Effect of Calcium Chelation on the Ion Content of Liver Mitochondria in Carbon Tetrachloride-poisoned Rats*

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SUMMARY

When toxic doses of CCl₄ were given to rats, there ensued marked accumulations of Ca⁺⁺ and inorganic orthophosphate by whole liver commencing 6 hours after treatment. When these livers were homogenized in 0.25 M sucrose, the Ca⁺⁺ and Pᵢ concentrations of the mitochondria subsequently harvested were elevated several fold, reflecting the higher levels of these ions in the whole tissue. The K⁺ concentration usually declined in the whole tissue and decreased in the mitochondria. In these mitochondria, respiratory rate and respiratory control were markedly depressed. If, on the other hand, the liver samples were homogenized in 0.25 M sucrose containing the Ca⁺⁺ chelator, ethylenediaminetetraacetate (or EGTA or DCTA), the mitochondria often contained much lower amounts of Ca⁺⁺ and Pᵢ, and the respiratory rate and respiratory control were closer to normal levels. Whether a chelator was used or not, the changes in Ca⁺⁺ and K⁺ concentration in the whole tissue or mitochondria did not appear to be closely related to each other. The effect of the chelators on ionic composition appeared to result from the prevention of accumulation of Ca⁺⁺ and Pᵢ by the mitochondria subsequent to the homogenization of the tissue. It is concluded that the major portion of the changes in content of Ca⁺⁺ of isolated liver mitochondria following CCl₄ poisoning which have been reported by others occurred during the isolation of these organelles and must be considered to be artifacts of the isolation procedure. The residual chelator-resistant changes in respiratory function and ionic composition may reflect actual qualitative changes in the mitochondria extant in these organelles in vivo.

It has been reported that among the morphological and biochemical changes in liver brought about by feeding CCl₄ are striking alterations in ionic composition of the whole liver and mitochondria (1-5). Thiers, Reynolds, and Vallee (2) showed that mitochondria isolated from livers of CCl₄-treated animals contained larger amounts of Ca⁺⁺ and lower amounts of K⁺ than did the control mitochondria. In addition, the respiratory rates and phosphorylative capacity of these mitochondria decreased as a function of the Ca⁺⁺:K⁺ ratio (3). The data on ion movements during CCl₄ poisoning have been used to support the concept that the changes observed in mitochondrial transport of these ions in vivo also occur in vitro, perhaps as part of a physiological regulatory process (6). In support of this concept are the extensive studies by Reynolds (4, 7, 8) on the morphology, cytochemistry, and ultrastructure of liver parenchymal cells, in which he concluded that in the latter stages of Ca⁺⁺ accumulation (16 to 24 hours following administration of CCl₄) the mitochondria in situ contained large amounts of Ca⁺⁺ which were actively accumulated.

Bearing on these findings is the recent report that kidney mitochondria obtained from tissue which was calcified as a consequence of parathyroid hormone treatment also contained high levels of Ca⁺⁺ as well as Pᵢ and exhibited inhibited respiration and phosphorylation, and impaired ion-translocating properties (9, 10). By homogenizing the kidney tissue in the presence of ethylenediaminetetraacetic acid—a technique introduced by Slater and Cleland (11, 12) to prevent Ca⁺⁺ translocation during the isolation of sarcosomes—it was concluded that a major portion of this Ca⁺⁺ was not present within the mitochondrion in situ but rather was sequestered during the isolation of the mitochondria. Because the total Ca⁺⁺ content of the whole liver increased after CCl₄ poisoning (1, 2), and because the behavior of the mitochondria subsequently harvested resembled that of kidney mitochondria following parathyroid hormone administration (9, 10), the possibility was raised that under the former conditions some Ca⁺⁺—perhaps a major portion—was present in the mitochondrion as an artifact of the preparatory procedure. Indeed Rees, Sinha, and Spector (5) had suggested that certain abnormalities in mitochondrial enzyme activities were artifacts arising from exposure of the mitochondria to high concentrations of calcium during isolation of the particles.

The present study was undertaken to evaluate this possibility and to determine quantitatively, if possible, the degree of internal redistribution of Ca⁺⁺ which might have occurred. The data presented below indicate that a major fraction of the Ca⁺⁺ found...
TABLE I

Ion content of whole liver from rats killed at various times after receiving CCl4

For each time period listed, each of four experimental rats received 0.5 ml of CCl4 in mineral oil per 100 g of body weight. Four untreated control rats received no treatment. At the specified time after treatment, all eight rats were killed; the livers were removed, chilled, and minced to ensure a homogeneous sample. Aliquots of mince, usually 3 g, were homogenized in 0.25 M sucrose or in 0.25 M sucrose + 0.01 M sodium EDTA. After samples were withdrawn for analysis, the homogenates were processed to obtain mitochondria, the assays of which are listed in Table II. The data listed in the present table are from analysis of the samples homogenized in sucrose only. Statistical analysis of the data in this table was conducted by means of Student's t test for independent samples. Each group of four experimental samples was compared with its own group of four control samples. For simplicity in listing the data, however, all assays of the 20 control samples were pooled to obtain the standard deviation.

<table>
<thead>
<tr>
<th>Hours after CCl4</th>
<th>Ion content (mean ± S.D.)</th>
<th>$\text{Ca}^{++}$</th>
<th>$P_i$</th>
<th>$K^+$</th>
<th>$Na^+$</th>
<th>$Mg^{++}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 ± 2</td>
<td>43 ± 12</td>
<td>418 ± 63</td>
<td>149 ± 25</td>
<td>43 ± 4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>18 ± 2b</td>
<td>57 ± 5</td>
<td>340 ± 23b</td>
<td>180 ± 28b</td>
<td>47 ± 3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>42 ± 18a</td>
<td>51 ± 15</td>
<td>270 ± 32b</td>
<td>260 ± 48b</td>
<td>44 ± 4b</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>130 ± 43</td>
<td>99 ± 9</td>
<td>270 ± 67b</td>
<td>441 ± 70b</td>
<td>52 ± 6b</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>237 ± 43</td>
<td>172 ± 34</td>
<td>337 ± 23b</td>
<td>358 ± 29b</td>
<td>52 ± 2b</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>115 ± 47</td>
<td>90 ± 18</td>
<td>259 ± 106</td>
<td>325 ± 306a</td>
<td>41 ± 8</td>
<td></td>
</tr>
<tr>
<td>* p &lt; 0.01.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$\text{b p &lt; 0.05.}$</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

in the liver mitochondria from CCl4-fed rats which were isolated from sucrose media that did not contain an adequate level of EDTA or other Ca++ chelator was sequestered post mortem and should not be attributed to an action of CCl4 on transport in vivo. Likewise, some loss in respiratory function appeared to be secondary to this movement of Ca++. The data also show that CCl4 treatment decreased the content of $K^+$ in the whole liver and mitochondria and that this change was not directly related to the accumulation of $Ca^{++}$.

METHODS

White, male rats of the A. R. Schmidt or Holtzman strains, usually weighing between 200 and 300 g, were treated orally with 0.5 or 0.25 ml/100 g of body weight as specified in the text below. In early studies the control animals received an equal volume of sucrose + 0.01 M sodium EDTA or sucrose (no chelator) and should not be attributed to an action of CCl4 on transport in vivo. Likewise, some loss in respiratory function appeared to be secondary to this movement of Ca++. The data also show that CCl4 treatment decreased the content of $K^+$ in the whole liver and mitochondria and that this change was not directly related to the accumulation of $Ca^{++}$.

RESULTS

Patterns of Ionic Change in Liver and Liver Mitochondria Produced by CCl4 Poisoning, and Effect of Chelating Agents—the results of CCl4 poisoning on the ion content of whole liver in one specific study are listed in Table I. Major increases in $Ca^{++}$, $P_i$, and $Na^+$ content and a decrease in $K^+$ content occurred by the 6th hour after treatment. $Ca^{++}$ and $P_i$ concentrations continued to increase and were, respectively, 24 and 4-fold higher than the control level 37 hours after treatment, after which they decreased and appeared to return toward the control values. The $K^+$ concentration decreased one-third by the 12th hour and remained depressed at that level throughout the remainder of the study. The $Na^+$ concentration reached a maximum by the 24th hour and then decreased, but was still more than twice the control concentration when the study was terminated. The Mg$^{++}$ concentration increased to a small but significant extent in the 12- to 37-hour study periods. There did not seem to be any consistent quantitative relationship between $K^+$ loss and $Na^+$, $Ca^{++}$, or $P_i$ gain, or between the gain in $Na^+$ and $Ca^{++}$. On the other hand, $P_i$ and $Ca^{++}$ were closely correlated, with about 0.5 mole of $P_i$ gained per mole of $Ca^{++}$.

The concentrations of these ions in the mitochondria isolated from these liver specimens are listed in Table II. When homogenization was conducted in 0.25 M sucrose only, that is, in the absence of a chelator such as EDTA, the changes in ionic composition following CCl4 usually reflected the changes which occurred in the whole tissue. For example, $Ca^{++}$ and $P_i$ concentrations increased to maximum values at the 37th hour, at which time the concentrations of these ions in the whole liver were also maximum. $K^+$ content decreased by the 6th hour, and was depressed throughout the remainder of the study. Mg$^{++}$ content changed little, if at all. One interesting discrepancy between the ionic composition of the mitochondria and that of whole tissue was noted in the case of $Na^+$ concentration. Whereas the mitochondrial content of $Na^+$ increased in the 6-, 12-, and 24-hour periods to a degree which closely paralleled a
The liver mitochondria from the CCl₄-poisoned rats also displayed inhibited respiration and loss of respiratory control (Table III). When chelator was omitted during homogenization, the mitochondria from CCl₄-fed animals contained several times greater concentrations of Ca²⁺ and P₁, and one-fifth less K⁺, had a respiratory rate about one-third that of normal, and did not exhibit respiratory control (ratio = 1). In this study EGTA and DCTA as well as EDTA were tested. When any of the chelators was present during homogenization, the mitochondria from CCl₄-poisoned animals contained several times greater concentrations of Ca²⁺ and P₁, and one-fifth less K⁺, had a respiratory rate about one-third that of normal, and did not exhibit respiratory control (ratio = 1). In this study EGTA and DCTA as well as EDTA were tested. 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of the CCl\textsubscript{4} mitochondria, it should be noted that EDTA and EGTA by themselves had a deleterious effect on the respiratory rate and respiratory control of the normal mitochondria. DCTA, on the other hand, stimulated respiration of the control mitochondria without lowering respiratory control.

Since Chance had shown that Mg\textsuperscript{++} could stimulate respiration of EDTA-treated mitochondria (15), this ion, alone or with ATP and succinate, was included together with the chelator during homogenization. These combinations had no effect over that of the chelator alone.

Studies on Mode of Action of Chelating Agents as Related to Ca\textsuperscript{++} and Pi Content of Mitochondria—In view of the known chelating properties of EDTA, we hypothesized that the chelator functioned in one of three ways: (a) by altering the characteristics of the mitochondrion so that Ca\textsuperscript{++}-containing mitochondria were either destroyed or else sedimented in a different fraction during differential centrifugation, e.g. debris or microsomes; (b) by removing from the mitochondrion Ca\textsuperscript{++} which had been deposited in vivo; or (c) by preventing extramitochondrial Ca\textsuperscript{++} from entering the mitochondrion following homogenization of the tissue. Each of these possibilities was examined, with the evidence favoring the last mode of action.

It was determined first whether the Ca\textsuperscript{++} in the liver mitochondria from the CCl\textsubscript{4}-fed animals harvested without chelator was confined to a small portion of the total mitochondrial fraction or was instead distributed in a general fashion. Fig. 1 portrays the results from one study, in which these mitochondria were partially sedimented through a linear sucrose gradient (0.25 M to 1.75 M). The mitochondria (defined by protein analysis) migrated as a broad band which spread over a large portion of the gradient, indicating by this behavior their heterogeneity. Ca\textsuperscript{++} was distributed in a similar manner, suggesting that it was associated with the majority of the mitochondria. Hence, the reduction in Ca\textsuperscript{++} content produced by chelator, often to a small fraction of that when chelator was not used, could not be attributed to loss or destruction of a specific Ca\textsuperscript{++}-containing fraction.

In order to determine the effect of chelator on the distribution of Ca\textsuperscript{++} within the various sedimentable fractions of the cell, livers from normal and CCl\textsubscript{4}-fed rats were homogenized and separated by differential centrifugation as described above. Table IV shows that, in the normal liver homogenized without EDTA, Ca\textsuperscript{++} was present in greatest amount in the debris plus nuclear fraction, followed by the supernatant, mitochondrial, and mitochondrial "wash" fractions, in that order. Inclusion of EDTA in the homogenizing medium had no effect on the Ca\textsuperscript{++} content of the debris plus nuclear fraction, but this ion was decreased in the mitochondrial and microsomal fractions and increased in the cell supernatant fraction. CCl\textsubscript{4} treatment led to major increases in Ca\textsuperscript{++} content in all fractions, with the debris plus nuclear fraction containing over two-thirds and the mitochondrial fraction containing about 10% of the total amount. When EDTA was present during the homogenization, the Ca\textsuperscript{++} content of all of the particulate fractions was drastically lowered, and over 75% of the Ca\textsuperscript{++} of the whole liver was found in the supernatant fraction. The data in this and the preceding paragraph, taken together, make it unlikely that the chelator functioned in accordance with Possibility a.

Data which indicate that under our experimental conditions EDTA was unable to remove the major content of Ca\textsuperscript{++} contained in mitochondria (Possibility b) are shown in Fig. 2. Livers from control and CCl\textsubscript{4}-fed rats were homogenized in 0.25 M sucrose. After the debris plus nuclear fraction was removed, the mitochondrial pellet was obtained and without

**Table IV**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ca\textsuperscript{++} content per fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Debris plus nuclei</td>
<td>6.3</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.6</td>
</tr>
<tr>
<td>Mitochondrial washes</td>
<td>0.7</td>
</tr>
<tr>
<td>Microsomes</td>
<td>3.1</td>
</tr>
<tr>
<td>Cell supernatant</td>
<td>4.3</td>
</tr>
<tr>
<td>Recovered in fractions</td>
<td>16.0</td>
</tr>
<tr>
<td>Homogenate, total</td>
<td>17.8</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>90</td>
</tr>
</tbody>
</table>

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The effect of suspension in EDTA on the Ca ++ content of liver mitochondria from control and CCl 4 -fed rats. The animals receiving CCl 4 (0.25 ml of CCl 4 in mineral oil per 100 g of body weight) were killed 18 hours after treatment. Other details are listed in the text.

Additional washing was resuspended in 0.25 M sucrose alone or in sucrose containing 0.01 and 0.03 M EDTA in a final volume equal to that of the original homogenate. These suspensions were shaken gently and continuously at 0 ° and were sampled over a 2-hour period. Experimental livers homogenized without chelator yielded mitochondria with a 6-fold increase in Ca ++ concentration compared to the control tissue. There was an initial, rapid loss of about 15% of the Ca ++ from the experimental mitochondria, followed by a gradual loss at an over-all rate which was little influenced by the concentration or presence of EDTA. At the end of the study, for example, the mitochondria from the CCl 4 -treated animals suspended in EDTA contained one-third less Ca ++ than at the start of the study, but this level was somewhat greater than in the mitochondria suspended in sucrose alone. Moreover, there was no discernible effect of the chelator on the levels of Ca ++ in the control mitochondria. These data seem to rule out Possibility b.

The data suggest, by elimination, that EDTA functioned according to Possibility c, by preventing the uptake of extramitochondrial Ca ++ by the mitochondrion subsequent to homogenization of the tissue. That normal mitochondria freed from the liver cell could sequester Ca ++ under the conditions of homogenization (0-3 °C, no added substrate) is indicated by the data shown in Fig. 3. Low levels of CaCl 2 (0.5 to 2.0 mM) were added to 0.25 M sucrose, and the liver was homogenized. The initial mitochondrial pellet was resuspended and washed in 0.25 M sucrose only, so that the only intentional exposure of the mitochondria to Ca ++ was during the initial part of the isolation procedure. As indicated in the figure, the concentration of Ca ++ in the isolated mitochondria was a direct function of the concentration of the Ca ++ in the homogenizing medium. The concentration to Ca ++ appeared to be P 1, since its accumulation by the mitochondrion was proportional to that of Ca ++. The concentration of the other ions measured did not change appreciably.

Additional evidence supporting the concept that the chelator functioned by complexation of Ca ++ and preventing its entry and that of P 1 into the mitochondrion during homogenization is shown in Fig. 4. In these experiments, livers from normal and CCl 4 -fed rats were pooled and homogenized in 0.25 M sucrose alone or containing either 0.5, 1.0, or 2.0 mM CaCl 2, and mitochondria were prepared as described in the text.
Ahmed, and McLean (1) and Reynolds, Thiers, and Vallee (2, 3), observed that in the liver of CC14 treated animals, a low content of K+, and a loss of respiratory function (2, 3), Reynolds conducted a careful study of the morphological and biochemical changes which are observed (1). Followed by a sustained accumulation of Ca++ beginning several hours later. The earlier rise in liver Ca++ content has been confirmed by Smuckler (16), but not by Judah, Ahmed, and McLean (1). The rat liver can be expected to explain the assimilation of biochemical and morphological changes which are observed (1). Following the isolation of liver mitochondria from CC14-poisoned rats and the demonstration that these organelles contained a high content of Ca++, a low content of K+, and a loss of respiratory function (2, 3), Reynolds conducted a careful study of the morphological and biochemical changes in liver tissue (4, 7, 8). He reported that there was rapid accumulation and release of Ca++ by the liver cell within a 2-hour period after poisoning, followed by a sustained accumulation of Ca++ beginning several hours later. The earlier rise in liver Ca++ content has been confirmed by Smuckler (16), but not by Judah, Ahmed, and McLean (1). The chelator had relatively little influence on the removal of Ca++ during the fractionation procedure when this ion was present in the medium in which the whole liver was homogenized. That the chelator lowered the Ca++ content of isolated mitochondria through complexation with extramitochondrial Ca++ and preventing its accumulation by the mitochondrion during the isolation procedure—the action assumed to occur by Slater and Cleland (11)—is supported by the following evidence.

1. The liver mitochondria were capable of rapid accumulation of Ca++ during the fractionation procedure when this ion was present in the medium in which the whole liver was homogenized. This confirms the similar observation of Reynolds, Thiers, and Vallee (3).

2. Three different chelators and different cationic forms of one chelator functioned in a qualitatively similar manner, suggesting that the factor in common was the Ca++-binding property of these compounds.

3. The concentration of chelator effective in yielding low Ca++ mitochondria was related to the total amount of Ca++ in the whole tissue.

4. The chelator had relatively little influence on the removal of Ca++ from normal mitochondria or mitochondria from CC14-fed animals, even during extended periods of exposure.

5. Since Ca++ was distributed in a general fashion throughout the mitochondrial fraction, destruction by chelator of a possible mitochondrial fraction, small in amount but heavily loaded with Ca++, could not account for the lowering of the Ca++ content of the mitochondria.

6. Inclusion of chelator during homogenization caused the majority of the Ca++ of the tissue to appear in the supernatant fraction, rather than in the debris and mitochondria, as occurred when chelator was omitted. This finding would rule out the possibility that the chelator slightly altered the sedimenting properties of the mitochondria so that the calcium-containing...
particles would sediment in the fraction just ahead of or just after the mitochondria (i.e. debris or microsomes).

It is apparent, therefore, that the earlier data (2, 3) on the large scale accumulation of Ca++ and the apparent inverse stoichiometric relationship between Ca++ gain and K+ loss in the mitochondria from CCL4-poisoned rats are misleading, since the reported changes in Ca++ content most likely occurred in large measure in vitro, not in vivo as assumed. On the other hand, since the experimental mitochondria contained higher than control levels of Ca++ and Pi and lower levels of K+ and respiratory function even when harvested in the presence of chelator, our results resemble the earlier data (2, 3) qualitatively, if not quantitatively. Whether the present data when chelator was used reflect the ionic composition of the mitochondria as they exist in vivo, or instead mean that the chelator as used was only partially effective in preventing artifactual translocation of Ca++ and Pi, must yet be determined.

REFERENCES

Effect of Calcium Chelation on the Ion Content of Liver Mitochondria in Carbon Tetrachloride-poisoned Rats
David V. Cohn, Roger Bawdon, Raymond R. Newman and James W. Hamilton


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