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SHORT COMMUNICATIONS

Sensitivity to acetaldehyde of pyruvate oxidation by mitochondria from liver, kidney, brain and muscle*

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Although acetaldehyde is rapidly metabolized within the liver, blood levels of acetaldehyde increase after ethanol administration [1]. The concentration of acetaldehyde in peripheral blood is considerably lower than in hepatic venous blood suggesting extrahepatic oxidation of acetaldehyde [2, 4]. Furthermore, alcohol dehydrogenase activity is found in tissues other than the liver [5, 6]. For example, the kidney produces $^{14}CO_2$ from ^{14}C-labeled ethanol [7, 8]. Mitochondria isolated from various organs of the rat could oxidize acetaldehyde, this metabolism being NAD^+ dependent and rotenone-sensitive [9]. Since circulating acetaldehyde must be metabolized, it was of interest to study the effect of acetaldehyde on the oxidation of respiratory substrates by these mitochondria. In liver mitochondria, oxygen consumption, oxidative phosphorylation, fatty acid oxidation and CO_2 production from citric acid cycle intermediates were depressed by high levels of acetaldehyde [10–13]. The physiological significance of these results was difficult to evaluate, since these concentrations of acetaldehyde (usually 1 mM and higher) were higher than blood levels of acetaldehyde usually found after ethanol administration (0.1 to 0.2 mM [1]). We report here that concentrations of acetaldehyde which may be considered physiological after ethanol consumption inhibit the oxidation of pyruvate, and that the pyruvate dehydrogenase complex from mitochondria from various organs of the rat appears to be especially sensitive to inhibition by acetaldehyde.

Liver and kidney mitochondria were isolated from male Sprague–Dawley rats [14], using 0.25 M sucrose–0.01 M Tris–HCl (pH 7.4)–0.001 M EDTA, and washed twice. Muscle mitochondria were prepared [15], using tissue excised from the hind limbs of the rats. Brain mitochondria were prepared by a modification of the method of Puszkin *et al.* [16]. The crude mitochondrial pellet was suspended in 3 per cent dialyzed ficoll in a buffer of 0.24 M mannitol–0.06 M sucrose–0.05 mM EDTA (Tris), pH 7.4, and layered over a 6 per cent ficoll solution in the same buffer. The mitochondrial pellet obtained after centrifugation at 10,000 rev/min in the ss-34 rotor of a RC-2B Sorvall centrifuge was suspended in 0.25 M sucrose–0.01 M Tris–HCl (pH 7.4)–0.001 M EDTA. Oxygen uptake was assayed at 30° using a Clark oxygen electrode and a Yellow Springs oxygen monitor, in a reaction mixture of 0.3 M mannitol, 0.01 M Tris–HCl, pH 7.4, 0.01 M potassium phosphate, pH 7.4, 0.01 M KCl, 0.003 M $MgCl_2$ and mitochondria (about 2–4 mg protein) in a final reaction volume of 3.0 ml. With brain mitochondria, bovine serum albumin (final concentration, 1%) was also added. State 3 respiration was initiated by the addition of 0.5 mM ADP. Substrates included glutamate (10 mM) plus malate (3 mM), pyruvate (10 mM) plus malate (3 mM), α-ketoglutarate (10 mM), β-hydroxybutyrate (10 mM), succinate (10 mM) (in the presence of 0.004 mM rotenone) and ascorbate (5 mM) plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (0.2 mM). The ADP/O ratio was calculated from the extra consumption of oxygen produced by the addition of ADP. Statistical analysis was performed by Student's *t*-test.

Acetaldehyde had no effect on the state 4 rate of oxygen uptake associated with the oxidation of pyruvate-malate by liver mitochondria (Table 1). However, the state 3 rate of pyruvate oxidation was inhibited by 0.2 to 0.3 mM acetaldehyde, with increasing inhibition occurring at higher concentrations of acetaldehyde (Table 1). By contrast, the oxidation of glutamate was considerably less sensitive to inhibition by acetaldehyde; 0.5 mM had no effect on the state 3 rate of glutamate oxidation while 1.0 mM acetaldehyde produced a decrease of 14 per cent (Table 1). Similar results were obtained with other NAD^+-dependent substrates, e.g. the state 3 rate of β-hydroxybutyrate oxidation was decreased 4, 14, 20 and 27 per cent by acetaldehyde concentrations of 0.5, 1.0, 2.0 and 3.0 mM, respectively; the state 3 rate of α-ketoglutarate oxidation was decreased 8, 16, 25 and 32 per cent by the same concentrations of acetaldehyde. Much higher concentrations of acetaldehyde are required to inhibit the oxidation of succinate or ascorbate [10]. Thus, the oxidation of pyruvate appears to be especially sensitive to inhibition by acetaldehyde. This inhibition is not limited specifically to ADP-stimulated oxygen uptake, since acetaldehyde depressed pyruvate oxidation stimulated by the ionophore valinomycin to the same extent as it depressed the state 3 rate of pyruvate oxidation (compare experiments A and B, Table 1). Similar to the results found with state 3 glutamate and pyruvate oxidation, acetaldehyde was much less inhibitory toward valinomycin-stimulated glutamate oxidation (−3, −13 and −23 per cent at acetaldehyde concentrations of 0.5, 1.0 and 2.0 mM, respectively) than it was toward valinomycin-stimulated pyruvate oxidation.

Acetaldehyde had no effect on the state 4 rate of pyruvate oxidation by mitochondria isolated from brain, muscle and kidney (Table 2). However, as with liver mitochondria, the state 3 rate of pyruvate oxidation was inhibited by 0.2 to 0.3 mM acetaldehyde (Table 2). That the oxidation of pyruvate is uniquely sensitive to acetaldehyde is indicated by the fact that the oxidation of glutamate, an NAD^+-dependent substrate, succinate (not shown) and ascorbate (not shown) by mitochondria from kidney, brain and muscle was not affected by concentrations of acetaldehyde which decreased the oxidation of pyruvate (Table 2). Since acetaldehyde inhibited the state 3 rate of pyruvate oxidation, without affecting the state 4 rate, the respiratory control ratio associated with the oxidation of pyruvate by mitochondria from liver (4.1), brain (5.7), muscle (11.2) and kidney (3.9) was decreased by acetaldehyde. With liver mitochondria, respiratory control was lowered by 18, 29 and 32 per cent by 0.2, 0.3 and 0.5 mM acetaldehyde respectively. With brain mitochondria, respiratory control was lowered by 22, 31 and 39 per cent by the same concentrations of acetaldehyde. The respiratory control ratio associated with the oxidation of glutamate by mitochondria from liver (4.7), brain (8.9), muscle (9.2) and kidney (5.6)

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Table 1. Effect of acetaldehyde on oxygen uptake by liver mitochondria*

Substrate	Concentration of acetaldehyde (mM)	Oxygen uptake (natoms/min/mg protein)		Effect (%)		P	
		State 4	State 3	State 4	State 3	State 4	State 3
Expt. A							
Pyruvate-malate	0 (20)	13.98 ± 0.83	56.82 ± 2.65				
	0.1 (4)	15.64 ± 1.46	59.67 ± 4.83	+12	+5	NS	NS
	0.2 (15)	14.97 ± 0.94	49.48 ± 2.82	+7	−13	NS	0.10 > P > 0.05 (0.0704)
	0.3 (17)	15.54 ± 1.07	44.53 ± 2.57	+11	−22	NS	<0.01
	0.5 (19)	14.49 ± 0.88	40.06 ± 1.93	+4	−29	NS	<0.001
	1.0 (15)	15.50 ± 1.00	38.02 ± 1.97	+10	−33	NS	<0.001
	2.0 (8)	15.55 ± 1.70	33.67 ± 1.92	+11	−41	NS	<0.001
Glutamate-malate	0 (7)	19.72 ± 2.18	93.10 ± 7.39				
	0.5 (7)	18.79 ± 2.19	89.97 ± 8.04	−5	−3	NS	NS
	1.0 (7)	19.23 ± 2.27	80.54 ± 7.49	−2	−14	NS	NS
	2.0 (7)	19.95 ± 1.56	76.09 ± 6.43	+1	−19	NS	<0.05
	3.0 (3)	18.25 ± 3.92	64.36 ± 7.72	−7	−31	NS	<0.05
Expt. B							
Pyruvate-malate	0 (7)		63.10 ± 4.91				
	0.2 (5)		56.47 ± 6.15		−11		NS
	0.3 (7)		49.92 ± 3.64		−21		<0.02
	0.5 (7)		44.93 ± 2.73		−29		<0.01
	1.0 (7)		40.92 ± 2.32		−35		<0.002

* Oxygen uptake by liver mitochondria was assayed in the absence (state 4) and presence (state 3) of ADP in Expt. A. In Expt. B, valinomycin (0.5 μM) was used to stimulate oxygen uptake. Results are expressed as mean ± S.E.M. The number of experiments is indicated in parentheses. NS = not significant.

was not affected by 0.5 mM acetaldehyde, and was depressed by about 10 and 20 per cent by 1 and 2 mM acetaldehyde respectively. The ADP/O ratio associated with the oxidation of pyruvate was depressed by 0.3 mM acetaldehyde (Table 3). These changes are very small, but because of the high reproducibility, they are significant. Kidney mitochondria appeared to be especially sensitive to the lowering of the ADP/O ratio by acetaldehyde. It is possible that the lowering of the ADP/O ratio associated with the oxidation of pyruvate may reflect the decreased flux of electrons through the respiratory chain caused by acetaldehyde inhibition of pyruvate dehydrogenase, rather than a true uncoupling effect by acetaldehyde. This would be consistent with the lack of stimulation of state 4 respiration or of ATPase activity by acetaldehyde (Tables 1 and 2, see also Ref. 10), and the fact that the ADP/O ratio associated with the oxidation of glutamate is not affected by 0.5 mM acetaldehyde (Table 3), a concentration which does not inhibit glutamate-linked oxygen uptake.

In liver mitochondria, high concentrations of acetaldehyde inhibit oxygen uptake linked to the oxidation of glutamate, α-ketoglutarate and β-hydroxybutyrate comparably, whereas oxygen uptake associated with succinate or ascorbate is not affected [10]. It was previously suggested that the similar inhibition of the NAD^+-dependent substrates by acetaldehyde may be due to an effect on a common intermediate in the oxidation of these substrates, e.g. NADH dehydrogenase, although an effect on the individual dehydrogenases was not to be ruled out [10]. The pyruvate dehydrogenase complex appears to be uniquely sensitive to acetaldehyde, since concentrations of acetaldehyde which do not affect the oxidation of other substrates depress the state 3 oxidation of pyruvate by mitochondria from liver, kidney, muscle and brain. The extent of inhibition by acetaldehyde increases only slightly as the acetaldehyde concentration is raised above 1 mM. This may reflect, in part, the ability of the acetaldehyde itself to serve as a substrate for the mitochondrial respiratory chain. Consequently, measurements of oxygen consumption may not necessarily reflect the true sensitivity to acetaldehyde, although the potent inhibition of pyruvate oxidation can be readily detected. In addition, acetaldehyde can be oxidized by mitochondria from all these tissues [9]. In parallel experiments, under conditions similar to those of the assays of oxygen uptake (3 mg protein, 5-min total reaction period), about 250 nmoles acetaldehyde are consumed by intact liver mitochondria. The concentrations reported here reflect the initial concentration of acetaldehyde added.

Since the concentration of pyruvate exceeds that of acetaldehyde by more than 20-fold, it is unlikely that acetaldehyde interaction with pyruvate (acetoin formation) limits the availability of pyruvate to its dehydrogenase. Acetaldehyde has been shown to inhibit the pyruvate dehydrogenase complex isolated from ox brain and ox kidney by a mechanism which is non-competitive with respect to pyruvate, thiamine pyrophosphate, coenzyme A and NAD^+ [17]. Furthermore, the α-ketoglutarate dehydrogenase complex was not affected by concentrations of acetaldehyde which inhibit the oxidation of pyruvate [17]. The K_i for acetaldehyde inhibition of the state 3 oxidation of pyruvate by brain mitochondria is about 0.5 to 1.0 mM (Table 2), which is close to the K_i reported for the partially purified pyruvate dehydrogenase complex from ox brain (values of 0.42 mM [18] and 0.13 mM [17] being reported). This further suggests that the inhibitory effect of acetaldehyde on pyruvate oxidation by intact mitochondria is related to inhibition of pyruvate dehydrogenase. In addition, aldehyde dehydrogenase activity is found in mitochondrial fractions from all these tissues [19–24], and acetaldehyde is readily utilized in these tissues, e.g. radioactive acetaldehyde but not radioactive ethanol results in the labeling of amino acids in incubated brain slices [25, 26]. Intact mitochondria isolated from these tissues all catalyze an NAD^+-dependent oxidation of acetaldehyde [9]. Of considerable interest is the possibility that the inhibitory action of acetaldehyde may involve interaction with sulfhydryl groups of the pyruvate dehydrogenase complex, since cysteine partially reverses the inhibition [17]. Cysteine has been shown to relieve the inhibition of several mitochondrial functions by acetaldehyde [12].

Table 2. Effect of acetaldehyde on oxygen uptake by mitochondria from brain, muscle and kidney*

Tissue	Substrate	Concentration of acetaldehyde (mM)	Oxygen uptake (natoms/min/mg protein) State 4	Oxygen uptake (natoms/min/mg protein) State 3	Effect (%) State 4	Effect (%) State 3	P State 4	P State 3
Brain	Pyruvate-malate	(7)	33.50 ± 3.67	192.38 ± 10.93				
		0.2 (7)	33.36 ± 4.60	149.47 ± 14.98	0	−22	NS	0.10 > P > 0.05
		0.3 (6)	31.12 ± 2.74	122.88 ± 6.23	−7	−36	NS	<0.01
		0.5 (7)	32.15 ± 3.76	112.71 ± 7.95	−4	−41	NS	<0.002
		1.0 (6)	34.67 ± 4.66	100.13 ± 9.91	+3	−48	NS	<0.002
		2.0 (3)	33.83 ± 2.52	96.17 ± 6.65	+1	−50	NS	<0.01
	Glutamate-malate	0 (3)	15.7	139.4				
		0.5 (3)	14.9	135.3	−5	−3		
		1.0 (3)	15.7	123.2	0	−12		
		2.0 (3)	15.7	109.7	0	−21		
		3.0 (3)	12.8	92.3	−18	−34		
Muscle	Pyruvate-malate	0 (5)	25.37 ± 3.82	283.46 ± 22.19				
		0.2 (5)	25.50 ± 3.98	241.06 ± 18.64	0	−15	NS	NS
		0.3 (5)	26.83 ± 4.78	222.00 ± 17.95	+6	−22	NS	<0.05
		0.5 (5)	26.51 ± 4.55	176.25 ± 17.72	+4	−38	NS	<0.01
		1.0 (5)	27.58 ± 2.93	115.67 ± 11.71	+9	−59	NS	<0.001
	Glutamate-malate	0 (5)	32.91 ± 3.57	302.15 ± 37.42				
		0.5 (2)	35.52	293.10	+8	−3	NS	NS
		1.0 (5)	29.81 ± 4.36	264.20 ± 25.21	−9	−13	NS	NS
		2.0 (5)	33.74 ± 4.07	238.81 ± 22.47	+3	−21	NS	<0.05
		3.0 (5)	30.72 ± 4.2	177.21 ± 33.50	−7	−41	NS	<0.01
Kidney	Pyruvate-malate	0 (10)	17.19 ± 1.10	66.37 ± 4.25				
		0.1 (5)	16.99 ± 1.59	61.92 ± 5.70	−1	−7	NS	NS
		0.2 (9)	17.50 ± 1.01	58.37 ± 3.60	−2	−12	NS	NS
		0.3 (9)	16.80 ± 1.33	50.34 ± 4.85	−2	−24	NS	<0.02
		0.5 (8)	16.99 ± 2.13	47.85 ± 6.38	−1	−28	NS	<0.01
		1.0 (8)	16.44 ± 1.65	37.79 ± 3.43	−4	−43	NS	<0.001
		2.0 (6)	13.46 ± 1.65	31.77 ± 4.59	−22	−52	0.10 > P > 0.05	<0.001
	Glutamate-malate	0 (3)	14.1	78.6				
		0.5 (1)	13.8	75.9	−2	−3		
		1.0 (3)	14.1	68.4	0	−13		
		2.0 (3)	13.8	59.8	−2	−24		
		3.0 (3)	12.6	51.3	−11	−35		

* Results are expressed as mean ± S.E.M. The number of experiments is indicated in parentheses. NS = not significant.

Table 3. Effect of acetaldehyde on oxidative phosphorylation*

Tissue	Substrate	Concentration of acetaldehyde (mM)	ADP/O ratio	Effect (%)	P
Liver	Pyruvate-malate	0 (17)	2.78 ± 0.08		
		0.1 (9)	2.84 ± 0.10	+2	NS
		0.2 (15)	2.59 ± 0.07	−7	NS
		0.3 (17)	2.47 ± 0.08	−11	<0.01
		0.5 (17)	2.43 ± 0.09	−13	<0.01
		1.0 (11)	2.23 ± 0.11	−20	<0.01
		2.0 (6)	2.14 ± 0.11	−23	<0.02
	Glutamate-malate	0 (5)	2.89 ± 0.13		
		0.5 (5)	2.89 ± 0.18	0	
		1.0 (5)	2.57 ± 0.19	−11	NS
		2.0 (5)	2.34 ± 0.10	−19	<0.05
Brain	Pyruvate-malate	0 (5)	2.79 ± 0.04		
		0.2 (5)	2.61 ± 0.07	−6	NS
		0.3 (5)	2.48 ± 0.08	−11	<0.02
		0.5 (5)	2.36 ± 0.08	−15	<0.01
		1.0 (5)	1.96 ± 0.08	−30	<0.001
Kidney	Pyruvate-malate	0 (7)	2.72 ± 0.15		
		0.2 (6)	2.45 ± 0.22	−10	NS
		0.3 (5)	2.21 ± 0.23	−19	0.10 > P > 0.05 (0.0783)
		0.5 (5)	2.01 ± 0.17	−26	<0.02
		1.0 (5)	1.76 ± 0.22	−35	<0.01

* Results are expressed as mean ± S.E.M. The number of experiments is indicated in parentheses. NS = not significant.

The strong inhibition by acetaldehyde of pyruvate oxidation by brain mitochondria has been shown in several reports [27–30]. Kiessling [22] originally observed that pyruvate oxidation is inhibited in mitochondria from several different tissues in the following order of sensitivity: cerebellum > muscle > cerebrum > kidney > liver; he concluded that the capacity of the tissue to metabolize acetaldehyde may be important especially with regard to pyruvate oxidation. These experiments required measuring oxygen uptake for 25 min [22], a period long enough for significant loss of acetaldehyde by mitochondrial oxidation of acetaldehyde. Thus, the greater the rate of acetaldehyde oxidation (which gives rise to oxygen consumption), the less the apparent inhibition of pyruvate oxidation. In this report, short polarographic experiments were employed, a procedure which avoids the previous problems. The oxidation of pyruvate by kidney, liver, muscle and brain mitochondria is inhibited by acetaldehyde to comparable extents in all tissues examined (Tables 1 and 2). That the oxidation of an important metabolite in intermediary metabolism is depressed by levels of acetaldehyde which circulate after ethanol administration and which can be found in the liver after ethanol administration [31, 32] suggests that acetaldehyde may contribute to the pathogenesis of ethanol toxicity in many organs. Moreover, the conspicuous increase in the concentration of circulating acetaldehyde after the administration of disulfiram [1] raises the possibility of a unique effect on pyruvate metabolism in all organs under those conditions. Further studies are required to determine the nature of the inhibition of pyruvate oxidation by acetaldehyde. Pyruvate dehydrogenase is a complex enzyme system which exists in active (phosphorylated) and inactive (phosphorylated) forms. Numerous components and factors regulate the distribution of pyruvate dehydrogenase between active and inactive forms, e.g. pyruvate itself, ADP, fatty acids, ATP/ADP ratio, NAD/NADH ratio and citrate [33–38].

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Effects of alanosine and hadacidin on enzymes using aspartic acid as a metabolite*

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Alanosine is an extracellular product of *Streptomyces alanosinicus* which has been shown to have antibiotic, antitumor and immunosuppressive activity [1–3]. The structure of the natural product is L-(−)-2-amino-3-(hydroxynitrosamino) propionic acid [4]. Studies of its mode of action suggest that it can inhibit both AMP and pyrimidine biosynthesis in microbes [5], and it was suggested that the drug affected both adenylosuccinate synthetase and aspartate transcarbamylase. Recently, Graff and Plagemann [6] have reported that in Novikoff hepatoma cells the inhibition is specifically for adenylosuccinate synthetase with no effect on pyrimidine biosynthesis.

Hadacidin (*N*-formyl hydroxyaminoacetic acid) is an antibiotic from *Penicillium frequentans* which also inhibits adenylosuccinate synthetase [7], causing a decrease in AMP biosynthesis without inhibition of GMP formation. The inhibition of adenylosuccinate synthetase is competitive with the natural substrate aspartate [8].

Gale and Smith [9] have compared the effect of alanosine and hadacidin on partially purified *Escherichia coli* adenylosuccinate synthetase. Hadacidin was a potent inhibitor competitive with aspartate as previously determined, but alanosine did not inhibit the enzyme. The similarity of the structure of alanosine to aspartate made this result somewhat unexpected, particularly in view of the more recently discovered specific inhibition *in vivo* of adenylosuccinate synthetase by alanosine [6]. Gale and Smith [9] have suggested that a metabolite of alanosine was responsible for the inhibition observed which may be what occurs in the studies of Graff and Plagemann [6].

To further evaluate the roles of alanosine and hadacidin, the inhibition toward *E. coli* adenylosuccinate synthetase, aspartase, asparaginase and aspartate transcarbamylase was studied. These enzymes all have aspartate as a substrate or product allowing conclusions to be drawn about the specificity of aspartate binding sites in the different proteins. Also, the ability of a mammalian adenylosuccinate synthetase to utilize alanosine as a substrate was tested.

DL-Alanosine and hadacidin were kindly provided by Merck, Sharp & Dohme Research Laboratories, Rahway, NJ. IMP, carbamylphosphate, aspartic acid, asparagine and GTP were supplied by Sigma, St. Louis, MO. All other chemicals were reagent grade. Frozen *E. coli* B cells were obtained from Grain Processing, Muscatine, Iowa.

Adenylosuccinate synthetase and aspartase were purified from frozen *E. coli* B cells as described previously [10, 11] and from rat liver [12]. Aspartate transcarbamylase was purified from the special strain of *E. coli* as described by Gerhart and Holoubek [13]. Asparaginase purified from *E. coli* was obtained from CalBiochem, La Jolla, CA.

Adenylosuccinate synthetase activity was determined with the spectrophotometric assay reported previously [14] or by separation of the reaction products using [^{14}C]IMP as a substrate on PEI-cellulose sheets as described by Crabtree and Henderson [15]. Aspartase activity was followed by either the spectrophotometric assay [11] or by measurement of the ammonia produced with Nessler's reagent [16]. Asparaginase activity was assayed also using Nessler's reagent [16]. Aspartate transcarbamylase activity was followed by monitoring H^+ production in a Radiometer pH-stat with pH 8.3 as an endpoint [17].

The inhibition of adenylosuccinate synthetase by hadacidin reported by Shigeura and Gordon [8] was confirmed (K_I = 4.2 μM for the *E. coli* enzyme and 6.3 μM for the

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