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Inhibition *in vitro* of acyl-CoA dehydrogenases by 2-mercaptoacetate in rat liver mitochondria

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In rat liver hypo-osmotically treated mitochondria, 2-mercaptoacetate inhibits respiration induced by palmitoyl-CoA, octanoate or butyryl-CoA only when the reaction medium is supplemented with ATP. Under this condition, NADH-stimulated respiration is not affected. In liver mitochondrial matrix, the presence of ATP is also required to observe a 2-mercaptoacetate-induced inhibition of acyl-CoA dehydrogenases tested with palmitoyl-CoA, butyryl-CoA or isovaleryl-CoA as substrate. As the oxidation of these substrates is also inhibited by the incubation medium resulting from the reaction of 2-mercaptoacetate with acetyl-CoA synthase, with conditions under which 2-mercaptoacetate has no effect, 2-mercaptoacetyl-CoA seems to be the likely inhibitory metabolite responsible for the effects of 2-mercaptoacetate. Kinetic experiments show that the main effect of the 2-mercaptoacetate-active metabolite is to decrease the affinities of fatty acyl-CoA dehydrogenases towards palmitoyl-CoA or butyryl-CoA and of isovaleryl-CoA dehydrogenase towards isovaleryl-CoA. Addition of *N*-ethylmaleimide to mitochondrial matrix pre-exposed to 2-mercaptoacetate results in the immediate reversion of the inhibitions of palmitoyl-CoA and isovaleryl-CoA dehydrogenations and in a delayed reversion of butyryl-CoA dehydrogenation. These results led us to conclude that (i) the ATP-dependent conversion of 2-mercaptoacetate into an inhibitory metabolite takes place in the liver mitochondrial matrix and (ii) the three fatty acyl-CoA dehydrogenases and isovaleryl-CoA dehydrogenase are mainly competitively inhibited by this compound. Finally, the present study also suggests that the inhibitory metabolite of 2-mercaptoacetate may bind non-specifically to, or induce conformational changes at, the acyl-CoA binding sites of these dehydrogenases.

It is now well established that the administration of 2-mercaptoacetate induces a fatty liver in the rat (Sabourault *et al.*, 1976). In previous studies, we showed that this compound increased the rate of peripheral lipolysis and depressed fatty acid oxidation in the liver (Sabourault *et al.*, 1979) through an inhibition of long-chain acyl-CoA dehydrogenase (Bauché *et al.*, 1981).

Further experiments performed *in vitro* indicated that 2-mercaptoacetate entered the mitochondrial matrix and that an energy-dependent activation was required to observe the inhibition of fatty acid oxidation (Bauché *et al.*, 1982).

The purpose of the present study was to determine the mechanisms of the inhibition of fatty acid oxidation induced by 2-mercaptoacetate *in vitro*. Since we observed an impairment of acyl-CoA dehydrogenations after 2-mercaptoacetate administration (Bauché *et al.*, 1981), it seemed interesting to determine whether the depressed fatty acid oxidation observed *in vitro* in the presence of 2-mercaptoacetate was due to an inhibition of acyl-CoA dehydrogenations or to other mechanisms.

In the present paper, we report evidence that a metabolite of 2-mercaptoacetate (probably 2-mercaptoacetyl-CoA) inhibits non-specifically the three fatty acyl-CoA dehydrogenases (long-chain, general and short-chain acyl-CoA dehydrogenases) as well as the branched-chain acyl-CoA dehydrogenase, namely isovaleryl-CoA dehydrogenase.

Materials and methods

Preparation of hypo-osmotically treated rat liver mitochondria

Overnight-starved female Wistar rats (200 ± 20 g) were killed by decapitation and liver mitochondria were prepared by the method of Beattie (1968). The mitochondrial pellet from two livers was washed three times by suspension and centrifugation in 0.25 M-sucrose at 4°C and was finally suspended in 2 ml of 0.1 M-sucrose. The suspension was left at room temperature for 2 h. At the end of this period, the resulting suspension was immediately used. Protein was determined by the method of Lowry *et al.* (1951).

Preparation of mitochondrial soluble extracts

Liver mitochondria from two rats were isolated by the procedure described above and were finally suspended in 5 ml of 0.05 M-potassium phosphate (pH 7.4). Then, this suspension was sonicated at 4°C for 6 × 15 s with a Branson Sonifier equipped with a microtip operating at maximal output. The resulting mixture was centrifuged at 130000 ***g*** for 60 min. The protein concentration of the supernatant was about 10 mg/ml.

Measurements of oxygen uptake of hypo-osmotically treated rat liver mitochondria

The respiration rate of hypo-osmotically treated mitochondria was measured as previously described (Bauché *et al.*, 1981) at 25°C with a Gilson oxygraph fitted with a Clark oxygen electrode. The reaction medium (pH 7.4) consisted of 58 mM-KCl, 25 mM-NaCl, 6 mM-$MgCl_2$, 13 mM-K_2HPO_4, 3 mM-KH_2PO_4 and hypo-osmotically treated mitochondria equivalent to 3.5–4 mg of protein (final volume 1.6 ml). Respiration rates were measured with 1 mM-NADH, 1 mM-NAD^+ + 25 μM-palmitoyl-CoA, 1 mM-NAD^+ + 50 μM-sodium octanoate or 1 mM-NAD^+ + 50 μM-butyryl-CoA as substrates. In some experiments, 0.4 mM-CoA and 1 mM-ATP were also added to the incubation medium. When present, 1 mM-2-mercaptoacetate was added 30 s before the addition of coenzymes and respiratory substrates. The rates of respiration are expressed in nmol of O_2 consumed/min per mg of mitochondrial protein added.

Enzyme assays

Effects of 2-mercaptoacetate. Butyryl-CoA dehydrogenase, palmitoyl-CoA dehydrogenase and isovaleryl-CoA dehydrogenase were measured with a double-beam spectrophotometer by following at 600 nm the acyl-CoA-dependent reduction of 2,6-dichloroindophenol in the presence of phenazine methosulphate as outlined by Hoskins (1969). The assay mixture (final volume 3.0 ml) contained 0.05 M-potassium phosphate (pH 7.4), 0.1 mM-2,6-dichloroindophenol, 50 μl of mitochondrial extract (approx. 0.5 mg of protein) and 25 μM-palmitoyl-CoA, 50 μM-butyryl-CoA or 50 μM-isovaleryl-CoA. In some experiments, 80 μM-$MgCl_2$, 80 μM-ATP and 0.2 mM-*N*-ethylmaleimide were also present in the assay mixture. When 2-mercaptoacetate was added, the concentration used was 25 μM. Since free thiol groups reduce 2,6-dichloroindophenol, it was necessary to correct the data for this non-specific dye reduction. Therefore, mitochondrial extracts and 2-mercaptoacetate were added to the two paired-cuvettes, whereas the substrate was added only to one cuvette. Under these conditions, the only difference between the two paired cuvettes was the absence (reference cuvette) or the presence (sample cuvette) of the substrate. The temperature was maintained at 25°C and the reaction was initiated by the addition of 0.5 mM-phenazine methosulphate, which was found necessary to observe dehydrogenation activities.

Effects of 2-mercaptoacetate preincubated with acetyl-CoA synthase. 2-Mercaptoacetate (5 mM) was incubated for 30 min at 37°C in a medium containing 0.1 M-Tris/HCl, pH 7.5, 10 mM-$MgCl_2$, 1 mM-ATP, 1 mM-CoA and acetyl-CoA synthase (4 units/ml of incubation medium). Control incubations were performed simultaneously without acetyl-CoA synthase. The reaction was stopped by addition of 7% $HClO_4$, neutralized to pH 6.0 and centrifuged. Supernatant (10 μl; control or assay) was added to the assay mixture described above without any cofactor addition (final volume 3.0 ml) and acyl-CoA dehydrogenase activities were determined as described previously.

Kinetic experiments. Kinetic experiments were performed as described above using various concentrations of substrates: 3.75–25 μM-palmitoyl-CoA, 6.25–75 μM-butyryl-CoA, 10–75 μM-isovaleryl-CoA in the absence or in the presence of 2-mercaptoacetate (2.5 or 25 μM). To obtain the best fit, K_m and $V_{max.}$ values were calculated on a computer using either the Michaelis–Menten equation or a non-linear regression program based on the theoretical model of 'one substrate, two enzymes' (Brown, 1980). Acyl-CoA dehydrogenase specific activity was calculated using 16.1 $mM^{-1} \cdot cm^{-1}$ as the extinction coefficient of 2,6-dichloroindophenol and was expressed as nmol of 2,6-dichloroindophenol reduced/min per mg of matrix mitochondrial protein.

Statistical analysis

Statistical significance of the data was analysed by Student's *t* test.

Materials

All reagents were of analytical grade or of highest purity available and were from Sigma

Chemical Co., St Louis, MO, U.S.A., or from E. Merck, Darmstadt, Germany.

Results

Effects of 2-mercaptoacetate on NADH and fatty acid oxidations by hypo-osmotically treated mitochondria

It is well known that intact mitochondrial membranes are poorly permeable to NADH, NAD^+ and CoA and that this poor permeability increases after treatment of mitochondria with hypo-osmotic sucrose solution (Lehninger, 1951). Therefore, hypo-osmotically treated mitochondria were used to study the effects of 2-mercaptoacetate on NADH and fatty acid oxidations. As shown in Table 1, exposure of these mitochondria for 30s to 1mM-2-mercaptoacetate had no effect on respiration stimulated by NADH, palmitoyl-CoA or butyryl-CoA. Essentially the same results were found when the period of exposure was prolonged to 3min (results not shown). In contrast, when the same experiments were repeated in a medium containing either 1mM-ATP (results not shown) or 0.4mM-CoA + 1mM-ATP (Table 1), 1mM-2-mercaptoacetate inhibited the respiration stimulated by palmitoyl-CoA (28 and 33% inhibition respectively), octanoate (38 and 32% inhibition) and butyryl-CoA (41 and 42% inhibition) but failed to affect the NADH-dependent respiration. Additions of 1mM-FAD and 1mM-GTP to the reaction medium or longer pre-incubation time (3min) gave similar results (results not shown). These observations are indicative of a specific inhibition of fatty acid oxidation by 2-mercaptoacetate independently of the fatty acid chain length. Furthermore, since these inhibitions occurred only in an ATP-supplemented medium and in the presence of the matrix mitochondrial CoA, it is reasonable to postulate that the conversion of 2-mercaptoacetate into its CoA derivative was necessary to observe such inhibitions. This suggestion is consistent with our previous observation (Bauché *et al.*, 1982) that the inhibition *in vitro* of the β-oxidation by 2-mercaptoacetate occurs with coupled but not with uncoupled mitochondria.

Table 1. *Effects of 2-mercaptoacetate on NADH and fatty acid oxidations by hypo-osmotically treated mitochondria*

The reaction chamber of the oxygen electrode contained 58mM-KCl, 25mM-NaCl, 6mM-$MgCl_2$, 13mM-K_2HPO_4, 3mM-KH_2PO_4 (pH 7.4) and hypo-osmotically treated mitochondria equivalent to 3.5–4.0mg of protein (final volume 1.6ml). When indicated, the assay mixture also contained 0.4mM-CoA and 1mM-ATP. Respiration was initiated with 1mM-NADH, 25μM-palmitoyl-CoA + 1mM-NAD^+, 50μM-butyryl-CoA + 1mM-NAD^+ or 50μM-sodium octanoate + 1mM-NAD^+. When present, 1mM-2-mercaptoacetate was added 30s before the addition of the respiratory substrates. Each value is the mean ± S.E.M. for the number of determinations indicated in parentheses.

Substrate	Cofactors added	O_2 consumption (nmol/min per mg of protein) Control	+2-Mercaptoacetate
NADH	None	17.4 ± 0.9 (5)	18.2 ± 1.8 (5) $P > 0.05$
	CoA + ATP	14.7 ± 1.3 (5)	17.4 ± 2.7 (5) $P > 0.05$
Palmitoyl-CoA	None	10.6 ± 1.3 (5)	9.5 ± 1.1 (5) $P > 0.05$
	CoA + ATP	10.3 ± 1.4 (5)	6.9 ± 0.8 (5) $0.001 < P < 0.01$
Butyryl-CoA	None	8.6 ± 0.8 (5)	7.4 ± 0.9 (5) $P > 0.05$
	CoA + ATP	7.3 ± 0.6 (5)	4.2 ± 0.5 (5) $P < 0.001$
Octanoate	None	Undetectable	Undetectable
	CoA + ATP	9.9 ± 1.4 (5)	6.7 ± 0.7 (5) $0.001 < P < 0.01$

Effects of 2-mercaptoacetate on acyl-CoA dehydrogenase activities

The above results as well as our previous findings that the β-oxidation inhibition occurring after administration of 2-mercaptoacetate was primarily due to an inhibition of acyl-CoA dehydrogenase (Bauché *et al.*, 1981) prompted us to directly investigate the effects *in vitro* of 2-mercaptoacetate on some of the mitochondrial acyl-CoA dehydrogenase activities. This was performed using palmitoyl-CoA, butyryl-CoA or isovaleryl-CoA as the substrates, under conditions where the electron-transferring flavoprotein was not operative (i.e. in the presence of phenazine methosulphate; see the Materials and methods section). As already observed in Table 1, 2-mercaptoacetate was found to elicit inhibitory effects on the dehydrogenations of these substrates only when the reaction medium was supplemented with ATP (Table 2).

Table 2 also indicates that the magnitude of the inhibitory effect of 2-mercaptoacetate was dependent on the substrate used: only 16% inhibition was seen with palmitoyl-CoA, but 36% and 63% inhibition were found with butyryl-CoA and isovaleryl-CoA respectively. The same results were also obtained when mitochondrial extract and inhibitor were pre-incubated together (in the presence of ATP) for up to 30min before substrate addition (results not shown). Because butyryl-CoA is mainly dehydrogenated by short-chain acyl-CoA dehydrogenase and also, but to a lesser extent, by general acyl-CoA dehydrogenase (Hall, 1978; Furuta *et al.*, 1981; Ikeda *et al.*, 1983), the above results do not enable us to decide which of these dehydrogenases

Table 2. *Effects of 2-mercaptoacetate on acyl-CoA dehydrogenase activities*

The assay mixture (final volume 3 ml) consisted of 0.05 M-potassium phosphate (pH 7.4), 0.1 mM-2,6-dichloroindophenol, 0.5 mM-phenazine methosulphate and 0.5 mg of matrix mitochondrial protein. When indicated, the assay mixture also contained 80 μM-$MgCl_2$ and 80 μM-ATP. Concentrations of acyl-CoA used were: 25 μM-palmitoyl-CoA, 50 μM-butyryl-CoA and 50 μM-isovaleryl-CoA. Assays were carried out as described in the Materials and methods section in the absence of 2-mercaptoacetate (control), in the presence of 25 μM-2-mercaptoacetate, in the presence of 10 μl of control pre-incubation medium (5 mM-2-mercaptoacetate + cofactors) or in the presence of 10 μl of assay pre-incubation medium (5 mM-2-mercaptoacetate + cofactors + acetyl-CoA synthase). Acyl-CoA dehydrogenase activity was expressed as nmol of 2,6-dichloroindophenol reduced/min per mg of protein. Each value is the mean ± S.E.M. for the number of determinations indicated in parentheses. Abbreviation used: DCIP, 2,6-dichloroindophenol.

		Acyl-CoA dehydrogenase activity (nmol of DCIP reduced/min per mg of protein)			
Substrates	Cofactors added	Control	+2-Mercapto-acetate	+10 μl of control incubation medium	+10 μl of assay incubation medium
Palmitoyl-CoA	None	15.5 ± 0.6 (5)	15.1 ± 0.7 (5) $P > 0.05$	15.5 ± 0.8 (6) $P > 0.05$	12.7 ± 0.8 (6) $P < 0.001$
	$MgCl_2$ + ATP	18.0 ± 0.3 (5)	15.1 ± 0.5 (5) $P < 0.001$		
Butyryl-CoA	None	22.6 ± 2.1 (4)	21.0 ± 3.4 (4) $P > 0.05$	22.2 ± 2.0 (6) $P > 0.05$	13.3 ± 1.1 (6) $P < 0.001$
	$MgCl_2$ + ATP	27.1 ± 1.7 (5)	16.9 ± 1.1 (5) $P < 0.001$		
Isovaleryl-CoA	None	7.1 ± 1.9 (3)	6.1 ± 0.8 (3) $P > 0.05$	6.8 ± 0.8 (6) $P > 0.05$	3.1 ± 0.4 (6) $P < 0.001$
	$MgCl_2$ + ATP	8.4 ± 1.2 (4)	3.1 ± 0.6 (4) $P < 0.001$		

was affected by the active metabolite of 2-mercaptoacetate. On the contrary, since palmitoyl-CoA and isovaleryl-CoA dehydrogenations are thought to be exclusively catalysed by long-chain acyl-CoA and isovaleryl-CoA dehydrogenases respectively (Ikeda *et al.*, 1983; Noda *et al.*, 1980), the inhibitory effects induced by 2-mercaptoacetate on these dehydrogenations indicate that the active metabolite of 2-mercaptoacetate is probably an inhibitor of both palmitoyl-CoA and isovaleryl-CoA dehydrogenases.

To further investigate the possible role played by 2-mercaptoacetyl-CoA in these inhibitions, acyl-CoA dehydrogenase activities were determined as previously described in the presence of 10 μl of a pre-incubated solution of 2-mercaptoacetate with acetyl-CoA synthase and cofactors. Under these conditions, palmitoyl-CoA, butyryl-CoA and isovaleryl-CoA oxidations were impaired (18%, 41% and 56% inhibition, respectively), whereas the assays performed in the presence of 10 μl of a control pre-incubation medium (2-mercaptoacetate + cofactors but without acetyl-CoA synthase) did not affect these oxidations (Table 2). These results thus indicate that 2-mercaptoacetate is a substrate for acetyl-CoA synthase and strongly suggest that the resulting compound that elicits acyl-CoA dehydrogenase inhibitions is 2-mercaptoacetyl-CoA.

Kinetic experiments

In order to get a better insight into the inhibitory actions of 2-mercaptoacetate on acyl-CoA dehydrogenase activities, kinetic analysis by the Lineweaver-Burk method was performed using palmitoyl-CoA, butyryl-CoA or isovaleryl-CoA as the substrates. Experiments were performed as above in the presence of ATP to allow the conversion of 2-mercaptoacetate into an inhibitory metabolite.

Fig. 1 shows the double-reciprocal plots of acyl-CoA dehydrogenase activities versus palmitoyl-CoA concentration in the absence and in the presence of 25 μM-2-mercaptoacetate. As can be seen, whereas the $V_{max.}$ values were not significantly different whether 2-mercaptoacetate was present or not, the K_m values increased from 5.02 ± 0.6 to 10.48 ± 2.73 μM ($n = 5$) in the presence of the inhibitor. Although these experiments do not allow calculation of the K_i value (the concentration of the inhibitory metabolite was unknown), they do indicate that under these conditions 2-mercaptoacetate induces a weak competitive inhibition of long-chain acyl-CoA dehydrogenase activity with respect to palmitoyl-CoA.

Fig. 2 shows that, with butyryl-CoA as the substrate, the plots were curvilinear with a downward concavity suggesting the presence of at least two different enzymic activities, one having a low K_m and being likely the short-chain acyl-CoA dehydrogenase and the other one having a high K_m, which probably represents the general acyl-CoA dehydrogenase. To verify this hypothesis, these kinetic data were subjected to computer analy-

	Control	+ 2-Mercaptoacetate
K_m (μM)	5.02 ± 0.60 (5)	10.48 ± 2.73 (5) $0.001 < P < 0.01$
$V_{max.}$ (nmol of DCIP reduced/min per mg of protein)	22.6 ± 0.90 (5)	22.4 ± 1.1 (5) $P > 0.05$

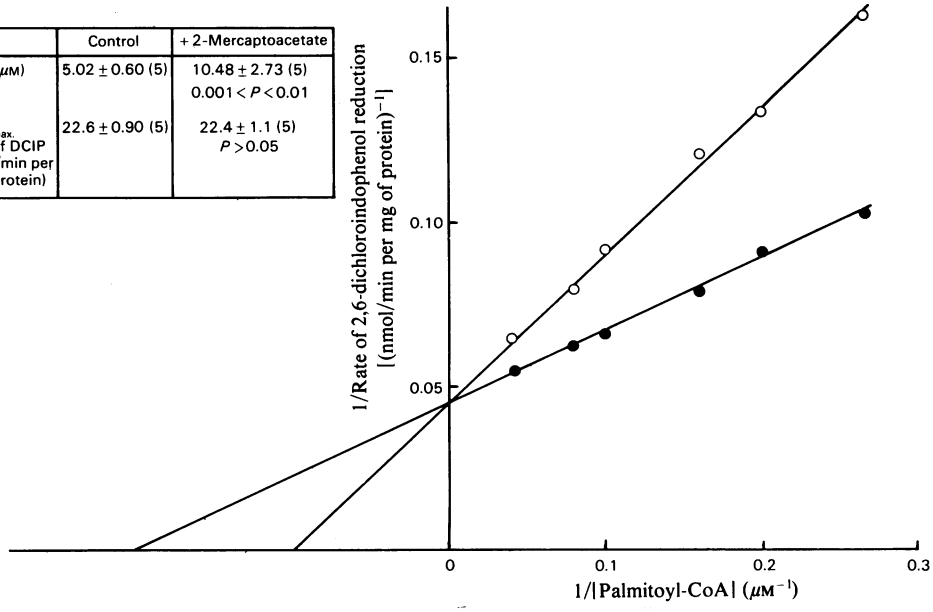

Fig. 1. *Lineweaver-Burk plot of inhibition of acyl-CoA dehydrogenase induced by 2-mercaptoacetate in rat liver mitochondrial matrix with palmitoyl-CoA as the substrate*

Acyl-CoA dehydrogenase activity is determined as described in the Materials and methods section in the absence (●) and in the presence (○) of 25 μM-2-mercaptoacetate. The points shown are means of five determinations. The inset shows the K_m and $V_{max.}$ values, calculated with a computer using the Michaelis–Menten equation. Values are means ± S.E.M. of five separate determinations. Abbreviation used: 2,6-dichloroindophenol.

sis to determine the K_m and $V_{max.}$ values and the results of this analysis indicated indeed that the curves best fitted the model of two enzymes, one substrate. By this method, in the absence of 2-mercaptoacetate, the $V_{max.}$ values were 16.81 and 16.84 nmol of 2,6-dichloroindophenol reduced/min per mg of protein and the K_m values were: 0.50 μM and 35.95 μM for the short-chain and the general acyl-CoA dehydrogenase respectively. When the same computer analyses were applied to the kinetic data obtained in the presence of 2-mercaptoacetate, the two kinetic parameters concerning short-chain acyl-CoA dehydrogenase were modified, leading to a slight decrease in $V_{max.}$ (13.89 nmol of 2,6-dichloroindophenol reduced/min per mg of protein) and to an increase in K_m (2.62 μM), indicating that the inhibition, although being mixed (competitive and non-competitive), was predominantly competitive in nature. Under the same conditions, 2-mercaptoacetate appeared to act mainly as a competitive inhibitor of the general acyl-CoA dehydrogenase with respect to butyryl-CoA, since the calculated K_m value increased to 195.0 μM, whereas the $V_{max.}$ value remained essentially unchanged (inset, Fig. 2).

In the next experiments, the effects of 2-mercaptoacetate on isovaleryl-CoA dehydrogenase were studied. As shown in Fig. 3, 2-mercaptoacetate slightly decreased the $V_{max.}$ value (6.05 versus 7.85 nmol of 2,6-dichloroindophenol reduced/min per mg of protein) but clearly increased the K_m value (13.00 versus 5.52 μM), indicating that the inhibition was a mixture of competitive and non-competitive types. Thus it seems that a common feature of the inhibitory effects induced by 2-mercaptoacetate on acyl-CoA dehydrogenases is a decrease in the affinity of these enzymes for their respective substrates.

In addition, the results presented in Table 3 suggest that the mechanisms involved in these inhibitions are probably different with respect to the nature of the acyl-CoA dehydrogenase tested. In fact, in experiments where the thiol-specific reagent *N*-ethylmaleimide was added to the reaction medium after the inhibitor, but just before the reaction was initiated, 2-mercaptoacetate had no more effect on palmitoyl-CoA or isovaleryl-CoA dehydrogenations, whereas a 33% decrease was observed with butyryl-CoA as the substrate. However, when *N*-ethylmaleimide was added 4 min before reaction initiation, 2-mercaptoacetate had no more inhibitory effect on butyryl-CoA oxidation (16.4 ± 2.0 nmol of 2,6-dichloroindophenol reduced/min per mg of protein, $n = 3$). Furthermore, when *N*-ethylmaleimide was added before 2-mercaptoacetate, no inhibitory effects could be seen with any of the substrates tested (results not shown). These results indicate that (i) the acyl-CoA dehydrogenase inhibitions induced by 2-mercapto-

Short-chain acyl-CoA dehydrogenase	Control	+ 2-Mercaptoacetate
K_m (μM)	0.50	2.62
$V_{max.}$ (nmol of DCIP reduced/ min per mg of protein)	16.81	13.89

General acyl-CoA dehydrogenase	Control	+ 2-Mercaptoacetate
K_m (μM)	35.95	195.00
$V_{max.}$ (nmol of DCIP reduced/ min per mg of protein)	16.84	16.56

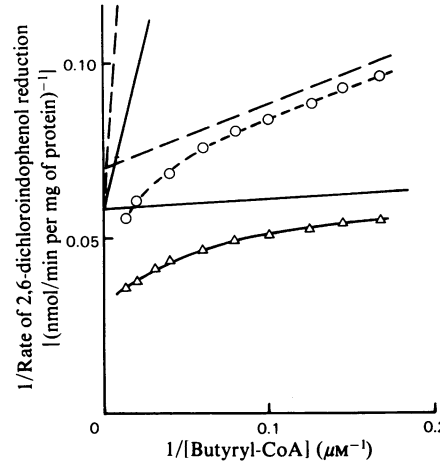

Fig. 2. *Lineweaver-Burk plot of inhibition of acyl-CoA dehydrogenase induced by 2-mercaptoacetate in rat liver mitochondrial matrix with butyryl-CoA as the substrate*

Acyl-CoA dehydrogenase activity is determined as described in the Materials and methods section in the absence (△) and in the presence (○) of 25 μM-2-mercaptoacetate. The points shown are means of four determinations and the lines (continuous lines, without inhibitor; broken lines, with inhibitor) are drawn from the computer data giving the best fit using a non-linear regression program based on the theoretical model of one substrate, two enzymes. The inset shows the K_m and $V_{max.}$ values calculated from the computer data.

	Control	+ 2-Mercaptoacetate
K_m (μM)	5.52 ± 0.47 (4)	13.00 ± 2.60 (4) $P < 0.001$
$V_{max.}$ (nmol of DCIP reduced/ min per mg of protein)	7.85 ± 0.75 (4)	6.05 ± 0.48 (4) $0.001 < P < 0.01$

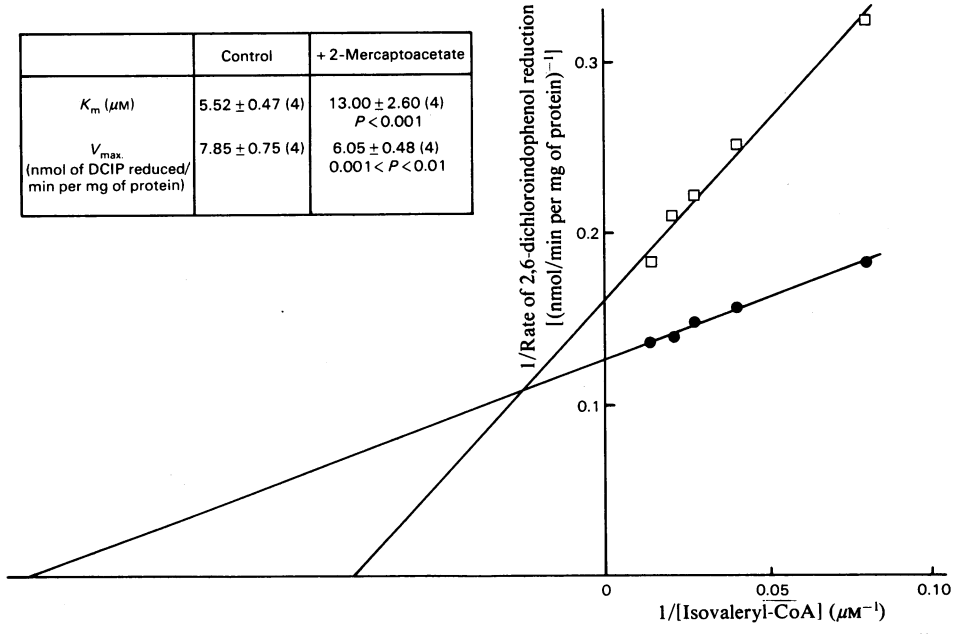

Fig. 3. *Lineweaver–Burk plot of inhibition of isovaleryl-CoA dehydrogenase induced by 2-mercaptoacetate in rat liver mitochondrial matrix with isovaleryl-CoA as the substrate*

Isovaleryl-CoA dehydrogenase activity is determined as described in the Materials and methods section in the absence (●) and in the presence (□) of 2.5 μM-2-mercaptoacetate. The points shown are the means of four determinations. The inset shows the K_m and $V_{max.}$ values, calculated as described in the legend to Fig. 1. The values are means ± S.E.M. of four separate determinations.

Table 3. *Effects of 2-mercaptoacetate on various acyl-CoA dehydrogenase activities in the presence of N-ethylmaleimide*

Experimental conditions and statistical analysis are as described in Table 2, except that 0.2mM-*N*-ethylmaleimide was added just before the reaction was initiated by the addition of phenazine methosulphate. Abbreviation used: DCIP, 2,6-dichloroindophenol.

Substrates	Cofactors added	Acyl-CoA dehydrogenase activity (nmol of DCIP reduced/min per mg of protein) Control	+2-Mercaptoacetate
Palmitoyl-CoA	None	12.2 ± 1.4 (3)	12.4 ± 0.5 (3) $P > 0.05$
	$MgCl_2$ + ATP	11.2 ± 0.5 (5)	11.2 ± 0.7 (5) $P > 0.05$
Butyryl-CoA	None	16.9 ± 0.8 (3)	17.6 ± 1.6 (3) $P > 0.05$
	$MgCl_2$ + ATP	16.5 ± 1.8 (5)	11.1 ± 1.8 (5) $0.001 < P < 0.01$
Isovaleryl-CoA	None	7.9 ± 0.3 (3)	8.5 ± 1.2 (3) $P > 0.05$
	$MgCl_2$ + ATP	8.0 ± 0.9 (4)	7.3 ± 0.6 (4) $P > 0.05$

acetate are reversible and (ii) that the inhibitory compound binds more tightly to one of the two enzymes involved in butyryl-CoA dehydrogenation than to palmitoyl-CoA or isovaleryl-CoA dehydrogenase. Since *N*-ethylmaleimide reacts unspecifically with thiol groups, it is not possible to determine whether the inhibition reversions observed in the presence of this compound (Table 3) are due to the binding of *N*-ethylmaleimide to the inhibitor or to the dehydrogenases. Therefore, no valid conclusions can be drawn from this experiment on the role of thiol groups in the molecular mechanisms of acyl-CoA dehydrogenase inhibition induced by 2-mercaptoacetate.

Discussion

In a previous report (Bauché *et al.*, 1981), we showed that the 2-mercaptoacetate-induced fatty liver was mainly due to an inhibition of acyl-CoA dehydrogenase activity and consequently to a marked depression of the β-oxidation pathway. Moreover, experiments performed *in vitro* indicated that 2-mercaptoacetate itself was able to enter the mitochondrial matrix and to induce an inhibition of fatty acid oxidation (Bauché *et al.*, 1982).

The present study was undertaken in order to determine whether 2-mercaptoacetate was also able to inhibit acyl-CoA dehydrogenation *in vitro* and, if so, to get a better insight into the mechanisms involved in this effect.

Experiments performed either with mitochondria made permeable to ATP, CoA and its thioesters or with mitochondrial matrix (Tables 1 and 2) further substantiate the hypothesis that conversion of 2-mercaptoacetate through an ATP-dependent reaction is required to observe a β-oxidation inhibition. In addition, this finding underlines the fact that the enzymic conversion of 2-mercaptoacetate into its inhibitory derivative takes place within the mitochondrial matrix. Since, in rat liver mitochondrial matrix, activation of short-chain fatty acids is catalysed by two separate ATP-dependent acyl-CoA synthetases having optimal activity on C_2 and C_4 substrates (Aas, 1971), it can be reasonably postulated that one of these two enzymes may also be involved in the activation of 2-mercaptoacetate. In such a case and as already suggested by us (Bauché *et al.*, 1982), the most likely metabolite resulting from this activation should be 2-mercaptoacetyl-CoA. The fact that 2-mercaptoacetate is a substrate for acetyl-CoA synthase and that the resulting compound induces acyl-CoA dehydrogenase inhibition (Table 2) further supports this hypothesis.

The data in the present paper clearly show that the inhibition of fatty acid oxidation observed in the presence of 2-mercaptoacetate results primarily from an inhibition of acyl-CoA dehydrogenation. This finding is in agreement with previous studies from this laboratory (Bauché *et al.*, 1981) showing that, after 2-mercaptoacetate administration, the rate of palmitoyl-CoA dehydrogenation was strongly depressed in rat liver. However, the results presented in Table 2 indicate that, in the presence of 25μM-2-mercaptoacetate, palmitoyl-CoA dehydrogenation was only slightly inhibited and, in any case, less affected than butyryl-CoA dehydrogenation. Thus the inhibitory action of 2-mercaptoacetate observed *in vitro* appears to be mainly specific for short-chain CoA esters. One possible explanation for these discrepant results is that the mitochondrial concentration of the inhibitory metabolite of 2-mercaptoacetate may be different *in vitro* and *in vivo*: *in vitro*, this concentration may be too low to substantially affect long-chain acyl-CoA dehydrogenase, whereas *in vivo*, and 3h after 2-mercaptoacetate administration, this concentration may have reached a sufficiently high level to cause a potent inhibition of this fatty acyl-CoA dehydrogenation. In fact, this might be especially the case for 2-mercaptoacetyl-CoA, which, because of the impermeability of mitochondria towards CoA esters, would have probably accumulated under these conditions.

As far as we know, the acyl-CoA dehydrogenations are catalysed by a system involving two different flavoproteins: a substrate-linked dehydrogenase and another flavoprotein called electron-transferring flavoprotein (Crane *et al.*, 1956; Crane & Beinert, 1956). The latter is an obligatory electron

carrier from acyl-CoA dehydrogenases to the electron transport chain or to an artificial electron acceptor such as 2,6-dichloroindophenol (Beinert, 1962). To couple acyl-CoA dehydrogenases to 2,6-dichloroindophenol, phenazine methosulphate can be used, thus replacing electron-transferring flavoprotein (Beinert, 1962; Engel & Massey, 1971). Since in the present study we found that the presence of phenazine methosulphate was required to obtain a 2,6-dichloroindophenol reduction (results not shown), this indicates that, under the experimental conditions used, the electron-transferring flavoprotein was ineffective. Thus, the decrease in fatty acyl-CoA dehydrogenation observed in the presence of 2-mercaptoacetate can only be accounted for by an inhibition of acyl-CoA dehydrogenases and not by an inhibition of the electron-transferring flavoprotein.

From the recent literature, there are three arguments that might explain some of the present findings and that strengthen further the assumption that 2-mercaptoacetyl-CoA is indeed the inhibitory metabolite of 2-mercaptoacetate. First, Hall *et al.* (1979) and Frerman *et al.* (1980) have indicated that the binding of acyl-CoA to acyl-CoA dehydrogenases involves a strong binding of the CoA moiety and only a weak binding of the hydrocarbon moiety of the acyl group. According to this mechanism, 2-mercaptoacetyl-CoA but not 2-mercaptoacetate would also bind to acyl-CoA dehydrogenase, thus explaining why 2-mercaptoacetate in itself is unable to inhibit acyl-CoA dehydrogenases and why its active metabolite behaves essentially as a competitive inhibitor. Secondly, Auer & Frerman (1980) have also suggested that the carbonyl moiety of acyl-CoA may interact with the acyl-CoA dehydrogenase flavin. In the case of 2-mercaptoacetyl-CoA, such an interaction may also occur and, if so, would add weight to the explanation of why the inhibition of short-chain acyl-CoA dehydrogenase observed in the presence of 2-mercaptoacetate is not only competitive but also non-competitive. Thirdly, considering the polymeric structure of acyl-CoA dehydrogenase (Thorpe *et al.*, 1979; Furuta *et al.*, 1981; Ikeda & Tanaka, 1983), it also cannot be excluded that the changes in the kinetic parameters of these enzymes may result from conformational changes due to the binding of 2-mercaptoacetyl-CoA at a site(s) different from the substrate-binding site(s).

Finally, the present paper also shows that, in addition to its effects on fatty acyl-CoA dehydrogenases, the active product of 2-mercaptoacetate is a strong inhibitor of isovaleryl-CoA dehydrogenase (Table 2, Fig. 3). This finding strengthens the similarities between the 2-mercaptoacetate- and the hypoglycin-induced inhibitory effects on mitochondrial metabolism. In fact, after transamination, hypoglycin is activated in the mitochondria into its inhibitory active metabolite methylenecyclopropylacetyl-CoA (Manchester, 1974), which is, as shown in the present study for the 2-mercaptoacetate metabolite, a strong inhibitor of isovaleryl-CoA and short-chain acyl-CoA dehydrogenases and a weaker inhibitor of the other fatty acyl-CoA dehydrogenases (Billington *et al.*, 1974, 1978; Kean, 1976).

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References

Aas, M. (1971) *Biochim. Biophys. Acta* **231**, 32–47
Auer, H. E. & Frerman, F. E. (1980) *J. Biol. Chem.* **255**, 8157–8163
Bauché, F., Sabourault, D., Giudicelli, Y., Nordmann, J. & Nordmann, R. (1981) *Biochem. J.* 196, 803–809
Bauché, F., Sabourault, D., Giudicelli, Y., Nordmann, J. & Nordmann, R. (1982) *Biochem. J.* **206**, 53–59
Beattie, D. S. (1968) *Biochem. Biophys. Res. Commun.* **31**, 901–907
Beinert, H. (1962) *Methods Enzymol.* **5**, 546–557
Billington, D., Kean, E. A., Osmundsen, H. & Sherratt, H. S. A. (1974) *IRCS Med. Sci.: Libr. Compend.* **2**, 1712
Billington, D., Osmundsen, H. & Sherratt, H. S. A. (1978) *Biochem. Pharmacol.* **27**, 2891–2900
Brown, R. J. (1980) *J. Theor. Biol.* **83**, 527–531
Crane, F. L. & Beinert, H. (1956) *J. Biol. Chem.* **218**, 717–731
Crane, F. L., Mii, S., Hauge, J. G., Green, D. E. & Beinert, H. (1956) *J. Biol. Chem.* **218**, 701–716
Engel, P. C. & Massey, V. (1971) *Biochem. J.* **125**, 879–887
Frerman, F. E., Miziorko, H. M. & Beckmann, J. D. (1980) *J. Biol. Chem.* **255**, 11192–11198
Furuta, S., Miyazawa, S. & Hashimoto, T. (1981) *J. Biochem. (Tokyo)* **90**, 1739–1750
Hall, C. L. (1978) *Methods Enzymol.* **53**, 502–518
Hall, C. L., Lambeth, J. D. & Kamin, H. (1979) *J. Biol. Chem.* **254**, 2023–2031
Hoskins, D. D. (1969) *Methods Enzymol.* **14**, 110–114
Ikeda, Y. & Tanaka, K. (1983) *J. Biol. Chem.* **258**, 1077–1085
Ikeda, Y., Dabrowski, C. & Tanaka, K. (1983) *J. Biol. Chem.* **258**, 1066–1076
Kean, E. A. (1976) *Biochim. Biophys. Acta* **422**, 8–14
Lehninger, A. L. (1951) *J. Biol. Chem.* **190**, 345–359
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Manchester, K. L. (1974) *FEBS Lett.* **40**, S133–S139
Noda, C., Rhead, W. J. & Tanaka, K. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2646–2650
Sabourault, D., Giudicelli, Y., Nordmann, R. & Nordmann, J. (1976) *Biochim. Biophys. Acta* **431**, 241–248
Sabourault, D., Bauché, F., Giudicelli, Y., Nordmann, J. & Nordmann, R. (1979) *FEBS Lett.* **108**, 465–468
Thorpe, C., Matthews, R. G. & Williams, C. H. (1979) *Biochemistry* **18**, 331–337