitochondria taken from rat liver 13 hours after carbon tetrachloride feeding were induced to swell in vitro by suspending them either in isotonic sucrose medium containing carbon tetrachloride, or in distilled water, citrate levels were reduced to approximately one-tenth of the levels in the nonswollen mitochondria. This finding indicates that the capacity to retain citrate exhibited by liver mitochondria of rats 13 hours after carbon tetrachloride poisoning is not due to irreversible binding of citrate by these mitochondria. The finding (18) that carbon tetrachloride poisoning leads to increased content of mitochondrial calcium raised the possibility of irreversible binding of citrate. Carbon tetrachloride poisoning resulted, after 13 hours, in an average increase of 23% in mitochondrial protein. This change is relatively small in comparison with changes in citrate content. It was on the basis of relative constancy of yield of mitochondrial protein that data of Table II were expressed as µg of total liver mitochondrial citrate per 100 g of rat body weight and not referred to corresponding protein content of the mitochondria. Liver fat 13 hours after carbon tetrachloride feeding was found to be 316 ± 27 (standard deviation) µg of total liver lipid per 100 g of rat body weight, as compared to 171 ± 16 for noncarbon tetrachloride-fed controls. In other experiments, for two female rats 13 hours after carbon tetrachloride poisoning, 72% of total liver lipid was triglyceride, as compared to 29% triglyceride in a single female control.

Glucose 6-Phosphatase and Mitochondrial ATPase 4 Hours After Carbon Tetrachloride Feeding—Mitochondria isolated from livers of rats 4 hours after carbon tetrachloride poisoning exhibited a normal response in the ATPase transformation test (Table IV). In the same rats, glucose 6-phosphatase activity was depressed 49%, and liver triglycerides were elevated 180%. In an earlier study it was found that glucose 6-phosphatase activity of livers of carbon tetrachloride poisoned rats was depressed 49%, and liver triglycerides were elevated 180%. The latter observation was the basis of our statement, made in an abstract (19), that 2 hours after carbon tetrachloride poisoning glucose-6-Pase activity was not depressed. Subsequently we have consistently found large depressions of glucose-6-Pase activity at 2 hours. (See Table V.)

### Table III

<table>
<thead>
<tr>
<th>Mitochondrial citrate levels after storage at 0°C</th>
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</thead>
<tbody>
<tr>
<td>Conditions: 90 ml of a nuclei-free homogenate of 6 g of liver was prepared as described in &quot;Experimental Procedure.&quot; Two 10-ml aliquots were centrifuged immediately at 7000 × g for 10 minutes. The centrifuged mitochondrial pellets were extracted with 5% trichloroacetic acid. The residual nuclei-free homogenate was stored at 0°C. At intervals of 1, 2, and 3 hours, additional duplicate 10-ml aliquots were centrifuged and processed for citrate analysis. The elapsed time from death of the rat until extraction of the first mitochondrial pellets was approximately 1 hour. Four rats were treated with carbon tetrachloride plus fluorocetate, and four rats with fluorocetate alone. The data are expressed as the means ± standard errors in µg of total liver mitochondrial citrate per 100 g of rat body weight. The doses of carbon tetrachloride and/or fluorocetate were as indicated in &quot;Experimental Procedure.&quot; The rats were killed 13 hours after carbon tetrachloride feeding.</td>
</tr>
<tr>
<td>Storage time</td>
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<td>--------------</td>
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<tr>
<td>hrs</td>
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<tr>
<td>1 (initial level)</td>
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<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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</tbody>
</table>

### Table IV

<table>
<thead>
<tr>
<th>Mitochondrial ATPase transformation test and glucose 6-phosphatase activity of rat liver 4 hours after carbon tetrachloride feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditions: Food was removed from male rats at 6 a.m.; carbon tetrachloride administered at 10 a.m.; rats killed at 2 p.m. All data given are means ± standard errors for four control and four carbon tetrachloride-poisoned rats.</td>
</tr>
<tr>
<td>ATPase transformation test</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>2,4-Dinitrophenol-ATPase, mg*</td>
</tr>
<tr>
<td>Mg++-ATPase, mg*</td>
</tr>
<tr>
<td>Glucose 6-phosphatase, mg†</td>
</tr>
<tr>
<td>Liver triglycerides, mg‡</td>
</tr>
</tbody>
</table>

* Number of mg of P1 hydrolyzed from ATP by total liver mitochondria per 100 g of body weight.
† Number of mg of P1 hydrolyzed from glucose-6-P per whole liver per 100 g of body weight.
‡ Number of mg of triglycerides per whole liver per 100 g of body weight.

In this earlier study it was observed that at 2 hours there was an average depression of 13% (4 to 16%) in glucose-6-Pase activity of experimental animals compared with control animals assayed on the same day. However, there was no difference, statistically, when values for experimental animals obtained 2 hours after carbon tetrachloride poisoning were compared with a grand mean of 43 animals used as controls for experiments ranging from 2 to 24 hours after intoxication and assayed over many days. The latter observation was the basis of our statement, made in an abstract (19), that 2 hours after carbon tetrachloride poisoning glucose-6-Pase activity was not depressed. Subsequently we have consistently found large depressions of glucose-6-Pase activity at 2 hours. (See Table V.)

### Table V

| Mitochondrial Respiratory Control, Octanoate Oxidization, and Glucose 6-Phosphatase Activity 2 Hours After Carbon Tetrachloride Feeding—Intact rat liver mitochondria exhibit respiratory control; i.e. a limited supply of ADP dampens the oxidative rate, even though oxygen and substrate are available (Table V). On addition of the uncoupling agent, 2,4-dinitrophenol, the rate of oxidation of glutamate was increased 3.16-fold. Two hours after carbon tetrachloride poisoning there was a small, statistically insignificant increase in the rate of glutamate oxidation, both in presence and absence of 2,4-dinitrophenol. For liver mitochondria from carbon tetrachloride-poisoned animals, uncoupling with 2,4-dinitrophenol resulted in a 3.18-fold increase in the rate of oxidation of glutamate. These data clearly indicate that no impairment of mitochondrial respiratory control has occurred at this time. Furthermore, the liver mitochondria of these rats 2 hours after carbon tetrachloride poisoning, exhibited no impairment in the capacity to oxidize fumarate, or octanoate plus fumarate. However, for these same rats, liver glucose 6-phosphatase activity was depressed 33%, and liver triglycerides were elevated 69%. The latter two deviations from comparable control values were both highly significant statistically.
of body weight.

whole liver protein.

chondrial protein.

0.002

means was estimated with "student's" t-test.

cuvette, would give an optical density reading of 0.2 at 520 nm in a Coleman junior spectrophotometer. Data given are means ± standard errors. There were five control and five CCl₄-fed rats. For one rat, glucose 6-phosphatase was not determined. Significance of differences between means was estimated with "student's" t-test.

<table>
<thead>
<tr>
<th>Glutamate oxidation</th>
<th>Controls (µl)</th>
<th>CCl₄-fed (µl)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 2,4-dinitrophenol</td>
<td>3.11 ± 0.133</td>
<td>3.56 ± 0.180</td>
<td>0.5 &gt; p &gt; 0.4</td>
</tr>
<tr>
<td>Plus 2,4-dinitrophenol, µl</td>
<td>9.82 ± 1.42</td>
<td>11.3 ± 1.31</td>
<td>0.5 &gt; p &gt; 0.4</td>
</tr>
</tbody>
</table>

Octanote oxidation

Fumarate alone, µl

8.18 ± 0.18

9.01 ± 0.18

0.4 > p > 0.2

Octanote + fumarate, µl

18.5 ± 3.99

21.1 ± 6.52

0.5 > p > 0.4

Glucose 6-phosphatase, µg

25.5 ± 9.15

17.1 ± 1.55

p < 0.005

Liver triglycerides, mg

20.8 ± 4.56

35.2 ± 3.90

p < 0.005

* Number of µl of O₂ taken up in 20 minutes by 1 mg of mitochondrial protein.
† Number of µl of O₂ taken up in 60 minutes by 1 mg of mitochondrial protein.
‡ Number of mg of liver triglycerides per whole liver per 100 g of body weight.

<table>
<thead>
<tr>
<th>Rat liver microsomal DPNH cytochrome c reductase activity after carbon tetrachloride poisoning</th>
</tr>
</thead>
<tbody>
<tr>
<td>After CCl₄ feeding</td>
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* Number of animals.
† After Mahler (22), 1 unit of DPNH cytochrome c reductase is that amount of enzyme which yields an initial rate of 1.00 optical density unit per minute at 550 nm and 22°C. Data given here are the same, except that the temperature was 30°C and measured activity, in units, was converted to that amount of activity present in the whole liver per 100 g of rat body weight.

**TABLE VII**

Effect of carbon tetrachloride in vitro on rat liver glucose 6-phosphatase

Conditions: For the preincubation step at 30°C, whole homogenate or isolated microsomes were suspended in sucrose-EDTA medium previously saturated with CCl₄ and placed in a Thunberg tube with 0.5 ml of CCl₄ in the side arm.

<table>
<thead>
<tr>
<th>Rat liver fraction</th>
<th>Temperature</th>
<th>Concentration of liver fraction</th>
<th>Glucose 6-phosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate (2)</td>
<td>0°C</td>
<td>1.5 ± 0</td>
<td>28.1 ± 16.2</td>
</tr>
<tr>
<td>Homogenate (2)</td>
<td>30°C</td>
<td>1.5 ± 9</td>
<td>27.6 ± 3.7</td>
</tr>
<tr>
<td>Homogenate (2)</td>
<td>60°C</td>
<td>6.0 ± 9</td>
<td>28.7 ± 28.3</td>
</tr>
<tr>
<td>Microsomes (1)</td>
<td>0°C</td>
<td>1.25 ± 8.75</td>
<td>22.0 ± 34.9</td>
</tr>
<tr>
<td>Microsomes (1)</td>
<td>2.5 ± 10</td>
<td>24.0 ± 42.1</td>
<td></td>
</tr>
<tr>
<td>Microsomes (2)</td>
<td>5.0 ± 10</td>
<td>27.6 ± 35.5</td>
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</tr>
<tr>
<td>Microsomes (2)</td>
<td>1.5 ± 9</td>
<td>14.2 ± 0.2</td>
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</tr>
<tr>
<td>Microsomes (2)</td>
<td>5.0 ± 9</td>
<td>21.6 ± 1.1</td>
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</tr>
<tr>
<td>Microsomes (1)</td>
<td>15.0 ± 9</td>
<td>26.7 ± 10.6</td>
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</tr>
<tr>
<td>Microsomes (1)</td>
<td>15.0 ± 9</td>
<td>24.0 ± 8.82</td>
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</tr>
</tbody>
</table>

* Number of eq mg per 1.5 ml of final reaction volume.
† Number of µg of P₃ hydrolyzed from glucose-6-P in 20 minutes at 30°C by the amount of whole homogenate or microsome fraction indicated.
‡ One drop of CCl₄ added during resuspension of microsomes.

**DISCUSSION**

The finding that 13 hours after carbon tetrachloride poisoning, rats can respond to injected fluoroacetate by building up the citrate content of their liver mitochondria, and the fact that this citrate was not lost on subsequent storage of the mitochondria, indicates that some measure of mitochondrial membrane selective permeability has persisted at this relatively late stage of the developing pathology. An earlier study (5) in which the capacity of potassium-depleted mitochondria to reaccumulate potassium was measured, also indicated a persistence, at least in large part, of mitochondrial membrane selective permeability up to 10½ hours after carbon tetrachloride poisoning. The data of Table IV indicate that the mitochondrial ATPase transformation, which is an enzymic correlate of altered mitochondrial structure,
has not occurred at a time when liver triglycerides are increasing rapidly. As indicated by our earlier work (4) and by the data of Table V, mitochondrial respiratory control is also maintained at a time when marked aberrations in hepatic lipid metabolism are evident. The results of all these tests support the view that mitochondrial damage is not a key factor in early stages of the developing pathology. Nevertheless, these findings did not rule out the possibility that mitochondrial enzymes more directly concerned with oxidation of fat may be selectively destroyed by carbon tetrachloride poisoning. Indeed, we observed that in some rats, oxidation of octanoate by isolated liver mitochondria was depressed to 50% of control levels as early as 4 hours after carbon tetrachloride poisoning. However, the finding (3) that liver triglycerides are increasing rapidly during the first several hours after intoxication, coincident in time with attainment of the peak concentration of carbon tetrachloride in the liver (2), reduces the question of impaired fat oxidation at later times to secondary importance. The data of Table V show that at 2 hours, when marked aberrations in other phases of hepatic lipid metabolism are evident, octanoate oxidation was unimpaired. It appears to us at the present time that the available data are overwhelmingly against the view (16, 17, 20) that the liver mitochondria are the primary loci involved in carbon tetrachloride hepatotoxicity. The functional integrity of mechanisms localized in the endoplasmic reticulum is another question. Neubert and Maibauer (21) showed that a microsomal enzyme system which catalyzed detoxication of aminopyrine was destroyed in livers of carbon tetrachloride-poisoned rats at a time when mitochondrial oxidative phosphorylation was intact. Data presented here have clearly shown that aberrations in other enzymic properties of the endoplasmic reticulum, as revealed by study of the isolated microsome fraction, are evident as early as 2 hours after carbon tetrachloride feeding when, by all tests so far employed, mitochondrial function was unimpaired. The finding that enzymes of the endoplasmic reticulum were undergoing pathological change at a time when the toxic agent was at its peak concentration in the liver, and the sensitivity of microsomal glucose 6-phosphatase to carbon tetrachloride in vitro, suggested to us that the key mechanism attacked by carbon tetrachloride was probably localized in this part of the cell. How the further finding that liver triglycerides were piling up rapidly at this time led to the formulation and testing of a simple hypothesis to explain the pathogenesis of carbon tetrachloride fat accumulation has been indicated above. The hypothesis that the primary lesion responsible for the pathological accumulation of liver fat involves inhibition or destruction of a hepatic triglyceride-secreting mechanism has so far been tested only with respect to carbon tetrachloride poisoning. Nevertheless, it appears to us that the general problem of the pathogenesis of hepatic fat accumulation has now reached a new level of development. Experiments are in progress to determine whether the hepatic triglyceride secretory mechanism, so elegantly revealed by Byers and Friedman (1), has been inhibited or destroyed in other pathological conditions leading to abnormal accumulation of liver fat.

**SUMMARY**

Studies of citrate retention, adenosine triphosphatase activity, and respiratory control, indicate that integrity of liver mitochondria is not seriously altered in the period from 4 to 10 hours after intragastric administration of carbon tetrachloride to rats. At 2 hours, mitochondrial respiratory control and oxidation of octanoate were unimpaired, whereas liver glucose 6-phosphatase was depressed 33% per cent and liver triglyceride content was increased 60%. Microsomal DPNH cytochrome c reductase activity of rat liver is significantly increased 2 hours after intoxication. Glucose 6-phosphatase is completely destroyed in vitro by solutions of carbon tetrachloride in isotonic sucrose at 30°C, but not at 0°C. The conclusion is drawn from these experiments that liver mitochondria are not the primary target for the attack by carbon tetrachloride. The clear-cut evidence of early involvement of the endoplasmic reticulum has led to the formulation and testing of a new hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation.

**REFERENCES**

Studies of Biochemical Changes in Subcellular Particles of Rat Liver and Their Relationship to a New Hypothesis Regarding the Pathogenesis of Carbon Tetrachloride Fat Accumulation
Richard O. Recknagel and Benito Lombardi


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