Hydrogen transfer between ethanol molecules during oxidoreduction *in vivo*

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Rates of exchange catalysed by alcohol dehydrogenase were determined *in vivo* in order to find rate-limiting steps in ethanol metabolism. Mixtures of [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol were injected in rats with bile fistulas. The concentrations in bile of ethanols having different numbers of ^2H atoms were determined by g.l.c.-m.s. after the addition of [$^2\text{H}_6$]ethanol as internal standard and formation of the 3,5-dinitrobenzoates. Extensive formation of [$^2\text{H}_4$]ethanol indicated that acetaldehyde formed from [2,2,2- $^2\text{H}_3$]ethanol was reduced to ethanol and that NADH used in this reduction was partly derived from oxidation of [1,1- $^2\text{H}_2$]ethanol. The rate of acetaldehyde reduction, the degree of labelling of bound NADH and the isotope effect on ethanol oxidation were calculated by fitting models to the found concentrations of ethanols labelled with 1–4 ^2H atoms. Control experiments with only [2,2,2- $^2\text{H}_3$]ethanol showed that there was no loss of the C-2 hydrogens by exchange. The isotope effect on ethanol oxidation appeared to be about 3. Experiments with (1S)-[1- ^2H]- and [2,2,2- $^2\text{H}_3$]ethanol indicated that the isotope effect on acetaldehyde oxidation was much smaller. The results indicated that both the rate of reduction of acetaldehyde and the rate of association of NADH with alcohol dehydrogenase were nearly as high as or higher than the net ethanol oxidation. Thus, the rate of ethanol oxidation *in vivo* is determined by the rates of acetaldehyde oxidation, the rate of dissociation of NADH from alcohol dehydrogenase, and by the rate of reoxidation of cytosolic NADH. In cyanamide-treated rats, the elimination of ethanol was slow but the rates in the oxidoreduction were high, indicating more complete rate-limitation by the oxidation of acetaldehyde.

Several metabolic effects of ethanol are considered to be due to the increased ratio between the concentrations of free NADH and free NAD^+ in the cytosol of the liver cell (Reitz, 1979). The mechanism behind this increase is not known, but the production of NADH in the first, cytosolic, step in ethanol metabolism makes it likely that the mechanism is related to this reaction. Thus the regulation of this reaction has been intensely debated (Dawson, 1983a; Kacser, 1983; Crow, 1983). The rate was first suggested to be determined by the rate of dissociation of the alcohol dehydrogenase–NADH complex (Theorell & Bonnichsen, 1951). The redox effects observed by Forsander *et al.* (1958) led to the idea that the reaction was limited by the rate of reoxidation of cytosolic NADH (Videla & Israel, 1970; Hillbom, 1971). It has also been suggested that acetaldehyde oxidation is the rate-limiting reaction, with the rate being determined by the concentration of

acetaldehyde, and with this concentration being set by an equilibrium in the alcohol dehydrogenase system (Krebs, 1969).

If the dissociation of the enzyme–NADH complex were rate-limiting, the rate of association would be slow relative to the rate of ethanol elimination. Indications that this might be the case have come from measurements of ^2H excess in compounds formed during metabolism of [1,1- $^2\text{H}_2$]ethanol. The ^2H excess was much higher in a reduction catalyzed by alcohol dehydrogenase than in other reductions (Cronholm, 1974; Curstedt, 1982), indicating incomplete equilibration of free and bound NADH.

If instead the oxidation of acetaldehyde were rate-limiting, the rate of acetaldehyde reduction would be high relative to the rate of ethanol elimination. This reduction has been observed *in vivo* in isotope experiments. Thus Gershman (1975) found that [^{14}C]acetaldehyde was reduced during

ethanol metabolism *in vivo*, and the rate of reduction was at least 2% of the rate of ethanol oxidation.

In the present study an attempt was made to identify the rate-limiting step under physiological conditions by simultaneously measuring the rates of NADH dissociation and acetaldehyde reduction in relation to the rate of ethanol elimination. This could be done by measuring the intermolecular hydrogen transfer at C-1 of suitably deuterated ethanol.

Materials and methods

Materials

[1,1- $^2\text{H}_2$]Ethanol (99.6 atom % ^2H) and [2,2,2- $^2\text{H}_3$]ethanol (99.0 atom % ^2H) were obtained from Rohstoff-Einfuhr G.m.b.H., Düsseldorf, Germany. The [2,2,2- $^2\text{H}_3$]ethanol used to study the retention of ^2H was a gift from Dr. A. L. Burlingame, San Francisco, CA, U.S.A. [$^2\text{H}_6$]Ethanol (more than 98 atom% ^2H) was obtained from Merck AG, Darmstadt, Germany. (1*S*)-[1- ^2H]Ethanol was prepared from [1,1- $^2\text{H}_2$]ethanol by exchange with yeast cells essentially as described by Günther *et al.* (1973). The isolated ethanol and the acetaldehyde formed in a reaction catalyzed by yeast alcohol dehydrogenase were analysed as previously described (Cronholm & Fors, 1976). The results showed that 6.7% was unlabelled, 2.0% was labelled only in the 1-*pro-R* position, 85.8% was labelled only in the 1-*pro-S* position, and 5.5% was [1,1- $^2\text{H}_2$]ethanol.

[1- ^{14}C]Ethanol (6.9 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Calcium cyanamide was obtained from American Cyanamide Company (Wayne, NJ, U.S.A.).

Animal experiments

Female Sprague-Dawley rats weighing about 220g were anaesthetized with diethyl ether and provided with bile fistulas and a tube into the peritoneal cavity to simplify injections. They were kept in restraining cages with free access to food pellets and a 0.5% (w/v) NaCl solution. After about 18h the deuterated ethanol were administered intraperitoneally as 20% (w/v) solutions in saline. Bile was collected in 0.5h fractions starting at the time of injection. The fractions were weighed and [$^2\text{H}_6$]ethanol was added as an internal standard. Bile (10 μl) was taken for quantification of ethanol by g.l.c. with propan-1-ol as internal standard on a column of 0.2% Carbowax 1500 on Carbowax C (Supelco, Inc., Bellefonte, PA, U.S.A.).

Radioactivity was determined in a scintillation counter with Instagel (Packard Instrument Co., Downers Grove, IL, U.S.A.) as scintillator.

Analysis of deuterated ethanol

The 3,5-dinitrobenzoate of ethanol was prepared by a modification of the method described by Holley & Holley (1952). Pyridine (5 μl), benzene (150 μl) and (in an ice bath) 0.55g of anhydrous K_2CO_3 were added to the bile sample (less than 0.5ml). Finally 25mg of 3,5-dinitrobenzoyl chloride (Merck AG) and 2ml of dichloromethane were added, and the mixture was stirred for 18h at room temperature. The dichloromethane phase was removed and the water phase was extracted twice with 2ml of dichloromethane. The dichloromethane phases were pooled and washed with 1ml of water, dried with anhydrous Na_2SO_4 and concentrated by evaporation of the solvent.

The ethyl 3,5-dinitrobenzoate was purified on an 8.2ml column of Lipidex 5000 (Packard Instrument Co.) in heptane. The 15–30ml eluate was concentrated by evaporation of the solvent and analysed by g.l.c.–m.s. in an LKB 9000 instrument equipped with a quartz capillary column (CP Sil 5 CB; Chrompack, Middelburg, The Netherlands) and a falling-needle injection system. The column temperature was 145°C and that of the ion source was 310°C. The electron energy was 22.5eV. Spectra were recorded on magnetic tape by repetitive accelerating voltage scanning over the region m/z 239– m/z 246 in 0.5s. The values were bunched (Axelson *et al.*, 1974) and the area of the central 30% of each mass spectrometric peak was used in the calculation of isotope excess in the molecular ion (Biemann, 1962).

Results

Evaluation of the analytical method

Aqueous 20mM-ethanol test samples (0.5ml) containing [1- ^{14}C]ethanol were treated by the method. The recovery of ^{14}C was about 10%. In order to study if there were isotope effects in the method and if the mass spectrometric response was the same for ethanol labelled with ^2H in different ways, the method was applied to 20mM samples of the deuterated ethanol which were used in the biological experiments. The response was essentially the same for the different ethanol. Thus, the ratio between the relative responses (m.s. response/amount added) for [$^2\text{H}_1$]– and [$^2\text{H}_6$]ethanol was 1.05. It was therefore considered possible to use the method for quantitative analysis in spite of the low recovery.

Retention of ^2H in [2,2,2- $^2\text{H}_3$]ethanol

Two rats were given [2,2,2- $^2\text{H}_3$]ethanol (1g/kg) intraperitoneally, and bile was collected. The ratio between dideuterated and trideuterated molecules was about 0.05 as in the injected ethanol and with

no tendency to increase. There were no molecules having one or four ^2H atoms. It was concluded that the acetaldehyde formed as intermediate in the reversible oxidoreduction of ethanol did not exchange ^2H for ^1H of the medium at a rate that was sufficient to affect the interpretation of the hydrogen transfer in experiments where $[1,1-^2\text{H}_2]\text{ethanol}$ and $[2,2,2-^2\text{H}_3]\text{ethanol}$ were simultaneously administered.

Rate of metabolism of (1S)-[1- $^2\text{H}_1$]ethanol relative to that of [2,2,2- $^2\text{H}_3$]ethanol

In order to study the isotope effect on the oxidation of acetaldehyde, two rats were given an intraperitoneal injection of (1S)-[1- $^2\text{H}_1$]ethanol (1 g/kg) together with [2,2,2- $^2\text{H}_3$]ethanol (1 g/kg) as reference. Quantification of differently labelled ethanol in bile was performed by g.l.c.-m.s. after addition of $[^2\text{H}_6]\text{ethanol}$ as internal standard. The ethanol in bile was mainly monodeuterated and trideuterated (Fig. 1). The small and about equal concentration of $[^2\text{H}_2]\text{ethanol}$ and unlabelled ethanol were ascribed to the presence of these in the (1S)-[1- $^2\text{H}_1$]ethanol used. The fraction of the excreted ethanol that was monodeuterated increased during the experiment, indicating a primary isotope effect on the oxidation of acetaldehyde and reduction of a fraction of the acetaldehyde. The isotope effect ($1/u$) was evaluated by comparisons with models. It was assumed that there was no secondary isotope effect on the oxidation of [2,2,2- $^2\text{H}_3$]ethanol, (1S)-[1- $^2\text{H}_1$]ethanol or [2,2,2- $^2\text{H}_3$]acetaldehyde. The rate (v) of decrease in concentration of the sum of mono- and trideuterated ethanol was assumed to be constant, and was evaluated by linear regression analysis. The rates of decrease with time (t) of the concentrations of $[^2\text{H}_1]\text{ethanol}$ (d_1) and $[^2\text{H}_3]\text{ethanol}$ (d_3) are then given by the following equations:

$$\frac{dd_1}{dt} = \frac{v}{1 + (d_3/ud_1)}$$

$$\frac{dd_3}{dt} = \frac{v}{1 + (ud_1/d_3)}$$

The calculations of models were performed by making the approximation:

$$\frac{\Delta d_n}{\Delta t} = \frac{dd_n}{dt}$$

using a Δt (0.01 h) that was sufficiently small to give similar results when smaller values were used. Models were calculated with u values differing by 0.01 and compared with the observed results by calculation of the sum of the squared deviations. The best fit was obtained with isotope effects of 1.37 and 1.16, respectively (Fig. 1).

Fig. 1. Detection of an isotope effect on the oxidation of acetaldehyde during ethanol metabolism *in vivo*. A mixture of (1S)-[1- $^2\text{H}_1$]ethanol (1 g/kg) and [2,2,2- $^2\text{H}_3$]ethanol (1 g/kg) was administered to a rat, and the concentrations in 0.5 h bile fractions of $[^2\text{H}_1]\text{ethanol}$ (○), $[^2\text{H}_2]\text{ethanol}$ (●) and $[^2\text{H}_3]\text{ethanol}$ (□) were measured. The $[^2\text{H}_2]\text{ethanol}$ was present as a contaminant in the (1S)-[1- $^2\text{H}_1$]ethanol. The curves give the calculated concentrations assuming an isotope effect of 1.37.

Exchange between [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol

A mixture of [1,1- $^2\text{H}_2$]ethanol (0.5 g/kg) and [2,2,2- $^2\text{H}_3$]ethanol (0.5 g/kg) was administered intraperitoneally to two rats, and the ethanol excreted in bile was quantified by g.l.c. and analysed with regard to isotopic composition. Molecules having one and four ^2H atoms appeared during the elimination of the ethanol, but no molecules having five ^2H atoms were detected. $[^2\text{H}_6]\text{Ethanol}$ could therefore be used as an internal standard, and was added to the bile in the other experiments. Three rats were given the approx. 1:1 mixture of the labelled ethanol in a total dose of 2 g/kg. The concentrations of $[^2\text{H}_1]\text{ethanol}$ and $[^2\text{H}_4]\text{ethanol}$ increased rapidly during the first 1 h after the administration, and at the same time the concentrations of $[^2\text{H}_2]\text{ethanol}$ and $[^2\text{H}_3]\text{ethanol}$ decreased rapidly (Fig. 2). This indicates extensive oxidoreduction of the ethanol with transfer of ^2H between molecules. During the last hours, the concentrations of $[^2\text{H}_1]\text{ethanol}$ and $[^2\text{H}_2]\text{ethanol}$ were essentially equal and the same was true for the concentrations of $[^2\text{H}_3]\text{ethanol}$ and $[^2\text{H}_4]\text{ethanol}$. Apparently the molecules had equilibrated to a large extent, with the ^2H content of the hydrogen that was introduced during reduction of acetaldehyde being about 50%. This is close to the fraction

Fig. 2. Intermolecular hydrogen transfer and oxidoreduction during ethanol metabolism *in vivo*

A mixture of [1,1- $^2\text{H}_2$]ethanol (1 g/kg) and [2,2,2- $^2\text{H}_3$]ethanol (1 g/kg) was administered to a rat, and the concentrations in 0.5 h bile fractions of [$^2\text{H}_1$]ethanol (○), [$^2\text{H}_2$]ethanol (●), [$^2\text{H}_3$]ethanol (□) and [$^2\text{H}_4$]ethanol (■) were measured. The curves give the calculated concentrations assuming the values given in Table 2 (expt. no. 5).

of molecules having four or two ^2H atoms, that should have been equal to the fraction of labelled coenzyme if the primary isotope effect on ethanol oxidation was small. This indicates that ^2H was lost only to a small extent by exchange of NADH bound to alcohol dehydrogenase with free, less labelled NADH.

Formation of [$^2\text{H}_1$]ethanol and [$^2\text{H}_4$]ethanol took place in all the experiments, but to different extents (Table 1). The mean concentration of [$^2\text{H}_4$]ethanol was about half that of [$^2\text{H}_1$]ethanol, consistent with a high degree of retention of ^2H during the oxidoreduction.

The decrease in the total ethanol concentration was essentially linear, and the rate (v) was calculated by linear regression analysis (Table 2). In the rats given 2 g/kg, the first value was higher than expected from a linear decrease, and this value was not used in the calculation of the rate. It appears possible that there was some direct transfer of ethanol from the peritoneal cavity into the bile.

The quantitative interpretation in terms of extent of oxidoreduction and transfer of hydrogen

Table 1. Areas under time-curves for concentrations of different deuterated ethanols in bile of rats given a 1:1 mixture of [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol, expressed as percentages of the sum of these areas

Expt. no.	Ethanol dose (g/kg)	Labelled ethanol in bile (%)			
		$^2\text{H}_1$	$^2\text{H}_2$	$^2\text{H}_3$	$^2\text{H}_4$
1	1	7.1	58.1	31.7	3.1
2	1	10.1	56.8	29.6	3.6
3	2	14.0	51.3	28.7	6.0
4	2	8.5	56.5	31.1	4.0
5	2	19.7	42.4	26.2	11.8

Table 2. Results on ethanol oxidation and NADH bound to alcohol dehydrogenase, obtained by fitting models to concentrations in bile of deuterated ethanols after administration of a 1:1 mixture of [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol to rats

The dose was 1 g/kg in expts. 1 and 2, and 2 g/kg in expts. 3–5. For details on the models, see the text.

Expt. no.	Net rate (mM/h)	Total rate divided by net rate	NADH labelling (fraction of maximum)	
			(fraction of maximum)	Isotope effect
1	12.8	1.7	0.64	2.6
2	12.9	2.1	0.65	2.7
3	12.3	4.2	0.57	4.6
4	14.0	2.1	0.50	3.2
5	10.8	7.4	0.75	4.2

was done by comparisons with models. These were calculated using the net rate (v) and the concentrations of the different deuterated ethanols at the beginning of the linear decrease in total concentration, and assuming different ratios between the total rate of ethanol oxidation and the net rate of ethanol elimination (f), different degrees of labelling of NADH, relative to the theoretical maximum when no exchange with free NADH takes place (y), and different isotope effects in the removal of the 1-*pro-R* hydrogen of ethanol ($1/q$). It was assumed that the rates of oxidation of the different molecular species were proportional to their concentrations, divided by the isotope effect in case of molecules having a ^2H in the 1-*pro-R* position, and that the rates of reduction of acetaldehyde molecules were proportional to their concentrations. The isotope effect in the removal of the 4-*pro-R* hydrogen of NADH (Bush *et al.*, 1973) was neglected in the models. The primary isotope effect in the oxidation of acetaldehyde (see above) was neglected in the first approximation. With d_n being the concentration of [$^2\text{H}_n$]ethanol, the theoretical maximum of NADH labelling is:

$$\frac{q(d_2 + d_4)}{d_1 + d_3 + q(d_2 + d_4)}$$

and multiplication by y gives the probability (a) for deuteration of an acetaldehyde molecule during reduction. The rates of increase with time (t) of the different molecular species are then given by the following equations, where $c = d_1 + d_3 + q(d_2 + d_4)$:

$$\frac{dd_1}{dt} = v \cdot \frac{(1-a)(f-1)(d_1 + qd_2) - fd_1}{c}$$

$$\frac{dd_2}{dt} = v \cdot \frac{a(f-1)(d_1 + qd_2) - fqd_2}{c}$$

$$\frac{dd_3}{dt} = v \cdot \frac{(1-a)(f-1)(d_3 + qd_4) - fd_3}{c}$$

$$\frac{dd_4}{dt} = v \cdot \frac{a(f-1)(d_3 + qd_4) - fqd_4}{c}$$

The calculation of models was performed as described above with the steps being 0.1 for f and 0.01 for y and q . The models with the best fit were in good agreement with the observed values (Fig. 2). The total rate of ethanol oxidation was 1.7–7.4 times higher than the net rate of ethanol elimination (Table 2), indicating significant reduction of acetaldehyde. The labelling of bound NADH was 0.62 ± 0.09 (S.D.) of the theoretical labelling in the absence of exchange with free NADH. In the rats with a high rate of acetaldehyde reduction, the labelling of bound NADH was also high. The primary isotope effect on ethanol oxidation was 3.4 ± 0.9 (S.D.).

Models were also calculated with account taken to the primary isotope effect on acetaldehyde oxidation ($1/u$). The rate of oxidation of acetaldehyde derived from $[^2\text{H}_1]$ - and $[^2\text{H}_2]$ ethanol as a fraction (g) of the total rate of acetaldehyde oxidation is then:

$$g = \frac{u(d_1 + qd_2)}{u(d_1 + qd_2) + d_3 + qd_4}$$

The rates of the changes in concentrations of deuterated ethanols could then be calculated as described above, if $f(d_1 + qd_2) - cg$ was substituted for $(f-1)(d_1 + qd_2)$ and $f(d_3 + qd_4) - c(1-g)$ was substituted for $(f-1)(d_3 + qd_4)$. Tests with this model in the manner described above yielded values for the isotope effect which were close to 1 (1.00–1.18), and values for y which were slightly higher than in the other model.

Effects of cyanamide

Two rats were given calcium cyanamide (30 mg/kg) intraperitoneally in 2 ml of saline, and after 2 h a mixture of $[1,1\text{-}^2\text{H}_2]$ ethanol (1 g/kg) and $[2,2,2\text{-}^2\text{H}_3]$ ethanol (1 g/kg) was given intraperiton-

eally. By linear regression analysis, the rate of decrease in total ethanol concentration was found to be 5.5 and 2.6 mm/h, respectively, in the two rats. $[^2\text{H}_1]$ - and $[^2\text{H}_4]$ ethanol appeared rapidly in bile, and after the first 1 or 2 h the concentrations of all four labelled ethanols decreased with approximately the same rates (Fig. 3). The results indicate rapid interconversion of ethanol and acetaldehyde. With d_n being the concentration of $[^2\text{H}_n]$ ethanol, the labelling of the hydrogen incorporated during acetaldehyde reduction in this steady state would be $d_4/(d_3 + d_4)$ or $d_2/(d_1 + d_2)$. This labelling relative to the maximal labelling ($d_2 + d_4$) was essentially constant after the first 2 h, the mean $\pm$ S.D. being 0.95 ± 0.01 and 1.03 ± 0.01 , respectively, in one rat (Fig. 3) and 0.93 ± 0.02 and 1.03 ± 0.01 , respectively, in the other rat. The values are close to 1, indicating that the loss by exchange of ^2H from the enzyme-bound NADH was slow. This makes it possible to calculate the rate of reduction of acetaldehyde from the estimated initial rates of appearance of $[^2\text{H}_4]$ - or $[^2\text{H}_1]$ ethanol and the maximal labelling of NADH ($d_2 + d_4$). The rate of acetaldehyde reduction (divided by the distribution volume for ethanol) was 120 and 70 mm/h in the two rats, respectively, which is about 25 times the net rate of ethanol elimination.

Fig. 3. Effect of cyanamide on the intermolecular hydrogen transfer and oxidoreduction during ethanol metabolism *in vivo*

Cyanamide (30 mg/kg) was given to a rat 2 h before a mixture of $[1,1\text{-}^2\text{H}_2]$ ethanol (1 g/kg) and $[2,2,2\text{-}^2\text{H}_3]$ ethanol (1 g/kg) was administered. The concentrations in 0.5 h bile fractions of $[^2\text{H}_1]$ ethanol ($\circ$), $[^2\text{H}_2]$ ethanol ($\bullet$), $[^2\text{H}_3]$ ethanol ($\square$) and $[^2\text{H}_4]$ ethanol ($\blacksquare$) were measured.

Although the loss of ^2H by exchange of enzyme-bound NADH was slow, as discussed above, it was not absent. The rate (divided by the distribution volume for ethanol) was calculated from the decrease in the sum of the concentrations of $[^2\text{H}_2]$ - and $[^2\text{H}_4]$ ethanol (determined by linear regression analysis) by subtracting the decrease due to ethanol elimination, and it was then found to be 0.6 and 0.5 mm/h, respectively, in the two rats.

Discussion

The method

Intermolecular hydrogen transfer during oxidation *in vivo* has been previously demonstrated for C_{19} -steroid sulphates by a method similar to the one used in the present study (Cronholm & Rudqvist, 1982). Oxidoreduction may also be detected by the decrease in $^3\text{H}/^{14}\text{C}$ ratio of a substrate labelled with ^{14}C and ^3H at the hydroxylated carbon. However, the degree of oxidoreduction is underestimated in such experiments, since the extent of intermolecular hydrogen transfer is not determined. The back-reaction with incorporation of labelled and unlabelled hydrogen atoms also have to be taken into account in studies on isotope effects, which otherwise might be underestimated. On the other hand, the changes in apparent isotope effect might be used to calculate the extent of the back-reaction (Damgaard, 1983; Lundquist & Iversen, 1984).

A disadvantage of using ^2H instead of a carbon isotope to label the molecule is that exchange reactions might take place with loss of ^2H . The absence of this type of exchange was demonstrated in the present study with the use of $[2,2,2\text{-}^2\text{H}_3]$ ethanol. There might also be secondary isotope effects when deuterated compounds are used, but these are usually small and were therefore neglected. The primary isotope effect on oxidation of acetaldehyde was found to be small and it was considered that the values obtained were more accurate when it was not determined and corrected for.

Deuterium was transferred from C-1 of ethanol to the acetaldehyde that was being reduced. In the quantitative evaluation it was assumed that the rate of ethanol elimination was linear, although it is now believed to obey Michaelis-Menten kinetics (Lundquist & Wolthers, 1958; Wilkinson, 1980). In view of the high ethanol concentrations used this would not introduce any serious error in the calculations, and it also seems quite possible that the elimination was in fact linear since factors other than saturation of alcohol dehydrogenase may determine the rate, as discussed below. Oxidation of ethanol catalysed by systems other than alcohol dehydrogenase would not seriously

affect the results if the reactions are irreversible, as is probably the case with both catalase and the microsomal ethanol-oxidizing system.

Hydrogen transfer between ethanol molecules

During metabolism of a mixture of $[1,1\text{-}^2\text{H}_2]$ - and $[2,2,2\text{-}^2\text{H}_3]$ ethanol, the calculated labelling of the NADH bound to alcohol dehydrogenase was about 62% of the labelling at C-1 in the ethanol (Table 2). This is in good agreement with the labelling of the bound NADH measured as the ^2H excess in the cyclohexanol formed from cyclohexanone in similar experiments, which was 67% of that in the $[1,1\text{-}^2\text{H}_2]$ ethanol (Cronholm, 1974).

With knowledge of the labelling of the free NADH pool used in binding to alcohol dehydrogenase it would be possible to calculate the rate of association of NADH to the enzyme in relation to the total rate of oxidation of ethanol. However, different values (18–37% of that in the $[1,1\text{-}^2\text{H}_2]$ ethanol) have been obtained for the labelling of the NADH used in different cytosolic reductions (Cronholm *et al.*, 1974; Cronholm & Fors, 1976; Curstedt, 1982). The highest value (37%) was observed for the hydrogen at C-2 of the glycerol moiety in newly formed phosphatidylcholines (Curstedt, 1982). Using this value and the labelling of bound NADH relative to that at C-1 in ethanol (y , Table 2) the rate of association of NADH in the five rats could be calculated from the expression $(1-y)/(y-0.37)$ to be 0.7–3.9 times the total rate of ethanol oxidation, or 2.3–9.0 times the net rate of ethanol elimination. The rate of association of NADH divided by the distribution volume for ethanol was then 29–113 mm/h.

The difference in ^2H excess of different pools of NADH makes it possible that the free NADH close to alcohol dehydrogenase had a higher ^2H excess than assumed above and that the rate of association of NADH thus was higher than calculated. On the other hand, the isotope effect on the reduction of acetaldehyde with (4R)-[4- ^2H]NADH causes underestimation of the ^2H content of bound NADH. With this isotope effect being 2.6 (Bush *et al.*, 1973) the ^2H excess of bound NADH would have been 72–89% instead of 50–75% (Table 2) of that in the ethanol, and the rate of association of NADH would have been 0.7–2.3 times the net rate of ethanol elimination, or, after division by the distribution volume, 9–28 mm/h. According to deJuan & Taylor (1976), there is no isotope effect on the binding of (4R)-[4- ^2H]NADH, which otherwise could have influenced the results.

The loss of ^2H by exchange of bound and free NADH was inhibited when aldehyde dehydrogenase was inhibited by cyanamide, as seen when the rate was calculated assuming absence of isotope

effect on the reduction of acetaldehyde and assuming that the labelling of free NADH was unaffected by cyanamide (Cronholm, 1985). Thus, the rate of association of NADH divided by the distribution volume for ethanol was 1.1 and 1.6 mm/h in two rats. This effect was probably due to a lower concentration of free NADH in the presence of the inhibitor, caused by the inhibition of the net elimination of ethanol.

Backward reaction during elimination of ethanol

The oxidation of ethanol was found to be accompanied by a rapid backward reaction. Reduction of administered [^{14}C]acetaldehyde during ethanol metabolism has previously been observed to occur *in vivo* to a limited extent (Gershman, 1975). Lundquist & Iversen (1983, 1984) have observed the reduction in incubations with isolated hepatocytes and concluded that it also occurs *in vivo*. In the present study, the backward rate was 0.7–6.4 times the net rate of ethanol elimination. This very rapid rate of acetaldehyde reduction was not expected from the concentration of acetaldehyde in the liver of rats given ethanol (10 μM) and the kinetic characteristics of purified rat liver alcohol dehydrogenase determined by Crabb *et al.* (1983). Thus, the data and equations in the paper by Crabb *et al.* (1983) could be used to calculate that the backward rate should be about 0.2% of the forward rate. The rate of ethanol elimination *in vivo* has been found to proceed at the rate expected from the activity of the isolated enzyme, but since the backward reaction has not been considered in these calculations, the present results support the opinion that the activity *in vitro* cannot readily be used to calculate the rate of elimination *in vivo* (Higgins, 1979).

Inhibition of ethanol elimination by addition or accumulation of acetaldehyde has previously been observed *in vitro* and *in vivo* (Lindros *et al.*, 1972; Tottmar & Marchner, 1976; Crow *et al.*, 1977; Cederbaum & Dicker, 1979, 1981; Dawson, 1981). The present results strongly suggest that the inhibition is due to reversal of the alcohol dehydrogenase reaction.

Administration of the aldehyde dehydrogenase inhibitor cyanamide further increased the rate in the backward reaction, in spite of the decreased concentration of free NADH (see above). This was probably due to an increase of the acetaldehyde concentration, indicating that the concentration is not normally high enough to saturate the alcohol dehydrogenase, and this is in agreement with the K_m value (37 μM) and concentration of acetaldehyde in the absence of inhibitor (10 μM) reported by Crabb *et al.* (1983). The net rate of ethanol elimination was much lower than in the absence of

the inhibitor, as previously observed by Tottmar & Marchner (1976). The total rate of ethanol oxidation was actually higher than in the absence of the inhibitor, indicating that the effect on the net rate was due to the backward reaction and not to inhibition of alcohol dehydrogenase by acetaldehyde (Dawson, 1983b) or cyanamide (Sharkawi, 1980). The increase in the total rate of ethanol oxidation might have been due to an elevated concentration of the binary complex containing NAD^+ , resulting from a rapid rate of reduction of acetaldehyde, and to the lower concentration of free NADH, caused by the lower net rate of ethanol elimination.

Rate-limiting of ethanol elimination

The rate of dissociation of NADH from alcohol dehydrogenase was at least 1.7–3.3 times the rate of net ethanol elimination. This is not in agreement with the contention that the rate of elimination is determined only by the rate of dissociation of the binary complex, as it often is *in vitro* (Theorell & Bonnichsen, 1951) and as it has been calculated to be *in vivo* from kinetic data obtained *in vitro* (Crabb *et al.*, 1983). It indicates an additional role for the concentrations of NADH and NAD^+ since these would determine the extent to which the free enzyme would bind the coenzymes. Thus, the rate of reoxidation of NADH would be of importance in determining the rate of ethanol elimination (Videla & Israel, 1970; Hillbom, 1971). On the other hand, results from studies with the redox couple cyclohexanol/cyclohexanone as indicator of the redox state of the coenzyme bound to alcohol dehydrogenase have shown that ethanol causes a greater change of the redox state of the bound than of the free coenzyme, indicating that the dissociation of NADH from, or association of NAD^+ to, alcohol dehydrogenase might be slow and contribute to the rate-limitation of ethanol elimination (Cronholm, 1983). In agreement with this, the present study has shown that the equilibration of free and bound NADH during ethanol elimination is incomplete.

The observed reduction of acetaldehyde could only occur if oxidation of acetaldehyde was at least partly rate-limiting for the net elimination of ethanol. Since the oxidation of ethanol appears to be rapid and reversible, the reoxidation of NADH could determine the concentration of acetaldehyde and thus its oxidation, as suggested by Krebs (1969). In cyanamide-treated rats, the ethanol–acetaldehyde interconversion was very rapid and the net rate of ethanol elimination was slow. Thus the oxidation of acetaldehyde became more completely rate-limiting for the elimination of ethanol in these rats.

It may be concluded that the oxidation of acetaldehyde, the oxidation of cytosolic NADH, and the dissociation of the alcohol dehydrogenase-NADH complex all contribute to the rate-limitation of ethanol elimination.

Isotope effects

Several of the reactions involved in hydrogen transfer between ethanol molecules during ethanol elimination may be influenced by isotope effects. In the present experiments there was competition between labelled and unlabelled molecules and they were thus influenced by apparent isotope effects on the ratio between $V_{\max}$ and K_m [$^D(V/K)$; Northrop, 1977]. The isotope effect on the removal of the 1-*pro-R* hydrogen of ethanol (about 3.4, Table 2) is in good agreement with that seen *in vitro* (3.0; Damgaard, 1981). The observation of smaller isotope effects *in vivo* (Cronholm, 1974; Damgaard, 1981) was probably due to lack of correction for oxidoreduction of ethanol with partial hydrogen retention (Damgaard, 1983).

The oxidation of [$1\text{-}^2\text{H}$]acetaldehyde was found to be subject to an isotope effect [$^D(V/K)$] of 1.2 and 1.4, respectively, in two rats. This is much less than the $^D(V/K)$ of about 3 found *in vitro* with the cytosolic enzyme from horse liver (Eckfeldt & Yonetani, 1976) or human liver (Vallari & Pietruszko, 1981). This could be due to the differences in kinetics of the cytosolic enzyme and the mitochondrial enzyme, since the latter is considered to be most important for acetaldehyde oxidation (Weiner, 1979).

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