Incorporation of the 1-*pro-R* and 1-*pro-S* hydrogen atoms of ethanol in the reduction of acids in the liver of intact rats and in isolated hepatocytes

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Ethanol oxidation causes redox effects. The coupling of this oxidation via NADH to intermediary metabolism was studied in order to reveal the underlying mechanisms. Isolated rat hepatocytes were incubated with [1,1- $^2\text{H}_2$], (1*R*)-[1- ^2H]- and (1*S*)-[1- ^2H]-ethanol and the ^2H incorporation was measured in lactate, β -hydroxybutyrate, fumarate, malate, succinate, α -oxoglutarate and citrate. The results differed in the following ways from results obtained in intact rats. Lactate became labelled to an increasing extent, and in more than one position, indicating labelling of pyruvate. A small and constant fraction of malate and fumarate was formed without access to [^2H]coenzyme. Addition of aspartate increased this fraction, which was concluded to be formed in the mitochondria. Citrate was essentially unlabelled. The ^2H from (1*R*)-[1- ^2H]ethanol contributed to malate to a larger extent and to β -hydroxybutyrate to a smaller extent, and ^2H from (1*S*)-[1- ^2H]ethanol contributed to lactate to a smaller extent. These results indicate that the exchange via shuttle systems was less efficient in isolated hepatocytes than in intact rats. The ^2H incorporation was independent of concentration of [1,1- $^2\text{H}_2$]ethanol when this was above 5 mM. Additions known to increase ethanol elimination, and cyanamide, which decreases it, had no marked effect on the ^2H incorporation. This indicates equilibration of the NADH bound to alcohol dehydrogenase with free NADH. Disulfiram and cyanamide caused a decrease in the relative incorporation from (1*S*)-[1- ^2H]ethanol into malate in liver of intact rats. Addition of cyanamide to incubations with hepatocytes resulted in a decrease of the contribution of ^2H from (1*S*)-[1- ^2H]ethanol in lactate, β -hydroxybutyrate and malate. This indicates that acetaldehyde was only oxidized in the mitochondrial compartment.

The interference of ethanol with the metabolism in liver is to a large extent due to the NADH produced in the oxidations catalysed by alcohol dehydrogenase and aldehyde dehydrogenase (Reitz, 1979). The utilization of this NADH in reductions of tricarboxylic-acid-cycle and related acids has been studied in intact rats with ethanol species having ^2H at C-1 (Cronholm *et al.*, 1974; Blomberg & Cronholm, 1979). The relative utilization of the 1-*pro-R* and 1-*pro-S* hydrogen atoms was different in the reductive formation of lactate, β -hydroxybutyrate and malate, indicating heterogeneity of the cytosolic and mitochondrial NADH pools. It was considered that similar experiments should be carried out with isolated hepatocytes, since this would eliminate heterogeneity due to differences in oxygenation in different parts of the liver lobuli, and since incubations permit addition of substrates and drugs.

Acetaldehyde is generally considered to be oxidized mainly in the mitochondria, but it is not known to what extent extramitochondrial aldehyde dehydrogenases might contribute (Weiner, 1979). Therefore inhibitors for the mitochondrial enzyme were used in experiments on intact rats and in incubations of hepatocytes in order to study if the 1-*pro-S* hydrogen atom would appear to a larger extent in products formed in cytosolic reductions, as would be the case if acetaldehyde were oxidized in the cytosol.

Methods

Materials

[1,1- $^2\text{H}_2$]Ethanol (96.7 atom % ^2H) was obtained from Rohstoff-Einfuhr G.m.b.H., Düsseldorf, Germany. (1*R*)-[1- ^2H]Ethanol and (1*S*)-[1- ^2H]ethanol were prepared, purified and analysed as

previously described (Cronholm & Fors, 1976). The ^2H excess in the former was 86.9 atom % in the 1-*pro-R* position and 11.3 atom % in the 1-*pro-S* position, and in the latter 7.2 atom % in the 1-*pro-R* position and 86.3 atom % in the 1-*pro-S* position.

Disulfiram (tetraethylthioperoxydicarbonic diamide), carboxymethyl-cellulose (sodium salt, high viscosity), noradrenaline (norepinephrine, arterenol), lithium acetoacetate, phenazine methosulphate, dihydroxyacetone, amino acids and collagenase (Type I) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Disulfiram was recrystallized from acetone/water and dried. Calcium cyanamide was obtained from American Cyanamide Co. (Wayne, NJ, U.S.A.) and 2,4-dinitrophenol was obtained from Chroma-Gesellschaft Schmid and Co., Stuttgart-Untertürkheim, Germany. Tricarboxylic-acid-cycle and related acids were obtained as previously reported (Blomberg & Cronholm, 1979).

Animals

Female Sprague-Dawley rats weighing about 220 g were used. They had free access to food pellets (R3; Ewos, Södertälje, Sweden) and water. Hepatocytes were isolated at 09:00–10:00 h, and experiments on intact rats were performed at about 12:00 h.

^2H -labelled ethanol species (1 g/kg) were administered by intraperitoneal injection of 20% (w/v) solutions in 0.9% (w/v) saline. The rats were anaesthetized with diethyl ether, and, 20 min after the injection, a part of a liver lobe was freeze-clamped *in situ* with tongs precooled with liquid N_2 (Wollenberger *et al.*, 1960).

Incubation with isolated hepatocytes

Rats were anaesthetized with diethyl ether and hepatocytes were isolated by a modification of the 'in situ' perfusion method described by Berry & Friend (1969). Details of the preparation and incubation procedure are given elsewhere (Cronholm & Curstedt, 1984). The incubations were stopped by addition of 1 ml of 3M- HClO_4 containing methoxyamine hydrochloride (10 mg/ml) (Eastman-Kodak Co., Rochester, NY, U.S.A.).

Analytical procedures

Tricarboxylic-acid-cycle and related acids were isolated and analysed for ^2H content by g.l.c.–m.s. with an LKB 9000 instrument, using repetitive accelerating voltage scanning as previously described (Axelson *et al.*, 1974; Blomberg & Cronholm, 1979).

The fragment ion that was used in the mass spectrum of dimethyl succinate, at m/z 115, was accompanied by an abundant ion at m/z 114. The origin of these ions was established by analysis of

$\text{di}[^2\text{H}_3]\text{methyl succinate}$, prepared from succinic acid in $^2\text{H}_4\text{methanol}$ and boron trifluoride ethyl etherate (Cronholm *et al.*, 1974). The mass spectrum showed that the ion at m/z 115 contained all four, and that at m/z 114 only three, of the methylene hydrogen atoms in succinate. Calculations of the abundances of succinate molecules having none to two ^2H atoms were based on the intensities of the ions at m/z 114–117 and the theoretical abundances of the isotope peaks at m/z 116 and 117, calculated from the isotopic composition of the elements.

The ^2H excess values obtained in experiments with chiral $[1-^2\text{H}]\text{ethanol}$ species were corrected for chiral impurity of these as previously described (Cronholm & Fors, 1976). Student's *t* test was used for statistical evaluation.

Results

Incorporation of ^2H from chiral $[1-^2\text{H}]\text{ethanol}$ species in intact rats

^2H atoms both at the 1-*pro-R* and the 1-*pro-S* positions of ethanol were incorporated into organic acids in the liver (Table 1). Administration of disulfiram or cyanamide orally before ethanol did not change the incorporation into lactate or β -hydroxybutyrate. Statistically significant decreases in the extent of incorporation from both positions were noted for fumarate, malate and citrate in cyanamide-treated rats. When corrected for the decrease in the transfer from the 1-*pro-R* position, it was found that the relative incorporation from the 1-*pro-S* position in malate was decreased by treatment with both cyanamide ($P < 0.001$) and disulfiram ($P < 0.01$). By the same method, the decreases in the ratio between incorporation from the 1-*pro-S* and the 1-*pro-R* positions in fumarate and citrate were not found to be statistically significant.

Incorporation of ^2H from $[1,1-^2\text{H}_2]\text{ethanol}$ in isolated hepatocytes

Hepatocytes from one rat were incubated with 20 mM- $[1,1-^2\text{H}_2]\text{ethanol}$ for 5–60 min. The isotopic composition of organic acids is given in Table 2. Fumarate and malate contained both mono- and di-deuterated molecules, as expected from incorporation in the malate dehydrogenase reaction and reversibility in this and the fumarase reaction. However, the ratio between the abundances of dideuterated ($^2\text{H}_2$) and monodeuterated ($^2\text{H}_1$) molecules was higher than expected from random labelling in two positions. This might be due to formation of a fraction ($1-f$) without access to $[^2\text{H}]\text{coenzyme}$. It was assumed that molecules formed with access to $[^2\text{H}]\text{coenzyme}$ had equal ^2H excess (a) in two positions, and f and a were

Table 1. *Effects of aldehyde dehydrogenase inhibitors on the incorporation of ^2H from (1R)-[1- ^2H]- and (1S)-[1- ^2H]ethanol in liver acids in intact rats*

A stomach tube was used for the administration of 0.3 ml of 1% (w/v) carboxymethyl-cellulose without (controls) or with 60 mg of disulfiram in suspension, or for the administration of a suspension of 6.6 mg of calcium cyanamide and 0.1 g of pulverized food pellets in 0.5 ml of water. After 24 h (controls and disulfiram) or 3 h (cyanamide) the rats were given intraperitoneal injections of [^2H]ethanol (1 g/kg) as 20% (w/v) solutions in saline, 20 min before freeze-clamping of the liver. The values are means $\pm$ s.d. ($n = 6$).

Analysed acid	Inhibitor	^2H atoms incorporated per 100 molecules from the:		Ratio between incorporation from 1- <i>pro-S</i> and 1- <i>pro-R</i> positions
		1- <i>pro-R</i> position	1- <i>pro-S</i> position	
Lactate	None	9.7 $\pm$ 2.3	2.3 $\pm$ 0.7	0.23
	Disulfiram	11.9 $\pm$ 3.1	2.5 $\pm$ 1.7	0.21
	Cyanamide	11.7 $\pm$ 3.9	2.4 $\pm$ 1.4	0.20
β -Hydroxybutyrate	None	2.3 $\pm$ 0.7	2.9 $\pm$ 0.5	1.26
	Disulfiram	2.6 $\pm$ 1.2	2.5 $\pm$ 0.8	0.96
	Cyanamide	1.8 $\pm$ 0.8	1.9 $\pm$ 1.5	1.06
Fumarate	None	26.0 $\pm$ 3.1	8.2 $\pm$ 0.7	0.31
	Disulfiram	23.4 $\pm$ 1.4	6.5 $\pm$ 1.6	0.28
	Cyanamide	16.5 $\pm$ 0.8*	4.3 $\pm$ 0.9*	0.26
Malate	None	31.4 $\pm$ 3.0	10.9 $\pm$ 0.8	0.35
	Disulfiram	29.6 $\pm$ 1.5	8.1 $\pm$ 0.9*	0.27
	Cyanamide	21.7 $\pm$ 0.7*	5.0 $\pm$ 0.8*	0.23
Citrate	None	6.0 $\pm$ 1.0	2.4 $\pm$ 0.5	0.39
	Disulfiram	4.6 $\pm$ 0.6	2.0 $\pm$ 0.4	0.44
	Cyanamide	3.4 $\pm$ 0.3*	1.1 $\pm$ 0.3*	0.32

* $P < 0.001$ when compared with corresponding control.

calculated by combining the expressions $^2\text{H}_1 = 2fa(1-a)$ and $^2\text{H}_2 = fa^2$. The fraction (f) formed with access to [^2H]coenzyme was essentially constant and 80–95% for both acids. The labelling (a) in one position of these molecules was also essentially constant, but it was lower in fumarate (31–36%) than in malate (41–46%).

Lactate consisted to an increasing extent of mono- and di-deuterated molecules. The results indicate labelling of at least two positions, and there is no reason to believe that these had the same ^2H excess. The ratio between the abundances of di- and mono-deuterated molecules increased. Thus the increase in total labelling could not be due only to a decrease in the fraction of molecules formed without access to [^2H]coenzyme. Rather, the marked increase of the fraction of dideuterated molecules indicates increase in the ^2H excess of one of the labelled positions. Since both these changes could have occurred, it was not possible to calculate the labelling in the individual positions or the fraction formed with access to [^2H]coenzyme.

β -Hydroxybutyrate and citrate contained small, but increasing, fractions of monodeuterated molecules. Succinate was usually unlabelled, but in some samples it contained mono- and di-deuterated molecules. In these, the abundance of di-deuterated molecules was high, and the results

were consistent with the presence of a small fraction that had 20–25% ^2H excess in two positions.

The effect of concentration on the incorporation of ^2H from [1,1- $^2\text{H}_2$]ethanol was investigated with hepatocytes from another rat (Fig. 1). The fraction of molecules formed with access to [^2H]coenzyme (f) and the ^2H excess in one position of these (a) were calculated for fumarate and malate as described above. It can be seen that the fraction f did not increase markedly with the concentration, whereas there was an increase in the ^2H excess in this fraction and in the labelling of lactate when the concentration was increased from 2 to 20 mM. As in the experiment described above, the ^2H excess in molecules formed with access to [^2H]coenzyme was lower for fumarate than for malate.

Effects of substrates and drugs on incorporation of ^2H from [1,1- $^2\text{H}_2$]ethanol

A series of incubations was performed in order to determine factors that regulate the incorporation of hydrogen from C-1 of ethanol into liver metabolites. The results from some of these experiments are given in Table 3. No significant ^2H excess was found in citrate, α -oxoglutarate or succinate.

Fig. 1. Incorporation of ^2H in organic acids during incubations of hepatocytes with different concentrations of $[1,1\text{-}^2\text{H}_2]\text{ethanol}$. Hepatocytes (100 mg) obtained from a female rat were incubated for 15 min with $[1,1\text{-}^2\text{H}_2]\text{ethanol}$. (a) Shows the abundance of monodeuterated β -hydroxybutyrate molecules ($\blacklozenge$) and the abundance of dideuterated lactate molecules ($\blacktriangle$). (b) Shows the ^2H excess at one position in fumarate ($\bullet$) and malate ($\blacksquare$) molecules formed with access to $[^2\text{H}]\text{coenzyme}$ and the size of this fraction for fumarate ($\circ$) and malate ($\square$). (b) Also shows the abundance of monodeuterated lactate molecules ($\blacktriangledown$). The values are means $\pm$ S.D. ($n = 4$).

This is in agreement with the apparent K_m for ethanol elimination in isolated hepatocytes, which is about 1 mM (Cornell *et al.*, 1983).

Malate formed during metabolism of $[1,1\text{-}^2\text{H}_2]\text{ethanol}$ in intact rats was labelled to about the same extent at C-2 and in the 3-*pro-S* position, indicating rapid reversible reactions in the sequence oxaloacetate-malate-fumarate (Cronholm *et al.*, 1974). The absence of ^2H loss during formation of malate and fumarate from $[1,1,3,3\text{-}^2\text{H}_4]\text{glycerol}$, presumably via pyruvate and oxaloacetate, indicated that the malate $\leftrightarrow$ oxaloacetate interconversion was slow in isolated hepatocytes (Cronholm & Curstedt, 1984). The extensive formation of dideuterated molecules of malate and fumarate in hepatocytes incubated with $[1,1\text{-}^2\text{H}_2]\text{ethanol}$ shows that these reactions were rapid and reversible as in intact rats. The difference between the results obtained in isolated hepatocytes with the two ^2H -labelled substrates is probably due to differences in the flux in glycolysis and gluconeogenesis and in redox state.

The relative abundances of dideuterated malate and fumarate molecules were actually higher than expected from random and equal labelling of two positions, indicating that a fraction had been formed without access to $[^2\text{H}]\text{coenzyme}$. This fraction was about 15% in hepatocytes from fed rats (Fig. 1, Table 3), which is the same as the

fraction of malate in mitochondria (Siess *et al.*, 1978; Brocks *et al.*, 1980). Thus the fraction formed without access to $[^2\text{H}]\text{coenzyme}$ was probably formed and present in the mitochondria, where NADH was essentially unlabelled, as seen from the low labelling of β -hydroxybutyrate. The intra-mitochondrial fraction was apparently increased by the addition of aspartate, and it may be concluded that the unlabelled acids formed in mitochondria accumulated in this compartment.

Addition of lactate decreased the labelling in malate and fumarate molecules formed with access to $[^2\text{H}]\text{coenzyme}$ without changing the fraction of these molecules. This was probably due to dilution of the labelled NADH by oxidation of the lactate.

Succinate became mono- and di-deuterated in some incubations with $[1,1\text{-}^2\text{H}_2]\text{ethanol}$, and the results were consistent with a small fraction having 20–25% ^2H in two positions. This indicates that succinate was formed from fumarate, as observed in the perfused rat liver under limiting oxygen supply (Hoberman & Prosky, 1967).

Only monodeuterated lactate is formed during metabolism of $[1,1\text{-}^2\text{H}_2]\text{ethanol}$ in intact rats (Cronholm *et al.*, 1974; Blomberg & Cronholm, 1979). In contrast, dideuterated molecules appeared in the incubations with isolated hepatocytes. This is explained by labelling of pyruvate, formed from malate or via phosphoenolpyruvate

Table 3. *Effects of substrates on the incorporation of ²H into organic acids during incubation of hepatocytes with [1,1-²H₂]ethanol*
Hepatocytes (100 mg) from fed (F) or 24 h-starved (S) female rats were incubated with 20 mM-[1,1-²H₂]ethanol. Values are means ± s.d. [n = 4, except *(n = 1), †(2) or ‡(3)]. Statistical significance: §P < 0.01 and ||P < 0.001 when compared with corresponding controls.

Hepatocyte preparation	Addition to medium	Incubation time (min)	² H atoms per 100 molecules of:		² H atoms in one position per 100 molecules formed with access to [² H]coenzyme of:		Fraction (%) formed with access to [² H]coenzyme	
			Lactate	β-Hydroxybutyrate	Fumarate	Malate	Fumarate	Malate
F1	None	15	41.5 ± 3.7	1.4 ± 0.0	38.1 ± 2.2	47.5 ± 4.4†	56.9 ± 14.4	84.1 ± 14.6†
F2	None	15	44.8 ± 4.0	1.7 ± 0.1	33.6 ± 9.6	40.1*	86.1 ± 16.3	85.3*
F3	None	15	37.8 ± 2.2	2.8 ± 0.6	42.1 ± 2.1	46.4 ± 3.1	83.1 ± 3.8	86.2 ± 4.9
F3	Alanine (10 mM)	15	42.0 ± 1.5	3.6 ± 0.2	40.5 ± 1.8	46.3 ± 6.2†	84.6 ± 4.2	87.6 ± 10.3‡
S1	None	15	19.5 ± 3.6	2.6 ± 0.1	41.4 ± 4.2	42.0 ± 1.7	60.7 ± 5.4	77.4 ± 4.4
S1	Lactate (10 mM)	15	9.6 ± 3.5	2.9 ± 0.9	16.3 ± 3.2	25.2 ± 3.4	93.2 ± 7.6	71.9 ± 3.0
F4	None	5	39.3 ± 1.3	1.8 ± 0.0	41.2 ± 2.9	50.2 ± 1.6†	86.4 ± 6.4	87.8 ± 2.0†
F4	Aspartate (10 mM)	5	34.1 ± 2.7†	1.8 ± 0.0†	32.3 ± 1.5§	44.6 ± 6.5	37.1 ± 8.6	30.4 ± 1.1
F4	Dihydroxyacetone (2.5 mM)	5	29.7 ± 2.8§	1.9 ± 0.1	30.5 ± 4.0§	39.5 ± 3.1§	92.8 ± 8.6	89.4 ± 5.5
F4	Aspartate (10 mM)	15	55.2 ± 3.6	2.0 ± 0.0	34.8 ± 0.6§	44.5 ± 0.3§	43.2 ± 2.4	40.8 ± 0.4
F4	Dihydroxyacetone (2.5 mM)	15	42.4 ± 2.2†	1.9 ± 0.2	36.3 ± 1.7†	45.8 ± 0.9†	82.2 ± 12.9†	86.2 ± 0.8†
S2	None	15	48.3 ± 11.3†	1.0 ± 0.3	36.7 ± 6.4	41.5 ± 2.1	56.1 ± 15.1	72.9 ± 1.7
S2	Aspartate (10 mM)	15	50.9 ± 9.2†	1.2 ± 0.2	34.2 ± 1.8	41.5 ± 2.2	28.5 ± 4.1	29.7 ± 0.8
S2	Dihydroxyacetone (2.5 mM)	15	27.6 ± 9.3	1.5 ± 0.3	36.4 ± 8.0	40.7 ± 7.8	62.5 ± 11.2	69.7 ± 8.5

Table 4. Incorporation of ^2H into organic acids in hepatocytes incubated with chiral $[1\text{-}^2\text{H}]$ ethanol species

Hepatocytes (100mg) from female rats were incubated with 20mM-(1R)- $[1\text{-}^2\text{H}]$ - or (1S)- $[1\text{-}^2\text{H}]$ -ethanol. Cyanamide (5mM) was added 10min before ethanol, and alanine (10mM) was present in the medium after resuspension of the cells. Values are means $\pm$ S.D. ($n = 4$). Significance: * $P < 0.01$ and † $P < 0.001$ when compared with corresponding controls.

Analysed acid	Hepatocyte preparation	Incubation time (min)	Addition to medium	^2H atoms incorporated per 100 molecules from the:		Ratio between incorporation from 1- <i>pro</i> -S and 1- <i>pro</i> -R positions
				1- <i>pro</i> -R position	1- <i>pro</i> -S position	
Lactate	1	5	None	6.9 ± 0.3	0.7 ± 0.1	0.10
	1	15	None	15.4 ± 0.5	0.4 ± 0.1	0.03
	2	15	Alanine	37.0 ± 6.2	1.3 ± 0.9	0.04
	3	15	None	28.1 ± 0.4	1.9 ± 1.0	0.06
	3	15	Cyanamide	$35.0 \pm 1.6^\dagger$	0.0 ± 0.4	0.00
β -Hydroxybutyrate	1	5	None	0.9 ± 0.3	2.0 ± 0.5	2.2
	1	15	None	1.0 ± 0.2	1.7 ± 0.2	1.7
	2	15	Alanine	0.1 ± 0.1	1.4 ± 0.3	12
	3	15	None	1.0 ± 0.2	1.9 ± 0.1	1.9
	3	15	Cyanamide	$1.9 \pm 0.3^*$	$0.3 \pm 0.2^\dagger$	0.2
Fumarate	1	5	None	40.9 ± 5.0	14.6 ± 3.0	0.36
	1	15	None	44.1 ± 0.9	9.2 ± 1.2	0.21
	2	15	Alanine	52.8 ± 0.4	11.3 ± 1.5	0.21
Malate	1	5	None	52.5 ± 2.6	13.9 ± 1.0	0.26
	1	15	None	53.7 ± 1.3	12.4 ± 0.9	0.23
	3	15	None	49.6 ± 5.2	16.1 ± 2.2	0.32
	3	15	Cyanamide	40.6 ± 2.6	$5.8 \pm 1.6^\dagger$	0.14

from oxaloacetate. Although the labelling in individual positions of lactate could not be determined, the increasing fraction of dideuterated lactate molecules indicates that the labelling of the pyruvate increased slowly during the incubation, whereas the labelling at C-2 was constant. Labelling of lactate both at C-2 and C-3 constitutes a problem in studies with (1R)- $[1\text{-}^3\text{H}]$ -ethanol or $[2\text{-}^3\text{H}]$ xylitol. Thus the slow increase in labelling of lactate observed by Vind & Grunnet (1982) might have been due to accumulation of label at C-3 rather than at C-2.

The mechanism of the labelling at C-3 of lactate was studied by addition of alanine and aspartate. These were expected to yield pyruvate, directly or via oxaloacetate, but the additions failed to change the labelling pattern. Aspartate increased the fraction of fumarate and malate formed without access to $[^2\text{H}]$ coenzyme, which was concluded to be formed and present in the mitochondria. The results indicate that, after addition of aspartate, the transport of malate out of the mitochondria was slow in comparison with the interconversion catalysed by extramitochondrial malate dehydrogenase, and supports the conclusion that malate formed without access to $[^2\text{H}]$ coenzyme was present in the mitochondria.

The addition of aspartate to hepatocytes from starved rats was expected to increase the labelling of all acids by increasing the rate of the malate shuttle, considered to be rate-limiting for ethanol oxidation in these cells (Rognstad, 1981). How-

ever, this was not seen, and dihydroxyacetone also failed to cause the increase in labelling expected from contribution to the glycerol 3-phosphate shuttle. Compounds shown to increase ethanol metabolism in isolated hepatocytes by various mechanisms also did not increase the ^2H incorporation into organic acids. These experiments were performed with cells from fed rats, since the concentrations of acids in cells from starved rats appeared to be unphysiologically low. This could explain the lack of effect of Mn^{2+} , noradrenaline and phenazine methosulphate, since their stimulation of ethanol oxidation has only been shown with hepatocytes from starved rats (Rognstad, 1981). However, 2,4-dinitrophenol, which increases ethanol oxidation in perfused liver from both fed and starved rats (Seiden *et al.*, 1974), also did not affect the labelling. Thus the ^2H excess in NADH appears to be independent of the rate of ethanol elimination when the concentration of ethanol was sufficient to saturate alcohol dehydrogenase. This could be the case if the oxidoreduction catalysed by alcohol dehydrogenase and those catalysed by other cytosolic dehydrogenases were in a state of near-equilibrium, with the rate of ethanol elimination being limited by the rate of elimination of acetaldehyde or the rate of reoxidation of NADH (Higgins, 1979).

The ^2H incorporation from $[1,1\text{-}^2\text{H}_2]$ ethanol into lactate, β -hydroxybutyrate, fumarate and malate was generally higher in isolated hepatocytes than *in vivo* (Cronholm *et al.*, 1974; Blomberg

& Cronholm, 1979). Since ethanol elimination is slower in isolated hepatocytes than in intact rats (Crow *et al.*, 1978), this supports the conclusion that there was no direct relation between rate of ethanol elimination and extent of ^2H incorporation. Citrate was much less labelled than *in vivo*. This was probably due to low labelling of intra-mitochondrial oxaloacetate, indicating slower exchange with labelled cytosolic malate in the malate shuttle.

The observed ^2H incorporation from chiral $[1-^2\text{H}]$ ethanol species into organic acids in liver in intact rats is in good agreement with the results of a previous study (Blomberg & Cronholm, 1979). The higher incorporation of ^2H from $[1,1-^2\text{H}_2]$ ethanol in malate and fumarate in isolated hepatocytes than *in vivo* was apparently due mainly to higher incorporation of ^2H from the 1-*pro-R* position. This could be due to lower efficiency of the shuttle systems in isolated hepatocytes than *in vivo* (Rognstad, 1981; Braggins & Crow, 1981). Further support for this interpretation is provided by the lower incorporation into β -hydroxybutyrate from $(1R)$ - $[1-^2\text{H}]$ ethanol in hepatocytes than *in vivo*.

The incorporation of ^2H at C-3 in lactate was concluded to be due to formation from malate. Since malate consistently had a high ratio between the incorporation from the 1-*pro-S* and 1-*pro-R* positions of ethanol, this ratio for C-2 in lactate was probably even lower than seen from Table 4, and thus much lower than *in vivo* (Table 1). This could also be explained by slower exchange in shuttle systems in isolated hepatocytes than *in vivo*.

As discussed above, most (85%) of the analysed malate was cytosolic. Thus the more efficient incorporation of ^2H from $(1S)$ - $[1-^2\text{H}]$ ethanol in malate (and fumarate) than in lactate both *in vivo* and in isolated hepatocytes indicates that lactate dehydrogenase and cytosolic malate dehydrogenase do not share a common coenzyme system that could equilibrate the two reactions (Blomberg & Cronholm, 1979).

One possible explanation for the extensive incorporation of ^2H from $(1S)$ - $[1-^2\text{H}]$ ethanol into malate is that the cytosolic malate dehydrogenase utilizes NADH formed by oxidation of acetaldehyde in the cytosol. This possibility was investigated with aldehyde dehydrogenase inhibitors. Disulfiram and cyanamide were given orally to rats in doses known to inhibit the aldehyde dehydrogenase activity in liver at the time of administration of the $[1-^2\text{H}]$ ethanol species (Marchner & Tottmar, 1978). The effects on the ^2H incorporation were of the same type after treatment with disulfiram and cyanamide, but only the latter drug caused statistically significant effects. This is in agreement with the greater effect of cyanamide on the mitochondrial 'low- K_m aldehyde dehydro-

genase' and on the elimination of ethanol *in vivo* (Marchner & Tottmar, 1978).

Both cyanamide and disulfiram decreased the incorporation of ^2H into malate from $(1S)$ - $[1-^2\text{H}]$ ethanol more than that from $(1R)$ - $[1-^2\text{H}]$ ethanol. These compounds mainly inhibit the 'low- K_m aldehyde dehydrogenase' of mitochondria, with no or lesser effects on the cytosolic and microsomal enzymes with a high K_m for acetaldehyde (Marchner & Tottmar, 1978). The results indicate that the ^2H incorporated from the 1-*pro-S* position was transferred to NADH by oxidation of $[^2\text{H}]$ acetaldehyde in mitochondria. The lack of an increase in relative incorporation from $(1S)$ - $[1-^2\text{H}]$ ethanol in lactate indicates that the acetaldehyde oxidation was not catalysed by extramitochondrial enzymes when the intramitochondrial enzyme was inhibited. Thus the extramitochondrial enzymes appear to be of little importance in the metabolism of acetaldehyde. Since it can be concluded that the incorporation of the 1-*pro-S* hydrogen took place in the mitochondria, the more extensive incorporation from $(1S)$ - $[1-^2\text{H}]$ ethanol in malate than in β -hydroxybutyrate indicates lack of equilibration of mitochondrial malate dehydrogenase and β -hydroxybutyrate dehydrogenase via a common coenzyme system.

In experiments with isolated hepatocytes, cyanamide inhibited the incorporation of ^2H from $(1S)$ - $[1-^2\text{H}]$ ethanol into lactate, malate and β -hydroxybutyrate (Table 4), as expected from inhibition of the acetaldehyde oxidation (Cederbaum & Dicker, 1981). The more pronounced effects in isolated hepatocytes than *in vivo* might have been due to evaporation of accumulated acetaldehyde in the former experiments. The incorporation of ^2H from $(1R)$ - $[1-^2\text{H}]$ ethanol in lactate and β -hydroxybutyrate increased, although the net ethanol oxidation was probably inhibited (Cederbaum & Dicker, 1981). Thus the exchange of ^2H from the 1-*pro-R* position of ethanol via NADH to the acids was increased, although the elimination of acetaldehyde was slow. The increase in ethanol oxidation to acetaldehyde in the presence of a decreased net ethanol elimination, i.e. an increased ethanol $\leftrightarrow$ acetaldehyde exchange, has also been observed *in vivo* in cyanamide-treated rats (Cronholm, 1985). The low incorporation of ^2H from $(1S)$ - $[1-^2\text{H}]$ ethanol into lactate in spite of the incomplete inhibition of aldehyde oxidation supports the conclusion that the extramitochondrial aldehyde dehydrogenase(s) lack an important role in the oxidation of acetaldehyde.

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