Effects of Maleate on the Content of CoA and Its Derivatives in Rat Kidney Mitochondria*

Anastasis Pacanis, Tadeusz Strzelecki, and Jerzy Rogulski
From the Department of Clinical Biochemistry, Institute of Pathology, Medical Academy, Gdnask 80-211, Poland

(Received for publication, January 7, 1981)

Effects of maleate on the content of CoA derivatives in isolated mitochondria and in the tissues of maleate-intoxicated rats have been studied. The addition of maleate to kidney mitochondria incubated with 2-oxoglutarate decreased CoA-SH and acid-soluble acyl-CoA concentrations while acid insoluble acyl-CoA content remained unchanged. As a result, a substantial loss (depletion) of the total CoA occurred. Similar changes in CoA content were found in vivo in the kidneys of maleate-treated rats. Neither in the isolated liver mitochondria nor in the liver of intoxicated animals have such changes been observed before. Acetoacetate, the substrate for CoA transferase, added to kidney mitochondria before maleate, abolished its inhibitory effect on oxidation of 2-oxoglutarate and prevented the decrease of CoA content. The data are in accord with the previous findings indicating that maleate can bind and sequester CoA in the form of a stable and metabolically inert compound.

It has been shown that maleic acid may act as CoA acceptor in the reaction catalyzed by the purified succinyl-CoA:3-oxo-acid coenzyme A transferase (EC 2.8.3.5.), referred to herein as CoA transferase (1–3). In the preceding paper we demonstrated that the most obvious product of this reaction, i.e. maleyl-CoA, reacts spontaneously with the SH groups of free CoA to form an addition compound (Reaction 1). This product turns, after the hydrolysis of an acyl bond, into a stable, inert thioether resulting in a complete inactivation of the molecule of CoA (Reaction 2).

We have postulated that the enzymatic formation of maleyl-CoA and its subsequent transformations to ether may provide a trapping system for mitochondrial CoA in in vivo conditions. This mechanism would explain satisfactorily the inhibitory effects of maleate on the oxidation of CoA-dependent substrates on the basis of sequestration of part of CoA from the metabolic pool. Several years ago, it was shown in a preliminary communication from our laboratory that maleate decreased mitochondrial CoA content significantly (4). No detailed studies on changes in CoA intermediates have, however, been reported. In the present work, the effects of maleate on CoA derivatives in kidney mitochondria and in the kidney tissue of maleate-treated rats have been studied.

**EXPERIMENTAL PROCEDURES**

*In Vivo Experiments—*Male Wistar rats of 180 to 250 g, fasted overnight, were used. Sodium maleate was administered intraperitoneally in a single dose of 400 mg/kg of body weight, and the animals were then killed, by cervical dislocation, at intervals ranging from 20 to 180 min after treatment. The noninjected animals were used as controls for the experiments. The kidneys and livers were gently excised and freeze-clamped in aluminum tongs precooled in liquid nitrogen. The frozen tissues were pulvverized and extracted twice with perchloric acid as described by Williamson and Corkey (5).

**Preparation and Incubation of Mitochondria—*Rat kidney and liver mitochondria were isolated in a medium containing 250 mM sucrose, 5 mM Tris-chloride buffer, pH 7.4, 5 mM potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, and 1.5 mM ADP. The anaerobic dismutation of 2-oxoglutarate and ammonia was assayed in the presence of antimony and rotenone as described previously (7). The reaction was started by the addition of mitochondria (0.2 ml) and ended at time intervals by the addition of 0.5 ml of cooled perchloric acid containing 100 mM dithiothreitol. Zero-time samples were obtained by adding perchloric acid to the media prior to the mitochondria.

**Isolation of Radioactive Succinohydroxamic Acid—*Labeled succinyl-CoA formed in a large scale mitochondrial experiment was separated by chromatography after a conversion to succinohydroxamic acid, essentially according to Hersh and Jencks (8). Rat kidney mitochondria (14 mg of protein/ml) in a total volume of 5.0 ml were incubated for 15 min at 30 °C with the medium described in Fig. 2, containing 5 mM 2-oxo-[5-14C]glutarate (specific activity, about 10,000 cpm/μmol). When present, maleate was added at 2 mM concentration. At the end of the incubation, an aliquot was taken for 2-oxoglutarate determination. 1 ml of 2% neutralized hydroxylamine was then added to the reaction mixture to convert any succinyl-CoA present to succinohydroxamic acid. The mixture was allowed to stand for 15 min at room temperature, then heated for 3 min at 100 °C, cooled and filtered. In a control sample, the addition of hydroxylamine and deproteinization had been done prior to the addition of labeled 2-oxoglutarate. Succinohydroxamic acid was then added (25 μmol/ml) to the filtrate as a carrier and the solution was placed on a Dowex 1-formate column (15 × 1.2 cm). The column was washed with 70 ml of water and successively eluted with 500 ml each of 0.005, 0.05, and 1.0 M acetic acid. 15-ml fractions were collected. The fractions containing hydroxamic acid were pooled, concentrated on a rotary evaporator, transferred to planchets, dried, and counted with an end window Ge-Mounter.

**Analytical Methods—*Following deproteinization and neutralization, the samples of tissue or mitochondrial extracts were assayed for...
CoA and its derivatives using fluorometric enzyme techniques, and the unknowns were compared with the internal standards. CoA-SH content was assayed by the 2-oxoglutarate dehydrogenase method of Garland et al. (9) in the presence of 16 mM dithiothreitol. Acetyl-CoA was determined by means of citrate synthase and maleate dehydrogenase in the assay system described by Herrera and Freinkel (10). The total acid-soluble CoA derivatives, i.e., acyl-soluble acetyl-CoA plus CoA-SH, were measured after an alkaline hydrolysis (10 min at 55 °C, pH 11 to 12) of an aliquot of the extract in the presence of 25 mM dithiothreitol. The content of the short chain acyl-CoA derivatives other than acetyl-CoA was calculated by the subtraction of the separately assayed contents of CoA-SH and acetyl-CoA from the content of the total acid-soluble CoA. Acid-insoluble acyl-CoA was determined as CoA after an alkaline hydrolysis of the pellet produced by perchloric acid extraction. The total content of CoA was measured after the alkaline hydrolysis of the whole sample containing both the perchloric acid extract and the pellet. The recoveries of CoA and acetyl-CoA added to the extracts or to the powdered tissue prior to the extraction were within 10% quantitative.

Pyruvate and 2-oxoglutarate were assayed enzymatically according to the methods given by Bergmeyer and Bernt (11) and Bucher et al. (12). Succinohydroxamic acid was prepared and determined according to the procedure of Lipmann and Tuttle (13). Protein was assayed by the method of Gornall et al. (14).

Materials—2-oxoglutarate dehydrogenase was prepared according to Sanadi et al. (15) and citrate synthase according to Srere (16). Sodium acetocetate was prepared by the method of Seeley (17). CoA-SH (85-90% pure), acetyl-CoA (90% pure), and 1-glutamate dehydrogenase were purchased from Sigma, and 2-oxoglu-[5-14C]tarate (specific activity, 80 mCi/mol) was obtained from the Radiochemical Centre (Amersham, Bucks, England). NADH was from Boehringer and Soehne GmbH (Mannheim, FRG), and maleic acid from v/o Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

Fig. 1 shows that the addition of 2-oxoglutarate to rat kidney mitochondria caused a decline of CoA-SH content during the first minute of incubation and an equivalent rise of acid-soluble acetyl-CoA. Subsequently, a partial release of CoA-SH and a slight decrease of acid-soluble acyl-CoA occurred. No changes in acid-insoluble acyl-CoA or in the total content of CoA were observed. The term “Total CoA” means enzymatically active CoA derivatives, i.e., free CoA-SH, acid-soluble, and acid-insoluble acyl-CoA metabolites.

The effect of maleate on the content of CoA derivatives in rat kidney mitochondria incubated in the presence of 2-oxoglutarate. Mitochondria (4-6 mg/ml) in a total volume of 1.0 ml were incubated with basic medium, 5 mM 2-oxoglutarate and 1.5 mM ADP. When present, maleate was added at 2 mM concentration. Incubations were ended at 0, 1, 2.5, and 5 min intervals by addition of 0.5 ml of perchloric acid (12.5%). The CoA derivatives were then assayed as described under “Experimental Procedures.” The changes induced by maleate are depicted by broken lines (---), while controls are depicted by solid lines (——). Values shown are the means of four to five separate experiments.

In the presence of maleate the CoA-SH content fell rapidly and continued to decrease with time to values as low as 10% of the original content. The increase of acid-soluble acyl-CoA derivatives during the first minute after maleate addition was much smaller relative to the values obtained in the absence of the inhibitor. Moreover, it was followed by a significant decrease to about 70% of the initial value. These changes resulted in a substantial decrease of free CoA-SH and short chain acyl-CoA derivatives from about 2.5 to about 0.8 nmol/mg of protein. No long chain, acid-insoluble acyl-CoA accumulated. As a consequence, there was a definite loss of about 65% of the total content of CoA in rat kidney mitochondria metabolizing 2-oxoglutarate in the presence of maleate.

Under the conditions of anaerobic dismutation of 2-oxoglutarate, the changes in the level of CoA intermediates corresponded mainly to the formation of succinyl-CoA and its relatively slow metabolism by the substrate-level phosphorylation. The fall in the CoA-SH content associated with maleate addition persisted during the whole incubation period (Fig. 2). A transient initial elevation was followed by a substantial decrease in acid-soluble acyl-CoA. These results indicate, therefore, a depletion of the total content of CoA by about 35%. The striking difference between the changes in CoA derivatives content as a function of time in the presence of maleate under aerobic and anaerobic conditions might be explained on the basis of succinate accumulation in the course of anaerobic dismutation of 2-oxoglutarate. As was shown, succinate is an effective competitor with maleate in CoA transferase reaction (3).
protein. In the presence of maleate, only 0.18% of radioactivity was recovered in succinohydroxamic acid, corresponding to about 0.65 nmol of succinyl-CoA/mg of mitochondrial protein. These results mean the minimum values of succinyl-CoA content possible, but nevertheless, they indicate that under the anaerobic dismutation of 2-oxoglutarate, maleate lowered the succinyl-CoA concentration by about 40%, which is in good agreement with the results presented in Fig. 2.

It has been demonstrated previously (7) that the presence of any substrate for CoA transferase greatly diminished or relieved the inhibitory effects of maleate on 2-oxoglutarate metabolism. The changes in 2-oxoglutarate utilization and in the total content of CoA in rat kidney mitochondria induced by maleate added before or after acetooacetate are shown in Fig. 3.

The addition of 2 mM maleate caused a decline of total CoA to about 53% of the initial value, which corresponded to about 56% of the inhibition of 2-oxoglutarate oxidation. The subsequent addition of 6 mM acetooacetate influenced the CoA content and the rate of 2-oxoglutarate metabolism only slightly. On the other hand, the presence of acetooacetate prior to maleate addition abolished its inhibitory effect on 2-oxoglutarate oxidation almost completely and prevented the decrease of CoA content. It is also interesting to mention that the inhibitory effects of maleate added along with the substrate were almost doubly strong in comparison with the addition after a prior period of incubation of mitochondria with 2-oxoglutarate alone.

Experiments with rat liver mitochondria pointed out that maleate did not at all affect the changes induced by 2-oxoglutarate in the contents of CoA intermediates or the total content of CoA, which was in accordance with the previous data indicating that maleate did not affect the oxidation of 2-oxoacids in the liver (18, 19).

Intraperitoneal administration of maleate to normal rats produced changes in tissue CoA derivatives similar to those found in isolated mitochondria (Table I). The most characteristic effect, observed in the kidney 3 h after the injection of maleate, was the loss of about half of the total CoA content. Again, the most affected was the content of CoA-SH, which was lowered to about 30%, whereas other short chain acyl-CoA derivatives were lowered by about 40%. Acid-insoluble CoA forms remained unchanged. In the liver, the moderate conversion of CoA-SH into a short chain acyl-CoA coincided with the significant decrease of acetyl-CoA by about 50% with no changes in the total CoA content.

In Fig. 4, changes of CoA-SH and acid-soluble acyl-CoA in the kidney at different intervals after the intoxication with maleate are depicted. As soon as 20 min after the injection the content of CoA-SH fell by about 50%. It decreased further, to about 30% of the control animals' values, and remained at this low level for the next 2 h. The content of acetyl-CoA in the kidneys of maleate-treated rats decreased almost linearly during the first 20 min after intoxication, to about 40% of the control rats' values. It remained at this level for at least 1 h and then rose slightly, up to 50% of the control values in the 3rd h after the injection. The decrease of the tissue levels of CoA coincided with the accumulation of large amounts of 2-oxoacids in the kidneys of maleate-treated rats. The increase of 2-oxoglutarate content with time follows the pattern exactly reciprocal to the changes of CoA. After 40 min the level of 2-

<table>
<thead>
<tr>
<th>Kidney</th>
<th>Control</th>
<th>Maleate-treated</th>
<th>Control</th>
<th>Maleate-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CoA</td>
<td>137 ± 13 (9)</td>
<td>74 ± 13 (6)</td>
<td>245 ± 18 (6)</td>
<td>234 ± 27 (5)</td>
</tr>
<tr>
<td>Free CoA-SH</td>
<td>48 ± 9 (10)</td>
<td>14 ± 2 (9)</td>
<td>110 ± 14 (9)</td>
<td>68 ± 11 (8)</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>29 ± 7 (4)</td>
<td>10 ± 4 (4)</td>
<td>71 ± 8 (4)</td>
<td>35 ± 4 (4)</td>
</tr>
<tr>
<td>Acid-soluble acetyl-CoA</td>
<td>71 ± 9 (10)</td>
<td>36 ± 6 (8)</td>
<td>96 ± 8 (8)</td>
<td>127 ± 28 (8)</td>
</tr>
<tr>
<td>Acid-soluble acyl-CoA, other than acetyl-CoA</td>
<td>42</td>
<td>26</td>
<td>25</td>
<td>92</td>
</tr>
<tr>
<td>Acid-insoluble acyl-CoA (c)</td>
<td>15 ± 3 (5)</td>
<td>17 ± 3 (4)</td>
<td>27 ± 4 (5)</td>
<td>29 ± 8 (5)</td>
</tr>
<tr>
<td>Calculated total CoA (a + b + c)</td>
<td>134</td>
<td>67</td>
<td>233</td>
<td>224</td>
</tr>
</tbody>
</table>

TABLE I

Content of CoA derivatives in the kidney and liver of untreated and maleate-treated rats

Animals were killed 3 h after intraperitoneal injection of maleate, 400 mg/kg body weight. Kidneys and livers were excised and freeze-clamped. Untreated animals were used as controls. Frozen tissues were pulverized, extracted, and assayed as described under “Experimental Procedures”. Values shown are the means ± S.E., with the number of animals given in parentheses.
Effects of Maleate on CoA Derivatives

Maleate-intoxicated rats.

with a simultaneous inhibition of gluconeogenesis in the kidney. Acetyl-CoA may contribute to the overall inhibition of gluco-

The addition of maleate to the isolated rat kidney mitochondria metabolizing 2-oxoglutarate was associated with three sequential events: (a) rapid and immense decline of CoA-SH; (b) small and transient rise of acid soluble acyl-CoA followed by a substantial fall; and (c) as a consequence of the above, a net loss of about 65% of the total CoA content. The amount of acid-insoluble acyl-CoA derivatives did not change appreciably. Similar changes in the CoA content were observed in vivo in the kidney of maleate-treated rats for at least 3 h after intoxication. Such profound disturbances in CoA metabolism affect several enzyme systems and may account for many inhibitory effects produced by maleate (7, 18, 19).

One of the earliest and most sensitive ones appears to be the inhibition of 2-oxoacid dehydrogenases, possibly caused by a deficiency of intramitochondrial CoA. A diminished flux through these steps may account for the decrease of short chain acyl-CoA, such as succinyl-CoA and acetyl-CoA, and for considerable accumulation of 2-oxoacids. A much more extensive accumulation of pyruvate as compared with 2-oxoglutarate may be explained on the basis of an increased glycolysis with a simultaneous inhibition of gluconeogenesis in the kidney of maleate-treated rats (20). The diminished level of acetyl-CoA may contribute to the overall inhibition of gluco-

eogenesis observed in the kidneys of those animals. The above findings agree well with the data of Scharer (et al.) (21) who reported 50% reduction of acetyl-CoA in the kidney of maleate-intoxicated rats.

The data of both in vitro and in vivo experiments demonstrated neither short nor long chain acyl-CoA derivatives accumulation, suggesting that the primary effect of maleate is to sequester cellular CoA in the form of an unusual derivative other than thioester. As we demonstrated in the preceding paper, maleate may be transformed by CoA transferase into an acyl derivative which, in turn, reacts by a double bond with the SH groups of free CoA. This intermediate turns, after slow hydrolysis of an acyl bond, into a thioester which is metabolically inert. This final product is stable and it would not release free CoA after the alkaline hydrolysis. The sub-

sequent metabolic inert. This final product is stable and it would not release free CoA after the alkaline hydrolysis. The sub-

getransferase peroxidase, into a thioether which is sequestered cellular CoA in the form of an unusual derivative other than thioester. As we demonstrated in the preceding paper, maleate may be transformed by CoA transferase into an acyl derivative which, in turn, reacts by a double bond with the SH groups of free CoA. This intermediate turns, after slow hydrolysis of an acyl bond, into a thioester which is metabolically inert. This final product is stable and it would not release free CoA after the alkaline hydrolysis. The sub-

getransferase peroxidase, into a thioether which is sequestered cellular CoA in the form of an unusual derivative other than thioester. As we demonstrated in the preceding paper, maleate may be transformed by CoA transferase into an acyl derivative which, in turn, reacts by a double bond with the SH groups of free CoA. This intermediate turns, after slow hydrolysis of an acyl bond, into a thioester which is metabolically inert. This final product is stable and it would not release free CoA after the alkaline hydrolysis. The sub-

Succinyl-CoA + E ——— E·CoA + succinate

Each molecule of enzyme · CoA that is formed can react with any acid substrate (succinate, acetoacetate, or maleate) to give the appropriate acyl-CoA, depending on the substrate concentration and affinity. It is therefore understandable that in the presence of a satisfactorily high concentration of ace-

toacetate or succinate, less maleyl-CoA may be formed, and furthermore, it can react with acetoacetate or succinate to give back maleate and appropriate acyl-CoA. This is in accord with the findings showing that the addition of acetoacetate before or together with maleate diminishes the inhibition of 2-oxoglutarate metabolism and prevents or decreases the loss of CoA, while the addition of acetoacetate after maleate has much smaller effects. It indicates that once maleyl-CoA has combined with CoA-SH to form a thioether derivative, the CoA molecule is sequestered and it cannot be recovered.

The liver is known to be the tissue deficient in CoA transferase activity (3, 24, 25). It means that the formation of maleyl-CoA, the reactive and toxic metabolite of maleate, does not take place in liver mitochondria. This explains satisfactorily the lack of sensitivity of the liver to the action of maleate. Changes in CoA metabolites observed in the liver of maleate-treated rats, i.e. decreases in CoA-SH and acetyl-CoA and increases in other short chain acyl-CoA derivatives, may be interpreted as those indicating the stimulation of liver fatty acids oxidation and ketogenesis. The inhibition of CoA-de-

pendent mitochondrial oxidations in the kidney resulted in an increase of plasma-free fatty acids, ketone bodies, and 2-oxoacids (20). A rise in liver ketogenesis is thought to be secondary to the increase in plasma fatty acids. It has been shown previously that 3 h after maleate administration, the level of plasma fatty acids increased by about 30% while blood acetoacetate was doubled (20).

Acknowledgment.—We wish to express our gratitude to Professor Dr. S. Angielski for his interest and helpful discussion in the course of this work.

REFERENCES

4. Angielski, S., Rogulski, J., Pacanis, A., Sztutowicz, A., and Wojci-

kowski, C. (1968) Proceedings of the Fifth FEBS Meeting, Prague, p. 98 (abstr.)

13, 434-513
8. Hersh, L. B., and Jencks, W. P. (1967) J. Biol. Chem. 242, 3481-

3488

97, 587-594

Chem. 177, 715-716

Chem. 197, 851-862

365

100, Hans Huber Publishers, Bern/Stuttgart/Vienna
23. Hersh, L. B., and Jencks, W. P. (1967) J. Biol. Chem. 242, 3486-

3488

(1971) Biochem. J. 121, 41-47

148, 382-390