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EFFECT OF CYANAMIDE ON THE METABOLISM OF ETHANOL AND ACETALDEHYDE AND ON GLUCONEOGENESIS BY ISOLATED RAT HEPATOCYTES

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Abstract—Previous experiments demonstrated that acetaldehyde stimulated glucose production from pyruvate, whereas gluconeogenesis from glycerol, xylitol and sorbitol was inhibited [A.I. Cederbaum and E. Dicker, *Archs Biochem. Biophys.* **197**, 415 (1979)]. To determine the mechanism whereby acetaldehyde affects glucose production from these precursors, and to evaluate the role of acetaldehyde in the actions of ethanol, experiments with cyanamide were carried out. The oxidation of acetaldehyde by isolated rat liver cells was inhibited by cyanamide after a brief incubation period. Associated with this inhibition of acetaldehyde oxidation was an inhibition of ethanol oxidation by cyanamide and an increase in the amount of acetaldehyde which arose during the oxidation of ethanol. Ethanol oxidation was decreased because of the ineffective removal of acetaldehyde in the presence of cyanamide. Cyanamide had no effect on hepatic oxygen uptake. The increase in the β-hydroxybutyrate/acetoacetate ratio produced by acetaldehyde was completely prevented by cyanamide, whereas the slight increase in the lactate/pyruvate ratio was not prevented by cyanamide. Cyanamide partially reversed the ethanol-induced increase in the lactate/pyruvate ratio, but it completely prevented the ethanol-induced increase in the β-hydroxybutyrate/acetoacetate ratio. The ethanol-induced change in the mitochondrial redox state may, therefore, be due primarily to the mitochondrial oxidation of the acetaldehyde which arises during the oxidation of ethanol. The inhibitory effects of acetaldehyde on gluconeogenesis from glycerol, xylitol and sorbitol, as well as the stimulation of acetaldehyde of glucose production from pyruvate, were completely prevented by cyanamide. These results indicate that the effects of acetaldehyde on gluconeogenesis represent metabolic effects, rather than direct effects of acetaldehyde. Changes in the cellular NADH/NAD^+ ratio as a consequence of acetaldehyde metabolism are postulated to be responsible for these actions of acetaldehyde. Ethanol stimulated glucose production from pyruvate, while inhibiting gluconeogenesis from glycerol, xylitol and sorbitol. Cyanamide, which prevented the effects of acetaldehyde on gluconeogenesis, also prevented the effects of ethanol on gluconeogenesis. This prevention by cyanamide may be suggestive for a role for acetaldehyde in the actions of ethanol on gluconeogenesis. The possibility cannot be ruled out, however, that the prevention of the effects of ethanol by cyanamide may be due to the partial inhibition of ethanol oxidation by cyanamide. These results indicate that cyanamide is an effective inhibitor of acetaldehyde oxidation by isolated liver cells and therefore can be used to determine the mechanism whereby acetaldehyde affects metabolic function. Depending on the reaction under investigation, acetaldehyde can have direct or indirect effects on cellular metabolism.

There is much current interest in evaluating the role of acetaldehyde in the actions of ethanol [1]. The effects of acetaldehyde on gluconeogenesis from various precursors [2, 3] has been studied recently. Acetaldehyde was found to stimulate glucose production from pyruvate, but to inhibit glucose production from xylitol, sorbitol and glycerol [2, 3]. Methylene blue, an artificial electron acceptor, partially prevented these effects of acetaldehyde, suggesting that the actions of acetaldehyde may require the metabolism of acetaldehyde with subsequent changes of the cellular NADH/NAD^+ redox ratio [2, 3]. The metabolism of ethanol caused similar effects on glucose production from pyruvate (stimulation) and from xylitol, sorbitol and glycerol (inhibition), as did acetaldehyde, leading to the suggestion that acetaldehyde may play some role in the actions of ethanol on gluconeogenesis from these substrates [2, 3].

To determine the mechanism whereby acetaldehyde alters glucose production from various precursors, as well as to evaluate a possible role of acetaldehyde in the actions of ethanol, studies with inhibitors of acetaldehyde metabolism were required. Effective inhibitors of aldehyde dehydrogenase include disulfiram [4–6], pargyline [7, 8] and cyanamide [9–12]. Disulfiram, lacks specificity [4] and can also inhibit the mitochondrial respiratory chain in a "rotenone-like" manner [13]. Inhibition of acetaldehyde oxidation by pargyline in isolated hepatocytes requires long incubation periods with the inhibitor and is incomplete [14]. In addition, pargyline itself inhibits the control rates of glucose production (unpublished observations). Cyanamide was used as an alternative to disulfiram in alcohol therapy [9]. Cyanamide, administered *in vivo*, has been shown to inhibit the activity of the low K_m mitochondrial aldehyde dehydrogenase [11, 12]. After a brief incubation period, cyanamide *in vitro*

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inhibits acetaldehyde oxidation by intact rat liver mitochondria [15]. These latter studies suggested that cyanamide may be a useful *in vitro* inhibitor of acetaldehyde metabolism in isolated cells.

The experiments described in the current manuscript were carried out to determine if the effects of acetaldehyde (and ethanol) on gluconeogenesis from various precursors represent a direct effect of acetaldehyde or require the metabolism of acetaldehyde. The effects of cyanamide *in vitro* on the oxidation of acetaldehyde and ethanol, the changes in the hepatic redox ratios produced by the metabolism of acetaldehyde and ethanol, the accumulation of acetaldehyde during the metabolism of ethanol, and hepatic oxygen consumption were first evaluated. After demonstrating that cyanamide effectively inhibits acetaldehyde metabolism by isolated hepatocytes, the ability of cyanamide to prevent the actions of acetaldehyde and ethanol on gluconeogenesis was determined.

EXPERIMENTAL PROCEDURES

Liver cells were prepared from 24-hr fasted male Sprague–Dawley rats weighing between 250 and 350 g, as previously described [16]. The buffer used was Krebs–Ringer bicarbonate, supplemented with a 10 mM phosphate buffer, pH 7.4, and 2.5% fatty acid-free bovine serum albumin. The buffer was gassed with 95 per cent O_2–5 per cent CO_2 prior to, but not during, the experiments. Viability of the cells was >95 per cent as determined by trypan blue exclusion and succinate-stimulated respiration.

The oxidation of acetaldehyde was assayed in a reaction medium containing the above buffer and about 10 mg of liver cell protein in a final volume of 3.0 ml. Pyrazole was added to a final concentration of 3 mM. The cells were incubated with buffer or the appropriate concentrations of cyanamide for 5 min at 37° before initiating the reaction with acetaldehyde (final concentrations of either 0.2, 0.4 or 0.67 mM). The flasks were sealed with serum caps containing center-well cups. The reaction was terminated after 5 or 10 min at 37° by the injection of trichloroacetic acid (final concentration of 4.5%) through the serum cap. Semicarbazide (0.015 M in 0.18 M phosphate buffer, pH 7.4) was injected into the center cup, and after an overnight diffusion period, the absorbance at 224 nm of aliquots from the center well was determined. Acetaldehyde uptake was determined by subtracting the remaining acetaldehyde from zero-time controls (acid added before the acetaldehyde) [14].

Ethanol oxidation was assayed in a reaction medium containing the above buffer and about 20 mg of liver cell protein in a final volume of 3.0 ml. Where indicated, pyruvate was added to a final concentration of 10 mM. The cells were incubated with buffer or cyanamide for 5 min at 37° before initiating the reaction with ethanol (final concentration of 10 mM). The reaction was terminated after 60 min by the addition of trichloroacetic acid, and the remaining ethanol was determined as previously described [16]. Zero-time controls contained acid added before the ethanol.

The accumulation of acetaldehyde during the metabolism of ethanol was assayed as described above for ethanol oxidation. Serum caps containing center-well cups filled with semicarbazide were employed to trap the acetaldehyde released during the oxidation of ethanol [14]. All values were corrected for acetaldehyde levels found in zero-time controls.

Glucose production from various precursors was assayed as previously described [2, 3], using the above buffer and 15–20 mg of liver cell protein in a final volume of 3.0 ml. Where indicated, 3 mM pyrazole was included in the reaction medium. The cells were incubated with buffer or 0.10 mM cyanamide for 5 min at 37° before the rapid addition of ethanol or acetaldehyde. The reaction was initiated by the addition of the appropriate substrate (final concentration of 10 mM). In view of the rapid oxidation of acetaldehyde by the liver cells, calculated to be 650 (0.2 mM acetaldehyde) or 870 (0.5 mM or 1 mM acetaldehyde) nmoles per 5 min per 17.5 mg protein, it was necessary to supplement the incubation medium with either 600 or 900 nmoles of acetaldehyde every 5 min to restore approximately the initial acetaldehyde concentrations of 0.2, 0.5 or 1.0 mM [2, 3]. After 30 min of incubation at 37° the reaction was terminated by rapid centrifugation and glucose was determined on 0.5 ml aliquots of the supernatant fraction as described [2, 3].

The cytosolic NADH/NAD^+ redox ratio was determined by assaying the lactate/pyruvate ratio, whereas the mitochondrial NADH/NAD^+ redox ratio was determined by assaying the β-hydroxybutyrate/acetoacetate ratio [17, 18]. The reaction medium contained the above buffer, 10–20 mg of liver cell protein, 3.3 mM lactate, 0.6 mM pyruvate, 3.3 mM acetoacetate and 0.6 mM β-hydroxybutyrate in a final volume of 3.0 ml. The cells were incubated with buffer or 0.10 mM cyanamide for 5 min before initiating the reaction with either ethanol (10 mM), acetaldehyde (0.2 or 1.0 mM) or buffer. The reactions were terminated after 30 min at 37° by the addition of ice-cold perchloric acid (final concentration of 7%). After centrifugation, neutralization with KOH and re-centrifugation, aliquots of the supernatant fraction were assayed for lactate and pyruvate [19] or for acetoacetate and β-hydroxybutyrate [20].

All incubations were carried out in duplicate or triplicate. Results refer to mean ± S.E.M. Statistical analysis was performed by Student's *t*-test. The number of experiments is indicated in the tables or table legends.

RESULTS

Effect of cyanamide on the oxidation of acetaldehyde. In the presence of pyrazole (to prevent reduction of acetaldehyde to ethanol via activity of alcohol dehydrogenase), liver cells oxidized acetaldehyde at rates of 7.5 to 10 nmoles $\cdot$ min^{-1} $\cdot$ $(mg\ protein)^{-1}$ (Table 1), which corresponds to rates of 1.6 to 2.2 μmoles $\cdot$ min^{-1} $\cdot$ $(g\ liver)^{-1}$ wet weight. Similar rates of acetaldehyde oxidation have been observed by others [21–24]. After incubating the liver cells with cyanamide for 5 min, the oxidation of acetaldehyde was strikingly reduced (Table 1). Significant inhibition was observed at 0.01 mM cyanamide and

Table 1. Effect of cyanamide on the oxidation of acetaldehyde by isolated rat liver cells*

Concn of cyanamide (mM)	Rate of acetaldehyde oxidation [nmoles · min^{-1} · (mg liver cell protein)$^{-1}$]					
	Concentration of acetaldehyde (mM)					
	0.2		0.4		0.67	
	Rate	Effect (%)	Rate	Effect (%)	Rate	Effect (%)
0	7.45 ± 0.92		9.90 ± 1.29		8.95 ± 1.73	
0.01	2.34 ± 0.37	−69†	4.45 ± 0.16	−55‡	4.44 ± 0.62	−50§
0.02	1.67 ± 0.20	−78†	3.31 ± 0.21	−67†	3.29 ± 0.47	−63§
0.05	1.35 ± 0.33	−82†	3.12 ± 0.37	−68†	2.76 ± 0.62	−69§
0.10	0.99 ± 0.32	−87†	2.11 ± 0.09	−79†	1.96 ± 1.32	−78§
0.33	0.83 ± 0.30	−89†	1.84 ± 0.22	−81†	1.71 ± 0.93	−81§

* The oxidation of acetaldehyde was assayed as indicated under Experimental Procedures in the presence of 3 mM pyrazole. Liver cells were incubated with cyanamide or buffer for 5 min before initiating the reaction with acetaldehyde. Results are from three experiments.
† $P < 0.005$.
‡ $P < 0.02$.
§ $P < 0.05$

Table 2. Effect of cyanamide on the oxidation of ethanol by isolated rat liver cells*

Concn of cyanamide (mM)	Minus pyruvate		Plus pyruvate	
	Ethanol oxidation [nmoles · min^{-1} · (mg liver cell protein)$^{-1}$]	Effect of cyanamide (%)	Ethanol oxidation [nmoles · min^{-1} · (mg liver cell protein)$^{-1}$]	Effect of cyanamide (%)
0	5.72 ± 0.83		9.50 ± 0.83	
0.02	4.33 ± 0.47	−24	5.42 ± 0.18	−43†
0.05	4.05 ± 0.67	−29‡	5.08 ± 0.37	−46†
0.10	3.65 ± 0.27	−36‡	4.38 ± 0.37	−54†
0.33	3.63 ± 0.32	−36‡	4.07 ± 0.15	−57†

* The oxidation of ethanol was assayed in the absence or presence of 10 mM pyruvate. Liver cells were incubated for 5 min with the listed concentrations of cyanamide (or buffer) before initiating the reaction with ethanol (final concentration of 10 mM). Results are from three experiments without pyruvate and from five experiments with pyruvate.
† $P < 0.001$.
‡ $P < 0.05$.

Table 3. Effect of cyanamide on the accumulation of acetaldehyde during the oxidation of ethanol*

Time (min)	Acetaldehyde accumulation (nmoles/mg liver cell protein)			
	Minus pyruvate		Plus pyruvate	
	Control	Plus cyanamide	Control	Plus cyanamide
15	3 ± 0.4	40 ± 7†	40 ± 6	111 ± 14†
30	4 ± 0.3	45 ± 8†	38 ± 6	138 ± 7†
60	10 ± 0.2	60 ± 14‡	22 ± 6	133 ± 6†
90	17 ± 0.4	65 ± 12‡	16 ± 4	128 ± 7†
120	21 ± 0.3	63 ± 12§	21 ± 2	130 ± 11†
150	26 ± 0.3	63 ± 11§	26 ± 5	122 ± 15†

* The accumulation of acetaldehyde was measured as described under Experimental Procedures. Where indicated, pyruvate was present at a final concentration of 10 mM. Liver cells were incubated with 0.1 mM cyanamide or buffer for 5 min before initiating the reaction with ethanol (final concentration of 10 mM). Results are from four experiments. Statistical analysis refers to the effects of cyanamide.
† $P < 0.002$.
‡ $P < 0.01$.
§ $P < 0.02$.

maximum inhibition was found at 0.10 mM. The oxidation of all three concentrations of acetaldehyde tested was inhibited by cyanamide, although cyanamide appeared to be especially inhibitory towards the oxidation of the lowest concentration of acetaldehyde (0.2 mM) studied (Table 1).

In the absence of an incubation period i.e. when the reaction medium contained acetaldehyde and cyanamide and the reaction was initiated by the addition of the cells, cyanamide was without any effect on the oxidation of 0.2 to 0.67 mM acetaldehyde. There was progressive inhibition of acetaldehyde oxidation by cyanamide as the incubation period of the cells with cyanamide increased. In a representative experiment, 0.10 mM cyanamide inhibited the oxidation of 0.2 mM acetaldehyde by 55, 65 and 79 per cent after 1.5, 3 or 5 min of incubation; the oxidation of 0.40 mM acetaldehyde was inhibited 47, 64 and 73 per cent, and the oxidation of 0.67 mM acetaldehyde was inhibited 43, 52 and 60 per cent by 0.10 mM cyanamide after 1.5, 3 or 5 min of incubation respectively.

Effect of cyanamide on the oxidation of ethanol. Since the oxidation of ethanol may be influenced by the rapid removal of acetaldehyde, the effect of cyanamide on ethanol oxidation was determined. Liver cells oxidized ethanol at a rate of 5.7 nmoles · min^{-1} · (mg liver cell protein)$^{-1}$, corresponding to a rate of about 1.25 μmoles · min^{-1} · (g liver)$^{-1}$, wet weight (Table 2). Concentrations of cyanamide that effectively inhibited the oxidation of acetaldehyde also produced some inhibition of ethanol oxidation (Table 2).

The rate of ethanol oxidation was increased 67 per cent by pyruvate, which acts as a cytoplasmic NADH-oxidizing agent (Table 2). In the presence of pyruvate the rate of ethanol oxidation corresponded to a value of about 2 μmoles · min^{-1} · (g liver)$^{-1}$, wet weight. Cyanamide was especially effective in inhibiting the accelerated rate of ethanol oxidation found in the presence of pyruvate (Table 2).

Effect of cyanamide on the accumulation of acetaldehyde during the oxidation of ethanol. Although acetaldehyde is metabolized rapidly by the liver, blood levels of acetaldehyde have been reported to increase during the oxidation of ethanol [25]. During the oxidation of ethanol by hepatocytes, acetaldehyde was found to accumulate in a time-dependent manner [14]. This accumulation is blocked by pyrazole, indicating that metabolism of ethanol is required and that there is no spontaneous formation of acetaldehyde from ethanol. Pyruvate, by increasing the rate of ethanol metabolism (acetaldehyde generation), increased the accumulation of acetaldehyde [14]. Table 3 summarizes similar experiments carried out in the absence and presence of cyanamide. In the absence of cyanamide (and pyruvate) low amounts of acetaldehyde accumulated in the reaction medium (about 2–3 per cent of the acetaldehyde generated from ethanol oxidation). In the presence of 0.10 mM cyanamide, the accumulation of acetaldehyde during the oxidation of ethanol increased 10-fold after a 15–30 min incubation period (Table 3). Significant increases were also found at the longer incubation periods.

In the presence of pyruvate alone, the accumulation of acetaldehyde during the oxidation of ethanol increased 10-fold after a 15–30 min incubation period (Table 3). The increase in acetaldehyde accumulation in the presence of pyruvate was not sustained at longer incubation times, probably due to the rapid utilization of pyruvate by the liver cells. The higher levels of acetaldehyde found after 15 or 30 min of incubation in the presence of pyruvate were further increased in the presence of 0.10 mM cyanamide (Table 3). After a 30-min period of ethanol metabolism in the presence of pyruvate, the levels of acetaldehyde found to accumulate in the incubation medium were about 150 μM in the absence of cyanamide and 500 μM in the presence of cyanamide (data calculated from Table 3). Cyanamide, therefore increased the accumulation of acetaldehyde despite decreasing the rate of acetaldehyde generation (ethanol oxidation). This probably reflects the ability of cyanamide to inhibit the oxidation of acetaldehyde to a considerably greater extent (>80 per cent) than it inhibited the oxidation of ethanol (<40 per cent).

Cyanamide, in concentrations up to 0.50 mM, had no effect on the rate of oxygen consumption by the liver cells [control rates of about 20 natom · min^{-1} · (mg liver cell protein)$^{-1}$.

Effect of cyanamide on the changes of the cellular NADH/NAD$^+$ redox ratio produced by ethanol and acetaldehyde. Ethanol and acetaldehyde are metabolized by NAD$^+$-dependent dehydrogenases. Many of the effects of ethanol are due to changes in the cellular NADH/NAD$^+$ redox ratio produced by the metabolism of ethanol [26–28]. In view of the localization of lactate dehydrogenase in the cytosol, changes in the cytosolic NADH/NAD$^+$ ratio may be expressed as changes in the lactate/pyruvate ratio. Since β-hydroxybutyrate dehydrogenase is exclusively a mitochondrial enzyme, changes in the mitochondrial NADH/NAD$^+$ ratio may be expressed as changes in the β-hydroxybutyrate/acetoacetate ratio [17, 18]. Ethanol increased the lactate/pyruvate ratio from a value of 11 to a value of 43 and increased the β-hydroxybutyrate/acetoacetate ratio from 0.75 to 1.85 (Table 4). These changes were completely prevented by 3 mM pyrazole or by 0.01 mM methylene blue, indicating that the changes reflect the metabolism of ethanol by alcohol dehydrogenase. Cyanamide (0.1 mM) by itself had no effect on the lactate/pyruvate or β-hydroxybutyrate/acetoacetate ratios. In the presence of cyanamide, ethanol increased the lactate/pyruvate ratio only 2-fold, as compared to the 4-fold increase found in the absence of cyanamide (Table 4). Cyanamide completely prevented the increase in the β-hydroxybutyrate/acetoacetate ratio caused by the metabolism of ethanol (Table 4).

Most of the acetaldehyde produced during the metabolism of ethanol is oxidized in the mitochondria by a low K_m aldehyde dehydrogenase [12, 21–24, 29]. When acetaldehyde was added to a suspension of liver cells, the β-hydroxybutyrate/acetoacetate ratio was elevated (Table 5). The increase was less than that found with ethanol (compare Tables 4 and 5) and may have reflected the difficulty in maintaining effective levels of acetaldehyde because of

Table 4. Effect of ethanol on the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios in the absence and presence of cyanamide*

Additions	Lactate/ Pyruvate	Effect of ethanol (%)	β-Hydroxybutyrate/ Acetoacetate	Effect of ethanol (%)
None	11 ± 0.8		0.75 ± 0.06	
Ethanol (10.0 mM)	43 ± 9.0	+290†	1.85 ± 0.18	+147‡
Cyanamide (0.1 mM)	11 ± 1.0		0.92 ± 0.06	
Ethanol (10.0 mM) + cyanamide (0.1 mM)	22 ± 3.0	+100†	1.00 ± 0.09	+9

* The lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios were determined as described under Experimental Procedures. Liver cells were incubated for 5 min with cyanamide or buffer before initiating the reaction by the addition of buffer or ethanol. Results are from six to seven experiments.
† $P < 0.005$.
‡ $P < 0.001$.

Table 5. Effect of acetaldehyde on the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios in the absence and presence of cyanamide*

Additions	Lactate/ Pyruvate	Effect of acetaldehyde (%)	β-Hydroxybutyrate/ Acetoacetate	Effect of acetaldehyde (%)
None	15 ± 2		0.83 ± 0.12	
Acetaldehyde (0.2 mM)	19 ± 3	+27	1.07 ± 0.02	+29
Acetaldehyde (1.0 mM)	21 ± 5	+40	1.36 ± 0.11	+64†
Cyanamide (0.1 mM)	13 ± 2		0.86 ± 0.07	
Acetaldehyde (0.2 mM) + cyanamide (0.1 mM)	17 ± 2	+30	0.84 ± 0.09	−2
Acetaldehyde (1.0 mM) + cyanamide (0.1 mM)	18 ± 3	+38	0.96 ± 0.05	+12

* The lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios were determined as described under Experimental Procedures. Liver cells were incubated for 5 min with cyanamide or buffer before initiating the reaction by the addition of either buffer or acetaldehyde. Results are from three (lactate/pyruvate) or four (β-hydroxybutyrate/acetoacetate) experiments. Pyrazole was present at a final concentration of 3 mM.
† $P < 0.02$.

Table 6. Effect of cyanamide on the stimulation of glucose production from xylitol and sorbitol by acetaldehyde in the absence of pyrazole*

		Glucose production			
		Minus cyanamide		Plus cyanamide	
Substrate	Concn of acetaldehyde (mM)	Rate [μmoles · (30 min)$^{-1}$ · (mg liver cell protein)$^{-1}$]	Effect of acetaldehyde (%)	Rate [μmoles · (30 min)$^{-1}$ · (mg liver cell protein)$^{-1}$]	Effect of acetaldehyde (%)
Xylitol	0	0.147 ± 0.018		0.146 ± 0.017	
	0.2	0.214 ± 0.025	+46†	0.259 ± 0.033	+77†
	0.5	0.237 ± 0.028	+61†	0.276 ± 0.040	+89†
	1.0	0.254 ± 0.029	+73†	0.275 ± 0.043	+88†
Sorbitol	0	0.189 ± 0.014		0.146 ± 0.011	
	0.2	0.230 ± 0.020	+22	0.227 ± 0.014	+55‡
	0.5	0.243 ± 0.018	+29†	0.227 ± 0.015	+55‡
	1.0	0.248 ± 0.023	+31†	0.221 ± 0.014	+51‡

* Glucose production from 10 mM xylitol or 10 mM sorbitol was assayed as described under Experimental Procedures. Where indicated, cyanamide was present at a final concentration of 0.1 mM. After a 5-min incubation of liver cells with buffer or with cyanamide, the reaction was initiated by the rapid addition of substrate and the indicated initial concentrations of acetaldehyde. Results are from four experiments. Pyrazole was not present in these experiments.
† $P < 0.05$.
‡ $P < 0.005$.

the rapid metabolism of this compound. Cyanamide completely prevented the elevation of the β-hydroxybutyrate/acetoacetate ratio produced by acetaldehyde (Table 5).

Acetaldehyde produced a slight increase in the lactate/pyruvate ratio, but it was not statistically significant (Table 5). The increase may have reflected some metabolism of acetaldehyde by the cytosolic aldehyde dehydrogenase. Cyanamide did not prevent the slight increase in the lactate/pyruvate ratio (Table 5), probably because the cytosolic aldehyde dehydrogenase has been shown to be less sensitive to cyanamide than the mitochondrial enzyme [12].

Effect of cyanamide and acetaldehyde (in the absence of pyrazole) on glucose production from xylitol and sorbitol. It was observed previously that acetaldehyde, in the absence of pyrazole, stimulates glucose production from xylitol and sorbitol [2]. Since gluconeogenesis from xylitol and sorbitol is regulated by the availability of NAD^+ [30–32], the stimulation by acetaldehyde in the absence of pyrazole was postulated to be a reflection of the reduction of acetaldehyde to ethanol via alcohol dehydrogenase (acetaldehyde + NADH $\rightleftharpoons$ ethanol + NAD^+) with the subsequent regeneration of NAD^+ [2]: experiments with cyanamide were carried out to test this. If the increase in glucose production from xylitol and sorbitol produced by acetaldehyde in the absence of pyrazole is mediated by alcohol dehydrogenase, and not aldehyde dehydrogenase, cyanamide should not block the stimulation by acetaldehyde. Table 6 shows that acetaldehyde produced an increase in the rate of glucose production from xylitol and sorbitol in the absence, as well as in the presence, of 0.10 mM cyanamide. In fact, the stimulatory action of acetaldehyde appeared to be greater in the presence, than in the absence, of cyanamide (Table 6). This concentration of cyanamide inhibited acetaldehyde oxidation by about 80 per cent (Table 1). These results indicate that the stimulatory effect by acetaldehyde in the absence of pyrazole is mediated via alcohol dehydrogenase and not aldehyde dehydrogenase. Cyanamide, by blocking acetaldehyde oxidation via aldehyde dehydrogenase, may divert utilization of acetaldehyde to the alcohol dehydrogenase pathway with a subsequent increase in the regeneration of NAD^+. This would result in a greater stimulation by acetaldehyde than occurs in the absence of cyanamide.

Effect of cyanamide and acetaldehyde on glucose production in the presence of pyrazole. In the presence of pyrazole, acetaldehyde inhibited glucose production from xylitol, sorbitol and glycerol (Table 7), while glucose production from pyruvate was stimulated (Table 7). Similar results have been reported previously [2, 3]. The inhibitory actions of acetaldehyde may reflect the oxidation of acetaldehyde with subsequent changes in the cellular NADH/NAD^+ ratio or a direct effect of acetaldehyde. For example, the oxidation of pyruvate, as

Table 7. Effect of acetaldehyde, in the presence of pyrazole, on glucose production from xylitol, sorbitol, glycerol and pyruvate, in the absence and presence of cyanamide*

		Glucose production			
		Minus cyanamide		Plus cyanamide	
Substrate	Concn of acetaldehyde (mM)	Rate [μmoles · (30 min)$^{-1}$ · (mg liver cell protein^{-1}]	Effect of acetaldehyde (%)	Rate [μmoles · (30 min)$^{-1}$ · (mg liver cell protein)$^{-1}$]	Effect of acetaldehyde (%)
Xylitol (4)	0	0.121 ± 0.017		0.115 ± 0.015	
	0.2	0.081 ± 0.019	−33	0.110 ± 0.013	−4
	0.5	0.064 ± 0.015	−50†	0.103 ± 0.012	−10
	1.0	0.053 ± 0.012	−56‡	0.102 ± 0.012	−11
Sorbitol (4)	0	0.151 ± 0.008		0.127 ± 0.010	
	0.2	0.098 ± 0.015	−35‡	0.120 ± 0.009	−6
	0.5	0.069 ± 0.014	−54§	0.113 ± 0.008	−11
	1.0	0.057 ± 0.013	−62§	0.107 ± 0.006	−16
Glycerol (7)	0	0.071 ± 0.005		0.069 ± 0.003	
	0.2	0.057 ± 0.004	−20†	0.066 ± 0.006	−4
	0.5	0.047 ± 0.005	−33‖	0.062 ± 0.006	−10
	1.0	0.043 ± 0.005	−39§	0.059 ± 0.006	−15
Pyruvate (5)	0	0.070 ± 0.003		0.074 ± 0.003	
	0.2	0.080 ± 0.003	+14†	0.068 ± 0.003	−8
	0.5	0.087 ± 0.002	+24§	0.066 ± 0.003	−11
	1.0	0.095 ± 0.002	+36§	0.071 ± 0.002	−4

* Glucose production from 10 mM substrate was assayed as described under Experimental Procedures in the presence of 3 mM pyrazole. Where indicated, cyanamide was present at a final concentration of 0.1 mM. After a 5-min incubation of liver cells with buffer or with cyanamide, the reaction was initiated by the rapid addition of substrate and the indicated initial concentrations of acetaldehyde.

† $P < 0.05$.

‡ $P < 0.02$.

§ $P < 0.001$.

‖ $P < 0.005$.

Table 8. Effect of ethanol on glucose production from xylitol, sorbitol, glycerol and pyruvate in the absence and presence of cyanamide*

Substrate	Concn of ethanol (mM)	Glucose production: Minus cyanamide — Rate [μmoles·(30 min)$^{-1}$·(mg liver cell protein)$^{-1}$]	Minus cyanamide — Effect of ethanol (%)	Plus cyanamide — Rate [μmoles·(30 min)$^{-1}$·(mg liver cell protein)$^{-1}$]	Plus cyanamide — Effect of ethanol (%)
Xylitol (4)	0	0.118 ± 0.021		0.117 ± 0.021	
	10	0.070 ± 0.011	−41†	0.105 ± 0.015	−10
	50	0.072 ± 0.012	−39†	0.093 ± 0.015	−20
Sorbitol (3)	0	0.166 ± 0.015		0.140 ± 0.014	
	10	0.076 ± 0.006	−54‡	0.121 ± 0.012	−14
	50	0.079 ± 0.006	−52‡	0.103 ± 0.009	−26§
Glycerol (6)	0	0.072 ± 0.007		0.076 ± 0.008	
	10	0.039 ± 0.003	−46‖	0.055 ± 0.008	−24§
	50	0.044 ± 0.003	−39‖	0.051 ± 0.006	−29§
Pyruvate (5)	0	0.087 ± 0.005		0.084 ± 0.005	
	10	0.129 ± 0.005	+48‡	0.098 ± 0.006	+17
	50	0.116 ± 0.006	+33‖	0.090 ± 0.008	+7

* Glucose production from 10 mM substrate was assayed as described under Experimental Procedures. Pyrazole was not present in these experiments. Where indicated, the final concentration of cyanamide was 0.10 mM. The liver cells were incubated with cyanamide or buffer for 5 min; ethanol was then added, followed by the appropriate substrate to initiate the reaction.

† $P < 0.05$.

‡ $P < 0.001$.

§ $0.10 > P > 0.05$.

‖ $P < 0.005$.

compared to other substrates, is especially sensitive to acetaldehyde [33]. Experiments were carried out with cyanamide to determine the mechanism of action of acetaldehyde. Cyanamide, at a concentration of 0.10 mM, nearly completely prevented the inhibitory effects of acetaldehyde on glucose production from xylitol, sorbitol and glycerol, as well as the stimulatory effect of acetaldehyde on glucose production from pyruvate (Table 7). Cyanamide by itself had no significant effect on the control rate of glucose production from any of these substrates.

Effect of cyanamide and ethanol on gluconeogenesis. Ethanol produced an increase in glucose production from pyruvate and inhibited glucose production from glycerol, xylitol and sorbitol (Table 8) [2, 3]. The effects of ethanol on glucose production from these substrates were prevented by pyrazole, indicating that metabolism of ethanol was required. The effects of acetaldehyde on glucose production from these substrates were similar to those produced by ethanol (Tables 7 and 8). Since cyanamide prevented the actions of acetaldehyde on glucose production, the effect of cyanamide on the actions of ethanol was evaluated. It was anticipated that, if acetaldehyde plays a role in the actions of ethanol, cyanamide would prevent these actions of ethanol. As shown in Table 8, cyanamide completely abolished the stimulatory effect of ethanol on glucose production from pyruvate, and it largely prevented the inhibitory effects of ethanol on glucose production from xylitol, sorbitol and glycerol.

DISCUSSION

In vivo administration of cyanamide results in an inhibition of the low K_m mitochondrial aldehyde dehydrogenase [11, 12] and in an accumulation of acetaldehyde during the metabolism of ethanol [11, 34]. A cyanamide derivative, present in commercial rodent laboratory diets, was shown to be responsible for high acetaldehyde blood levels during the oxidation of ethanol *in vivo* [35–37]. The mechanism of inhibition of aldehyde dehydrogenase by cyanamide is not known [38]. Cyanamide, *in vitro*, does inhibit the low K_m rat liver mitochondrial aldehyde dehydrogenase [12, 36]. Cyanamide, *in vitro*, is an effective inhibitor of mitochondrial aldehyde dehydrogenase activity, and of acetaldehyde oxidation, by intact rat liver mitochondria [15]. Cyanamide did not affect the oxidation of other mitochondrial substrates, indicating that cyanamide was not acting as an inhibitor of the respiratory chain or in some non-specific manner [15].

Our interest in characterizing the effectiveness of cyanamide as an *in vitro* inhibitor of acetaldehyde oxidation reflects the need to determine whether the effects of acetaldehyde on certain reactions are due to a direct toxic action of acetaldehyde, or require the metabolism of acetaldehyde. For example, in the previous mitochondrial studies with cyanamide [15], the inhibition of state 3 pyruvate oxidation by acetaldehyde was found not to be prevented by cyanamide. Since cyanamide blocked acetaldehyde oxidation more than 90 per cent, these results suggested a direct toxic effect of acetaldehyde on the pyruvate dehydrogenase complex [15]. Previous studies showed that acetaldehyde affected gluconeogenesis from various precursors [2, 3]. To determine whether these effects require the metabolism of acetaldehyde or reflect direct actions of acetaldehyde, an effective, specific inhibitor of acetaldehyde oxidation by the

liver cells was required. Results in this manuscript demonstrate that cyanamide can be used as an effective inhibitor of acetaldehyde oxidation by liver cells (Table 1). The requirement of a brief incubation period of the cells with cyanamide for effective inhibition has been observed before with isolated mitochondria [15]. Since acetaldehyde protects aldehyde dehydrogenase against inhibition by cyanamide [12], the ineffectiveness of cyanamide as an inhibitor without an incubation period may reflect this protective action by acetaldehyde. A brief period of incubation may also be necessary to allow penetration of cyanamide into the cells or for interaction with the enzyme. Inhibition of acetaldehyde oxidation by cyanamide appeared to be specific, since hepatic oxygen consumption and control rates of gluconeogenesis from various precursors were not affected by the inhibitor. The somewhat greater effectiveness of cyanamide in inhibiting the oxidation of low rather than high, concentrations of acetaldehyde (e.g. compare effects on 0.2 and 0.67 mM acetaldehyde oxidation, Table 1) may reflect the greater sensitivity to inhibition by cyanamide of the low K_m mitochondrial aldehyde dehydrogenase in comparison with the high K_m cytosolic aldehyde dehydrogenase [12].

Associated with the inhibition of acetaldehyde oxidation by cyanamide was an inhibition of ethanol oxidation. The inability to effectively remove acetaldehyde in the presence of cyanamide resulted in a decrease in the rate of ethanol oxidation, probably due to the kinetic characteristics of the alcohol dehydrogenase reaction which favors reduction of acetaldehyde back to ethanol. Cyanamide has also been shown to inhibit ethanol oxidation *in vivo* [12, 36].

The marked increase in the accumulation of acetaldehyde, during the oxidation of ethanol in the presence of cyanamide, was due to the inhibition of acetaldehyde oxidation by cyanamide. This was expected since cyanamide inhibited the oxidation of low concentrations of acetaldehyde, i.e. concentrations which arose during the metabolism of ethanol. The increased accumulation of acetaldehyde was due to the fact that acetaldehyde oxidation was inhibited by cyanamide to a greater extent than was ethanol oxidation. This is similar to results previously found with pargyline [14]. It is apparent that the liver cells efficiently removed most of the acetaldehyde that was generated by the oxidation of ethanol, since the amount of acetaldehyde that accumulated was only about 3 per cent of the amount that was generated by the oxidation of ethanol. This was due to the higher rates of acetaldehyde oxidation [about 7.5 to 10 nmoles·min^{-1}·(mg liver cell protein)$^{-1}$] compared to ethanol oxidation [about 5.7 nmoles·min^{-1}·(mg liver cell protein)$^{-1}$]. When this 2-fold excess of the acetaldehyde oxidation rate over the ethanol oxidation rate was lost, e.g. by the addition of cyanamide to inhibit acetaldehyde oxidation or by the addition of pyruvate to increase ethanol oxidation, the accumulation of acetaldehyde was increased markedly (Table 3).

Of interest is the observation that cyanamide completely prevented the ethanol-induced increase in the β-hydroxybutyrate/acetoacetate ratio, but only partially prevented the ethanol-induced increase in the lactate/pyruvate ratio. The latter probably was a reflection of the decrease in the rate of ethanol oxidation produced by cyanamide. The complete prevention by cyanamide of the reduction of the mitochondrial redox state during ethanol metabolism suggests that the NAD$^+$-dependent oxidation of the acetaldehyde, which arose during the oxidation of ethanol, was an important contributor to the change of the mitochondrial redox state during ethanol metabolism. These results confirm the recently reported *in vivo* experiments which indicated that cyanamide altered the effects of ethanol on the hepatic mitochondrial redox state [39].

Ethanol is known to inhibit gluconeogenesis from various precursors, with reduction of the hepatic NAD$^+$/NADH redox ratio playing the major role in these effects of ethanol [28]. It is not known what role, if any, acetaldehyde may play in the actions of ethanol on gluconeogenesis. Both ethanol and acetaldehyde stimulated glucose production from pyruvate, inhibited glucose production from glycerol, xylitol, and sorbitol, and had no effect with fructose [2, 3] (Tables 7 and 8). The similarities in the actions of ethanol and acetaldehyde on glucose production from these substrates suggested the possibility that acetaldehyde may contribute to the effects of ethanol on gluconeogenesis from these substrates [2, 3]. The mechanism of action of acetaldehyde on glucose production was not determined in those experiments. Since the oxidation of acetaldehyde also resulted in changes of the hepatic redox state, it is possible that acetaldehyde or its metabolism contributed to the effects of ethanol on gluconeogenesis. Alternatively, acetaldehyde may have directly affected the gluconeogenic pathway, e.g. high levels of acetaldehyde inhibited the activity of phosphoenolpyruvate carboxykinase in rat liver cytosol fractions [40]. Cyanamide was used to determine whether the effects of acetaldehyde on gluconeogenesis were direct actions, or were metabolic effects related to the oxidation of acetaldehyde.

Results in this article demonstrate that all the effects of acetaldehyde (in the presence of pyrazole) on glucose production can be prevented by concentrations of cyanamide that nearly totally inhibit the oxidation of acetaldehyde. These results suggest that the mechanism responsible for the inhibitory effects of acetaldehyde involves NAD$^+$-dependent oxidation of acetaldehyde via aldehyde dehydrogenase with the subsequent generation of NADH. Gluconeogenesis from glycerol, xylitol or sorbitol requires NAD$^+$-dependent oxidation to dihydroxyacetone, D-xylulose or fructose respectively. This step could be inhibited by acetaldehyde (plus pyrazole), especially since gluconeogenesis from these substrates has been shown to be regulated by the reoxidation of NADH generated from the primary dehydrogenase reaction [30–32]. Since low concentrations of acetaldehyde are oxidized in the mitochondria by a high affinity aldehyde dehydrogenase [21–24], the inhibitory effects of 0.2 mM acetaldehyde may have reflected mitochondrial oxidation of acetaldehyde with subsequent generation of reducing equivalents interfering with the transfer and reoxidation of reducing equivalents produced in the cytosol by the

α-glycerophosphate, xylitol or sorbitol dehydrogenase reactions. The stimulation of glucose production from pyruvate by acetaldehyde probably reflected the provision of NADH, generated by the oxidation of acetaldehyde, for the triose phosphate dehydrogenase reaction. The possibility that acetaldehyde inhibition of pyruvate dehydrogenase diverts utilization of pyruvate from an oxidative pathway to a gluconeogenic pathway [3] can now be ruled out. Although acetaldehyde inhibits pyruvate oxidation [33], this inhibition was not prevented by cyanamide [15], indicating that the inhibition was a direct effect of acetaldehyde. The ability of cyanamide to prevent the stimulation by acetaldehyde of glucose production from pyruvate would therefore be consistent with a metabolic effect involving NADH rather than a direct effect on pyruvate dehydrogenase. Acetate, the end product of acetaldehyde oxidation, had no effect on glucose production from these substrates [2, 3], further indicating that changes in the cellular $NADH/NAD^+$ ratio are responsible for these metabolic effects of acetaldehyde on gluconeogenesis.

In addition to providing an explanation for the mechanism of action of acetaldehyde on gluconeogenesis, cyanamide was utilized to help evaluate the role of acetaldehyde in the actions of ethanol on gluconeogenesis. If acetaldehyde plays a role in the effects of ethanol, cyanamide, by preventing the effects of acetaldehyde, should also prevent the effects of ethanol. At first glance, this suggestion appears to have been fulfilled as cyanamide indeed prevented the stimulation of glucose production from pyruvate by ethanol, and cyanamide diminished the extent of inhibition of glucose production from glycerol, xylitol and sorbitol by ethanol (Table 8). Cyanamide, however, also lowered the rate of oxidation of ethanol and partially reversed the ethanol-induced increase in the lactate/pyruvate ratio (Table 2 and 4). Hence, the ability of cyanamide to prevent or attenuate the effects of ethanol on gluconeogenesis may involve effects by cyanamide on the metabolism of ethanol, in addition to effects on the metabolism of acetaldehyde. At present, we cannot distinguish between these two possibilities, although they are clearly related, i.e. cyanamide inhibition of acetaldehyde oxidation also resulted in inhibition of ethanol oxidation. These results suggest that caution should be used when interpreting experiments involving the use of inhibitors of acetaldehyde oxidation, since effects on ethanol oxidation and on the ethanol-induced changes of the cellular redox states may also be altered by the inhibitor.

Other studies indicated that the inhibition of pyruvate oxidation by acetaldehyde was not prevented by cyanamide [15]. The current study indicates that the effects of acetaldehyde on gluconeogenesis are prevented by cyanamide (Table 7). These results demonstrate that acetaldehyde can have direct as well as indirect effects on cellular metabolism, depending on the reaction being investigated.

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