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PROTECTIVE EFFECT OF CYSTEINE ON THE INHIBITION OF MITOCHONDRIAL FUNCTIONS BY ACETALDEHYDE*

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Abstract—Acetaldehyde, the primary metabolite of ethanol oxidation, inhibited a number of mitochondrial functions *in vitro*. Cysteine, *in vitro*, afforded protection against the depression of CO_2 production from palmitate, octanoate and α-ketoglutarate by acetaldehyde. Relief occurred when the concentrations of cysteine and acetaldehyde were equimolar; greater relief was produced in the presence of excess cysteine. Acetaldehyde had no effect on glutamate-linked state 4 oxygen consumption, whereas the state 3 rate was inhibited. Cysteine almost completely relieved the inhibition of state 3 oxygen uptake, while the state 4 rate was slightly increased. Similar results were obtained with several other NAD^+-dependent substrates. The oxidation of succinate was inhibited only by much higher concentrations of acetaldehyde than those which inhibited the oxidation of NAD^+-dependent substrates. This inhibition was not affected by cysteine. Thiols containing free amino and free sulfhydryl groups in close proximity were the most effective in relieving the inhibition by acetaldehyde. Although a small protective effect was observed, cysteine did not significantly prevent the inhibition of oxidative phosphorylation by acetaldehyde. However, the mitochondria remained coupled in the presence of acetaldehyde plus cysteine. The ability of cysteine and acetaldehyde to interact was demonstrated by several criteria. Cysteine may exert its protective effect by forming a complex with acetaldehyde, thereby preventing acetaldehyde from interacting with the mitochondria.

Acetaldehyde, which is produced during ethanol metabolism in the liver, is a toxic compound, with numerous effects on mitochondrial functions [1–4]. Most of the acetaldehyde is believed to be oxidized by the mitochondria [5–7]. Acetaldehyde depressed oxygen consumption with NAD^+-dependent substrates and inhibited energy transduction and utilization by the mitochondria [8,9]. In addition, acetaldehyde depressed CO_2 production from fatty acids [10] and several citric acid cycle intermediates.†

Many of the effects of acetaldehyde on mitochondrial functions are similar to those reported for other agents capable of reacting with mitochondrial thiol groups, e.g. Cu^{2+} [11], compounds with a thiuram structure [12] and 5,5′-dithionitrobenzoic acid [13]. Thiols apparently participate in many mitochondrial active sites and functions, e.g. oxidative phosphorylation [14,15], active accumulation of Mg^{2+} [16], the active site of coupling factor B [17], NADH [18,19] and succinic [20,21] dehydrogenases, the active site of the phosphate carrier [22] and the adenine nucleotide translocase [23]. It has also been claimed that acetaldehyde interacts with CoASH [24]. It seemed possible, therefore, that some of the effects of acetaldehyde on mitochondrial functions may be related to a reaction between acetaldehyde and thiols.

Aldehydes react quite readily with mercaptans [25–27]. In the case of cysteine, ring closure can occur with the consequent formation of thiazolidines [25,26]. Cysteine reacts nonenzymatically with formaldehyde, forming thiazolidine-4-carboxylic acid [25–27]. Cysteine could complex with acetaldehyde to form the hemiacetal, which upon cyclization, would form 2-methylthiazolidine-4-carboxylic acid [25,28,29]. It has recently been suggested that such a complex may be a nontoxic detoxification product, since cysteine was claimed to protect against death from acetaldehyde toxicity *in vivo* [30]. In this report, we studied the ability of cysteine to reverse the acetaldehyde-induced inhibition of several mitochondrial functions.

MATERIALS AND METHODS

Preparations. Rat liver mitochondria were prepared as previously described [8]. The mitochondria were washed and suspended in 0·25 M sucrose–0·01 M Tris–HCl, pH 7·4–0·001 M EDTA. All radioactive counting procedures were performed in 0·7% 2,5-diphenyloxazole and 0·05% 1,4-bis-2(5-phenyloxazoly)-benzene in toluene. Protein was determined according to Lowry *et al.* [31]. Bovine serum albumin was depleted of fatty acids by the charcoal treatment of Chen [32]. The acetaldehyde was characterized as previously described [8] and shown to be identical with a chromatographically pure acetaldehyde standard. A 0·01 per cent impurity could have been detected under these conditions. Acetaldehyde was prepared by dilution with ice-cold H_2O and used immediately. The concentrations of acetaldehyde given are the amounts initially added, i.e. there is no correction for loss of acetaldehyde via volatilization or oxidation by the mitochondria. The thiol reagents were also freshly prepared and purged with nitrogen. Thiols were neutralized and kept on ice in well-stoppered flasks. We found it essential that only freshly

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prepared cysteine in the reduced form be used in these studies. As the cysteine aged or was exposed to air, it became inhibitory to several mitochondrial functions, presumably owing to its oxidation to the disulfide, cystine.

Oxygen consumption. Oxygen uptake was assayed at 30° using a Clark oxygen electrode and a Yellow Springs oxygen monitor. The reaction system consisted of 0·3 M mannitol; 10 mM Tris–HCl, pH 7·4; 10 mM potassium phosphate, pH 7·4; 2·5 mM $MgCl_2$; 10 mM KCl; and mitochondria equivalent to 3–4 mg protein in a final volume of 3·0 ml. Substrates (10 mM final concentration) included succinate, glutamate, α-ketoglutarate, β-hydroxybutyrate and pyruvate-malate. ADP (1 mM) was added to initiate state 3 conditions. When calculating the ADP/O ratio, 0·9 μmole ADP was added (final concentration of 0·3 mM).

Oxidative phosphorylation. The P/O ratio was assayed using a Gilson differential manometer, as previously described [8,33]. β-Hydroxybutyrate served as the substrate in all experiments. Oxygen uptake was determined manometrically, whereas the rate of phosphorylation was determined from the rate of synthesis of glucose 6-phosphate [34].

$^{14}CO_2$ production. $^{14}CO_2$ production was assayed using either palmitate[1-^{14}C], octanoate[1-^{14}C] or α-ketoglutarate[1-^{14}C] (New England Nuclear) as the substrates. The reaction was carried out in center well flasks containing 0·3 ml hydroxide of hyamine in the center well. The reaction system for the fatty acid experiments consisted of 100 mM KCl, 0·4 mM malate, 1 mM $MgCl_2$, 3 mM ATP, 3 mM carnitine, 10 mM potassium phosphate (pH 7·4), 6 mg of fatty acid depleted bovine serum albumin, 3·33 mM ADP and about 2 mg of mitochondrial protein in a final volume of 3·0 ml. For the experiments with α-ketoglutarate, the same reaction mixture as described for the oxygen consumption experiments was used, with 3·3 mM ADP also being added. The reaction was initiated by the addition of labeled fatty acid or α-ketoglutarate (final concentrations of 67 μM or 6·7 mM, respectively; 0·33 μCi). After 15 min, the reaction was terminated by the addition of 1 ml of 2 N HCl. CO_2 was allowed to collect in the center well for 30–60 min. The contents of the center well were collected, the center well was washed three times with 0·3 ml aliquots of scintillation fluid, and the sample plus washings was counted in a liquid scintillation counter.

Interaction of acetaldehyde and cysteine. Cysteine (400 and 600 nmoles) was incubated in 4 ml of 100 mM Tris–HCl, pH 8·5, with various amounts of acetaldehyde (cysteine/acetaldehyde ratio 0·1 to 2·0) for 2, 5, 10 or 20 min. 5,5′-Dithionitrobenzoic acid (final concentration 0·2 mM) was then added, and the absorbance at 412 nm was recorded in a Gilford 240 spectrophotometer. A millimolar extinction coefficient of 13·6 was used to calculate the concentrations of cysteine. Blanks included samples containing acetaldehyde but lacking cysteine.

Acetaldehyde (360 and 900 nmoles) was incubated in 4 ml of 100 mM potassium phosphate, pH 7·0, with various amounts of cysteine (cysteine/acetaldehyde ratio 0·5 to 5·0) for 2, 5, 10 or 20 min. Semicarbazide (15 mM in 160 mM phosphate buffer) was then added, and the absorbance at 224 nm was recorded [35]. A millimolar extinction coefficient of 9·4 was used to calculate the concentration of acetaldehyde. Blanks included samples containing cysteine but lacking acetaldehyde. Preliminary experiments indicated that similar results were obtained in the mannitol or KCl medias described for the oxygen uptake or CO_2 production experiments as in the simpler Tris or phosphate media described above.

Statistics. All values represent the mean ± standard error of the mean. The number of experiments is indicated in the individual tables. Statistical analysis was performed by Student's *t*-test. In the case of samples containing acetaldehyde plus cysteine, the analysis was made against controls which lacked these two compounds, as well as samples which contained only acetaldehyde.

RESULTS

Effect of cysteine on the acetaldehyde-induced inhibition of $^{14}CO_2$ production from octanoate and palmitate. Acetaldehyde is a potent inhibitor of $^{14}CO_2$ production from fatty acids [10] (Tables 1 and 2). This inhibition is not due merely to dilution of $^{14}CO_2$ by unlabeled CO_2 derived from acetaldehyde oxidation by the mitochondria, since acetaldehyde was considerably more inhibitory toward fatty acid oxidation than were comparable concentrations of acetate [10]. Cysteine itself (1 to 3·3 mM) had little effect

Table 1. Effect of cysteine on acetaldehyde-induced inhibition of $^{14}CO_2$ production from palmitate*

Acetaldehyde concn (mM)	Cysteine concn (mM)	$^{14}CO_2$ production (cpm/mg protein)	Effect on control (%)	P (control)	Effect on acetaldehyde (%)	P (acetaldehyde)
		23,463 ± 2104				
0·75		10,337 ± 1742	−56	0·004		
0·75	1·0	17,973 ± 3546	−23	NS	+73	0·10 > P > 0·05
0·75	3·3	22,614 ± 3660	−4	NS	+118	<0·02
0·75	10·0	20,417 ± 3463	−13	NS	+97	<0·02
		16,182 ± 3085				
1·5		4294 ± 1182	−73	0·004		
1·5	1·0	7759 ± 2149	−52	0·04	+80	NS
1·5	3·3	10,198 ± 2486	−37	NS	+137	0·04
1·5	10·0	11,339 ± 2296	−30	NS	+164	0·03

* $^{14}CO_2$ production from palmitate[1-^{14}C] was assayed as described in Materials and Methods in the absence or presence of acetaldehyde and cysteine. The number of experiments is four for 0·75 mM acetaldehyde and six for 1·5 mM.

Table 2. Effect of cysteine on acetaldehyde-induced inhibition of $^{14}CO_2$ production from octanoate*

Acetaldehyde concn (mM)	Cysteine concn (mM)	$^{14}CO_2$ production (cpm/mg protein)	Effect on control (%)	P (control)	Effect on acetaldehyde (%)	P (Acetaldehyde)
		25,521				
1·0		15,910	−38			
1·0	1·0	25,588	0		+61	
1·0	3·3	24,534	−4		+54	
1·0	10·0	21,020	−18		+32	
		27,995				
2·0		12,946	−54			
2·0	1·0	20,053	−28		+54	
2·0	3·3	24,594	−12		+89	
2·0	10·0	19,876	−29		+53	
		19,580 ± 3655				
3·0		6902 ± 1580	−65	<0·001		
3·0	1·0	8886 ± 1581	−55	0·02	+28	NS
3·0	3·3	12,676 ± 2171	−35	NS	+83	0·04
3·0	10·0	13,111 ± 1645	−33	NS	+89	0·02

* $^{14}CO_2$ production from octanoate[1-^{14}C] was assayed as described in Materials and Methods in the absence or presence of acetaldehyde and cysteine. Results represent the mean of two individual experiments for 1·2 mM acetaldehyde and the mean of six for 3 mM.

on octanoate or palmitate oxidation; with octanoate the control activity was 25,251 cpm/mg of protein in the absence of cysteine, and 25,639 and 25,638 cpm/mg in the presence of 1 and 3·3 mM cysteine respectively. With palmitate, the control activity was 22,738 cpm/mg of protein in the absence of cysteine, and 22,350 and 21,334 cpm/mg in the presence of 1 and 3·3 mM cysteine respectively. Some inhibition of fatty acid oxidation was observed using 10 mM cysteine (about 10–20 per cent). CO_2 production from palmitate[1-^{14}C] was depressed 56 and 73 per cent by 0·75 and 1·5 mM acetaldehyde respectively (Table 1). The addition of 1 mM cysteine afforded considerable relief of the inhibition of $^{14}CO_2$ production caused by 0·75 mM acetaldehyde (Table 1), while 3·3 and 10 mM yielded almost complete relief. At the higher concentration of 1·5 mM acetaldehyde, cysteine also protected against the depression of CO_2 production (Table 1).

Acetaldehyde depressed CO_2 production from octanoate[1-^{14}C] 38, 54 and 65 per cent at acetaldehyde concentrations of 1, 2 and 3 mM respectively (Table 2). Cysteine almost completely prevented the inhibition observed at 1 and 2 mM acetaldehyde (taking into consideration that 10 mM cysteine itself produced about 15 per cent inhibition of CO_2 production from octanoate). Even at 3 mM acetaldehyde, cysteine (3·3 and 10 mM) still yielded significant protection against the inhibition by acetaldehyde.

Effect of cysteine on the acetaldehyde-induced inhibition of $^{14}CO_2$ production from α-ketoglutarate. Part of the mechanism for the inhibition of CO_2 production from fatty acids by acetaldehyde involves inhibitory effects of acetaldehyde on the activity of the citric acid cycle. Acetaldehyde depressed $^{14}CO_2$ production from several citric acid cycle intermediates, with a major site of inhibition in the α-ketoglutarate–succinate span of the cycle.* Acetaldehyde depressed $^{14}CO_2$ production from α-ketoglutarate[1-^{14}C] by 31 and 47 per cent at acetaldehyde concentrations of 1 and 3 mM respectively (Table 3). Cysteine itself had little effect on CO_2 production from α-ketoglutarate, but almost completely relieved the inhibition produced by 1 mM acetaldehyde, and partially relieved the inhibition with 3 mM acetaldehyde (Table 3).

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Table 3. Effect of cysteine on acetaldehyde-induced inhibition of $^{14}CO_2$ production from α-ketoglutarate[1-^{14}C]*

Acetaldehyde concn (mM)	Cysteine concn (mM)	$^{14}CO_2$ production (cpm/mg protein)	Effect (%)	P (control)
		17,462		
1·0		12,049	−31	
1·0	3·3	15,174	−13	
1·0	10·0	16,288	−7	
		19,906 ± 2749		
3·0		10,648 ± 3244	−47	0·01
3·0	3·3	13,584 ± 2642	−32	NS
3·0	10·0	15,875 ± 1946	−20	NS

* $^{14}CO_2$ production was assayed as described in the legend to Table 1. Results are from either four (3 mM acetaldehyde concentrations) or two individual experiments.

Table 4. Effect of cysteine on glutamate and succinate oxidation*

Substrate	Cysteine concn (mM)	Oxygen consumption (natoms/min/mg protein) State 4	State 3	Effect (%) State 4	State 3
Glutamate		14·05	72·0		
	1·0	14·63	74·3	+4	+3
	3·0	15·99	76·9	+14	+7
	10·0	22·13	69·4	+58	−4
Succinate		23·0	111·0		
	1·0	22·5	103·5	−2	−7
	3·0	29·3	105·0	+27	−5
	10·0	33·0	117·8	+43	+6

* Oxygen consumption was assayed as described in Materials and Methods in the presence or absence of cysteine. Results are from three separate experiments.

Effect of cysteine on the oxidation of glutamate and succinate. As a control in the experiments to be described below, we measured the effect of cysteine on the oxidation of glutamate and succinate by isolated mitochondria. Cysteine had no effect on the state 3 rate of oxidation of either of these substrates (Table 4). However, the state 4 rate of oxygen consumption was stimulated with both substrates, particularly at 10 mM cysteine. This suggests the possibility of a slight uncoupling effect by cysteine. As a consequence, the respiratory control ratio associated with the oxidation of glutamate and succinate was depressed 38 and 27 per cent, respectively, by 10 mM cysteine. The addition of 3 or 10 mM cysteine to the mitochondria in the absence of a substrate gave rise to some oxygen uptake in excess of the endogenous rate (about 2 natoms/min/mg of protein).

Effect of cysteine on acetaldehyde-induced inhibition of glutamate oxidation. State 4 oxygen consumption with glutamate as the substrate was not affected by 3 mM acetaldehyde, whereas energy-dependent (state 3) oxygen uptake was depressed 34 per cent (Table 5). This inhibition was to a large extent prevented by cysteine (Table 5). The state 4 rate of oxygen uptake was stimulated to a comparable extent by cysteine in the presence or absence of acetaldehyde, suggesting that the stimulation is due to cysteine itself. One mM cysteine had no effect on the inhibition of oxygen uptake caused by 3 mM acetaldehyde (Table 5). However, with 1–2 mM acetaldehyde, 1 mM cysteine afforded considerable protection against the inhibition of state 3 oxygen uptake, and 3·3 mM cysteine completely prevented the inhibition (Table 6). This suggests that, in general, amounts of cysteine equimolar to those of acetaldehyde are required for complete protection. Very high concentrations of acetaldehyde inhibited numerous mitochondrial functions, apparently owing to nonspecific interactions and damage to the mitochondria [8–10]. The inhibition of state 3 glutamate oxidation by 12 mM acetaldehyde was not prevented even by 10 mM cysteine (Table 6).

In these experiments, the mitochondria were incubated with cysteine for 1–2 min in the polarograph chamber. Acetaldehyde was then added and oxygen uptake was recorded. ADP was subsequently added to initiate state 3 conditions. When mitochondria were first incubated with acetaldehyde and ADP, the subsequent addition of cysteine provided considerably less relief than in experiments in which cysteine was initially present in the incubation medium. Thus, cysteine probably interacts with acetaldehyde; the longer the period of interaction, the greater the relief of the acetaldehyde-induced inhibition. Apparently the inhibition by acetaldehyde is not easily reversible, possibly because acetaldehyde forms a strong complex with a mitochondrial receptor. In this respect, the inhibition of energized Ca^{2+} uptake by acetaldehyde *in vitro* was only partially reversed after several washings of the mitochondria [8].

Table 5. Effect of cysteine and acetaldehyde on oxygen consumption associated with the oxidation of glutamate*

No. of expts.	Respiratory state	Acetaldehyde concn (mM)	Cysteine concn (mM)	Oxygen uptake (natoms/min/mg protein)	Effect on control (%)	P (control)	Effect on acetaldehyde (%)	P (acetaldehyde)
22	4			13·89 ± 0·80				
22		3·0		14·00 ± 0·94	+1	NS		
13		3·0	1·0	13·51 ± 1·13	−5	NS	−8	NS
18		3·0	3·3	18·01 ± 1·67	+29	0·01	+34	0·012
12		3·0	10·0	21·53 ± 1·69	+60	<0·001	+69	<0·001
25	3			72·09 ± 3·46				
25		3·0		47·39 ± 1·68	−34	<0·001		
14		3·0	1·0	50·93 ± 2·99	−29	<0·001	+13	NS
20		3·0	3·3	62·90 ± 3·73	−12	<0·05	+32	<0·001
15		3·0	10·0	63·63 ± 2·73	−9	NS	+34	0·001

* Oxygen uptake was assayed as described in Materials and Methods in the presence of cysteine. Results are compared to the controls for the indicated experiments.

Table 6. Effect of cysteine on inhibition of glutamate oxidation by acetaldehyde*

Acetaldehyde concn (mM)	Cysteine concn (mM)	Oxygen uptake (natoms/min/mg protein)	Effect (%)
		63·1	
1·0		48·3	−20
1·0	1·0	59·2	−6
1·0	3·3	63·5	+1
2·0		44·9	−29
2·0	1·0	58·1	−8
2·0	3·3	61·2	−3
3·0		39·9	−37
3·0	1·0	49·4	−22
3·0	3·3	53·1	−16
12·0		15·5	−75
12·0	3·3	16·1	−74
12·0	10·0	15·0	−76

* State 3 oxygen uptake was assayed as described in Materials and Methods. Results are means of two to three individual experiments.

Effect of cysteine on acetaldehyde-induced inhibition of state 3 oxidation of NAD$^+$-dependent substrates and succinate. Acetaldehyde was previously shown to be particularly inhibitory toward energy-dependent oxygen consumption associated with the oxidation of NAD$^+$-dependent substrates [8]. Acetaldehyde comparably depressed state 3 oxygen uptake associated with the oxidation of β-hydroxybutyrate, α-ketoglutarate, pyruvate-malate and glutamate (Table 7) [8]. Cysteine relieved the inhibition of the state 3 oxidation of all NAD$^+$-dependent substrates tested (Table 7).

Significant inhibition of the state 3 oxidation of succinate was observed only at very high levels of acetaldehyde (Table 7) [8]. Under these conditions, cysteine afforded no relief of the acetaldehyde-induced inhibition, even at concentrations up to 20 mM (Table 7).

Effect of other thiols on inhibition of oxygen uptake by acetaldehyde. Thiols, other than cysteine, were also

Table 7. Effect of cysteine on inhibition of state 3 oxidation of NAD$^+$-dependent substrates and succinate by acetaldehyde*

Substrate	Acetaldehyde concn (mM)	Cysteine concn (mM)	Oxygen uptake (natoms/min/mg protein)	Effect (%)
β-Hydroxybutyrate			80·4	
	3·0		59·7	−26
	3·0	1·0	66·3	−18
	3·0	3·3	70·5	−12
	3·0	10·0	74·1	−8
α-Ketoglutarate			78·8	
	3·0		56·3	−29
	3·0	3·3	65·5	−17
	3·0	10·0	78·0	−1
Pyruvate-malate			52·9	
	3·0		31·3	−41
	3·0	3·3	43·4	−18
	3·0	10·0	45·5	−14
Succinate			83·0	
	3·0		70·7	−15
	3·0	3·3	71·4	−14
	3·0	10·0	73·9	−11
	6·67		63·5	−23
	6·67	3·3	65·8	−21
	6·67	10·0	66·0	−20
			92·5	
	12·0		66·6	−28
	12·0	5·0	67·5	−27
	12·0	10·0	63·8	−31
	12·0	15·0	57·4	−38
	12·0	20·0	55·5	−40

* Oxygen consumption was assayed as described in Materials and Methods. Results are from two to three individual experiments.

Table 8. Effect of thiols on the inhibition of glutamate oxidation by acetaldehyde*

Acetaldehyde	Addition	Oxygen consumption (natoms/min/mg protein) State 4	State 3	Effect on control (%) State 4	State 3	Effect on thiol control (%) State 4	State 3
–		14·7	72·1				
+		13·7	45·7	−7	−37		
–	Cysteine (3 mM)	16·7	73·5	+14	+2		
+	Cysteine (3 mM)	16·1	66·4	+10	−8	−4	−10
–	Mercaptoethylamine (3 mM)	18·6	76·4	+27	+6		
+	Mercaptoethylamine (3 mM)	19·2	73·9	+31	+2	+3	−3
–	Mercaptoethylamine (10 mM)	19·2	77·2	+31	+7		
+	Mercaptoethylamine (10 mM)	19·2	75·8	+31	+5	0	−2
–	Mercaptoethanol (3 mM)	15·9	74·9	+8	+3		
+	Mercaptoethanol (3 mM)	16·5	56·1	+12	−22	+4	−25
–	Mercaptoethanol (10 mM)	17·7	76·2	+20	+6		
+	Mercaptoethanol (10 mM)	17·3	52·7	+18	−27	−2	−31
–	Glutathione (3 mM)	19·5	64·0	+33	−11		
+	Glutathione (3 mM)	19·5	51·5	+33	−29	0	−20
–	Glutathione (10 mM)	17·2	62·4	+17	−13		
+	Glutathione (10 mM)	16·2	49·1	+10	−32	−6	−21
–	Dithiothreitol (1 mM)	15·9	66·3	+8	−8		
+	Dithiothreitol (1 mM)	14·7	40·7	0	−44	−8	−39
–	Dithiothreitol (3 mM)	15·9	58·9	+8	−18		
+	Dithiothreitol (3 mM)	17·3	29·2	+18	−60	+9	−50

* Oxygen uptake was assayed as described in Materials and Methods in the presence or absence of 3 mM acetaldehyde, and the indicated thiols. Results are from two to three individual experiments.

tested to determine if they could protect against the inhibition of glutamate oxidation by acetaldehyde. Similar to cysteine, mercaptoethylamine, in the absence of acetaldehyde, had no effect on the state 3 rate of oxygen uptake, whereas the state 4 rate was stimulated (Table 8). Mercaptoethylamine completely relieved the inhibition of state 3 oxygen uptake by acetaldehyde (Table 8). Mercaptoethanol also had no effect by itself on state 3 oxygen uptake, whereas the state 4 rate was slightly stimulated. There was a slight protective effect by mercaptoethanol (Table 8). Glutathione, in the absence of acetaldehyde, resembled cysteine in stimulating state 4 oxygen uptake. A slight decrease in the state 3 rate of glutamate oxidation was observed in the presence of 3 and 10 mM glutathione (Table 8). Glutathione exerted no protective effect. However, since glutathione itself caused a slight depression of the state 3 rate, when the results were compared to the glutathione control rate, a slight protective effect by glutathione was noted (Table 8, column 8). In any event, the slight protective effect of mercaptoethanol or glutathione was considerably less than that observed for cysteine and mercaptoethylamine. Dithiothreitol had little effect on state 4 or state 3 oxygen uptake at a 1 mM concentration, whereas some inhibition of the state 3 rate was observed at a 3 mM concentration (Table 8). Dithiothreitol did not protect aginst the inhibition of state 3 glutamate oxidation by acetaldehyde. In fact, the slight inhibition of glutamate oxidation by dithiothreitol appears to be additive to that of acetaldehyde (Table 8). Cystine (3·3 mM) produced no relief of the inhibition by acetaldehyde. Another disulfide-generating agent, 5,5′-dithionitrobenzoic acid, produced severe inhibition of state 3 glutamate oxidation (85 per cent at 0·3 mM) by itself. Inhibition of energy production and utilization by disulfide-generating agents has been shown by others [13–15].

Effect of cysteine on the inhibition of oxidative phosphorylation by acetaldehyde. Since acetaldehyde depressed state 3 oxygen consumption without any effect on the state 4 rate, the respiratory control ratio associated with the oxidation of glutamate was depressed by acetaldehyde [8] (Table 9). As mentioned above, cysteine itself caused some depression of the respiratory control ratio because it stimulated state 4 respiration, without any effect on state 3 respiration. Consequently, the respiratory control ratio was depressed to the same extent in the presence of cysteine plus acetaldehyde as it was in the presence of acetaldehyde alone (Table 9). In fact, in the presence of acetaldehyde plus 10 mM cysteine, the ratio was slightly, but significantly, decreased beyond that observed in the presence of acetaldehyde itself (Table 9, columns 8 and 9). Thus, any potential protective effect by cysteine on the inhibition by acetaldehyde is masked by an inhibitory effect of cysteine itself. The ADP/O ratio was depressed 23 per cent by 3 mM acetaldehyde (Table 9). Cysteine caused a slight reduction in the extent of inhibition by acetaldehyde. However the ADP/O ratio was still significantly depressed in the presence of acetaldehyde plus cysteine (Table 9, columns 6 and 7). Similar results were obtained when assaying the P/O ratio of oxidative phosphorylation with β-hydroxybutyrate as the substrate. The depression of the P/O ratio by acetaldehyde was not significantly relieved by cysteine (Table 9). The rate of phosphorylation was depressed 38 per cent by 3 mM acetaldehyde. Cysteine provided modest relief of this inhibition by acetaldehyde.

Effect of inhibitors of mitochondrial functions on oxygen uptake in the presence of glutamate, acetaldehyde and cysteine. Oxygen uptake in the presence of glutamate, acetaldehyde and cysteine was strongly inhibited by the typical inhibitors of the mitochondrial respiratory chain, rotenone, antimycin and cyanide

Table 9. Effect of cysteine on the acetaldehyde-induced inhibition of oxidative phosphorylation*

No. of Expts.	Reaction	Acetaldehyde concn (mM)	Cysteine concn (mM)	Ratio or reaction rate	Effect on control (%)	P (control	Effect on acetaldehyde (%)	P (acetaldehyde)
22	Respiratory			5·62 ± 0·27				
22	control ratio	3·0		3·53 ± 0·16	−37	<0·001		
13		3·0	1·0	3·78 ± 0·23	−36	<0·001	+3	NS
18		3·0	3·3	3·43 ± 0·23	−37	<0·001	+1	NS
12		3·0	10·0	3·01 ± 0·13	−46	<0·001	−14	0·04
12	ADP/O ratio			2·61 ± 0·06				
12		3·0		2·02 ± 0·05	−23	<0·001		
9		3·0	1·0	2·20 ± 0·09	−15	0·005	+8	NS
10		3·0	3·3	2·22 ± 0·08	−14	0·002	+9	NS
11		3·0	10·0	2·24 ± 0·08	−13	0·002	+10	0·10
11	P/O ratio			2·82 ± 0·14				
11		3·0		2·26 ± 0·15	−20	0·03		
10		3·0	1·0	2·33 ± 0·18	−15	<0·05	+5	NS
11		3·0	3·3	2·36 ± 0·14	−14	<0·05	+5	NS
10		3·0	10·0	2·38 ± 0·10	−13	<0·05	+9	NS
12	Rate of			221 ± 18				
12	phosphorylation	3·0		137 ± 12	−38	<0·01		
11		3·0	1·0	172 ± 13	−22	0·028	+21	NS
12		3·0	3·3	166 ± 15	−25	0·024	+21	NS
11		3·0	10·0	171 ± 20	−23	0·046	+23	NS

* The respiratory control ratio associated with the oxidation of glutamate was calculated from the state 4 and 3 rates of oxygen consumption shown in Table 5. The ADP/O ratio was determined by adding limiting amounts of ADP and calculating the extra consumption of oxygen produced by the addition of ADP. The P/O ratio was assayed as described in Materials and Methods. The rate of phosphorylation refers to net nmoles glucose 6-phosphate produced/min/mg of mitochondrial protein.

(Table 10). The effect of rotenone confirms the fact that electrons enter the respiratory chain prior to the rotenone-sensitive site, as would be expected with NAD^+-dependent substrates (glutamate and acetaldehyde). The inability of cysteine to relieve the inhibition of energy transduction by acetaldehyde suggested the possibility that, in the presence of cysteine and acetaldehyde, the mitochondria may be in a partially uncoupled state. Thus, the inhibition of oxygen uptake by acetaldehyde, but not energy transduction, would be significantly relieved by cysteine. If this were so, oxygen uptake would not be very sensitive to atractyloside, since this inhibitor of adenine nucleotide translocation would only block coupled or ADP-linked oxygen uptake and not uncoupled oxygen consumption. However, atractyloside completely abolished the stimulation of oxygen uptake by ADP to the same extent in controls and in samples incubated with acetaldehyde plus cysteine, whereas the state 4 rate remained unaffected (Table 10). Furthermore, oligomycin, which inhibits phosphorylation-linked oxygen consumption, without any effect on uncoupled or resting respiration [36], was as effective in blocking state 3 respiration in the presence of glutamate, acetaldehyde and cysteine as in controls containing glutamate alone (Table 10). Oligomycin did not affect the state 4 rate of oxygen uptake. Thus, the mitochondria remain coupled in the presence of acetaldehyde plus cysteine. Further evidence for this conclusion was provided by the observation that cysteine, alone

Table 10. Effect of inhibitors on oxygen consumption*

Acetaldehyde concn (mM)	Cysteine concn (mM)	Inhibitor	Oxygen consumption (natoms/min/mg protein) State 4	State 3
			11·0	68·3
3			11·0	47·3
3	3·3		12·0	59·0
3	10·0		16·0	66·7
3	10·0	Rotenone (0·002 mM)	2·5	3·4
3	10·0	Antimycin (0·002 mM)	2·5	3·0
3	10·0	Cyanide (1 mM)	0	0
3	10·0	Atractyloside (0·05 mM)	12·0	10·5
3	10·0	Oligomycin (0·0016 mM)	10·0	9·0
3	10·0	Oligomycin (0·004 mM)	12·0	9·0
		Oligomycin (0·004 mM)	8·8	7·2

* Oxygen uptake was assayed as described in Materials and Methods using glutamate as the substrate. Rotenone, antimycin and oligomycin were dissolved in ethanol, the final ethanol concentration being 0·05%. This concentration of ethanol had no effect on oxygen consumption.

Table 11. Reaction of cysteine and acetaldehyde*

Cysteine (nmoles)	Acetaldehyde (nmoles)	Ratio of cysteine/ acetaldehyde	Amount of cysteine detected (nmoles) Time of incubation (min) 2	5	10	20
400			384	396	377	369
	200	2	376	358	254	244
	400	1	373	311	230	140
	800	0·5	339	246	182	70
	1200	0·3	226	151	64	15
	4000	0·1	0	0	0	0
600			575	570	552	443
	300	2	526	493	435	235
	600	1	251	359	286	87
	1200	0·5	168	153	125	12
	1800	0·3	30	28	21	6
	6000	0·1	0	0	0	0

* Cysteine was incubated with various amounts of acetaldehyde for 2, 5, 10 or 20 min, as described in Materials and Methods. Dithionitrobenzoic acid was added, and the absorbance at 412 nm was recorded.

or in the presence of acetaldehyde, did not stimulate latent or Mg^{2+}-ATPase activity, whereas dinitrophenol, a typical uncoupler, stimulated this activity.

Interaction of acetaldehyde and cysteine. Cysteine could complex with acetaldehyde to form 2-methylthiazolidine-4-carboxylic acid [28–30]. Free sulfhydryl groups can be readily detected by use of the reagent, dithionitrobenzoic acid (Ellman's reagent) [37]. Interaction of the sulfhydryl groups of cysteine with acetaldehyde should decrease the number of free sulfhydryl groups that can be detected with Ellman's reagent. Cysteine was incubated with different amounts of acetaldehyde, Ellman's reagent was then added, and the absorbance at 412 nm was recorded. In the absence of acetaldehyde, the amount of cysteine detected decreased with longer periods of incubation, presumably because of auto-oxidation of cysteine to cystine (Table 11). The addition of increasing amounts of acetaldehyde progressively decreased the detectable content of cysteine (Table 11), particularly at longer incubation periods. Under conditions used in studies of oxygen uptake (incubation times of about 5 min) 30–40 per cent of the added cysteine was not detectable at equimolar amounts of cysteine and acetaldehyde, whereas under the conditions of the experiments in which CO_2 production was measured (10–20 min incubation), 60–80 per cent was not detected at equimolar amounts of cysteine and acetaldehyde.

Acetaldehyde readily interacts with semicarbazide to form a semicarbazone, which is detected by its absorption at 224 nm. Interaction of acetaldehyde with cysteine would be expected to decrease the amount of acetaldehyde that can be detected as the semicarbazone complex. Acetaldehyde was incubated with different amounts of cysteine, semicarbazide was then added, and the absorbance at 224 nm was recorded. The addition of increasing amounts of cysteine progressively decreased the detectable content of acetaldehyde, particularly after prolonged incubation (Table 12). The cysteine–acetaldehyde complex appeared stable, since there was no release of acetaldehyde from the complex, in the presence of semicarbazide even after prolonged periods of incubation. Further verification for the interaction of acetaldehyde and cysteine was provided by using gas chromatography to determine the concentration of acetaldehyde in the gas phase, in equilibrium with an aqueous solution of acetaldehyde. After incubating 30 min with cysteine, the detectable acetaldehyde content in the gas phase was depressed 11, 42 and 99 per cent, when the cysteine to acetaldehyde ratios were 0·33, 1·0 and 3·33 respectively.

DISCUSSION

Acetaldehyde has numerous toxic effects on mitochondrial functions. It is a strong inhibitor of NAD^+-dependent state 3 oxygen consumption; glutamate oxidation is depressed to a greater extent than that of succinate. Energy utilization is also inhibited by acetaldehyde, as evidenced by the decrease in respiratory control, P/O and ADP/O ratios, as well as the decrease in the rate of phosphorylation. Fatty acid oxidation is also reduced by acetaldehyde, owing to a variety of effects, including inhibition of β-oxidation, citric acid cycle activity, and the respiratory-phosphorylation chain [10]. That the activity of the citric acid cycle is affected is suggested by the depression of CO_2 production from α-ketoglutarate[1-^{14}C] (Table 3).*

It has been suggested that thiols are involved in many mitochondrial functions [12–24]. We therefore entertained the possibility that acetaldehyde may inhibit mitochondrial functions by interacting with essential thiols: thus, externally added thiols might be effective in protecting against the inhibition by acetaldehyde. In this report, cysteine reversed the inhibition by acetaldehyde of CO_2 production from fatty acids and α-ketoglutarate, as well as the depression of state 3 oxygen consumption. Cysteine was most effective when it was present before acetaldehyde was added to the mitochondria. Cysteine may form a

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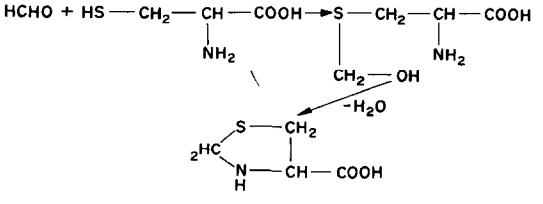
Table 12. Reaction of acetaldehyde and cysteine*

Acetaldehyde (nmoles)	Cysteine (nmoles)	Ratio of cysteine/ acetaldehyde	Amount of acetaldehyde detected (nmoles) Time of incubation (min) 2	5	10	20
360			344	348	376	360
	180	0·5	332	329	297	272
	360	1·0	322	296	272	256
	540	1·5	322	278	247	166
	900	2·5	303	252	221	154
	1800	5·0	263	227	141	102
900			831	837	813	841
	450	0·5	758	753	751	638
	900	1·0	631	577	483	379
	1350	1·5	657	493	403	324
	2250	2·5	555	369	303	162
	4500	5·0	464	264	182	210

* Acetaldehyde was incubated with various amounts of cysteine for 2, 5, 10 or 20 min, as described in Materials and Methods. Semicarbazide was added, and the absorbance at 224 nm was recorded.

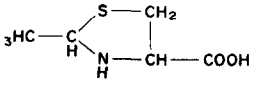
complex with acetaldehyde, thereby preventing the latter from interacting with the mitochondria. It is possible that complex formation may not entirely explain the protection by cysteine, since the inhibition of succinate oxidation by acetaldehyde is not relieved by cysteine. However, higher concentrations of acetaldehyde are required to inhibit the oxidation of succinate than the oxidation of NAD^+-dependent substrates. At these higher concentrations, acetaldehyde may have numerous nonspecific effects on mitochondrial functions. In this regard, the depression of oxygen consumption by 12 mM acetaldehyde, when glutamate was the substrate, could not be prevented by cysteine.

Semimercaptals are formed by a nonenzymatic reaction between aldehydes and mercaptans [25–27]. The reaction sequence has been particularly well characterized in the case of formaldehyde.



Thiazolidine-4-carboxylic acid

In the case of acetaldehyde, 2-methylthiazolidine-4-carboxylic acid is formed.



It has been shown that, in the presence of excess cysteine (cysteine/acetaldehyde ratio of 2 or more), djenkolic acid is produced [38]. However, this reaction proceeds very slowly at neutral pH and probably does not play a significant role under our conditions. We obtained direct evidence for the interaction of acetaldehyde and cysteine by studying the effect of acetaldehyde on the reaction of cysteine and Ellman's reagent and the effect of cysteine on the reaction of acetaldehyde and semicarbazide. The results suggest that a free SH and a free amino group in close proximity protect against inhibition by acetaldehyde. Thus, β-mercaptoethylamine is as effective as cysteine in relieving the inhibition by acetaldehyde. The carboxyl group of cysteine apparently plays no significant role in the interaction with acetaldehyde. Vicinal dithiols such as dithiothreitol do not exert any protective action. The need for the thiol group is suggested by the fact that compounds with free amino and free carboxyl groups (glycine or alanine) provide no relief of the acetaldehyde-induced inhibition. The importance of the amino group is suggested by the observation that thiols with free hydroxyl groups (mercaptoethanol or thioglycerol) are less effective than cysteine or β-mercaptoethylamine in providing a protective effect. Glutathione, which has a free SH and a free amino group, is not as effective as cysteine. The amino and thiol groups are further apart in glutathione than in cysteine, suggesting the necessity for the proximity of both ligands for maximum protection.

These findings that cysteine can protect against acetaldehyde toxicity on a molecular level may explain, at least in part, the observation that both cysteine and the complex of acetaldehyde and cysteine, 2-methylthiazolidine-4-carboxylic acid, *in vivo*, protected against death from acetaldehyde toxicity [30]. In subsequent studies, the protective effect of cysteine was mimicked by other thiols, whereas disulfides were ineffective [39]. The inhibition of the pyruvate dehydrogenase complex from ox brain by 1 mM acetaldehyde was reduced to about 50 per cent by 5 mM cysteine. There was no protection against the acetaldehyde inhibition by 5 mM glutathione, mercaptoethanol and dithiothreitol [40]. Essentially similar results were observed here, as these thiols were not as effective as cysteine in protecting against the inhibition of mitochondrial functions by acetaldehyde. It is also interesting that the reduction in cholinesterase activity in the brain of rats after chronic ethanol treatment has been reported to be prevented by 1% cysteine [41]. It is possible that this effect of chronic ethanol treatment may be mediated, in part, by acetaldehyde. In a similar manner, the decrease in glu-

tathione content found after a single administration of a large amount of alcohol [42] may be due, in part, to interaction of glutathione with acetaldehyde generated from the oxidation of ethanol.

The addition of cysteine alone gave rise to some oxygen consumption (about 2 natoms/min/mg of mitochondrial protein). In manometric experiments, practically no oxygen was consumed in the presence of cysteine for the first 20 min [29]. After this lag period, oxygen uptake occurred at a rate of about 5–10 natoms/min/mg of protein (calculated from the data of Fig. 1 and Table 1, Ref. 29). The complexation product of formaldehyde and cysteine, thiazolidine-4-carboxylic acid, was also oxidized by liver mitochondria at a rate of about 10–20 natoms oxygen/min/mg of protein [28,29]. Thus, it is possible that the 'relief' of the acetaldehyde-induced inhibition of glutamate-linked oxygen uptake may represent mitochondrial oxidation of 2-methylthiazolidine-4-carboxylic acid. However, the 2-methyl derivative is oxidized at a rate only 25 per cent of that of thiazolidine-4-carboxylic acid [28,29]. Thus the maximum theoretical increase of oxygen uptake would correspond to about 2–5 natoms/min, a rate considerably less than the increase in oxygen uptake (about 15–20 natoms/min) which actually occurs.

A trend toward slight relief by cysteine of acetaldehyde-induced inhibition of energy transduction was observed. However, the ADP/O, P/O and respiratory control ratios, and the rate of phosphorylation still remained depressed in the presence of acetaldehyde plus cysteine, suggesting the possibility that the mitochondria are uncoupled in the presence of cysteine plus acetaldehyde. Hence oxygen uptake, but not the associated energy-coupling reactions, would be elevated; this oxygen uptake would respresent an uncoupled rate, and not an energy-coupled state 3 rate. However, glutamate-supported oxygen uptake in the presence of acetaldehyde and cysteine was depressed by atractyloside and oligomycin, inhibitors which do not affect uncoupled respiration. Furthermore, ATPase activity was not elevated in the presence of acetaldehyde and cysteine, and state 4 respiration was only increased about 50 per cent. True uncouplers, such as dinitrophenol, increase ATPase activity and state 4 respiration more than 5-fold. Thus, the inability of cysteine to relieve the inhibition of oxidative phosphorylation by acetaldehyde remains unexplained. It is possible that, in the presence of acetaldehyde and cysteine, the mitochondria are only partly coupled. While the rate of oxygen uptake in the presence of acetaldehyde is increased by cysteine, part of this increase may represent uncoupled oxygen uptake, since the state 4 rate itself is somewhat increased by cysteine. Another complicating factor is that cysteine itself causes a very slight decrease in oxidative phosphorylation which may mask any potential relief of the acetaldehyde-induced inhibition.

The inhibition of mitochondrial functions by acetaldehyde *in vitro* resembles that found after chronic ethanol consumption [8–10,43]. These concentrations of acetaldehyde (0·75 to 3 mM) are higher than those usually found in blood after ethanol administration [44]. However, blood levels of acetaldehyde may reach levels of 0·5 to 1 mM or greater after administration of ethanol with certain drugs, e.g. disulfiram [44,45], pyrogallol [46] or pargylline [47]. Moreover levels of acetaldehyde in the liver considerably exceed those in the blood [48].* Most of the above studies were performed with acute ethanol administration. Chronic exposure to lower levels of acetaldehyde may result in effects similar to those seen in acute experiments. After chronic exposure to ethanol, acetaldehyde blood levels increased in man [49], and rat hepatic mitochondrial oxidation of acetaldehyde was depressed [50]. It would, therefore, be of interest to determine the effect of cysteine *in vivo* during chronic ethanol intoxication, which results in chronic exposure to acetaldehyde.

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