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NAD^+-Dependent Ethanol Oxidation: Redox Effects and Rate Limitation

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CRONHOLM, T. *NAD^+-dependent ethanol oxidation: Redox effects and rate limitation.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 229–232, 1983.—Effects of ethanol on interconversion of cyclohexanol and cyclohexanone was studied in isolated hepatocytes. Oxidation and reduction catalyzed by alcohol dehydrogenase were markedly inhibited and stimulated, respectively. The changed ratio between the rates indicated that the ratio of NAD^+ to NADH bound to alcohol dehydrogenase decreased several hundred times. This is much more than for the NAD^+ system used by, e.g., lactate dehydrogenase, and deuterium from [1,1-$^2H_2$] ethanol was incorporated in cyclohexanol much more than in, e.g., lactate. These results indicate that the coenzyme bound to alcohol dehydrogenase is not equilibrated with free coenzyme. Thus, the dissociation of NADH might be rate-limiting for ethanol oxidation. Deuterium transfer from chiral [1-^{2}H] ethanols and [2-^{2}H] glycerol in hepatocytes indicated that cytosolic malate dehydrogenase and lactate dehydrogenase were not completely equilibrated, whereas there was no difference in the utilization of NADH formed at alcohol dehydrogenase and at glycerol-3-phosphate dehydrogenase. Fluxes in redox reactions during ethanol oxidation may be too high for equilibration of cytosolic dehydrogenases.

NAD^+ systems Alcohol dehydrogenase Ethanol

METABOLISM of ethanol in the liver results in a more reduced redox state of the free, cytosolic NAD^+-NADH system [3], and this causes several metabolic effects [11]. However, the relationship between ethanol oxidation and redox state of the coenzyme is incompletely understood [2, 3, 8]. It has been suggested that a near-equilibrium situation exists at the alcohol dehydrogenase and that the rate-limiting step in ethanol elimination is reoxidation of NADH in shuttle systems or oxidative phosphorylation (see [8]). Alternatively, the rate-limiting step is one of the events at the alcohol dehydrogenase [2], e.g. dissociation of the binary complex [14], and accumulation of NADH is a secondary event [2]. We have previously observed that *in vivo* deuterium from (1*R*)-[1-^{2}H] ethanol labels NADH bound to alcohol dehydrogenase to a much higher extent than it labels NADH used by lactate dehydrogenase, indicating lack of equilibration via the NAD^+-NADH system of these two dehydrogenases [1,5]. The aim of the present study was to investigate the effect of ethanol on the redox state of the alcohol dehydrogenase-coenzyme complex and correlate this with the deuterium transfer between substrates via NADH in order to localize the rate-limiting step in ethanol oxidation.

METHOD

Preparation and Incubation of Hepatocytes

Female Sprague-Dawley rats weighing 220 g were given food pellets and water ad lib and were anesthesized with ether in the morning. Liver cells were prepared [13] and were incubated in 4 ml of Krebs-Henseleit bicarbonate buffer containing bovine serum albumin(1.1 g/l) and 11 mM glucose under an atmosphere of O_2/CO_2 19:1 [7]. Incubations were stopped by addition of 1 ml 3 M perchloric acid which contained methoxyamine hydrochloride (10 mg/ml) when organic acids were to be analyzed.

Quantitative Analysis

The quenched incubation mixture was neutralized with 3 M KOH and centrifuged. The supernatant was analyzed by gas chromatography using 0.2% Carbowax 1500 on Carbopack C (Supelco, Inc., Bellefonte, PA) at 150°C with 1-hexanol as internal standard.

Analysis of Deuterium Excess

Cyclohexanol was extracted with 3×1 ml methylene chloride. The pooled extract was dried with Na_2SO_4 and concentrated. Organic acids were isolated from the quenched incubation mixture and analyzed as previously described by gas chromatography/mass spectrometry [1]. A similar analytical procedure was used for cyclohexanol, using the fragment ion M-18 [5].

RESULTS

Interconversion of Cyclohexanol and Cyclohexanone

Cyclohexanol and cyclohexanone were interconverted when incubated with hepatocytes. There was no decrease in the sum of the amounts, and the results are expressed as the fraction present as cyclohexanol (Table 1). The data from the cyclohexanol incubations could be used to estimate the sum of the first-order rate constants for the oxidation (k_1) and reduction (k_{-1}) by the expression

$$k_1 + k_{-1} = -1/t \times \ln (1 - a_t)/(1 - a_{eq}))$$

TABLE 1
INTERCONVERSION OF CYCLOHEXANOL AND CYCLOHEXANONE IN ISOLATED HEPATOCYTES*

Compounds in incubation†	Cyclohexanol (mole fraction, %) after incubation‡ 2 min	5 min	15 min	30 min
Cyclohexanol	57.2 ± 5.9	25.7 ± 6.5	8.2 ± 2.1	—§
Cyclohexanol, ethanol	96.9 ± 0.2	96.4 ± 0.6	96.3 ± 0.7	96.2 ± 0.5
Cyclohexanone	13.7 ± 3.6	17.2 ± 2.8	14.2 ± 2.1	—§
Cyclohexanone, ethanol	63.9 ± 5.5	81.8 ± 1.2	97.2 ± 1.0	—§
Cyclohexanol, 4-methylpyrazole	—§	98.1 ± 0.2	95.2 ± 0.8	92.2 ± 0.8
Cyclohexanone, 4-methylpyrazole	—§	9.4 ± 2.2	24.0 ± 2.6	36.8 ± 1.8

*100 mg in 4 ml.
†Initial concentrations: cyclohexanol: 0.25 mM, cyclohexanone: 0.25 mM, ethanol: 20 mM, 4-methylpyrazole: 1.7 mM.
‡Mean value (three parallel incubations) ±S.D.
§Not determined.

where a_t and a_{eq} are the fractions of cyclohexanol remaining at time t and at equilibrium, respectively. The smallest variation among the results was obtained when a_{eq} was 0.07, and this gave the rate constants $k_1 = 2.8$ min^{-1} × g cells^{-1} and $k_{-1} = 0.2$ min^{-1} × g cells^{-1}. When cyclohexanone was used the reaction reached completion rapidly. The rate constants could not be estimated from these experiments, but they were apparently much higher than when cyclohexanol was used as substrate. At apparent equilibrium the mixture contained slightly more cyclohexanol when cyclohexanone was used as substrate.

Effect of 4-Methylpyrazole

In the presence of 4-methylpyrazole the metabolism of cyclohexanol and cyclohexanone proceeded towards an equilibrium where the mixture contained 83% cyclohexanol (Table 1). The first-order rate constants for oxidation and reduction were 0.04 min^{-1} × g cells^{-1} and 0.18 min^{-1} × g cells^{-1}, respectively.

Deuterium Incorporation from [1,1-$^2H_2$]Ethanol in Cyclohexanol

When 20 mM [1,1-$^2H_2$] ethanol (96.7 at .% excess, from Rohstoff-Einfuhr GmbH, Düsseldorf, Germany) was added to hepatocytes (100 mg) before cyclohexanone (0.25 mM) the cyclohexanol produced during 5–30 min contained a constant level of 60–70% deuterium. When cyclohexanol (0.25 mM) was used instead of cyclohexanone, the deuterium content increased slowly in the cyclohexanol (Fig. 1). The first-order rate constant for the incorporation was calculated from the steady-state part of the experiment to be 0.12–0.15 min^{-1} × g cells^{-1}, which should be equal to the rate constant for oxidation (k_1) in the presence of ethanol.

Effect of Ethanol on the Reduction of Cyclohexanone

The rate of net oxidation of cyclohexanol was too slow in the presence of ethanol to allow calculation of the rate constants, but the rates of reduction of cyclohexanone could be used (Table 1). The sum of the first-order rate constants for this experiment was calculated as described above to be 3.8–5.5 min^{-1} × g cells^{-1}. The mixture appeared to contain 96% cyclohexanol at equilibrium, and thus the rate constants for reduction and oxidation were 3.6–5.3 min^{-1} × g cells^{-1} and 0.15–0.23 min^{-1} × g cells^{-1}, respectively.

Deuterium Incorporation in Organic Acids

Hepatocytes were incubated with chiral [1-^{2}H] ethanols [5] or [2-^{2}H] glycerol (prepared by Dr. Tore Curstedt). The deuterium excess in some organic acids was measured (Table 2). There was good agreement between the degree of labelling of malate and fumarate. A small fraction of the deuterium in lactate was present at C-3, and a correction could not be applied. However, the results show that the relative incorporation of the 1-*pro-R* and the 1-*pro-S* hydrogens of ethanol in lactate and malate is clearly different. It is also seen that β-hydroxybutyrate was less labelled from (1*S*)-[1-^{2}H] ethanol than was the case with malate. The relative deuterium incorporation in different acids from (1*R*) [1-^{2}H]-ethanol and [2-^{2}H] glycerol was about the same.

DISCUSSION

Redox State of the Alcohol Dehydrogenase-Coenzyme Complex

It was previously observed that cyclohexanone is efficiently reduced in the rat *in vivo*, and that this reaction probably was catalyzed by alcohol dehydrogenase [4,5]. It was therefore considered possible to use the rates in the interconversion of cyclohexanol and cyclohexanone to study the redox state of the alcohol dehydrogenase-coenzyme complex and changes in redox state during ethanol oxidation. At equilibrium, the ratio between the concentrations of cyclohexanol and cyclohexanone should be determined by the ratio free NAD^+/free NADH in the cytosol. From the equilibrium constant [6] and the redox state of the NAD^+-NADH system in hepatocytes calculated from the lactate-pyruvate ratio [14] it can be calculated that at equilibrium the ratio of cyclohexanol to cyclohexanone should be 0.025. The observed ratio of 0.07 is not very far from the expected ratio if it is considered that the equilibrium constant was determined at room temperature and pH and ionic strength are not accurately known. However, the difference might also be due to the catalytic activity of NADP-dependent alcohol dehydrogenases, also called "aldehyde reductases." At least one isoenzyme in rat liver cytosol catalyzes the NADPH-

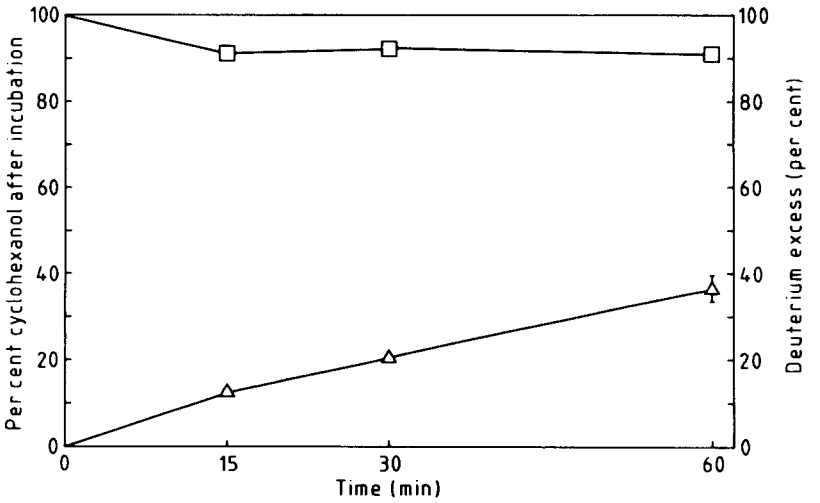

FIG. 1. Metabolism of cyclohexanol (0.25 mM) in hepatocytes (100 mg) in the presence of [1,1-$^2H_2$] ethanol (20 mM). Mole fraction (%) present as cyclohexanol (□) and deuterium excess in cyclohexanol (△) after incubation. Mean values (three parallel incubations). S.D. is shown when above 2%.

TABLE 2

DEUTERIUM INCORPORATION IN ORGANIC ACIDS OF ISOLATED HEPATOCYTES*

Deuterated substrate‡	Relative deuterium excess in one position (%)†		
	(1*R*) [1-^{2}H]ethanol	(1*S*) [1-^{2}H]ethanol	[2-^{2}H]glycerol
Organic acid			
Lactate§	15.4 ± 0.5	0.4 ± 0.1	5.1 ± 0.5
β-Hydroxybutyrate	1.0 ± 0.2	1.7 ± 0.2	0.6 ± 0.2
Malate	26.9 ± 0.6	6.2 ± 0.5	6.2 ± 0.5
Fumarate	22.0 ± 0.5	4.6 ± 0.6	6.0 ± 0.1

*100 mg in 4 ml, 15 min incubation.
†Relative to deuterium excess in substrate. Mean value (three–four parallel incubations) ±S.D.
‡Initial concentrations: ethanol: 20 mM, glycerol: 5 mM.
§Total deuterium excess.

dependent reduction of cyclohexanone [9]. This enzyme was not inhibited by pyrazole [9] and thus presumably not by 4-methylpyrazole which is an efficient inhibitor of alcohol dehydrogenase [16]. In the presence of the inhibitor, the metabolism of cyclohexanol and cyclohexanone proceeded towards an apparent equilibrium with a ratio of cyclohexanol to cyclohexanone of 4.9, i.e., about 70 times higher than in the absence of the inhibitor. The difference is in agreement with $NADP^+$ being used as coenzyme by the 4-methylpyrazole insensitive activity, since the $NADP^+$-NADPH system is much more reduced than the NAD^+-NADH system [14].

In order to correct for the presence of "aldehyde reductase," the first-order rate constants for the reactions catalyzed by this enzyme were subtracted from those determined in the absence of inhibitor. Both in the absence and the presence of inhibitor, the metabolism of both compounds appeared to proceed according to first-order kinetics, as expected from published K_m values [9,10]. In the absence of inhibitor, the rates appeared to be much higher when cyclohexanone was used than when cyclohexanol was used. The reason for this is not known, but since the final equilibrium compositions were about the same in both kinds of experiment, the conclusions regarding ethanol effect are essentially unaffected.

Effect of Ethanol on the Redox State of Bound Coenzyme

The most reliable estimate of the first-order rate constant for the oxidation reaction in the absence of ethanol was obtained in the experiments with cyclohexanol and was 2.8 $min^{-1} \times g\ cells^{-1}$. The rate constant in the presence of ethanol was 0.12–0.15 $min^{-1} \times g\ cells^{-1}$ as determined from deuterium incorporation, a value which was close to the values obtained in the incubations with cyclohexanone. After correction for oxidation by "aldehyde reductase" it is seen that ethanol caused a 25-fold decrease of the rate constant and presumably in the concentration of the alcohol dehydrogenase-NAD^+ complex.

The rate constant for reduction of cyclohexanone in the absence of ethanol could be determined from the cyclohexanol incubations to be about 0.2 $min^{-1} \times g\ cells^{-1}$. In the

presence of ethanol it was 3.6–5.3 $min^{-1} \times g\ cells^{-1}$ as determined from cyclohexanone incubations, or 2.9–3.6 $min^{-1} \times g\ cells^{-1}$ as determined from deuterium incorporation and final equilibrium composition. Presence of 4-methylpyrazole did not significantly decrease the rate constant, indicating that in the absence of ethanol essentially all of the cyclohexanone reduction is catalyzed by "aldehyde reductase." In the presence of ethanol, the reduction is mainly catalyzed by alcohol dehydrogenase, as seen from the rate constants and the efficient deuterium transfer into cyclohexanol from the 1-position of ethanol. The increase in the rate is at least 15-fold, and probably several orders of magnitude higher.

The total effect of ethanol on the alcohol dehydrogenase-catalyzed interconversion is thus to increase the equilibrium ratio between cyclohexanol and cyclohexanone at least 375 times. This should be equal to the change in the ratio between NADH and NAD^+ bound to the enzyme, and it is seen that this change is more pronounced than in any other redox couple used to study effects of ethanol [3]. The lactate-pyruvate ratio in isolated hepatocytes increases 8 times during ethanol metabolism [11]. Thus, it is concluded that the free cytosolic NAD^+-NADH system does not appear to be in equilibrium with the coenzyme bound to alcohol dehydrogenase. The bound NADH does not exchange rapidly with NADH in the rest of the cytosolic compartment as seen from the very high labelling of cyclohexanol formed during metabolism of [1,1-$^2H_2$] ethanol. If the presence of "aldehyde reductase" is taken into account the labelling of cyclohexanol was at least 70% of that in the ethanol. This is much more than the corresponding labelling of NADH used in the lactate dehydrogenase or malate dehydrogenase reactions. Thus, the slow dissociation of NADH from alcohol dehydrogenase may limit the amount of enzyme available for binding NAD^+, and thus constitute a rate-limiting step in ethanol oxidation. It may also be concluded that the redox state of the free NAD^+-NADH system cannot affect the rate of ethanol metabolism, since it is very different from that at alcohol dehydrogenase. The difference indicates dynamic compartmentation of the NAD^+-NADH system in the cytosol. This kind of compartmentation can also be studied with the aid of deuterated substrates. Thus, chiral [1-^{2}H] ethanols were used to label NADH at alcohol dehydrogenase and at aldehyde dehydrogenase with the same rates. Comparison of the degree of incorporation in different substrates indicates that lactate dehydrogenase and malate dehydrogenase are not rapidly exchanging with a common NADH pool. This indicates that the reactions catalyzed by these enzymes were slow in comparison with the net flux in redox reactions during ethanol metabolism. In contrast, there was no indication of a difference in the utilization of NADH formed at the alcohol dehydrogenase and at the glycerol-3-phosphate dehydrogenase.

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