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INHIBITORY EFFECT OF ACETALDEHYDE ON THE OXIDATION OF ETHANOL BY A HIGH-SPEED SUPERNATANT FRACTION OF RAT LIVER

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INTRODUCTION

In a recent review, Higgins [1] considered the possible role of acetaldehyde as a regulator of ethanol metabolism *in vivo*. He pointed to two ways in which acetaldehyde might influence the rate of oxidation of ethanol by alcohol dehydrogenase. First, because it is a product of the reaction, acetaldehyde contributes to the equilibrium factor. Second, studies with horse liver alcohol dehydrogenase indicate that acetaldehyde acts as a 'non-competitive' or 'mixed' inhibitor of the enzyme [2, 3]. However, present knowledge of the inhibitory effects of acetaldehyde is based entirely on experiments conducted under non-physiological conditions and Higgins [1] emphasized that, because a number of physico-chemical parameters strongly influence the reaction catalysed by alcohol dehydrogenase [4], there was a need to obtain data on the effects of acetaldehyde under near physiological conditions of temperature, pH and ionic strength. The present study was therefore aimed in that direction.

MATERIALS AND METHODS

Preparation of liver extract. Livers from adult female Wistar rats were homogenized in 1.5 vol of 0.1 m KCl containing 10 mM potassium phosphate buffer, pH 7.2. The homogenate was centrifuged at 30,000 *g* for 20 min and the resulting supernatant fluid was centrifuged at 50,000 *g* for 1 hr. The supernatant from this spin was dialysed for 16 hr against 200 vol of fresh KCl/potassium phosphate medium and was then recentrifuged at 50,000 *g* for 1 hr to remove remaining traces of particulate material. The clear red supernatant was retained on ice and was used for assay only on the day of its preparation. All preparative procedures were carried out at 0–4°.

Incubations. The oxidation of ethanol by the liver extract was followed spectrophotometrically at 340 nm using a Varian DM90 Recording Spectrophotometer operating in the split-beam mode and with the cuvette holders thermostated to 37°. The initial contents of the sample cuvette were 0.1 M KCl containing 10 mM potassium phosphate buffer, pH 7.2, liver extract (0.4–0.7 mg protein), NAD^+ (1 or 4 mM) and acetaldehyde (0–0.62 mM). The reference cuvette contained only KCl/potassium phosphate buffer and liver extract. Under these conditions no increase in absorbance was detected, indicating a negligible rate of reduction of NAD^+ by endogenous or added substances. Reduction of NAD^+ was observed when ethanol (34 or 50 mM) was added to the sample cuvette and the rate of increase in absorbance was followed by setting the recorder to give a full scale deflection of 0.1 Absorbance units.

In some experiments the KCl/potassium phosphate medium was replaced with a medium comprising 100 mM potassium phosphate, pH 7.2, 10 mM NaCl and 5 mM $MgCl_2$. It was thought that such a medium might resemble more closely the intracellular environment in which alcohol dehydrogenase normally exists, and that its use might also avoid possible inhibition of alcohol dehydrogenase by high concentrations of chloride ion [5].

Experiments were also performed at 23° instead of 37° to determine whether the effects of acetaldehyde on ethanol oxidation were temperature dependent.

Determination of acetaldehyde and NAD^+. The concentration of NAD^+ in a stock solution was determined using the cyanide addition method [6]. The concentration of acetaldehyde in a stock solution was measured spectrophotometrically by following the disappearance of NADH in the presence of purified yeast alcohol dehydrogenase [7].

Determination of the equilibrium constant for the alcohol dehydrogenase reaction. The equilibrium constant of the reaction under the experimental conditions employed in this study was determined using purified yeast alcohol dehydrogenase with varying initial concentrations of NAD^+ (0.4–1.6 mM) and ethanol (34–136 mM). The pH of each system was accurately measured and the increase in absorbance at 340 nm was followed until equilibrium was established. In calculating the equilibrium constant it was assumed that the final concentration of acetaldehyde was equal to that of NADH. Although the boiling point of acetaldehyde (21°) is well below the experimental temperature, in no case was there any detectable upward drift in absorbance during a 10–15 min period following the attainment of equilibrium. This suggested that evaporative loss of acetaldehyde from the cuvette was negligible, and was confirmed by other experiments in which a known amount of acetaldehyde was preincubated with alcohol dehydrogenase for 15 min at 37° prior to its reduction by NADH.

RESULTS AND DISCUSSION

Higgins [1] pointed out that, in general, the rate of an enzymatic reaction can be expressed as

$$v = \phi V_m Eq,$$

where v is the actual velocity, V_m is the maximum velocity, ϕ is the rate-factor function and Eq is the equilibrium factor.

The rate-factor function, ϕ, depends on the concentrations of all modifiers except the enzyme concentration but, when the concentration of only one modifier is varied, ϕ indicates the effect of that modifier.

The equilibrium factor, Eq, is defined by

$$Eq = 1 - Q^1 \quad \text{where } Q^1 = Q/Keq.$$

Q is the reaction quotient (the multiplicative product of all reaction product concentrations divided by the multiplicative product of all the substrate concentrations) and at equilibrium its value is equal to that of the equilibrium constant, Keq. Hence, at equilibrium Eq is zero whereas for reactions operating far from equilibrium Q, and Q^1, are essentially zero and Eq is unity.

The results given below are for two different experimental approaches, one in which the equilibrium factor was always unity and so had no restraining effect on the rate of reaction, and one in which the equilibrium factor varied between 0.68 and unity and was therefore assumed to influence the reaction rate accordingly.

In the first series of experiments the effect of acetaldehyde on ethanol oxidation was measured in the presence of relatively high concentrations of NAD^+ (4 mM) and ethanol (50 mM). Only the initial rate of NADH formation was measured in each experiment, so the equilibrium factor had a value of 1 in each case. The results shown in curve (a) of Fig. 1 demonstrate that acetaldehyde strongly inhibited the rate of ethanol oxidation, as little as 0.12 mM acetaldehyde causing 20 per cent inhibition and 0.6 mM causing over 50 per cent inhibition. This effect could be physiologically important as the intrahepatic concentration of acetaldehyde following the administration of ethanol to experimental animals usually lies in the 0.1–0.4 mM range [8–12], and similar levels might also occur in human subjects [13–15] when it is remembered that the concentration of acetaldehyde in the liver may be several times higher than that in the peripheral venous blood [10].

In the second series of experiments in which the effect of acetaldehyde on ethanol oxidation was measured, lower concentrations of NAD^+ and ethanol were used in order to approach more closely the conditions that are found *in vivo* after a heavy ethanol load. The chosen ethanol concentration (34 mM) is one easily reached or exceeded in humans under experimental and non-experimental conditions [13, 15, 16] while the chosen NAD^+ concentration (1 mM) is near that found in the *in vivo* liver [17–20]. In contrast to the first series of experiments, initial rates of NADH formation were not measured. Instead rates were determined when the NADH concentration had reached 0.005 mM (ie. 0.03 Absorbance); at this point the NAD/NADH ratio was 200, the value calculated by Christensen and Higgins [20] for the hepatic redox ratio during ethanol metabolism *in vivo*. Thus, NAD^+, NADH and ethanol were at the same fixed concentrations in all experiments and only the concentration of acetaldehyde varied. The equilibrium factor was calculated for each system using a value for the equilibrium constant which was determined experimentally as

$$Keq = \frac{[\text{acetaldehyde}][\text{NADH}][H^+]}{[\text{ethanol}][\text{NAD}]}$$
$$= 1.78 \pm 0.04 \times 10^{-11}\text{M}$$
(mean ± S.E.M. for 5 determinations).

This value is consistent with those reported by other workers or obtained by extrapolation from their data (range $1.66–1.94 \times 10^{-11}$M) [4, 20–22]. Hence, at pH 7.20, the experimental pH, equilibrium was reached when,

$$\frac{[\text{Acetaldehyde}][\text{NADH}]}{[\text{ethanol}][\text{NAD}]} = 2.82 \times 10^{-4}.$$

Figure 1 (b) shows the effect of acetaldehyde on the reaction rate under the conditions outlined above. The degree of inhibition was consistently greater than that seen in the first series of experiments [Fig. 1(a)] but, as shown in Table 1, this was fully explained by the influence of the equilibrium factor.

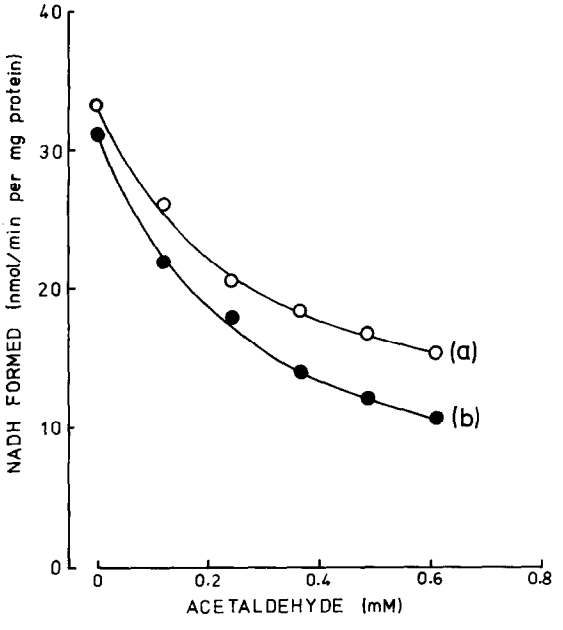

Fig. 1. Inhibition of ethanol oxidation by acetaldehyde. High-speed supernatant fraction of rat liver homogenate (0.62 mg protein) was incubated at 37° in 2.5 ml of medium comprising 100 mM KCl and 10 mM potassium phosphate buffer, pH 7.2, together with ethanol, NAD and acetaldehyde. Curve (a) shows the initial rate of NADH formation in systems containing 4 mM NAD, 50 mM ethanol and up to 0.61 mM acetaldehyde. Curve (b) shows the effect of acetaldehyde on the production of NADH in systems containing 1 mM NAD, 0.005 mM NADH and 34 mM ethanol.

Table 1. Effect of acetaldehyde on the ethanol-dependent reduction of NAD

Acetaldehyde (mM)		NADH formation nmol/min per mg protein			
Added	Total		v/V_m	Eq	ϕ
0	0.005	31.1	1.00	1.00	1.00
0.122	0.127	21.9	0.70	0.94	0.74
0.243	0.248	17.9	0.58	0.88	0.66
0.365	0.370	13.9	0.45	0.81	0.56
0.487	0.492	12.1	0.39	0.75	0.52
0.608	0.613	10.7	0.34	0.68	0.50

High-speed supernatant fraction of rat liver (0.62 mg protein) was incubated at 37° in 2.5 ml of medium comprising 100 mM KCl, 10 mM potassium phosphate buffer pH 7.2, 1 mM NAD, 34 mM ethanol, and up to 0.608 mM acetaldehyde. The rate of NADH formation was measured at an NADH concentration of 0.005 mM. The values of v/V_m are given in column 4 where v is the measured rate of formation of NADH and V_m is the maximum rate, taken to be that which occurred in the absence of added acetaldehyde. Eq and ϕ are the equilibrium factor and the rate factor function as defined in the text. ϕ was calculated by using the equation $\phi = v/(V_m\, Eq)$.

The rate-factor function ϕ, for whose calculation the maximum velocity was taken to be that which occurred in the absence of added acetaldehyde, decreased as the acetaldehyde concentration increased, indicating an inhibitory pattern very similar to that which was observed in the first series of experiments and again pointing to the potentially important role of acetaldehyde as a physiological regulator of alcohol dehydrogenase.

In order to check that the inhibitory effect of acetaldehyde was not due to an irreversible inactivation of the enzyme, liver extract was incubated at room temperature for up to 20 min in the presence or absence of 1 mM acetaldehyde. Samples of the acetaldehyde-treated and untreated extracts were then tested for alcohol dehydrogenase activity under the conditions used in the first series of experiments. As the extract was diluted 100-fold when introduced into the reaction mixtures, the residual acetaldehyde concentration in the mixture containing the treated extract was only 0.01 mM. The rates at which treated and untreated extracts oxidised ethanol differed by less than 3 per cent and it was therefore concluded that the acetaldehyde effect was fully reversible. It was also noted that the rates of ethanol oxidation by the treated and untreated extracts were identically inhibited by added acetaldehyde.

When both series of experiments were repeated using the potassium phosphate/NaCl/$MgCl_2$ medium the results were almost identical to those obtained with the KCl/potassium phosphate medium and the data are not presented here. Also, when the experiments were performed at 23°, the inhibitory effect of acetaldehyde was virtually the same as at 37°, though the reaction rates were only one-third of those recorded at the higher temperature.

The results presented above demonstrate that, under near physiological conditions of temperature, pH and ionic strength, acetaldehyde reversibly inhibits the NAD-dependent oxidation of ethanol by rat liver extract, and it is tempting to suggest that acetaldehyde could be important in regulating ethanol metabolism *in vivo*. There is evidence that after a large ethanol load the acetaldehyde concentration in the blood remains virtually constant for several hours while the ethanol concentration falls steadily [13]. This indicates that the rate of production of acetaldehyde is equal to its rate of elimination, implying that the two processes are geared to each other by some regulatory mechanism. Control of alcohol dehydrogenase by acetaldehyde could constitute such a mechanism since, if acetaldehyde is initially produced at a faster rate than it is eliminated, its concentration will rise and it will inhibit its own production. The steady-state concentration of acetaldehyde will be that which is sufficient to keep its rate of production equal to its rate of elimination.

Evidence from *in vivo* studies that could support the view that acetaldehyde regulates ethanol metabolism other than through its contribution to the equilibrium factor are scarce. However Tottmar and Marchner [8] observed that, in rats, inhibition of hepatic aldehyde dehydrogenase by disulfiram led to an increase in the hepatic acetaldehyde concentration and a fall in the rate of ethanol elimination, though there was no direct effect of disulfiram on alcohol dehydrogenase activity. The same workers [9] observed similar effects when rats were subjected to alterations in their dietary regimes. Work by Crow *et al.* [23] on isolated hepatocytes showed an association between an increase in the acetaldehyde concentration and a decrease in the rate of ethanol oxidation when the cells were exposed to disulfiram. Higgins [1], who reanalysed the data of Crow *et al.* [23], concluded that acetaldehyde exerted its influence only through the equilibrium factor but this conclusion is not unequivocally borne out by the data. Finally, it is worth alluding to the investigation of Lindros *et al.* [22] into the effects of acetaldehyde on the rate of elimination of ethanol by perfused rat liver. Calculations made on the assumption that the intrahepatic concentration of acetaldehyde was equal to that in the inflowing perfusion medium, lead to the conclusion that acetaldehyde acted only through the equilibrium factor. However calculations based on the assumption that the intrahepatic concentration was equal to that in the effluent medium suggest

that changes in the equilibrium factor could not adequately account for the inhibitory effect of acetaldehyde.

To summarize the work presented here, it was found that in experiments conducted under near-physiological conditions of temperature, pH and ionic strength, acetaldehyde, at concentrations similar to those found in liver after high doses of ethanol, acted as a reversible inhibitor of the NAD-dependent oxidation of ethanol by a high-speed supernatant fraction of rat liver. If acetaldehyde were to inhibit hepatic alcohol dehydrogenase similarly *in vivo* it could serve as a physiologically important regulator of ethanol metabolism.

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