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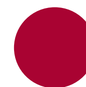

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Deuterium Isotope Effects on Ethanol Oxidation in Perfused Rat Liver and in Rats and Rabbits *in Vivo:* Application to Determine the Contribution of Various Pathways

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Abstract: The kinetic deuterium isotope effect, D(V/K), on ethanol oxidation was measured by the radiometric, competitive method using ^{14}C-labelled ethanol containing deuterium in the $(1-R)$ position. Acetate was isolated and used for the determination. Experiments were performed on rats either anaesthetized and laparotomized, or provided with indwelling catheters in *a. carotis, v. cava* and *v. portae*. Experiments were also made on perfused liver from rats pretreated with acetone, or a mixture of acetone and phenobarbital. Finally, intact non-anaesthetized rabbits were used. The apparent isotope effect in all *in vivo* experiments decreased rapidly in the presence of acetaldehyde as a consequence of the reversibility of the ADH reaction. In the case of rabbits and catheterized rats this problem was tackled by taking blood samples in quick succession, thus permitting extrapolation of the apparent isotope effect to the time of injection of the labelled ethanol. In anaesthetized rats injection of the ADH inhibitor isobutyramide was used to reduce the concentration of acetaldehyde and thereby the rate of decline of the apparent isotope effect. At high doses of isobutyramide the isotope effect was constant with time at about 1.9 suggesting the presence of non-ADH activity. In all three kinds of *in vivo* experiments the isotope effect ranged from 2.66 to 2.93. In the case of anaesthetized rats the mean value was 2.89 ± 0.05 (S.D.). This figure is significantly different from that of rat liver ADH, $P < 0.001$. As the figures for the initial isotope effects are minimum values the contribution of non-ADH ethanol oxidizing systems is likely to be small, probably less than 10 percent.

A number of enzymes appear to contribute to the oxidation of ethanol to acetaldehyde. Quantitatively most important is alcohol dehydrogenase (ADH), but peroxidation by catalase, aerobic oxidation by a number of cytochrome P_{450} species, and possibly oxidation catalyzed by iron complexes may also play a role. The kinetic deuterium isotope effects, D(V/K) in the nomenclature of Northrop (1975 & 1977), on these reactions differ widely. ADH and catalase are stereospecific for the $1-R$ position in ethanol with isotope effects of 3.0 and 1.9, respectively (Damgaard 1980 & 1981). Some ethanol oxidizing cytochrome P_{450} species are non-stereospecific with isotope effects from 2.7 to 4.0 (Ekström *et al.* 1987; Lundquist & Hansen (1989). The iron catalyzed reaction demonstrated by adding $FeSO_4$+EDTA to microsomes, is non-stereospecific with an isotope effect of 1.6.

Determination of the relative contributions of these reactions to the overall ethanol oxidation in the intact animal (or even establishing which contributions may be considered negligible) is a complex problem.

Measurement of the activity of the various ethanol metabolizing enzymes of rat liver microsomes was attempted (Lundquist & Hansen 1989) and the deuterium isotope effect in perfused liver from normal and phenobarbital treated rats was also measured (Lundquist *et al.* 1986). The present work comprises liver perfusion experiments on acetone treated and acetone+phenobarbital treated rats, experiments on anaesthetized rats, on unanaesthetized rats with indwelling catheters, and experiments on intact rabbits.

Materials and Methods

Animals. Female Wistar rats fasted for 24 hr, weighing about 200 g were used for both perfusion and *in vivo* experiments. Acetone treated rats were given one percent acetone in the drinking water offered *ad libitum* for 6–8 days. Acetone+phenobarbital treated rats were given both acetone (1%) and phenobarbital (0.8 g/l) in the drinking water for 4–7 days. White laboratory rabbits strain Ssc:CPH (female) weighing about 2.5 kg were fasted for 24 hr before being used in *in vivo* experiments. In cases when anaesthesia were used, pentothal was injected about 30 min. before the actual experiment.

Chemicals. $(1-R)[1\text{-}^2H, 1\text{-}^{14}C]$-ethanol (specific activity 50 Ci/mol) and $[2\text{-}^3H]$-ethanol (specific activity 0.25 Ci/mol) were prepared from $[1\text{-}^{14}C]$-ethanol and $[6\text{-}^3H]$-glucose as described by Damgaard (1981). $[1\text{-}^{14}C]$-ethanol, $[2\text{-}^3H]$-acetate and $[6\text{-}^3H]$-glucose were from Amersham, U.K. The radiochemical purity of the preparations was found to be adequate. Other chemicals were analytical or best available grade.

Analytical methods. Ethanol was determined according to Bonnichsen (1962), acetaldehyde according to Iversen & Damgaard (1983), acetate was isolated by diffusion in Conway units (Lundquist *et al.* 1961) before measurement of ^3H and ^{14}C radioactivity.

Perfusions. The procedure was as earlier described (Lundquist *et al.* 1986), cf. legend to table 1. Non-recirculating perfusion with Krebs-Henseleit bicarbonate solution equilibrated with a mixture of oxygen and carbon dioxide (95/5), pH 7.4, was used. The medium also contained 10 mM ethanol, $[2\text{-}^3H]$-ethanol and $(1-R)[1\text{-}^2H, 1\text{-}^{14}C]$-ethanol. The flow was about 25 ml/min., temperature at the outflow from the liver was about 35°. Oxygen consumption was measured

continuously by electrodes placed at the entrance and exit from the liver. The direction of flow could be reversed by means of a 4-port valve (Quistorff 1985). In the standard procedure three consecutive 7 min. periods were used, normal-reverse-normal direction of flow. Samples were taken from the outflowing medium every min. and analyzed for ethanol, acetaldehyde, ^{3}H- and ^{14}C-radioactivity of ethanol, and ^{3}H- and ^{14}C-radioactivity of acetate and non-volatile metabolic products (including acetate). For technical details cf. Lundquist *et al.* (1986). At the end of the experiment the liver was used for preparation of microsomes for determinations of isotope effect and activity of microsomal ethanol oxidizing enzymes. Thus the perfusion data and the data on isolated microsomes were obtained from the same liver.

In connection with each perfusion experiment to determine the isotope effect a rat from the same group of animals was used for measuring the fraction of ethanol metabolized further than to acetate. The technical procedure was as described above, but the perfusion medium contained 10 mM ethanol, [1-^{14}C]-ethanol+[2-^{3}H]-acetate. The ratio (f) of [3H]-acetate in the outflow to [3H]-acetate in the inflow in supposed to represent the fraction of the [14C]-acetate originating from ethanol which is not further metabolized. The rates of ethanol metabolism calculated from the ^{3}H-radioactivity in the perfusion experiments with double-labelled ethanol were corrected by dividing with the ratio f.

In vivo experiments.

Anaesthetized rats. Preliminary experiments showed that the measured isotope effect decreased within 30 min. to about 1.0. The following procedure was therefore introduced. Female rats fasted for 24 hr were anaesthetized with pentothal and given an intraperitoneal injection of 2 ml 5 percent (w/v) ethanol containing 0.9% NaCl. The animal was laparotomized and after 25 min. a mixture of $(1-R)$[1-^{2}H, 1-^{14}C]-ethanol (20×10^{6} cpm) and [2-^{3}H]-ethanol (60×10^{6} cpm) in 0.9% NaCl solution (about 0.8 ml) purified as previously described (Lundquist & Hansen 1989) was injected into the portal vein in the course of about 100 sec. Two min. after completion of the injection a blood sample from *vena cava* (about 0.5 ml) was collected into a weighed tube containing 0.5 ml semicarbazide-thiourea reagent (Iversen & Damgaard 1983). Similar samples were taken at 5–10–20 and in some experiments 30 min. after completion of the injection of the isotope mixture. Deproteinization was performed by adding 0.15 ml 3 M perchloric acid to 0.75 ml of the samples with thorough mixing. After 15 min. at 0° the precipitate was removed by centrifugation and radioactive CO_2 washed out by bubbling CO_2 through the samples, which were then neutralized by addition of M K_2CO_3. The protein free extracts were used for determination of ethanol, acetaldehyde, and for measurement of the $^{3}H/^{14}C$ ratio in ethanol and acetate as described above. In some experiments injection of the labelled ethanol was made in *vena cava* and samples for analysis taken from the portal vein. In experiments when isobutyramide was used to inhibit alcohol dehydrogenase the rate of ethanol oxidation was calculated from the steady state incorportaion of ^{3}H into acetate. From the specific radioactivity of ^{3}H-ethanol the concentration of radioactive acetate was calculated. Assuming that the concentration of acetate is proportional to the rate of ethanol oxidation, when this is low, i.e. the rate of acetate metabolism follows a first order reaction (Lundquist 1962), the rate of the inhibited reaction relative to the normal reaction equals the steady state concentration of radioactive acetate divided by the corresponding value for normal animals which have not received isobutyramide. The times used in the calculations were measured from the start of injection of the isotope mixture to the middle of the period used for withdrawal of blood samples.

Non-anaesthetized rats. The animals were provided with thin (PE 10) polyethylene catheters placed in the portal vein, *vena cava* (at the entrance of *v. hepatica*), and the external carotic artery. The catheters were exteriorized subcutaneously through a saddle in the interscapular region of the back. This arrangement permits the animals to move freely around in their cage, and blood samples to be obtained later without touching the animal. For details see Huang & Lardy (1981) and Huang & Veech (1988). The animals were used 24 hr after operation, when they seemed to be in good condition although still not eating or drinking.

Ethanol (3 ml, 1 M) was administered through the arterial catheter in the course of 2 min. After about 30 min. a sample of 0.2 ml venous blood was taken for ethanol determination. This and all subsequent blood samples were collected in weighed plastic centrifuge tubes containing 0.8 ml icecold freshly prepared solution of M perchloric acid and 50 mM thiourea. The tubes were stoppered, shaken and placed in ice for about 15 min. before centrifugation. At about 35 min. a mixture of [2-^{3}H]-ethanol (150×10^{6} cpm) and $(1-R)$[1-^{2}H, 1-^{14}C]-ethanol (50×10^{6} cpm) in about 0.5 ml was injected into the artery over about 30 sec. Simultaneous samples of about 0.2 ml were taken from both venous catheters again at inter-

Table 1.

Perfusion of liver from acetone treated and acetone+phenobarbital treated rats. The perfusion medium contained two radioactive species of ethanol *viz.* $(1-R)$[1-^{2}H, 1-^{14}C]-ethanol and [2-^{3}H]-ethanol. The ^{3}H-activity of acetate isolated from the effluent relative to the specific ^{3}H activity of the infused ethanol is a measure of the rate of ethanol metabolism, while the ^{14}C-radioactivity similarly determined is a measure of the rate of [1-^{2}H]-ethanol metabolism. The isotope effect is calculated as the ratio of these two 1. order velocities. Four experiments as described in Methods were made on each group of rats. The correction factor, f, was measured in separate experiments (4 in each group) by measuring the rate of [2-^{3}H]-acetate metabolism in the presence of ethanol as described in Methods. V_N and V_R are the rates of ethanol metabolism observed with respectively normal direction and reverse direction (i.e. from *v. cava* to *v. portae*) of perfusion. The rate of microsomal ethanol oxidation is given as percent of the overall ethanol oxidation measured in the corresponding perfusion experiments. Figures in parenthesis represent the range.

	Acetone treated rats	Acetone+ phenobarbital treated rats
Wet weight of liver (g)	5.6 (5.3–6.2)	6.6 (6.3–7.1)
Correction factor (f)	0.84 (0.81–0.88)	0.89 (0.84–0.93)
Oxygen uptake (μmol × (g liver)$^{-1}$ × min.$^{-1}$)		
Normal perfusion	2.17 (1.5–2.8)	2.69 (2.5–2.9)
Reverse perfusion	2.33 (1.6–3.1)	2.97 (2.8–3.2)
Rate of ethanol metabolism (μmol × (g liver)$^{-1}$ × min.$^{-1}$)		
V_N	0.45 (0.33–0.52)	0.61 (0.51–0.75)
V_R	0.59 (0.42–0.66)	0.74 (0.61–0.96)
V_R/V_N	1.31 (1.25–1.42)	1.21 (1.12–1.28)
Rate of microsomal ethanol oxidation		
Percent of V_N	5.8 (4.3–6.7)	5.9 (4.5–6.9)
Percent of V_R	4.4 (3.3–5.3)	4.9 (3.7–5.7)
Deuterium isotope effects		
On acid metabolites		
Normal perfusion	2.30 (2.15–2.46)	2.43 (2.33–2.56)
Reverse perfusion	2.29 (2.15–2.41)	2.41 (2.31–2.55)
On acetate		
Normal perfusion	2.57 (2.51–2.63)	2.68 (2.65–2.72)
Reverse perfusion	2.60 (2.54–2.67)	2.66 (2.60–2.71)
On microsomal ethanol oxidation	1.68 (1.63–1.69)	1.60 (1.57–1.63)

vals of 1 or 2 min. up to 9 min. after completion of the injection of labelled ethanol. Time was measured from the beginning of injection of the isotope mixture to the middle of the period used for withdrawal of blood samples. At 10 min. one large sample of venous blood was taken in the same way, after which the animal was killed and the liver removed and weighed. The same analyses as described for anaesthetized rats were made on the blood samples.

Intact rabbits. The animals received an intraperitoneal injection of 30 ml 2 M ethanol. Blood samples (2 ml) were taken from an ear vein 60, 90, and 120 min. later for determination of acetaldehyde and ethanol. At 125 min. a mixture of [2-^{3}H]-ethanol (6×10^8 cpm) and $(1-R)$[1-^{2}H, 1-^{14}C]-ethanol (2×10^8 cpm) was injected intravenously. Two min. after completion of the injection a blood sample (2 ml) was taken from the other ear and transferred to a weighed plastic centrifuge tube containing perchloric acid and thiourea as described above. Samples were taken again at 5, 10, and 15 min. At 20 min. a 5 ml sample was taken. The times were measured from the start of the injection of labelled ethanol to the midpoint of the period required for withdrawal of the blood sample. The animal was killed by injection of pentothal, and the liver perfused with KCl solution untill colourless. The liver was cut in small pieces and frozen for later determination of the isotope effect of rabbit liver ADH by the method of Damgaard (1981). The blood samples were used for determiuation of ethanol, acetaldehyde, radioactivity (^{3}H and ^{14}C) in ethanol, in acetate after diffusion in Conway units, and in total non-volatile radioactivity (which includes acetate) after evaporation to dryness at pH 9–10 followed by thorough chasing with non-radioactive ethanol.

Results

Table 1 summarizes the results of the perfusion experiments. The earlier observation (Lundquist *et al.* 1986) that ethanol oxidation was more rapid at reverse perfusion was confirmed also in rats treated with acetone or acetone+phenobarbital. Again no significant change in the magnitude of the isotope effect was observed when the flow direction was reversed.

Oxygen uptake was also larger when the direction of perfusion was from *v. cava* to the portal vein. The extra oxygen uptake at reverse perfusion was slightly more than necessary to account for the extra ethanol oxidation. Only about 25% of the total oxygen uptake is required to account for the measured ethanol oxidation.

Acetaldehyde concentration in the medium leaving the liver was determined in several samples from each perfusion period. In no case was the concentration above 1 μM. The concentration of acetaldehyde in pieces of liver freeze clamped immediately after stopping the perfusion was also determined in most experiments. Values of 2–6 nmol per g liver were recorded, but control experiments in which ethanol was added after deproteinization showed that artefactual aldehyde production took place to a considerable extent, so these analyses give only a possible maximum value for the intracellular acetaldehyde concentration.

The ethanol oxidation measured in microsomes isolated from the liver after completion of the perfusion experiments was 3–7 percent of the total ethanol oxidation, when experimental periods with both normal and reverse perfusion are included (table 1). It should therefore only have a very limited influence on the isotope effect measured in perfused liver. When the overall isotope effect as measured in the perfusion experiments is corrected for the microsomal contribution measured *in vitro*, the isotope effect, I_e, for the extra-microsomal contribution may be calculated to 2.66 for the acetone treated animals and 2.77 for the phenobarbital+acetone treated rats as compared to 2.58 and 2.67 respectively for the overall effect, I (table 1).

The formula used (see Damgaard 1982) is,

$$1/I = 0.95/I_e + 0.05/I_m$$

where 0.95 is the relative extra-microsomal ethanol oxidizing activity, I_e is the extra-microsomal isotope effect, 0.05 is the relative microsomal activity and I_m is the isotope effect measured on isolated microsomes. I is the overall isotope effect measured in perfusion experiments. Assuming that under the experimental condition, especially the low acetaldehyde concentration, the back reaction of alcohol dehydrogenase is negligible it is possible to calculate the contributions of ADH and catalase to the ethanol oxidation. However, if the back reaction does take place even to a small extent the calculation of the contribution of catalase becomes very uncertain, but maximum values for the contribution of catalase may be calculated to about 14 and 21 percent in respectively acetone treated and acetone+phenobarbital treated rats. It should be added that the microsomal contribution may also include some catalatic ethanol oxidation (Lundquist & Hansen 1989).

Anaesthetized rats. Experiments in which inhibitors were not used invariably showed a rapid, non-linear decline in the measured (apparent) isotope effect. The initial course of this decline was so steep that a reasonable extrapolation to the time of the start of injection of isotopically labelled ethanol was impossible (fig. 1).

As this decline seems to be caused by the back reaction

Table 2.

Effect of isobutyramide (IBA) on the deuterium isotope effect on ethanol oxidation *in vivo*. Female, fasted, anaesthetized rats were used. The acetaldehyde concentrations given were measured 2 min. after completion of the administration of labelled ethanol. The rates of ethanol oxidation were estimated from the radioactivity of [2-^{3}H]-acetate as described in Methods. Time was measured from the start of injection of labelled ethanol to the middle of the period required for withdrawal of blood samples. Isotope effects were measured on acetate. The initial isotope effects were calculated by linear extrapolation from the two first measured values to the time of the start of injection of labelled ethanol, except in the case of the experiments with 50 mg IBA, when all points were used for extrapolation. The isobutyramide was injected intraperitoneally together with non-radioactive ethanol about 30 min. before injection of the double labelled ethanol.

IBA (mg)	n	Acetaldehyde (μM)	Rate of ethanol oxidation (est.) (percent of contr.)	Isotope effect	I_0 (calc.)
50	2	1	8	2.00	–
10	1	1	20	2.48	2.88
5	6	0.4–0.7	50±14 (S.D.)	2.70±0.05	2.84
0	8	6 (2–12)	100	–	–

of ADH in the presence of acetaldehyde the effect of the non-competitive ADH inhibitor isobntyramide was tried. Under such conditions the concentration of aeetaldehyde is low (table 2) and the decline in apparent isotope effect is much reduced (fig. 1). Injection of 5 mg isobutyramide causcd a reduction in the rate of cthanol oxidation to about half the normal and the decline of the apparent isotope effect with time was reduced sufficiently to permit extrapolation to the initial value. The two first measurements of the apparcnt isotope effect (at 2 and 5 min.) were used for linear extrapolation to the start of injection of labelled ethanol. The theoretical curve for the decline in apparent isotope effcct is, however, steep at the beginning, so the value of I_0 arrived at will be a minimum value. Isobutyramide does not inhibit catalase, but inhibits microsomal ethanol oxidation somewhat at high concentrations (Lundquist & Hansen 1989). Howcver, thc inhibition of that system at the maximal intracellular concentrations expected after injection of 5 mg isobutyramide is insignificant. This procedure therefore allows the calcnlation of the minimum isotopc effect, I_0, in animals which have not rcceived inhibitor, in the following way.

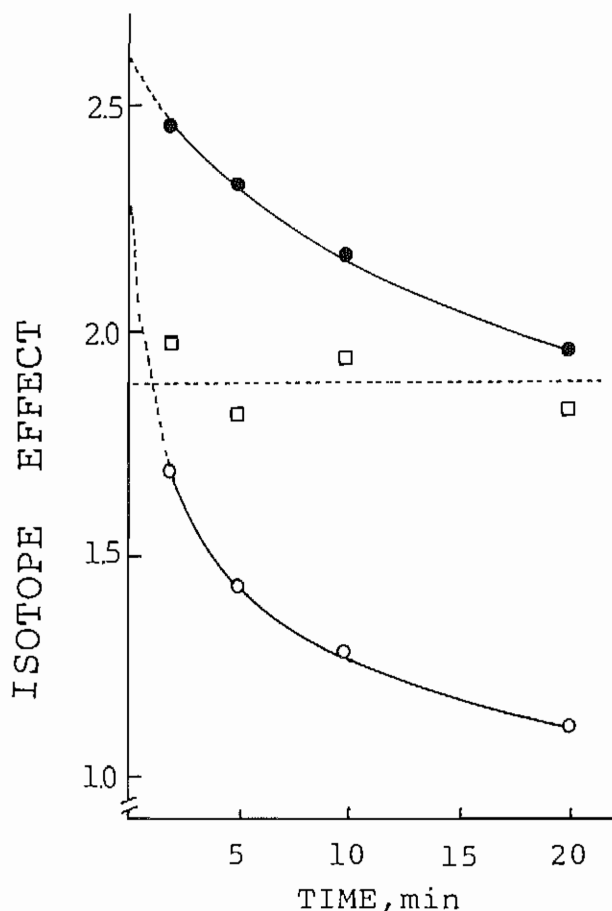

Fig. 1. Declinc of the apparent isotope effect with time in anesthetized female rats in absence or presence of the non-competitive alcohol dehydrogenase inhibitor isobutyramide (IBA). The inhibitor was injected together with non-radioactive cthanol 30 min. before injection of thc labelled ethanol mixture (at time zero). Blood ethanol concentration was between 10 and 14 mM. Open circles represent a control experiment without IBA, filled circles represent an experiment with injection of 5 mg IBA and squares injection of 50 mg IBA.

If a is the ethanol oxidizing activity of ADH, A the isotope effect of ADH, b the combined activity of catalase and cytochrome P_{450} systems, B the corresponding compound isotope effect, then the desired isotope effect, I_0, is

$$I_0 = (a+b)/(a/A + b/B) \quad (1)$$

From the isotope effect of the inhibited reaction (I_i) the following expression is derived for the relative contribution (a/v_0) of ADH activity to the cthanol oxidation in thc uninhibited reaction (v_0 = rate of ethanol oxidation).

$$a/v_0 = [I_i(1/B - 1/2A) - 0.5]/I_i(1/B - 1/A) \quad (2)$$

The relative eontribution of the b system (catalase + cytochrome P_{450}) is then,

$$b/v_0 = 1 - a/v_0, \quad \text{as} \quad v_0 = a + b$$

Combination of (1) and (2) gives the general epression,

$$I_0^{-1} = nI_i^{-1} + (1-n)A^{-1},$$

where n is the factor by which the ethanol oxidation velocity is reduced by the inhibitor. The magnitude of B does not appear in this formula. For the special case of $n = 0.5$ as used in the experiments (table 2) the formula reduces to

$$I_0^{-1} = 0.5(A^{-1} + I_i^{-1})$$

From the value of 2.70 for thc initial isotope effect when ADH is 50% inhibited, I_0 is calculated to 2.84 ± 0.05 (S.D.). This value is significantly different from the isotopc effect of rat liver ADH, 3.12 ± 0.09 (Damgaard 1981), $P < 0.001$. Thc single experiment with 10 mg IBA similarly gave $I_0 = 2.88$. In the two experiments with injection of 50 mg IBA the 8 individual measurements of the apparent isotope effect showed the following correlation,

$$I_t = 2.00 - 0.0073t, \quad r = -0.65,$$

indicating the presence of non-ADH activity, probably catalase.

Unanaesthetized rats in vivo.

Three experiments were made by meaus of the technique of Huang & Lardy (1981) and Huang & Veech (1988) (see Methods). Thc blood samplcs from *v. cava* and *v. portae* were taken simultaneously. This was considered correct as the transit time through the livcr is small compared to the time requircd for taking the blood samples (1–2 min.). The transit time has been measured to about 5 sec. in dogs (Goresky 1963) and about 3 sec. in rats, howcver, with considerable heterogeneity of flow (Daniel & Prichard 1951). Thc differcnces over the liver in ^3H-radioactivity per g blood of non-volatile reaction products divided by the corresponding difference in ^{14}C-radioactivity was used to calculate the isotope effect on ethanol oxidation in the liver by division with the $^3H/^{14}C$ ratio for the blood ethanol.

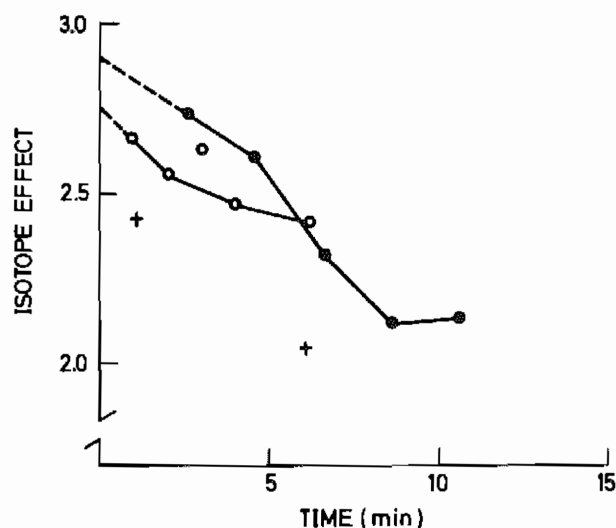

Fig. 2. Experiments on non-anaesthetized rats with indwelling catheters. Isotope effects were calculated from the porta cava differences of the ^{3}H-labelled ethanol metabolites divided by the porta cava differences of ^{14}C-labelled metabolites. Time was measured from the start of injection of labelled ethanol to the middle of the period used for withdrawal of blood samples. I_0, the initial isotope effect was measured by linear extrapolation of the first two measurements. Acetaldehyde was measured in the last blood sample only. The acetaldehyde concentration was in experiment 1 (open circles) 23 μM, in experiment 2 (filled circles) 12 μM and in experiment 3 (crosses) 32 μM. For further details see Methods.

There is a rapid decline of the apparent isotope effect with time (fig. 2), but as the blood samples were taken in rapid succession it is still possible to extrapolate to a minimum isotope effect at the time of the start of the injection of the labelled ethanol. The values found by extrapolation from the first two samples, 2.8 and 2.9 are in good agreement with those observed with the more primitive experiments on anaesthetized rats. The use of the radioactivity in the non-volatile reaction products instead of in acetate would tend to give a little lower values for the isotope effect as found in experiments on perfused liver (Lundquist *et al.* 1986).

Rabbits in vivo.

Three experiments with intact rabbits were made by the technique described. One illustrative experiment is depicted in fig. 3. The results are given in table 3. The isotope effect on ethanol oxidation by rabbit liver alcohol dehydrogenase was measured on extracts of liver by the procedure of Damgaard (1981). The values recorded in two livers were 3.01 and 3.03. The isotope effects of cytochrome P_{450} species and catalase were assumed to be similar to those of rats, 1.46 and 1.9 respectively, when the mono-deuterated substrate was used (table 4).

Discussion

The order in which the four series of experiments are described indicates increasing approximation to physiological conditions. The perfusion experiments are quite far from physiological as a simple aqueous solution was used at very high flow rate and subnormal temperature. This technique was aimed at keeping the acetaldehyde concentration at a very low level in order to prevent the back reaction of ADH, which causes the apparent isotope effect to decrease (see below). The effluent medium had indeed a low acetaldehyde concentration, but it cannot be excluded that the concentration within the liver at the site of ADH activity was considerably higher (4–7 μM) than in the medium. Unfortunately, reliable measurements of liver acetaldehyde were not possible owing to artefactual formation from ethanol during the analytical procedure (Iversen & Damgaard 1983). Intracellular presence of acetaldehyde could be the reason why the measured isotope effect was somewhat lower than that

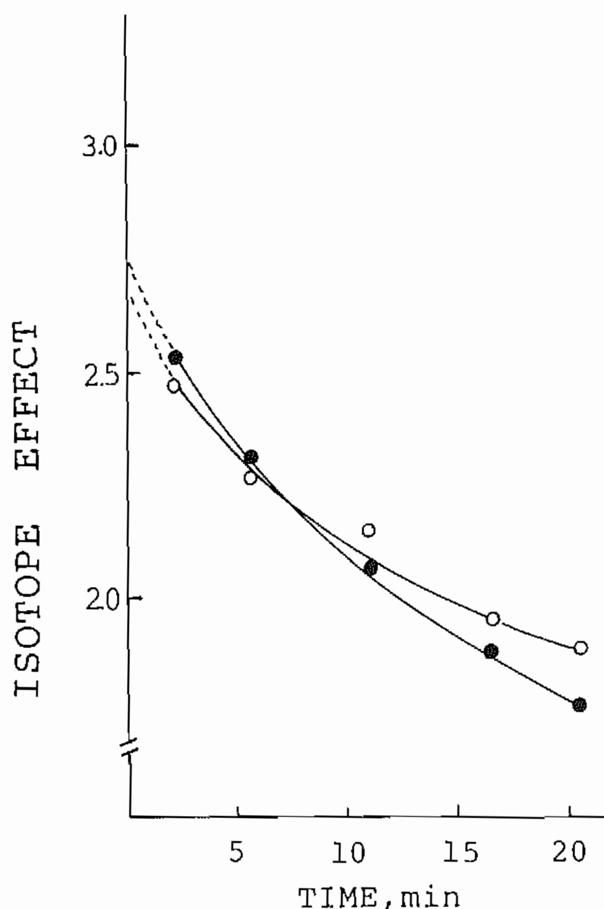

Fig. 3. Determination of the deuterium isotope effect on ethanol metabolism in a female rabbit. Experimental details are described in Methods. Non-radioactive ethanol was given intravenously to the animal 2 hr before injection of the mixture of [2-^{3}H]-ethanol and $(1-R)$[1-^{2}H, 1-^{14}C]-ethanol, which started at time zero and lasted for 0.25 min. Open circles, isotope effect measured on acetate, filled circles, isotope effect measured on non-volatile acid reaction products. Ethanol concentration 15–16 mM, acetaldehyde concentration, 12–18 μM. The I_0 values given in table 3 were obtained by linear extrapolation from the 2- and 5 min. measurements (see discussion). In this experiment the I_0 values arrived at in this way were 2.62 (from acetate) and 2.68 (from non-volatile reaction products).

Table 3.

Ethanol metabolism and deuterium isotope effect in female rabbits. The isotope effects were measured on acetate isolated from samples of ear vein blood taken at intervals during the first 2–20 min. after injection of double labelled ethanol (cf. fig. 3). Time was measured from the start of the injection of labelled ethanol to the middle of the period required for withdrawal of samples. I_0 is calculated by linear extrapolation from the first two blood samples to time zero. In experiments 2 and 3 the isotope effects were also measured on total acid metabolites. The values were 2.80 and 2.68 respectively. The metabolic rates for ethanol, v, were calculated from the decline in ethanol ^{3}H-radioactivity and the specific radioactivity of the blood ethanol assuming a volume of distribution for ethanol of 0.7 times the body weight.

Expt.	Ethanol (mM)	Acetaldehyde (μM)	I_0	v (μmol/min. × g liver)
1	18	7	2.79	3.0
2	15	10–14	2.72	1.7
3	16	12–18	2.62	1.7

found in living animals, thus leading to an overestimation of the microsomal contribution to ethanol oxidation (table 4). As the experiments were made on rats in which microsomal ethanol oxidation was induced the relatively low isotope effects could be caused by increased contribution of microsomal oxidation. The rates of ethanol oxidation measured in microsomes isolated from the liver after perfusion were, however, considerably lower than those measured in freshly prepared microsomes from acetone or phenobarbital treated rats (Lundquist & Hansen 1989). The contribution of microsomal ethanol oxidation could therefore be both overestimated and underestimated.

In vivo experiments in all cases showed a marked decrease of the apparent isotope effect with time. A strong correlation between the rate of decline and the concentration of acetaldehyde was observed both in these experiments and in experiments with isolated hepatocytes (unpublished). The mechanism of this phenomenon seems to be that the rate of ethanol oxidation catalysed by the reversible ADH system is the resultant of fast back and forth reactions. Hereby ^{14}C-acetaldehyde formed by oxidation $(1-R)[1\text{-}^2H, 1\text{-}^{14}C]$-ethanol is reduced to ^{14}C-ethanol lacking deuterium and therefore not subject to isotope effect (Lundquist & Iversen 1983 & 1984; Cronholm 1985).

Table 4.

Calculated maximal contribution of non-ADH enzymes to the ethanol oxidation in the four types of experiment performed. I is the apparent isotope effect measured. I_n is the assumed isotope effect of non-ADH when monodeuterated ethanol is used. $I_n = 1.46$ corresponds to the exclusive presence of the non-stereospecific cytochrome P_{450} species predominant in non-induced and barbiturate induced rats (true isotope effect = 2.7), $I_n = 1.6$ (true isotope effect = 4.0) indicates the exclusive presence of the cytochrome P_{450} induced by ethanol and acetone, and $I_n = 1.9$ indicates the exclusive presence of catalase.

		Percent 'non-ADH' activity		
Experiments	I	$I_n = 1.46$	$I_n = 1.6$	$I_n = 1.9$
Perfused liver				
Acetone treated	2.6	15	18	26
Acetone + phenobarbital treated	2.7	11	13	19
Anaesthetized rats	2.8	7	8	12
Non-anaesthetized rats	2.8–2.9	4–7	5–8	6–12
Non-anaesthetized rabbits	2.6–2.8	7–15	8–18	12–26

Determination of the initial isotope effect. As the apparent isotope effect declines rapidly following a non-linear time curve, which is steep at the beginning, determination of the initial value becomes crucial. The complex mathematical formula describing this curve does, unfortunately, not lend itself to extrapolation. It was therefore decided in all three groups of *in vivo* experiments to use linear extrapolation from the first two measurements to the time of starting the administration of labelled ethanol. Evidently this means that the initial isotope effect will be underestimated to a degree depending on the concentration of acetaldehyde. In consequence hereof calculations of the statistical significance of the difference between the extrapolated magnitude of the isotope effect and that of alcohol dehydrogenase would seem to be of dubious value except in the case when the acetaldehyde concentration is very low as in the experiments with inhibition of ADH by isobutyramide (Results and fig. 1).

The initial rate of decline of the overall isotope effect showed some variation between individual animals, probably caused by different blood acetaldehyde concentrations (fig. 2).

Experiments on anaesthetized rats. In these experiments the acetaldehyde concentration was reduced to about 1 μM by inhibition of alcohol dehydrogenase by injection of 5 mg IBA, which reduced the overall rate of ethanol oxidation to about 50 percent. It is noteworthy that on injection of 50 mg IBA, when ADH is nearly completely inhibited the measured isotope effect is constant and near the isotope effect of catalase (1.9), the only ethanol oxidizing system not inhibited by IBA. Unfortunately, the rate of ethanol oxidation was too low to be measured by the techniques available.

Experiments with non-anaesthetized rats. In these rats, having chronically implanted catheters in the portal vein, *vena cava*, and an artery the problem of the declining apparent isotope effect was partly solved by using smaller time intervals for sampling blood. These experiments are technically difficult, and individual measurements show considerable spreading (fig. 2). However, the procedure is believed to give a good approximation to physiological conditions (Huang & Veech 1988), and the results are in good agreement with those from anaesthetized rats. The same is true of the rabbit experiments, in which the only interference was blood sampling from the ear veins.

The many precautions necessary in studies of isotope effects by the competitive radiochemical method used have been adequately pointed out by Alderman *et al.* (1987). One

possibility for errors would be exchange of the tritium in [2-^{3}H]-ethanol with protium at some stage during the oxidation to acetate. However, experiments in which a mixture of [2-^{3}H]-ethanol aud [1-^{14}C]-ethanol was used showed no measureable decrease in the ratio of $^3H/^{14}C$ in acetate during the time periods used in the present experiments. This result at the same time excludes the presence of disturbing isotope effects caused by tritium in the methyl group.

One goal of this and similar studies has been to devise a method for the quantitative determination – without using inhibitors – of the contribution of the three (or more) enzyme systems capable of oxidizing ethanol. There is, however, one serious obstacle to achieve this goal apart from the declining apparent isotope effect since measurements of the overall isotope effect permit only the determination of two components. In the case of microsomes this problem was solved by using parallel experiments with $(1-R)$[1-^{2}H]-ethanol and [1, 1-$^2H_2$]-ethanol taking advantage of the presence of one or more non-stereospecific enzymes (Lundquist & Hansen 1989). Unfortunately this procedure is not directly applicable to *in vivo* systems because the oxidation of acetaldehyde containing deuterium at C1 to acetate is also subject to an isotope effect. In the case of crude mitochondrial aldehyde dehydrogenase this was found to 4–5 (unpublished results).

Contribution to ethanol oxidation of enzymes other than alcohol dehydrogenase. The directly measured rate of ethanol oxidation in microsomes isolated from fasted female rats contributed 3–7 percent of the total ethanol oxidation in the isolated, perfused liver of rats treated with acetone or acetone + phenobarbital nnder the experimental conditions used. In anaesthetized rats the figure calculated for the contribution of microsomal oxidation when absence of catalase is assumed is 7 percent, which may he taken as a maximum value in non-indnced animals. Therefore the activity of catalase would appear to be very small in these animals. Similar figures were calculated for non-anaesthetized rats and rabbits (table 4). This observation does not exclnde the possibility that catalase may play a more important role nnder conditions when the rate of production of hydrogen peroxide is large, e.g. at high concentrations of fatty acids, believed to be partly oxidized by peroxisomes with formation of hydrogen peroxide. Handler & Thurman (1987) thus found a very considerable increase in ethanol oxidation and hydrogen peroxide formation in recirculating liver perfusion with 4 percent albumin, when ADH was suppressed by methyl pyrazole. In unpublished, preliminary experiments with non-recirculating perfusion in the presence of 1 mM oleate and 4 percent defatted albumin in the medium, however, we did not find any significant decline of the isotope effect.

The possible occurrence in the organism of ethanol oxidation by superoxide, catalysed by iron complexes (see Lundquist & Hansen 1989) has been neglected in this investigation, as there is at present no indication that it contributes measurably to ethanol oxidation in the animal species studied. The isotope effect of this system measured with mono-deuterated ethanol is low (about 1.25), and even a small contribution hy this catalyst would lower the overall isotope effect siguificantly. For instanee 5 percent of this activity + 95 percent ADH activity would result in an isotope effect of 2.8, i.e. the magnitude found *in vivo*, leaving no room for either catalase or cytochrome P_{450}. As cytochrome P_{450} oxidation alone seems to occupy about 5 percent of the total activity, the role of iron-complexes (apart from cytochromes) would appear negligible.

The experimental uncertainty of the measurements in rats and rabbits is largely caused by the presence of acetaldehyde in blood and liver. In pigs and man the acetaldehyde concentration during alcoholaemia is much lower. These species may therefore be suitable for more accurate determination of the *in vivo* deuterium isotope effect. In these species advantage might also be taken of the dependence of cytochrome P_{450} catalysed ethanol oxidation on the ethanol concentration (K_m about 10 mM) to provide sufficient information to determine the contribntions of all three ethanol oxidizing components.

In vivo experiments on ethanol or acetone treated animals shonld also be performed before the possibilities of the approach by means of deuterium isotope effects may be considered exhausted.

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