Effect of ethanol on the redox state of the coenzyme bound to alcohol dehydrogenase studied in isolated hepatocytes

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Hepatocytes were isolated from fed female rats and incubated with a redox indicator system consisting of cyclohexanone and unlabelled or perdeuterated cyclohexanol. The concentrations and deuterium contents of these were measured by g.l.c. and g.l.c.–m.s. of oxime t-butyldimethylsilyl derivatives. The equilibrium composition represented the redox state of the coenzyme bound to alcohol dehydrogenase, since 4-methylpyrazole inhibited the interconversion. Reduction appeared to be catalysed to a small extent also by an NADPH-dependent aldehyde reductase. The NADH/NAD⁺ ratio on alcohol dehydrogenase was 3 orders of magnitude higher in the presence of ethanol than in its absence. This redox shift has the degree expected from reported kinetic constants. The shift was due both to a decreased rate of oxidation and to an increased rate of reduction in the indicator system. The results indicate that the redox effect of ethanol on the free NAD system is due to efficient removal of acetaldehyde from a near-equilibrium system consisting of ethanol, acetaldehyde and bound coenzymes, together with dissociation of NADH from the enzyme. The effect on the redox state of the bound coenzyme was less marked when the ethanol was deuterated at C-1, indicating an isotope effect. The ³H excess in the cyclohexanol formed was about 70% of that in the [1,1-²H₂]ethanol. This dilution, which is caused by binding of free NADH to the enzyme, indicates that reoxidation of cytosolic NADH partly limits the rate of ethanol oxidation.

INTRODUCTION

Metabolism of ethanol in the liver shifts the free NAD⁺/NADH system in the cytosol towards a more reduced redox state (Christensen & Higgins, 1979), and this change causes several of the metabolic effects of ethanol (Reitz, 1979). The shift might be due to a shift in the redox state of the coenzyme bound to alcohol dehydrogenase. This would be consistent with a more pronounced change in the redox state of the bound than the free coenzyme system, as also observed in a preliminary study (Cronholm, 1983).

The present paper describes the use of an artificial redox couple to study the redox state of the coenzyme bound to alcohol dehydrogenase and the shifts caused by ethanol metabolism. The equilibrium constant for interconversion of cyclohexanol and cyclohexanone (Dalziel & Dickinson, 1966) and a free NAD⁺/free NADH ratio of about 800 (Siess *et al.*, 1977) indicated that at equilibrium about 2.5% of the couple should be reduced. This should make it possible to determine an at least 1000-fold increase in the NADH/NAD⁺ ratio on the enzyme. The relevant *K_m* values for these compounds are about 1 mM (Dalziel & Dickinson, 1966; Markovic *et al.*, 1971), and thus the presence of the couple at low concentrations should not affect the redox state of the system.

MATERIALS AND METHODS

Materials

Cyclohexanol and cyclohexanone (Merck, Darmstadt, Germany) and hexan-1-ol (Analabs, North Haven, CT, U.S.A.) were of g.l.c. reference grade. [1-²H]-Cyclohexanol was prepared in diethyl ether by reduction of cyclohexanone with LiAl²H₄ (min. 98 atom %; Merck). [1,1-²H₂]Ethanol (96.7 atom %, or, in

the experiments with [²H₁₂]cyclohexanol, 99.6 atom %) and [²H₁₂]cyclohexanol (99.6 atom %) were obtained from Alfred Hempel G.m.b.H., Düsseldorf, Germany. 4-Methylpyrazole hydrochloride was kindly given by Dr. B. Sjöberg, AB Astra, Södertälje, Sweden.

The silylating mixture was 0.5 M-t-butyldimethylchlorosilane (Jansen, Beerse, Belgium) and 1.25 M-imidazole (Merck) in pyridine (Cronholm & Norsten, 1985).

Incubation procedure

Hepatocytes were isolated from female Sprague–Dawley rats weighing about 220 g and incubated in 4 ml of the bicarbonate buffer described by Krebs & Henseleit (1932). The methods used have been described by Cronholm & Curstedt (1984). The hepatocytes (usually 100 mg wet wt.) in 2 ml were incubated with 1 ml of buffer with or without ethanol for 5 min, and the substrate was then added in 1 ml of buffer. 4-Methylpyrazole was added 5 min before ethanol. Incubations were terminated with 1 ml of 3 M-HClO₄, and samples (50 µl) were taken during incubations into tubes containing 10 µl of 3 M-HClO₄. The samples were neutralized with 3 M-KOH and centrifuged for 5 min at 1370 g.

Formation of derivatives

The supernatants obtained from the terminated incubations with [²H₁₂]cyclohexanol were treated with 1.6 g of hydroxylamine hydrochloride (Cason & Harris, 1959). Anhydrous Na₂CO₃ was added slowly and, when the gas formation had stopped, the mixture was extracted with 3 × 2 ml of CH₂Cl₂. The pooled CH₂Cl₂ phases were washed with 1 ml of water and dried with anhydrous Na₂SO₄, and the volume was decreased to about 50 µl under a stream of nitrogen. The t-butyldimethylsilyl derivatives were formed by addition of 0.1 ml of the silylating mixture described above and heating for 1 h at

60 °C (Quilliam & Westmore, 1978). The products were extracted with 2 ml of hexane, and the organic phase was washed with 1 ml of water and dried over anhydrous Na_2SO_4 . The volume was decreased under a stream of nitrogen.

G.l.c. analysis

Portions of the supernatants obtained from terminated incubations were mixed with hexan-1-ol (internal standard), and cyclohexanol and cyclohexanone were quantified by g.l.c. either at 65–100 °C on a surface-coated open-tubular (SCOT) capillary column (32 m, i.d. 0.5 mm) coated with Carbowax 20 M, grade C (Scientific Glass Engineering, N. Melbourne, Australia), or at 100–150 °C on a packed column with 0.2% Carbowax 1500 on Carbowax C (Supelco, Bellefonte, PA, U.S.A.). In the former case a pre-column of Chromosorb W HP at 260 °C was used in an inlet splitter system (German & Horning, 1973).

G.l.c./mass spectrometry

Unmodified cyclohexanol was analysed at 60–110 °C in a glass capillary column (20 m, i.d. 0.34 mm) coated with SP-1000 (LKB-Produkter AB, Bromma, Sweden) and the inlet splitter system described above. The derivatives of cyclohexanol and cyclohexanone were analysed on fused-silica capillary columns (25 m, i.d. 0.32 mm) with cross-linked methyl silicone (0.25 μm) as stationary phase (Quadrex Co., New Haven, CT, U.S.A.) and with falling-needle solid-injection systems where the solvent was not allowed to evaporate completely. The mass spectrometers used were the LKB 9000 (electron energy 22.5 eV, ion source at 290 °C) or Finnigan 4000 instruments (electron energy 70 eV, ion source at 200 °C). The intensities of positive ions at m/z 82 (cyclohexanol, loss of H_2O), m/z 157 (cyclohexyl *t*-butyldimethylsilyl ether, loss of *t*-butyl radical), m/z 170 (cyclohexanone *t*-butyldimethylsilyl oxime, loss of *t*-butyl radical), and corresponding ions containing heavy isotope were used to calculate the isotopic composition (Biemann, 1962). Correction was made for the low-intensity fragment at m/z 81 in the analysis of free cyclohexanol.

The precision and accuracy of the determination of ^2H excess in free cyclohexanol was studied by triplicate analysis of six mixtures of unlabelled and $1\text{-}^2\text{H}$ -labelled cyclohexanol containing 4–77 mol% of the latter as determined by g.l.c. of the stock solutions. The determined mean ^2H excess deviated by less than 0.6 atom% from that expected, and the s.d. of the triplicates was 0.1–0.7 atom%.

RESULTS

Interconversion of cyclohexanone and cyclohexanol

Cyclohexanone was reduced to cyclohexanol during incubation with isolated hepatocytes (Fig. 1). The extent of reduction was markedly elevated by the presence of ethanol. During the course of the incubation, the sum of the concentrations of cyclohexanol and cyclohexanone slowly decreased, and the rates of decrease appeared to be the same for both compounds. After about 30 min, equilibrium was apparently reached in the interconversion. The ratio between the concentrations of cyclohexanol and cyclohexanone was about 600 times higher in the presence than in the absence of ethanol after

Fig. 1. Metabolism of cyclohexanone in isolated hepatocytes

Hepatocytes (100 mg) isolated from two livers were incubated with cyclohexanone (0.25 mM) in the absence or presence of ethanol (20 mM). The concentrations of cyclohexanone in the absence (○) and presence (●) of ethanol, and the concentrations of cyclohexanol in the absence (□) and presence (■) of ethanol, were measured by g.l.c. The values are mean values from triplicate incubations, and s.d. values over 2% are indicated.

60 min, and about 450 times higher in the presence than in the absence of ethanol after 120 min. The equilibrium was reached more rapidly when the initial and final compositions were similar than when they were markedly different, as expected from enzyme kinetics.

The final equilibrium was therefore studied by incubations with mixtures of compositions expected to be close to the final ones. The initial fraction of cyclohexanol was 15 mol% in experiments without and 90 mol% in experiments with ethanol (20 mM), and triplicate incubations were carried out for 15 min, with the concentrations of the redox couple being 0.025, 0.05, 0.125, 0.25 and 0.50 mM. The ratio between cyclohexanol and cyclohexanone was about 620 (mean value) times higher in the presence than in the absence of ethanol. The effect of ethanol (range 300–920-fold increase) was unrelated to the concentration of the redox couple.

The initial rate of reduction of cyclohexanone (0.25 mM) was studied by incubating 10 mg of hepatocytes for 2, 5, 10 and 15 min. Starting in the 2 min samples there were small amounts of cyclohexanol or another contaminant which were not detected in the 0 min samples. By linear-regression analysis of the results from four separate experiments, the rates after the first 2 min were found to be 0.73 ± 0.15 and 2.82 ± 0.52 $\mu\text{mol/min}$ per g of cells in the absence and the presence of ethanol (20 mM) respectively.

The initial rate of oxidation of cyclohexanol was studied in similar experiments. Hepatocytes (10 mg) were incubated for 4, 8, 12 and 16 min with 0.05, 0.125, 0.25 and 0.50 mM-cyclohexanol. An initial oxidation appeared to be an artefact, as described above, and the rates after 2 min were consistent with Michaelis–Menten kinetics, as seen from a linear Lineweaver–Burk plot. Linear-regression analysis of the results from four separate experiments gave a K_m of 0.15 mM and a V of about 1.63 $\mu\text{mol/min}$ per g of cells. The coefficients of variation

Fig. 2. Inhibition by 4-methylpyrazole of the interconversion of cyclohexanol and cyclohexanone

Isolated hepatocytes (100 mg) were incubated, and the fraction of the cyclohexanone/cyclohexanol couple present as cyclohexanol was determined by g.l.c. Cyclohexanol (0.25 mM) was incubated in the presence of (□) 1.7 mM- or (■) 34 mM-4-methylpyrazole. Cyclohexanone was incubated in the presence of (○) 1.7 mM- or (●) 34 mM-4-methylpyrazole. The values are mean values $\pm$ S.D. of results obtained with hepatocytes from four livers.

were 11–28% for the rates at different concentrations, and below 3% for the slope and ordinate intercept in the Lineweaver–Burk plot. The rate of oxidation was much lower in the presence of ethanol, and had to be measured by another method (see below).

Effects of 4-methylpyrazole on the interconversion of cyclohexanol and cyclohexanone

When 4-methylpyrazole was added to the incubations, the rates of conversion were considerably slower than in the absence of inhibitor. The initial rate of reduction of cyclohexanone was about the same with the inhibitor concentrations 1.7 mM and 34 mM, about 0.07 and 0.11 μ mol/min per g of cells respectively (Fig. 2). In contrast, the inhibition of the oxidation of cyclohexanol was much more marked with 34 mM-4-methylpyrazole, giving an initial rate of about 0.01 μ mol/min per g of cells, compared with an initial rate of about 0.05 μ mol/min per g of cells with 1.7 mM-4-methylpyrazole. Although equilibrium was not reached in the experiments, it is evident from Figs. 1 and 2 that this was much more displaced towards the reduced side in the presence than in the absence of 4-methylpyrazole.

Incorporation of ^2H into cyclohexanol from $[1,1-^2\text{H}_2]\text{ethanol}$

When $[1,1-^2\text{H}_2]\text{ethanol}$ (20 mM; ^2H excess 96.7 atom %) was added before cyclohexanone in the incubations, ^2H was incorporated into the cyclohexanol formed. The ^2H excess did not change during the incubation (Fig. 3), and it was the same when 3, 10, 30 or 100 mg of hepatocytes were used. The mean value ($\pm$ S.D.) from 40 incubations

Fig. 3. Metabolism of cyclohexanone in the presence of $[1,1-^2\text{H}_2]\text{ethanol}$

Isolated hepatocytes (100 mg) were incubated with cyclohexanone (0.25 mM). The mole fraction of the cyclohexanone/cyclohexanol couple present as cyclohexanol after incubation in the presence of 20 mM-ethanol (●) or $[1,1-^2\text{H}_2]\text{ethanol}$ (○) was determined by g.l.c. The ^2H excess in cyclohexanol (◇) formed in the latter case was determined by g.l.c.–m.s. All experiments were made with hepatocytes from one liver. The values are mean values of triplicate incubations, and S.D. values over 2% are indicated.

carried out with hepatocytes from four different livers was 66.6 ± 5.7 atom % excess. The effect of $[1,1-^2\text{H}_2]\text{ethanol}$ concentration was studied by incubations for 5, 15 and 30 min, in each case with hepatocytes from two different livers. When the concentration was 2 mM, the ^2H excess was lower (42.8 ± 2.4 atom %) than when the concentration was 10 or 20 mM (65.6 ± 3.6 atom %). The ^2H excess was also slightly lower when the concentration was 80 mM (58.7 ± 2.7 atom %). The rate of reduction was lower when the ethanol used was deuterated than when it was unlabelled, indicating an isotope effect (Fig. 3).

The isotope effect of $[1,1-^2\text{H}_2]\text{ethanol}$ on the equilibrium between cyclohexanol and cyclohexanone was studied by incubations of 0.25 mM mixtures of these containing 95% cyclohexanol. Hepatocytes from three livers were incubated for 60 and 90 min with 20 mM-ethanol or $[1,1-^2\text{H}_2]\text{ethanol}$ (99.6 atom %). The final cyclohexanone/cyclohexanol ratio was $52 \pm 22\%$ (mean $\pm$ S.D., $n = 6$) higher with the labelled than with the unlabelled ethanol.

Cyclohexanol incubated with isolated hepatocytes in the presence of $[1,1-^2\text{H}_2]\text{ethanol}$ incorporated ^2H . The rate of incorporation was higher when the concentration of cyclohexanol was lower, as exemplified in Fig. 4. It was assumed that the maximal ^2H excess (a_{max}) was that obtained when cyclohexanone was incubated and that

Fig. 4. ^2H exchange into cyclohexanol from $[1,1-^2\text{H}_2]\text{ethanol}$

Isolated hepatocytes (100 mg) were incubated in the presence of $[1,1-^2\text{H}_2]\text{ethanol}$ (20 mM) with cyclohexanol, 0.025 mM ($\circ$, $\square$, $\triangle$) or 0.5 mM ($\bullet$, $\blacksquare$, $\blacktriangle$). The ^2H excess in cyclohexanol was determined by g.l.c.-m.s. The hepatocytes used were from three different livers, indicated by different symbols.

the incorporation followed first-order kinetics. The rate constants were calculated from the ^2H excess (a atom %) by taking the ratio between the sum of the expressions $-\ln(1 - a/a_{\text{max}})$ and the sum of the incubation times for each concentration. The results were used to construct a Lineweaver-Burk plot, and the regression line indicated that the K_m and V values for the oxidation of cyclohexanol were 0.14 mM and 0.26 $\mu\text{mol}/\text{min}$ per g of cells respectively. The coefficients of variation for the slope and ordinate intercept in the Lineweaver-Burk plot were 5% and 14% respectively.

Incubations with $[^2\text{H}_{12}]\text{cyclohexanol}$

In studies on intermolecular hydrogen transfer and interconversion in the couple cyclohexanone/cyclohexanol, $[^2\text{H}_{12}]\text{cyclohexanol}$ was used in combination with unlabelled cyclohexanone. The analysis of ^2H excess then had to be improved to allow the determination of cyclohexanol having 0–11 ^2H atoms and cyclohexanone having 0–10 ^2H atoms. This was achieved by using *t*-butyldimethylsilyl derivatives.

The concentrations of cyclohexanol and cyclohexanone in the incubation mixtures were calculated from the areas under the ion-current chromatograms representing the individual isotopic species. The cyclohexanol/cyclohexanone ratio increased about 400–2500-fold during ethanol metabolism (Table 1). The effect was maximal after 30 min or less, and it was the same if the initial substrate was cyclohexanol or a 1:1 mixture of cyclohexanol and cyclohexanone. When $[1,1-^2\text{H}_2]\text{ethanol}$ was used instead of unlabelled ethanol, the cyclohexanol/cyclohexanone couple was less reduced, indicating an isotope effect.

The near-equilibrium state was also seen from the origin of cyclohexanol and cyclohexanone in the incubation mixtures in experiments where $[^2\text{H}_{12}]\text{cyclohexanol}$ and unlabelled cyclohexanone were incubated together. In the absence of ethanol, after 15 min about 50% of the cyclohexanol was derived from cyclohexanone and about 50% of the cyclohexanone was derived from $[^2\text{H}_{12}]\text{cyclohexanol}$. The results were about the same in experiments with ethanol present, but the cyclohexanone isolated after 15 min was derived from $[^2\text{H}_{12}]\text{cyclohexanol}$ only to the extent of $34 \pm 9\%$ (mean $\pm$ S.D., $n = 4$).

The oxidoreduction of $[^2\text{H}_{12}]\text{cyclohexanol}$ in the presence of ethanol caused a loss of ^2H from C-1, amounting to about 50% after 2 h. Since the rate of this loss is limited by the rate of oxidation of the $[^2\text{H}_{12}]\text{cyclohexanol}$, the latter could be calculated as described above under the assumption that the decrease in ^2H

Table 1. Redox shifts caused by ethanol in incubations with $[^2\text{H}_{12}]\text{cyclohexanol}$ and cyclohexanone

Isolated hepatocytes (100 mg) were incubated with $[^2\text{H}_{12}]\text{cyclohexanol}$ (0.25 mM) or a 1:1 mixture (0.25 mM) of $[^2\text{H}_{12}]\text{cyclohexanol}$ and cyclohexanone. The redox shift was calculated as the difference between the log of the ratios between cyclohexanol and cyclohexanone in incubations with and without ethanol, determined by g.l.c.-m.s. of oxime *t*-butyldimethylsilyl ethers. Each experiment was carried out with hepatocytes from four livers. The values are means $\pm$ S.D. ($n = 3-4$), or individual values shown where $n = 2$.

Expt.	Incubation with mixture	Time (min)	Fraction of cyclohexanol (mol %) after incubation with:			Calc. redox shift (no. of values)
			No ethanol	Ethanol (20 mM)	$[1,1-^2\text{H}_2]\text{ethanol}$ (20 mM)	
1	—	30	4.5 ± 2.8	97.8 ± 1.4		3.1 ± 0.2 (4)
	—	60	2.6 ± 1.1	97.4 ± 1.7		3.4 ± 0.6 (3)
	—	90	2.7 ± 1.5	95.4 ± 4.2		3.3, 3.4
	—	120	2.2, 5.3	94.7 ± 5.0		3.0, 3.1
2	—	15	8.7 ± 1.8	97.3 ± 2.6		2.7 ± 0.3 (4)
	—	60	3.7 ± 1.1	97.0 ± 1.1	91.5 ± 4.7	3.0 ± 0.3 (4)
	—	120	3.8 ± 1.1	96.4 ± 2.8	89.5 ± 2.4	2.9 ± 0.3 (3)
	+	15	6.9 ± 2.1	93.8 ± 4.5	90.6 ± 3.9	2.4 ± 0.5 (3)
	+	60	4.0 ± 0.9	94.1 ± 6.0	94.0 ± 2.1	2.9 ± 0.4 (4)
	+	120	5.2 ± 1.9	95.6 ± 0.9	91.1 ± 2.1	2.6 ± 0.2 (4)

excess is a first-order process. When the results from both experiments (see Table 1) were used, the rate of oxidation was found to be $0.070 \pm 0.026 \mu\text{mol/min per g}$ of cells (mean $\pm$ S.D., $n = 8$).

In the 15 min incubations of [$^2\text{H}_{12}$]cyclohexanol together with cyclohexanone in the absence of ethanol, there were small amounts of monodeuterated cyclohexanol. The ^2H excess in the otherwise unlabelled cyclohexanol, i.e. cyclohexanol formed from unlabelled cyclohexanone, was 1.4–12.5% in the four experiments. These results show that hydrogen removed from one cyclohexanol molecule may be transferred to another that is formed by reversible oxidoreduction.

The ^2H excess in the polydeuterated cyclohexanone decreased slowly in experiments carried out in the absence of ethanol. Thus, from an initial abundance of [$^2\text{H}_{11}$]cyclohexanol of 94.7% of molecules having 9–11 ^2H atoms, the abundance of [$^2\text{H}_{10}$]cyclohexanone among cyclohexanone molecules having 9–10 ^2H atoms had decreased to 90.2–92.0% after 15 min and to 78.7–81.3% after 120 min. This indicates a slow rate of keto–enol tautomerization of cyclohexanone during incubation. This decrease was not seen in experiments where ethanol was present, probably owing to the low concentration of cyclohexanone in these experiments.

When [$^2\text{H}_{12}$]cyclohexanol was incubated with hepatocytes in the presence of [$1,1\text{-}^2\text{H}_2$]ethanol, the abundance of [$^2\text{H}_{11}$]cyclohexanol among molecules having 9–11 ^2H atoms decreased from the initial value of 94.7% to $88.5 \pm 0.7\%$ after 15 min, $73.5 \pm 4.1\%$ after 60 min and $72.0 \pm 2.4\%$ after 120 min (mean $\pm$ S.D., $n = 3\text{--}4$). Thus it approached the ^2H excess observed in cyclohexanol in experiments with unlabelled cyclohexanone and [$1,1\text{-}^2\text{H}_2$]ethanol.

DISCUSSION

On the basis of equilibrium and kinetic constants, the cyclohexanol/cyclohexanone couple was chosen for the measurement of the effect of ethanol on the redox state of the coenzyme bound to alcohol dehydrogenase (see the Introduction). Further metabolism of these compounds might introduce an error. Cyclohexanol may be conjugated with glucuronic acid *in vivo* (Elliott *et al.*, 1959; Cronholm, 1974). The slow decrease in the concentration of the redox couple during incubation was probably due to evaporation rather than to conjugation, since the ketone disappeared at about the same rate as the alcohol. Lack of glucuronide formation in hepatocytes could be due to the incubation conditions, which are critical for optimal rates (Mizuma *et al.*, 1985). It is also possible that the analysis of metabolites in bile overestimated the rate of glucuronidation *in vivo*, since this might be necessary for excretion (Cronholm, 1974).

Enzymes other than alcohol dehydrogenase which use cyclohexanol and cyclohexanone as substrates include the NADPH-dependent aldehyde reductases (Sawada & Hara, 1979) and cytochrome *P*-450 (Gorsky & Coon, 1985). Experiments with the alcohol dehydrogenase inhibitor 4-methylpyrazole (Theorell *et al.*, 1969) indicated that aldehyde reductase might contribute only 10–15% of the reduction in the absence of ethanol and 4 times less in its presence. The inhibition of cyclohexanol oxidation was dependent on the concentration of 4-methylpyrazole, indicating incomplete inhibition of alcohol dehydrogenase. At least 96% of the oxidation

was dependent on alcohol dehydrogenase in the presence of ethanol, and much more in its absence. Thus the contribution from aldehyde reductase and cytochrome *P*-450 to the oxidation was minor or absent. The equilibrium in the cyclohexanol/cyclohexanone interconversion was much more towards the reduced side in the presence than in the absence of the inhibitor. This is in agreement with catalysis by the NADPH-dependent aldehyde reductase, since the redox state of the NADP system is about 5 orders of magnitude more to the reduced side than the NAD system in the cytosol of the liver cell (Christensen & Higgins, 1979).

The much higher rates of interconversion catalysed by alcohol dehydrogenase than by other systems made it possible to use the cyclohexanol/cyclohexanone couple to measure changes in the redox state of the coenzyme bound to alcohol dehydrogenase. Thus the effect of ethanol was the same in measurements with different concentrations of the redox couple, and near-equilibrium was seen from rapid interconversion of labelled and perdeuterated components. The shift in the cyclohexanol/cyclohexanone ratio caused by ethanol should be equal to the shift in the NADH/NAD $^+$ ratio on the enzyme. By determination of the concentrations at equilibrium with unlabelled substrates, it was found that the ratio was about 600 times higher in the presence than in the absence of ethanol (Fig. 1), and this was confirmed with deuterated substrates (Table 1). This shift is about the same as the 1000-fold increase that can be calculated from the kinetic constants and concentrations given by Crabb *et al.* (1983), by using the equations for the distribution of enzyme given by Segel (1975).

Effects of ethanol on the enzyme–coenzyme complex were also studied by measurements of initial rates. The initial rate of reduction of 0.25 mM-cyclohexanone was about 4 times higher in the presence than in the absence of ethanol. Whereas the K_m values for cyclohexanol oxidation were similar in the absence and presence of [$1,1\text{-}^2\text{H}_2$]ethanol (0.14–0.15 mM), V values were 1.63 and 0.26 $\mu\text{mol/min per g}$ of cells respectively. This decrease is smaller than the calculated 23-fold decrease in the fraction of enzyme binding NAD $^+$, which might be due to an isotope effect in the experiments with [$1,1\text{-}^2\text{H}_2$]ethanol. Thus the rate of the redox change (Fig. 3) and the final cyclohexanol/cyclohexanone ratio were lower with labelled than with unlabelled ethanol. Better agreement was seen between calculated and observed (15-fold) decrease in V when the rate of oxidation in the presence of ethanol was determined from the loss of ^2H from [$^2\text{H}_{12}$]cyclohexanol if K_m for this and unlabelled cyclohexanol were assumed to be the same, but also in this case isotope effects might be significant. The results show that ethanol changes the rates of both oxidation and reduction.

The effect of ethanol on the redox state of the bound coenzyme was used to calculate the concentration of acetaldehyde, assuming equilibrium in the ethanol–acetaldehyde interconversion with bound coenzyme. The calculated concentration in the experiments described by Crabb *et al.* (1983) was 14 μM , which is about the same as the measured concentration. This is in agreement with the rapid ethanol–acetaldehyde exchange observed for rats *in vivo* (Cronholm, 1985a) and in isolated hepatocytes (Vind & Grunnet, 1984; Cronholm, 1987).

Binding of free NADH to the enzyme was studied by measurements of the ^2H excess in cyclohexanol formed

by reduction during oxidation of $[1,1-^2\text{H}_2]\text{ethanol}$. This was about 69% of that in the $[1,1-^2\text{H}_2]\text{ethanol}$. The highest value obtained for ^2H excess in reduced compounds formed during metabolism of $[1,1-^2\text{H}_2]\text{ethanol}$ in isolated hepatocytes is 46% (Cronholm, 1985b) of that in the ethanol. If this represents the ^2H excess of the free NADH, the rate of association of NADH divided by the total rate of reduction of NAD^+ on alcohol dehydrogenase is $1.34 [(1-0.69)/(0.69-0.46)]$ (Cronholm, 1985a). When the ethanol-acetaldehyde exchange is taken into account, the rate of dissociation can be calculated to be only about 20% higher than the rate of association. Even if this value should be slightly higher, owing to an isotope effect in the reduction of cyclohexanone by (4*R*)-[4- ^3H]NADH (Bush *et al.*, 1973; Cronholm, 1985a), it is obvious that free and bound NADH were rapidly exchanged. However, the results also show that the equilibration was incomplete, as also seen from the transfer of ^2H from $[^2\text{H}_{12}]\text{cyclohexanol}$ to cyclohexanone.

The ratio between the rates of association and dissociation of NADH could be used with the kinetic constants given by Crabb *et al.* (1983) and the expressions for rates given by Segel (1975) to calculate the concentration of free NADH during ethanol oxidation. This was found to be $5.5\text{ }\mu\text{M}$, which is in good agreement with the value determined from the rate and extent of the ethanol-induced change in the lactate-pyruvate system (Bücher, 1970; Christensen & Higgins, 1979).

The redox effect on the cytosolic NAD system as measured *in vivo* and in isolated hepatocytes with several different redox couples in the rat and human is at most a 10-fold increase in the NADH/NAD^+ ratio (Forsander, 1970; Christensen & Higgins, 1979; Mørland *et al.*, 1980), which is at least 2 orders of magnitude less than the shift in redox state of the bound coenzyme. This difference indicates that the primary change is that of the bound coenzyme. It is unlikely that this is due to slow reoxidation of NADH, since it can be calculated that, even in the absence of any change in the concentration of free NADH, the redox shift in the bound coenzyme would still be a 400-fold increase. The rapid ethanol-acetaldehyde exchange indicates that efficient elimination of acetaldehyde causes the redox change in the bound coenzyme. The resulting accumulation of enzyme-NADH complex may cause the redox effect in the cytosol, since bound and free NADH are rapidly exchanged. This mechanism could explain the attenuated redox effect of ethanol after chronic ethanol treatment (Christensen & Higgins, 1979), since this treatment might decrease acetaldehyde elimination (Lieber, 1982).

As pointed out by Higgins (1979) and Kacser (1983), no single factor is rate-limiting for the elimination of ethanol. In agreement with this, the present study demonstrates the importance of several factors. Thus the ethanol-acetaldehyde exchange indicates that acetaldehyde oxidation is important (Krebs, 1969; Cronholm, 1985a), and the incomplete equilibration of bound and free NADH indicates a rate-determining role for the dissociation of NADH. Furthermore, the rapid association of NADH is in agreement with a rate-determining role for reoxidation of cytosolic NADH.

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