4-Bromocrotonic Acid, an Effective Inhibitor of Fatty Acid Oxidation and Ketone Body Degradation in Rat Heart Mitochondria

ON THE RATE-DETERMINING STEP OF $\beta$-OXIDATION AND KETONE BODY DEGRADATION IN HEART*

(Received for publication, August 13, 1981)

Yetunde Olowe and Horst Schulz
From the Department of Chemistry, City College of the City University of New York, New York, New York 10031

4-Bromocrotonic acid was found to effectively inhibit respiration supported by either palmitoylcarnitine or acetoacetate in coupled rat heart mitochondria. Partial inhibition was observed when 3-hydroxybutyrate served as a substrate, whereas pyruvate-supported respiration was unaffected by the inhibitor. Thus, 4-bromocrotonic acid inhibits fatty acid oxidation and ketone body degradation. When the enzymes of $\beta$-oxidation and ketone body degradation were assayed in mitochondria preincubated with 4-bromocrotonic acid, only 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase were found to be inactive. Evidence is presented for the enzymatic conversion of 4-bromocrotonic acid to 3-keto-4-bromobutyryl-CoA which effectively inhibits both thiolases. A kinetic evaluation of the inhibitions caused by 4-bromocrotonic acid in coupled rat heart mitochondria demonstrated that 3-ketoacyl-CoA thiolase and respiration supported by palmitoyl carnitine are inactivated at equal rates. However, acetoacetyl-CoA thiolase was inactivated more rapidly than was respiration supported by acetoacetate. It is suggested that the thiolase-catalyzed step is rate-limiting in $\beta$-oxidation or is as slow as other reactions are. In contrast the thiolytic cleavage of acetoacetyl-CoA does not seem to be rate-limiting in ketone body degradation.

The regulation of fatty acid oxidation is still poorly understood partly because the rate-limiting step of this pathway has not yet been definitely determined. It has previously been shown that 4-pentenoic acid inhibits fatty acid oxidation in rat heart mitochondria by specifically inactivating thiolase (1). Since the rates of inactivation of thiolase and of fatty acid oxidation were found to be nearly identical, it was suggested that the thiolase-catalyzed reaction may be rate-limiting in $\beta$-oxidation or may be as slow as other steps are (1). Unfortunately, attempts to evaluate the rate of the thiolase cleavage relative to the rate of ketone body metabolism in rat heart mitochondria by the same approach failed because 4-pentenoic acid is a good respiratory substrate in the presence of a-ketoglutarate which was used in place of malate to stimulate respiration supported by ketone bodies. 2-Bromocrotonic acid, an effective and specific inhibitor of thiolase and thus of palmitoylcarnitine-supported respiration in rat liver mitochondria (2, 3), was in our hands ineffective in rat heart mitochondria. Thus, we have tested various compounds for their ability to specifically inactivate thiolase and consequently inhibit respiration supported by either palmitoylcarnitine or ketone bodies in rat heart mitochondria. 4-Bromocrotonic acid was found to be such an inhibitor.

In this publication we report our observations and conclusions on the metabolism of 4-bromocrotonic acid in rat heart mitochondria and on the inhibition of the mitochondrial thiolases by metabolites of this compound. An evaluation of the rates of inhibition of the two thiolases as well as of respiration supported by either palmitoylcarnitine or ketone bodies leads us to suggest that the thiolase-catalyzed reaction may be rate-limiting in fatty acid oxidation but not in ketone body degradation.

**EXPERIMENTAL PROCEDURES**

MATERIALS—CoASH, NAD, NADH, butyryl-CoA, decanoyl-CoA, and palmitoyl-CoA were purchased from P-L Biochemicals. 4-Bromocrotonic acid was obtained from Chemical Procurement Laboratories, Inc., College Point, N.Y. [3H]CoASH was bought from New England Nuclear. 2-Decenoyl-CoA, di-3-hydroxybutyric acid, lithium acetate, 1-malate, and 3-hydroxyacyl-CoA dehydrogenase were obtained from Sigma. 4-Bromocrotonic acid was obtained by chemical synthesis (4). Microchondrial 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase were isolated from pig heart and purified as described by Staack et al. (5). Crotonyl-CoA (6) and acetoacetyl-CoA (7) were prepared according to standard procedures. 2-Decenoyl-CoA, 4-bromocrotonyl-CoA, 4-bromobutyryl-CoA, and 4-bromocrotonyl-[3H]CoA were synthesized from the corresponding free acids and CoASH by the method of Goldman and Vagelos (8). 3-Ketodecanoyl-CoA, 3-keto-4-bromobutyryl-CoA and 3-keto-4-bromomethyl-CoA (9) were prepared enzymatically from 2-decenoyl-CoA, 4-bromocrotonyl-CoA, and 4-bromocrotonyl-[3H]CoA, respectively, by the procedure of Steubert et al. (9). The concentrations of all CoA derivatives except for 3-keto-decanoyl-CoA and 3-keto-4-bromobutyryl-CoA were determined by the method of Elman (10) after cleaving the thioester bond with hydroxylamine at pH 7. The concentrations of 3-ketodecanoyl-CoA and 3-keto-4-bromocrotonyl-CoA were measured by following the oxidation of NADH at 340 nm in the presence of 3-hydroxyacyl-CoA dehydrogenase at pH 7.

Enzyme Assays—All enzyme assays were performed at 25 °C. Enzyme activities were determined spectrophotometrically on a Gilford recording spectrophotometer. Butyryl-CoA dehydrogenases [EC 1.3.99.2] and acyl-CoA dehydrogenases [EC 1.3.99.3] were assayed spectrophotometrically at 600 nm as described in principle by Hoskins (11). The assay mixture contained 0.1 M KP, (pH 7.6), 28 μM 2,6-dichlorophenolindophenol, 0.65 mM phenazine methosulfate, 20 μM acyl-CoA, 0.2 mM N-ethylmaleimide, 0.45 mM KCN, and 0.09% Triton X-100. The reaction was initiated by the addition of phenazine methosulfate. Enoyl-CoA hydratase [EC 4.2.1.17] was measured spectrophotometrically at 263 nm as described (12), except that the assay mixture contained 0.2 M KP, (pH 8), bovine serum albumin (0.1 mg/ml), 0.08% Triton X-100, and either 30 μM crotonyl-CoA or 30 μM 2-decenoyl-CoA. 3-Hydroxyacyl-CoA dehydrogenase [EC 1.1.1.35] was assayed spectrophotometrically at 340 nm. The standard assay mixture contained 0.05 M KP, (pH 7), 0.06% Triton X-100, 0.12 mM NADH, and bovine serum albumin (0.11 mg/ml). The reaction was started by the addition of acetoacetyl-CoA to a final concentration of...
were determined by spectrophotometrically the disappearance of the Mg$^{2+}$-enolate complex at 303 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 8.2), 25 mM MgCl$_2$, 30 mM KCl, 0.06% Triton X-100, bovine serum albumin (0.13 mg/ml), 70 µM CoA, and either 33 µM acetoacetyl-CoA or 10 µM 3-ketodecanoyl-CoA. Molar extinction coefficients of 21.400 and 13.900 cm$^{-1}$·M$^{-1}$ were used to calculate the rates determined with acetoacetyl-CoA and 3-ketodecanoyl-CoA, respectively. 3-Hydroxybutyrate dehydrogenase [EC 1.1.1.30] was assayed spectrophotometrically as described by Lehninger (13). The assay mixture contained 50 mM Tris-HCl (pH 8.2), 12.5 mM MgCl$_2$, 15 mM KCl, 50 mM nicotinamide, 10 mM cysteine, 2 mM NAD, 29 mM 3-hydroxybutyrate, 50 µM KCN, and 20 µM antimycin. Optimal activity of 3-hydroxybutyrate dehydrogenase was obtained only in the presence of nicotinamide and cysteine. The reaction was initiated by the addition of 3-hydroxybutyrate. 3-Ketoacid-CoA transferase [EC 2.8.3.51] was assayed as described by Stern (14). The assay mixture contained 67 mM Tris-SO$_4$ (pH 8.1), 5 mM MgSO$_4$, 10 mM succinate, and 30 µM acetoacetate-CoA. The disappearance of acetoacetate-CoA Mg$^{2+}$-enolate was measured spectrophotometrically at 303 nm. A molar extinction coefficient of 9000 cm$^{-1}$·M$^{-1}$ was used to calculate transferase activities.

Isolation of Mitochondria and Preparation of Mitochondrial Extracts—Heart mitochondria were isolated by the procedure of Chappell and Hanaford (15). The isolation buffer contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl (pH 7.4), and 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N″-tetraacetic acid. Protein concentrations were determined by the biuret method (16). For the determination of purity, heart mitochondria were completely inactivated, whereas none of the other oxidation enzymes was significantly affected. Thus, we conclude that the observed inhibition of fatty acid oxidation by 4-bromocrotonic acid is a consequence of the inactivation of both thiolases present in rat heart mitochondria. For experimental details see under “Experimental Procedures.”

Oxygen uptake measurements are described under “Experimental Procedures.”

Enzyme Activities in Mitochondria Preincubated with 4-Bromocrotonic Acid—Mitochondria were preincubated with varying concentrations of 4-bromocrotonic acid (0-20 µM) for 1, 2, or 3 min. Aliquots of the mitochondrial suspension (50-200 µl) were rapidly frozen in dry ice and stored at −76 °C until enzyme activities were assayed as described above. To insure the complete disruption of mitochondria, Triton X-100 (0.06%) was added to all assay mixtures except when 3-hydroxybutyrate dehydrogenase was assayed.

**Binding Study**—Purified pig heart 3-ketoacyl-CoA thiolase (3 µg) in 0.1 ml of 0.7 M Tris-HCl (pH 8.2) containing 10% glycerol and 10 mM mercaptoethanol was incubated with either 8 µM 4-bromocrotonyl-[14C]CoA (45,000 cpm) or 8 µM 3-keto-4-bromobutyryl-[14C]CoA (55,000 cpm) for 20 min at 25 °C. The reaction mixture was then rapidly filtered through Sephadex G-50 fine (1 ml) equilibrated with the incubation buffer as described in principle by Penefsky (18). Thiolase activities were determined before and after filtration. The radioactivity associated with thiolase was determined by liquid scintillation counting.

**RESULTS**

**The Effect of 4-Bromocrotonic Acid on Fatty Acid Oxidation in Rat Heart Mitochondria**—Both palmitoyl carnitine and pyruvate support high rates of respiration in isolated rat heart mitochondria as illustrated in Fig. 1 A and C. When mitochondria were preincubated for 3 min in the presence of 20 µM 4-bromocrotonic acid, oxidation supported by palmitoyl carnitine was completely inhibited (see Fig. 1B), whereas pyruvate-dependent respiration was unaffected (see Fig. 1D). This observation is indicative of the specific inhibition of fatty acid oxidation by 4-bromocrotonic acid. Since this compound also inhibited respiration supported by octanoic acid (data not shown), which enters mitochondria in a carnitine-independent manner (19), the same at which the inhibitor blocks fatty acid oxidation must be within the β-oxidation cycle. In order to identify the site of inhibition, we have assayed the enzymes of β-oxidation present in an extract of mitochondria preincubated with 20 µM 4-bromocrotonic acid for 3 min. As shown in Table II, both thiolases present in rat heart mitochondria were completely inactivated, whereas none of the other oxidation enzymes was significantly affected. Thus, we conclude that the observed inhibition of fatty acid oxidation by 4-bromocrotonic acid is a consequence of the inactivation of both thiolases present in rat heart mitochondria. For experimental details see under “Experimental Procedures.”

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Control</th>
<th>4-Bromocrotonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Activities in Mitochondria Preincubated with 4-Bromocrotonic Acid</td>
<td>$\mu$mol/min/mg protein</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Palmitoyl carnitine</td>
<td>Butyryl-CoA</td>
<td>0.072</td>
<td>100</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Decanoyl-CoA</td>
<td>0.064</td>
<td>100</td>
</tr>
<tr>
<td>Octanoate</td>
<td>Palmitoyl-CoA</td>
<td>0.086</td>
<td>100</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>Crotonyl-CoA</td>
<td>2.12</td>
<td>100</td>
</tr>
<tr>
<td>3-Hydroxyacyl-CoA dehydrogenase</td>
<td>Acetoacetyl-CoA</td>
<td>0.915</td>
<td>0.915</td>
</tr>
</tbody>
</table>

*Activities measured in the absence of 4-bromocrotonic acid.
of 3-ketoacyl-CoA thiolase. Since this inhibition occurred with coupled, but not with uncoupled mitochondria, it is likely that the inhibitor must first be activated by conversion to its CoA derivative and possibly must be further metabolized before it becomes inhibitory to thiolase. Of interest is the observation that 4-bromobutyric acid did not inhibit respiration supported by palmitoylcarnitine (data not shown). This inefficacy of 4-bromobutyric acid as an inhibitor of \( \beta \)-oxidation is most likely due to the observed inactivity of butyryl-CoA dehydrogenase toward 4-bromobutyryl-CoA (data not shown).

The inhibition of palmitoylcarnitine-supported respiration and the inactivation of the two thiolases were studied as a function of the concentration of 4-bromocrotonic acid. As illustrated in Fig. 2A acetoacetyl-CoA thiolase, which is believed to function only in ketone body degradation (5, 20), was inhibited more severely \((ID_{50} = 2 \mu M)\) than was 3-ketoacyl-CoA thiolase \((ID_{50} = 4 \mu M)\) which functions in fatty acid oxidation. The latter enzyme is completely inhibited by 15 \( \mu M \) of the inhibitor. The inhibition curve obtained for 3-ketoacyl-CoA thiolase parallels the inhibition pattern observed for palmitoylcarnitine-dependent respiration (see Fig. 2A). This finding supports the previous suggestion (1) that the thiolase-catalyzed reaction may be rate-limiting in fatty acid oxidation or at least may be as slow as other steps are.

The inhibition of respiration by 4-bromocrotonic acid is also a function of time (see Fig. 2B). In the presence of 20 \( \mu M \) inhibitor respiration is completely inhibited within 2 min.

The Effect of 4-Bromocrotonic Acid on Ketone Body Degradation in Rat Heart Mitochondria—The effect of 4-bromocrotonic acid on respiration supported by either 3-hydroxybutyrate or acetoacetate was studied with coupled rat heart mitochondria. As can be seen in Fig. 3 the inhibitor (at a concentration of 20 \( \mu M \) and after 3 min of preincubation) caused the complete inhibition of acetoacetate-supported respiration, whereas respiration sustained by 3-hydroxybutyrate was reduced by 80\%. Assays of individual enzymes of ketone body degradation proved that only acetoacetyl-CoA thiolase was inhibited (see Table III). The inhibition of acetoacetyl-CoA thiolase and of respiration supported by 3-hydroxybutyrate as a function of the 4-bromocrotonate concentration is shown in Fig. 4A. Clearly, acetoacetyl-CoA thiolase (at low concentrations of the inhibitor) is more severely inhibited than is respiration. The residual rate of respiration not sensitive to 4-bromocrotonate is probably sustained by NADH formed during the dehydrogenation of 3-hydroxybutyrate to acetoacetate. The inactivation of acetoacetyl-CoA thiolase by

![Fig. 2. Inhibitions by 4-bromocrotonic acid of thiolases and respiration supported by either palmitoylcarnitine or pyruvate in rat heart mitochondria. A, inhibitions of palmitoylcarnitine-supported respiration, 3-ketoacyl-CoA thiolase, and acetoacetyl-CoA thiolase as a function of the concentration of 4-bromocrotonic acid. Coupled rat heart mitochondria were preincubated for 5 min with the inhibitor and assayed for palmitoylcarnitine-dependent respiration (■), pyruvate-dependent respiration (Δ), 3-ketoacyl-CoA thiolase (●), and acetoacetyl-CoA thiolase (●) as described under “Experimental Procedures.” B, inhibition of palmitoylcarnitine-supported respiration by 4-bromocrotonic acid as a function of the preincubation time. Coupled rat heart mitochondria were preincubated with 20 \( \mu M \) 4-bromocrotonic acid and assayed for palmitoylcarnitine-supported inhibition (●) and pyruvate-supported respiration (Δ) as described under “Experimental Procedures.”](http://www.jbc.org/content/5410/7/5410/F2)

![Fig. 3. Effect of 4-bromocrotonic acid on respiration supported by ketone bodies in coupled rat heart mitochondria. For experimental details see under “Experimental Procedures.” OHC, 3-hydroxybutyrate (1 mM); AcAcOH, acetoacetate (1 mM); 4BrCt, 4-bromocrotonic acid (20 \( \mu M \)). The numbers represent the rates of respiration in nanomol O_2/min and 2 mg of protein.](http://www.jbc.org/content/5410/7/5410/F3)

![Fig. 4. Inhibitions of acetoacetyl-CoA thiolase and respiration supported by either 3-hydroxybutyrate or acetoacetate as a function of the concentration of 4-bromocrotonic acid. Coupled rat heart mitochondria were preincubated for 3 min with the inhibitor and assayed as described under “Experimental Procedures.” A, 3-hydroxybutyrate-dependent respiration (●); acetoacetyl-CoA thiolase (●). B, acetoacetate-dependent respiration (Δ); acetoacetyl-CoA thiolase (●).](http://www.jbc.org/content/5410/7/5410/F4)

**Table III**

<table>
<thead>
<tr>
<th>Enzyme Substrate</th>
<th>Control *</th>
<th>+ 4-Bromocrotonic acid</th>
<th>Remaining activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetoacetyl-CoA thiolase</td>
<td>Acetoacetyl-CoA</td>
<td>0.52</td>
<td>0</td>
</tr>
<tr>
<td>3-Ketoacid-CoA thiolase</td>
<td>Succinate + acetoacetyl-CoA</td>
<td>1.67</td>
<td>1.67</td>
</tr>
<tr>
<td>3-Hydroxybutyrate dehydrogenase</td>
<td>3-Hydroxybutyrate</td>
<td>0.021</td>
<td>0.021</td>
</tr>
</tbody>
</table>

* Activities measured in the absence of 4-bromocrotonic acid.
4-bromocrotonic acid is also more rapid than is the inhibition of respiration supported by acetoacetate (see Fig. 4B). Although the difference between the inhibition of the enzyme and that of the overall process was slight, it was reproducible and significant.

Metabolism of 4-Bromocrotonic Acid in Rat Heart Mitochondria—The observation that 4-bromocrotonic acid inhibits the two thiolases and consequently fatty acid oxidation in coupled, but not in uncoupled rat heart mitochondria, suggests that the acid must be activated in an energy-dependendent reaction before it becomes inhibitory. 4-Bromocrotonic acid is most likely converted to 4-bromocrotonyl-CoA by medium chain acyl-CoA synthetase which is located in the mitochondrial matrix (21). 4-Bromocrotonyl-CoA has been shown to be an inhibitor of isolated thiolase (22). When an extract of rat heart mitochondria was incubated with 20 μM 4-bromocrotonyl-CoA, a partial inhibition of thiolase activities was detected with either acetoacetyl-CoA (see Fig. 5A) or 3-ketodecanoyl-CoA (see Fig. 5B) as substrates. However, when additionally, NAD was present in the incubation mixture, the inhibition of the two thiolase activities was much more rapid and complete within 15 min. This observation suggests that 4-bromocrotonyl-CoA is metabolized, most likely via β-oxidation, and that one of its metabolites is an efficient inhibitor of both thiolases present in heart mitochondria. Experiments with purified enzymes demonstrated that 4-bromocrotonyl-CoA is acted upon by crotonase (see Fig. 6) as has been reported by Steinman and Hill (23). The rapid hydration of 4-bromocrotonyl-CoA by crotonase may be the reason why inhibition of thiolase present in a mitochondrial extract stops after a few minutes (see Fig. 5). The product of the crotonase-catalyzed reaction, presumably L-3-hydroxy-4-bromobutyryl-CoA, is a substrate for L-3-hydroxyacyl-CoA dehydrogenase as evidenced by the formation of NADH (see Fig. 6). The expected formation of both NADH and 3-keto-4-bromobutyryl-CoA is illustrated in Fig. 7A, curve 1, which shows the overlapping absorbance peaks of NADH and the Mg2+-enolate complex of 3-keto-4-bromobutyryl-CoA. Addition of EDTA to this solution resulted in a large decrease in absorbance due to the disappearance of the Mg2+-enolate complex (see Fig. 7A, curve 2). When 3-keto-4-bromobutyryl-CoA was generated enzymatically from 4-bromocrotonyl-CoA in the presence of pyruvate and lactate dehydrogenase to reoxidize NADH, the spectrum of only the Mg2+-enolate complex of 3-keto-4-bromobutyryl-CoA was observed. As shown in Fig. 7B the formation of 3-keto-4-bromobutyryl-CoA is time-dependent and the maximum absorbance of its Mg2+-enolate complex is observed at 310 nm. Since the absorbance maximum of the corresponding complex of acetoacetyl-CoA is at 303 nm (24), the bromine substituent causes a red shift of 7 nm. In Fig. 8 we have summarized our conclusion regarding the metabolism of 4-bromocrotonic acid in rat heart mitochondria. 4-Bromocrotonic acid is converted to its CoA ester, hydrated by crotonase, and dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase to 3-keto-4-bromobutyryl-CoA which is an effective inhibitor of both acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase.

Fig. 5. Inactivation of thiolases present in an extract of rat heart mitochondria. Soluble mitochondrial proteins (0.2 mg/ml) in 2 ml of 0.2 M Tris-HCl (pH 8.2) containing 25 mM MgCl2, 30 mM KCl, 20% glycerol, and 10 mM mercaptoethanol were incubated in the presence of either 20 μM 4-bromocrotonyl-CoA (A) or 20 μM 4-bromocrotonyl-CoA plus 0.23 μM NAD (B). Aliquots were assayed for thiolase activities with acetoacetyl-CoA (A) or 3-ketodecanoyl-CoA (B) as substrates as described under "Experimental Procedures."

Fig. 6. Spectrophotometric evidence for the conversion of 4-bromocrotonyl-CoA to 3-keto-4-bromobutyryl-CoA. The reaction was started by the addition of crotonase (4 minute) to 4-bromocrotonyl-CoA (11 nmol) in 0.6 ml of 0.1 M Tris-HCl (pH 8.2) containing 25 mM MgCl2 and 30 mM KCl. After completion of the hydration reaction NAD (0.14 μmol) and 3-hydroxyacyl-CoA dehydrogenase (1.5 units) were added to the assay mixture.

Fig. 7. The spectrum of the Mg2+-enolate complex of 3-keto-4-bromocrotonyl-CoA. A, 1) The spectrum obtained when 4-bromocrotonyl-CoA (10 mmol) in 1 ml of 0.1 M Tris-HCl (pH 8.2) containing 25 mM MgCl2 and 30 mM KCl was incubated with crotonase (7 units), NAD (0.23 μmol), and 3-hydroxyacyl-CoA dehydrogenase (2.4 units). 2) The spectrum after addition of EDTA (45 μmol) to the above described reaction mixture. B, the spectrum obtained when the reaction mixture described under A (1) contained additionally sodium pyruvate (1 μmol) and lactate dehydrogenase (4 units) to reoxidize NADH. 1) 10 min, (2) 20 min, and (3) 60 min after the start of the reaction.
lytic cleavage product of 3-keto-4-bromobutyryl-CoA was incubated with a mitochondrial extract, neither of the two thiolases was inhibited.

**Mechanism of Inhibition of Thiolas by 3-Keto-4-bromobutyryl-CoA**—When purified 3-ketoacyl-CoA thiolase was preincubated and assayed in the presence of 2 μM 3-keto-4-bromobutyryl-CoA, its activity decreased by two thirds in less than 10 s. The enzyme was completely inactivated within 20 min (see Fig. 9A). In contrast, 2 μM 4-bromocrotononyl-CoA caused only a slight inhibition which did not increase with time (see Fig. 9A). A similar result was obtained when 3-ketoacyl-CoA thiolase was preincubated with 6 μM 3-keto-4-bromobutyryl-CoA, but assayed in the presence of only 50 nM of the inhibitor (see Fig. 9B). Most important was the observation that 1 μM acetoacetyl-CoA effectively protected 3-ketoacyl-CoA thiolase against inactivation by 6 μM 3-keto-4-bromobutyryl-CoA (see Fig. 9B). Thus, the inhibitor seems to inactivate thiolase after first binding to the active site of the enzyme. This conclusion is not surprising because the inhibitor has all the structural elements of a substrate for thiolase and is highly effective at low concentrations. The time dependence of the inhibition of thiolase by 3-keto-4-bromobutyryl-CoA suggests that the inhibition is a consequence of a covalent modification of the enzyme. To provide direct evidence for the hypothesis 3-ketoacyl-CoA thiolase was inactivated in the presence of 3-keto-4-bromobutyryl[3H]CoA. After separating thiolase from the incubation medium by rapid filtration through Sephadex G-50, the enzyme was found to be completely inactive, but no radioactivity was associated with it. This observation is not compatible with a simple reversible inhibition of thiolase by 3-keto-4-bromobutyryl-CoA or with the covalent attachment of the complete inhibitor molecule to the enzyme. When a large amount of purified pig heart 3-ketoacyl-CoA thiolase (30 μg) was incubated in the presence of 3-keto-4-bromobutyryl-CoA and CoASH, spectrophotometric evidence for the disappearance of a stoichiometric amount of inhibitor was obtained (data not shown).

**DISCUSSION**

4-Bromocrotonic acid was found to be an effective inhibitor of respiration supported by either fatty acids or ketone bodies. Since pyruvate oxidation was unaffected by this inhibitor, neither the tricarboxylic acid cycle nor oxidative phosphorylation are inhibited by 4-bromocrotonic acid. Also the levels of CoASH and NAD are not lowered dramatically in other pathways of pyruvate oxidation would be inhibited. The impaired oxidation of octanoate, which enters mitochondria independent of carnitine (19), does not agree with the mitochondrial uptake of fatty acids as the primary site of inhibition. Consequently one of the steps of β-oxidation must be the site of inhibition. When the enzymes of oxidation were assayed, only 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase, which are believed to function in fatty acid oxidation and ketone body degradation respectively (5, 20), were found to be inhibited by 4-bromocrotonic acid. Thus, we conclude that the specific inactivation of the thiolas is the cause for the inhibition of fatty acid oxidation and ketone body degradation in rat heart mitochondria.

The repeated observation of a lag phase in the 4-bromocrotonate-dependent inhibition of respiration (see Fig. 2B) led us to suspect that 4-bromocrotonic acid must first be metabolized before it becomes inhibitory to thiolase. Since the inhibitor affected respiration in coupled, but not in uncoupled mitochondria, it is most likely first converted intramitochondrially to its CoA derivative in an ATP-dependent reaction. The resulting 4-bromocrotonyl-CoA is rapidly hydrated by crotonase, presumably to L-3-hydroxy-4-bromobutyryl-CoA, which in turn is dehydrogenated by NAD in the presence of L-3-hydroxyacyl-CoA dehydrogenase to yield 3-keto-4-bromobutyryl-CoA. Of the metabolites of 4-bromocrotonic acid, 4-bromocrotonyl-CoA has been reported to be an inhibitor of purified thiolase (22). We expected 3-keto-4-bromobutyryl-CoA to be an even more effective inhibitor because it should bind to thiolase as a substrate and should be very susceptible to a nucleophilic substitution at the γ-carbon. The results presented in this communication demonstrate that 3-keto-4-bromobutyryl-CoA of all the metabolites of 4-bromocrotonic acid is by far the most effective inhibitor of thiolase.

Preliminary experiments performed by us to elucidate the mechanism by which 3-keto-4-bromobutyryl-CoA inhibits 3-ketoacyl-CoA thiolase led us to the following conclusions and suggestions. 3-Keto-4-bromobutyryl-CoA binds first noncovalently to thiolase at its active site as suggested by the observed protection against inhibition in the presence of acetoacetyl-CoA. However, the inhibition of thiolase is not just a consequence of a noncovalent interaction with the inhibitor, because filtration of thiolase preincubated with 3-keto-4-bromobutyryl-[3H]CoA through Sephadex G-50 yielded the inactive enzyme devoid of radioactivity. The absence of radioactivity in the inactive enzyme also eliminates the possibility...
that a covalent enzyme-inhibitor complex may have been formed via a simple nucleophilic displacement of bromine by either the sulfhydryl group or another nucleophilic group present at the active site. Spectrophotometric evidence of the disappearance of the inhibitor in amounts stoichiometric to the added enzyme agrees best with the thiolytic cleavage of 3-keto-4-bromobutyryl-CoA to yield the bromoacetylated enzyme. This enzyme form itself may be inactive or it may undergo a secondary reaction involving the nucleophilic displacement of the bromine residue that would lead to the inactivation of the enzyme.

The availability of a compound which specifically inhibits both thiolases present in coupled rat heart mitochondria, has provided us with the opportunity to determine whether the rates of fatty acid oxidation and ketone body degradation in isolated mitochondria are determined by the activities of 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase, respectively. In a multienzyme system, in which intermediates are not channeled from one active site to the next, partial inactivation of the enzyme that catalyzes the rate-limiting step would result in a proportional inhibition of the overall pathway. In contrast, partial inactivation of an enzyme that catalyzes an equilibrium reaction would result in a lesser or no inhibition of the pathway. The observation of parallel inactivations of 3-ketoacyl-CoA thiolase and palmitoylcoenzyme A-supported respiration agrees with our previous suggestion that the thiolase-catalyzed reaction is either rate-limiting in \( \beta \)-oxidation or is as slow as other reactions are (1). Although this hypothesis agrees with our observation, it is not sufficiently supported by it, if channeling of \( \beta \)-oxidation intermediates occurs as has been suggested (25). The suggestion that 3-ketoacyl-CoA thiolase may catalyze the rate-limiting step in \( \beta \)-oxidation had prompted us to study the regulation of thiolases by mitochondrial coenzymes and metabolites. We have observed and reported that acetyl-CoA at low concentrations of CoASH is an effective inhibitor of 3-ketoacyl-CoA thiolase (28). Since the concentrations of acetyl-CoA and CoASH in heart change in response to changes in the energy demand (27), we have proposed that the rate of \( \beta \)-oxidation is tuned to the rate of energy consumption via the regulation of 3-ketoacyl-CoA thiolase by the acetyl-CoA/CoASH ratio (29).

A comparison of the rate, at which acetoacetate-supported respiration is inhibited by 4-bromocrotonic acid, with the rate of inactivation of acetoacetyl-CoA thiolase leads to the conclusion that this step is not rate-limiting in ketone body degradation. The incomplete inhibition of 3-hydroxybutyrate-supported respiration by 4-bromocrotonic acid is most likely due to the unaffected oxidation of 3-hydroxybutyrate to acetoacetate. Continuation of this process requires the mitochondrial uptake of 3-hydroxybutyrate and efflux of acetoacetate to be at least as rapid as the dehydrogenation of 3-hydroxybutyrate is, which was observed to be 12.5 nmol/min/mg of protein. In an uninhibited mitochondria 3-hydroxybutyrate is completely oxidized to CO\(_2\); the observed rate of respiration corresponds to a rate of 3-hydroxybutyrate breakdown of 7 nmol/min/mg of protein. Since this value is lower than the values of 12.5 nmol/min/mg of protein observed with mitochondrial containing inactive thiolase, we conclude that both the uptake and dehydrogenation of 3-hydroxybutyrate are not rate-limiting in ketone body degradation in normal heart mitochondria. Since the thiolase-catalyzed step does not seem to be rate-limiting either and since the capacity of rat heart mitochondria to oxidize acetate units greatly exceeds their ability to degrade ketone bodies, the activation of acetoacetate catalyzed by 3-ketoacid CoA-transferase appears to be the rate-limiting step in ketone body degradation in heart. The rate of acetoacetate activation and consequently that of the overall pathway is most likely regulated by the availability of succinyl-CoA as has been suggested by Hatefi and Fakouhi (28).

REFERENCES
4-Bromocrotonic acid, an effective inhibitor of fatty acid oxidation and ketone body degradation in rat heart mitochondria. On the rate-determining step of beta-oxidation and ketone body degradation in heart.
Y Olowe and H Schulz


Access the most updated version of this article at http://www.jbc.org/content/257/10/5408

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/257/10/5408.full.html#ref-list-1