Isotope effect in peroxidation of deuterium-labelled ethanol by liver catalase

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The kinetic V/K isotope effect upon peroxidation of (1R)-[1- $^2\text{H}_1$]ethanol by liver catalase was studied by using a radiochemical assay with both ^3H and ^{14}C as tracers. The value of 1.9 found is in agreement with the value of 2.52 for peroxidation of (1R)-[1- $^3\text{H}_1$]ethanol according to the 'Swain equation' [Swain, Stivers, Ruver & Schaad (1958) *J. Am. Chem. Soc.* **80**, 5885–5893]. There was no isotope effect on V , the maximal rate of peroxidation of (1R)-[1- $^2\text{H}_1$]ethanol, at rates of H_2O_2 generation/catalase haem up to 500 min^{-1} . Even at rates 20-fold higher the isotope effect on V may not be different from 1. A detailed description of the synthesis of the ^2H - and ^3H -substituted ethanol compounds and the analysis of these has been deposited as Supplementary Publication SUP 50110 (5 pages) at the British Library Lending Division, Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) **169**, 5.

The intrinsic primary isotope effect in an enzymic reaction sequence involving transfer of hydrogen may be calculated when the kinetic V/K isotope effects for both ^2H and ^3H are known (Northrop, 1975). The magnitude of the intrinsic primary isotope effect contributes to an understanding of the hydrogen transfer transition state (Jencks, 1969; More O'Ferrall, 1975). Reliable information about the intrinsic primary isotope effect can be obtained only when the values for the ^3H and ^2H V/K isotope effect are very accurate. In the peroxidation of (1R)-[1- $^3\text{H}_1$]ethanol by catalase from liver the V/K isotope effect was found to be 2.52 under near-physiological conditions (Damgaard, 1977). For peroxidation of (1R)-[1- ^2H]ethanol by catalase from liver the only isotope effect reported is 1.9–2.0 (Schonbaum & Chance, 1976). These authors used [1,1- $^2\text{H}_2$]ethanol, but no further experimental details are given and it is hence not certain whether their values are V/K or V isotope effects or hybrids of these two. As the V/K ^2H isotope effect is a prerequisite to obtain a measure of the intrinsic primary isotope effect, this value for liver catalase-mediated peroxidation of (1R)-[1- ^2H]ethanol has been determined. For this purpose a new radiochemical technique was employed. This technique is more sensitive and accurate than conventional enzyme kinetic methods and at the same time it offers the possibility of measurements of the V/K ^2H isotope effect of the enzymic reaction *in vivo*. In this last respect the new method is non-destructive as it

only requires analysis of the radioactivity in a small arbitrary fraction of reaction product, i.e. acetaldehyde or acetate.

By the use of conventional enzyme kinetics a complete absence of an isotope effect on V over a wide range of rates of H_2O_2 generation relative to haem concentration was established. This observation lends support to the view that the maximal rate of ethanol peroxidation by catalase is determined solely by the rate of formation of compound I. Catalase-mediated ethanol peroxidation *in vivo* at significant concentrations of ethanol is normally limited by the endogenous H_2O_2 generation (Oshino *et al.*, 1975). Therefore, at saturating concentrations of highly enriched (1R)-[1- ^2H]ethanol *in vivo*, no isotope effect is expected. However, when this compound or the corresponding ^3H -labelled compound is present in trace amounts in normal ethanol, the V/K isotope effect is obtained under all conditions (Simon & Palm, 1966).

Materials and methods

Enzymes

Catalase (EC 1.11.1.6) from female Wistar rat liver was prepared and tested as described by Price *et al.* (1962). Catalase from ox liver, alcohol dehydrogenase (EC 1.1.1.1) from yeast and glucose oxidase (EC 1.1.3.4) grade I were purchased from Boehringer, Mannheim, Germany.

Isotopes

[1-¹⁴C]Ethanol (27 Ci/mol) and D-[6-³H]glucose (500 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [U-²H]Ethanol (99% pure; Uvasol grade) was purchased from Merck, Darmstadt, Germany. The content of ¹H in the methylene position was checked by ¹H n.m.r. with an acetone standard in ²H₂O [δ (p.p.m.) 2.12]. The signal obtained at 3.49 p.p.m. was compatible with a protium content of less than 0.5% in this position. [1,2-¹⁴C]Acetaldehyde (8 Ci/mol) in the form of paraldehyde and ³H₂O (25 mCi/g) were purchased from New England Nuclear Corp., Boston, MA, U.S.A.

(1R)-[1,2-¹⁴C,1-²H]Ethanol was synthesized in the following way. After hydrolysis of the [1,2-¹⁴C]-paraldehyde the [1,2-¹⁴C]acetaldehyde was reduced to (1R)-[1,2-¹⁴C,1-²H]ethanol by enzymic transfer of ²H from L-[2-²H]lactate. This L-[2-²H]lactate had been synthesized from pyruvate by enzymic transfer of ²H from [U-²H]ethanol. The overall yield based on acetaldehyde was 75%. According to the ²H n.m.r. spectrum of the preparation with (²H)-acetone as standard (signal at 2.1 p.p.m.) the ²H content in the methylene position was 45–50% judged from the signal obtained at 3.5 p.p.m. Details of this synthesis are deposited as Supplementary Publication SUP 50110 (5 pages) with the British Library Lending Division, Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) **169**, 5.

[2-³H]Ethanol was made by fermentation of D-[6-³H]glucose with bakers yeast (De danske Spritfabrikker, Copenhagen, Denmark) and was purified by repeated distillations. The yield was 71% based on glucose. Less than 0.7% of the ³H was present in the methylene position and less than 0.03% in water in the final [2-³H]ethanol product. Details of this procedure and of the analysis of the final product are deposited with the B.L.L.D. as described above.

Chemicals were of analytical grade when not otherwise specified.

Enzymic assays

Ethanol (Bonnichsen, 1962) and acetaldehyde (Bergmeyer, 1962) were determined by the methods indicated.

Counting of radioactivity

Aqueous samples (1 ml) in 10 ml of scintillation liquid prepared as described by Anderson & McClure (1973) were counted simultaneously for ³H and ¹⁴C with efficiencies near to 50% for both isotopes in a dual channel liquid-scintillation spectrometer. No ³H counts appeared in the ¹⁴C channel; corrections were made for a reproducible overlap of

50% from the ¹⁴C channel into the ³H channel. In each experiment internal standards of [1-¹⁴C]-ethanol and ³H₂O were counted. The results of these controls did not indicate the need for further corrections. For the lowest radioactivities counting was performed long enough to ensure s.d. values of 2–5%. Generally, s.d. values of 0.5% or lower were obtained.

Radiochemical assay for the ²H isotope effect

As substrate the following mixture was used. Aqueous solutions of (1R)-[1,2-¹⁴C,1-²H]ethanol and [2-³H]ethanol were mixed with unlabelled ethanol in such proportions that the final mixture had specific radioactivities for ³H of 27–80 and for ¹⁴C of 8–40 c.p.m./nmol of ethanol and an enrichment of ²H of less than 1% in the 1-*pro-R* position of the ethanol. To lower the blank value obtained in the assay when this mixture was used as substrate, traces of acetaldehyde were removed by incubation with semicarbazide at a concentration of 15 mM at pH 6.5–7.5. After 24 h, ethanol and water were collected in frozen form on a 'cold finger' containing solid CO₂ in a closed glass tube. In control experiments the substrate mixture was prepared in the same way, but (1R)-[1,2-¹⁴C,1-²H]ethanol was replaced by [1-¹⁴C]ethanol.

The reaction mixture was 2 ml of 0.1 M-potassium phosphate, pH 7.0 at 22°C, containing 0.13–13 nmol of catalase haem and the substrate mixture at ethanol concentrations between 4 and 24 mM. The reaction mixture was kept at 37°C in a water jacketed glass vessel equipped with efficient magnetic stirring. The peroxidation of ethanol was initiated by immersion of the tip of a thin (1.0 mm) plastic tube from which a freshly diluted stock solution of H₂O₂ was delivered at a rate of 10 μ l/min controlled by a Unita IIb piston pump. During the incubation the air over the reaction mixture was slowly removed by aspiration and was led through 2 ml of ethanol contained in a glass tube at –70°C to trap the acetaldehyde. This tube was changed at various intervals. Portions (1.5 ml) of each of these samples were transferred to quartz spectrophotometer cuvettes (1.0 cm light path). The increase in A_{223} after addition of 0.50 ml of a 9 mM solution of semicarbazide hydrochloride in water was measured in a spectrophotometer. Blanks and standards in ethanol were measured simultaneously. The formation of the acetaldehyde semicarbazone (ϵ 8.8 mm⁻¹·cm⁻¹) was usually completed within 10–15 min, whereafter the A_{223} became constant. The content of each cuvette was then mixed with 135 μ mol of semicarbazide in 0.35 ml of 0.05 M-potassium phosphate, pH 5.0, in a counting vial. The content was evaporated to dryness under a gentle stream of N₂, redissolved in 1.0 ml of water and the radioactivity was counted. The concentration of

ethanol and the radioactivities of both ^3H and ^{14}C were determined after deproteinization with perchloric acid and neutralization with KOH. The ratio between the ^3H and ^{14}C radioactivities never deviated from that measured directly in the substrate mixture.

Non-radioactive assays for the ^2H isotope effect

These assays, assays I and II, were made in a conventional enzyme-kinetic way by comparing the two types of ethanol, i.e. normal and $[\text{U-}^2\text{H}]\text{ethanol}$, as substrates for the peroxidation of ethanol by catalase. The acetaldehyde formed in this reaction oxidized NADH in the presence of alcohol dehydrogenase, and the decrease in A_{340} was taken as a measure of ethanol peroxidation. H_2O_2 was generated at a constant rate by glucose oxidase and was used by catalase for peroxidation of ethanol. No reaction was observed in the absence of catalase. The procedure was essentially that of the standard assay for the ^3H isotope effect determination as used by Damgaard (1977). The reactions were carried out in a series of cuvettes, with a light path of 1.0 cm. The contents of the new cuvettes differed only in ethanol type and concentration. At the end of measurement one sample was withdrawn from each cuvette for determination of ethanol. From double reciprocal plots (Lineweaver & Burk, 1934) K_m and V with either ethanol or $[\text{U-}^2\text{H}]\text{ethanol}$ under identical conditions were obtained.

At high concentrations of ethanol non-linear plots were obtained in assay I. Therefore, a direct comparison of the measured rates of peroxidation of ethanol—was made in assay II. The buffered (0.1 M-potassium phosphate, pH 7.0 at 22°C)

solutions, containing a dialysed mixture of the three enzymes in the desired proportions plus NADH, were divided between two identical spectrophotometer cuvettes. The first one of these received ethanol and the second received $[\text{U-}^2\text{H}]\text{ethanol}$ in amounts to yield closely similar concentrations. The A_{340} was constant until glucose was added, when it decreased at a constant rate. After the initial peroxidation rate had been determined, the rate of H_2O_2 generation (dx/dt) was determined by adding extra catalase, to yield a concentration of 50 nm-haem. In this way the affinity for ethanol was increased to render the system completely peroxidatic at the high concentrations of ethanol employed (Oshino *et al.* 1973, 1974). To check that the activity of alcohol dehydrogenase was at least 100-fold higher than the measured rate of peroxidation, 3.5 μmol of acetaldehyde in 5 μl of water was added at the end of the measurement.

Results and discussion

The results of a single experiment by the radiochemical assay are shown in Table 1. After the start of infusion of H_2O_2 radioactivity appears in the samples. Considering the possibility of loss of acetaldehyde during the evaporation procedure there is a fair agreement between the amount of acetaldehyde determined from the A_{223} and from the ^3H radioactivity. The delay imposed on the conversion of the ^{14}C -labelled molecular species by the presence of ^2H in the 1-*pro-R* position is apparent, and the increase in the ratio between ^3H and ^{14}C radioactivity in acetaldehyde (R_p) compared with the ratio in ethanol (R_s) allows calculation of the

Table 1. Determination of the primary first-order kinetic ^2H isotope effect on the catalase-catalysed peroxidation of ethanol

Incubation and sampling were as described in the Materials and methods section. The reaction mixture contained 1.31 nmol of ox liver catalase haem. The concentration of ethanol decreased from 4.0 to 2.3 mM during the experiment. From 45 min and to the end of the experiment H_2O_2 was infused at a rate of 320 nmol/min. Specific radioactivities of the substrate were, for ^3H , 27.4 c.p.m./nmol and, for ^{14}C , 8.4 c.p.m./nmol of ethanol. The substrate ratio (R_s) was thus 3.25. The product ratio (R_p) measured in each sample is shown in a separate column. The average isotope effect (R_p/R_s) determined in samples 4–9 was 1.922.

Sample no.	Period (min)	Acetaldehyde in sample (nmol) calculated from:			Product ratio (R_p)	Isotope effect (R_p/R_s)
		A_{223}	^3H radioactivity	^{14}C radioactivity		
1	0–15	0.5	0.0	0.0	—	—
2	15–30	0.2	0.2	0.2	2.311	0.711
3	30–45	4.2	1.8	0.8	7.26	2.233
4	45–75	44.3	16.2	8.4	6.308	1.941
5	75–90	42.5	25.0	13.2	6.200	1.907
6	90–105	41.3	30.2	15.6	6.268	1.928
7	105–120	37.1	32.6	17.2	6.175	1.900
8	120–137	44.1	38.6	19.8	6.351	1.954
9	137–152	36.2	30.6	16.0	6.178	1.901

isotope effect. In this experiment the average V/K isotope effect for samples 4–9 was 1.922. In two control experiments, like the one shown in Table 1, the average isotope effects obtained were 0.987 ± 0.025 and 1.054 ± 0.054 .

Table 2 shows similar experiments with catalase from rat or ox liver. Variation of the rate of H_2O_2 generation per catalase haem did not alter the V/K isotope effect. This observation is further corroborated by the lack of effect of azide on the V/K isotope effect. In two experiments with 0.4 mM-NaN₃ in the reaction medium, the V/K isotope effects obtained in the radiochemical assay were 1.88 and 1.89. In these experiments only about 3% of the catalase activity remained. In the presence of azide, the concentration of catalase haem available

for catalysis is greatly diminished (Nicholls, 1964; Oshino *et al.*, 1974), and hence the rate of H_2O_2 generation per active haem becomes very large.

The results obtained with the non-radioactive assays are shown in Table 3. The V/K 2H isotope effects measured with assay I are close to those obtained with the radiochemical assay, although the variation is larger. The values are also close to those obtained by Schonbaum & Chance (1976), who found almost identical isotope effects with [1,1- 2H_2]ethanol and [U- 2H]ethanol. The complete absence of isotope effect upon V is apparent from Table 3 for values of $(1/e) \cdot (dx/dt)$ up to about 500 min^{-1} .

With increasing rates of H_2O_2 generation per catalase haem, $(1/e) \cdot (dx/dt)$, the affinity for ethanol

Table 2. 2H primary isotope effect upon peroxidation of ethanol catalysed by liver catalase at 37°C

The mean and S.D. of the isotope effects obtained from ten experiments similar to the one described in Table 1 are shown. The haem concentration (e) and the rate of H_2O_2 infusion (dx/dt) were varied within the ranges indicated.

Enzyme source	$(1/e) \cdot (dx/dt)$ (min^{-1})	[Ethanol] (mM)	Isotope effect	Number of experiments
Rat liver	100–900	5–24	1.906 ± 0.052	5
Ox liver	24–3600	4–8	1.912 ± 0.072	5

Table 3. Isotope effect upon maximal rate of [U- 2H]ethanol peroxidation by ox liver catalase

Rates of ethanol peroxidation were determined in a spectrophotometer at 340 nm; the presence of alcohol dehydrogenase allowed the oxidation of NADH by the acetylaldehyde formed in the peroxidation of ethanol. The technical details of the two assays are given in the Materials and methods section. The temperature in the reaction cuvette was 37°C unless otherwise indicated. e , The concentration of catalase haem; dx/dt , the rate of H_2O_2 generation; V and K_m , the maximal rate of ethanol peroxidation and the concentration of ethanol giving half maximal rate of peroxidation, respectively; $D_{(V/K)}$, the 2H isotope effect upon the first-order rate constant of the reaction with respect to ethanol, was determined as the ratio between the slopes obtained with [U- 2H]ethanol and ethanol in the Lineweaver–Burk plot; $D_{(V)}$, the 2H isotope effect upon V , i.e. the ratio between the V obtained with ethanol and the V obtained with [U- 2H]ethanol; v_H and $v_{^2H}$, the rates of ethanol peroxidation measured directly in assay II. The apparent isotope effect is the ratio between the two. No peroxidation of ethanol could be detected in absence of added catalase.

Assay I

Temperature (°C)	e (nM)	dx/dt ($\text{nM} \cdot \text{min}^{-1}$)	$(1/e) \cdot (dx/dt)$ (min^{-1})	V ($\text{nM} \cdot \text{min}^{-1}$)	K_m (mM)	$D_{(V/K)}$	$D_{(V)}$
22	159	419	2.6	490	0.2	2.05	1.0
22	159	833	5.2	606	0.9	1.84	1.0
22	159	1667	10.6	2000	1.3	1.89	1.0
37	16	8333	526	8200	40	2.00	1.0

Assay II

Expt. no.	e (nM)	dx/dt ($\text{nM} \cdot \text{min}^{-1}$)	$(1/e) \cdot (dx/dt)$ (min^{-1})	[Ethanol] (mM)	v_H ($\text{nM} \cdot \text{min}^{-1}$)	$v_{^2H}$ ($\text{nM} \cdot \text{min}^{-1}$)	$v_H/v_{^2H}$
1	281	4824	17	311	5025	5147	0.98
2	115	5016	46	311	5178	5178	1.00
3	12.8	5121	421	311	4338	4139	1.05
4	0.6	5199	8772	311	2027	1146	1.78
5	0.4	4110	10024	600	2799	2205	1.27
6	0.4	4435	11088	1080	1995	1774	1.12
7	0.4	2900	7073	1009	1543	1307	1.18

decreases (Oshino *et al.*, 1973) and very high concentrations of ethanol are required to obtain rates of ethanol peroxidation sufficiently close to V to make the graphical determination of this parameter precise enough for determination of the V isotope effect. A limit is set by the fact that concentrations of 100–300 mM-ethanol inhibit the assay system. Ethanol concentration is the independent parameter in assay I and at high concentrations non-linear double-reciprocal plots were obtained. Therefore, at high values of $(1/e) \cdot (dx/dt)$, assay II, in which the concentration of ethanol was kept constant at saturating or nearly saturating concentrations, was used. In Table 3, assay II, Expts. 1, 2 and 3 confirm the absence of isotope effect on V . In these experiments the concentration of ethanol is saturating and the rates of peroxidation obtained with $[U-^2H]$ ethanol, v_{H_2} , and with ethanol, v_H , are both equal to the rate of H_2O_2 generation (dx/dt) and are therefore V rates. In Expt. 4, the concentration of haem is decreased 21-fold with a corresponding increase in $(1/e) \cdot (dx/dt)$. Under this condition the concentration of ethanol is no longer saturating, as seen by the fact that v_H is much smaller than dx/dt . Hence the isotope effect v_H/v_{H_2} of 1.78 is a hybrid between the V and the V/K isotope effect. In Expts. 5, 6 and 7 where the concentration of ethanol is increased in an attempt to approach a V condition at this extreme value of $(1/e) \cdot (dx/dt)$, $(1/e) \cdot (dx/dt)$ is approximately the same as in Expt. 4.

It can be seen that the isotope effect, v_H/v_{H_2} , decreases as the V condition is approached. Inhibition of the assay system by ethanol at these concentrations can be seen by the concomitant decrease in v_H . Most probably catalase itself is inhibited since the mere addition of more catalase (see assay II) allowed measurement of a significantly higher rate of H_2O_2 generation, dx/dt . Extrapolation of the small isotope effects obtained under these extreme kinetic conditions to an infinite concentration of ethanol suggests a still smaller, or even absent, V isotope effect. For the range of physiological $(1/e) \cdot (dx/dt)$ values (rates of H_2O_2 generation per catalase haem) (Oshino *et al.*, 1975) it may safely be concluded that the V isotope effect is absent.

A kinetic analysis of the difference between V/K and V isotope effects has been given by Northrop (1977). Both isotope effects reflect the intrinsic primary isotope effect, but this is not usually expressed fully due to the existence of other steps in the catalytic reaction sequence. The existence of one or more steps slower than the 'bond-breaking step' at which the intrinsic primary isotope effect occurs can completely prevent this effect from being expressed in the net catalytic rate. In this respect, the V/K isotope effect and the V isotope effect do not depend on the same reaction steps in the catalytic reaction

sequence. In this irreversible reaction, the V/K isotope effect depends only on the steps situated between association of the labelled ethanol and the 'bond-breaking step'. The V isotope effect depends on the steps leading from the 'bond-breaking step' and to regeneration of the enzyme species (compound I) which reacts with the labelled ethanol. Among these steps are the association of H_2O_2 with the enzyme and the subsequent formation of compound I, which have been suggested to be rate-limiting for ethanol peroxidation (Schonbaum & Chance, 1976). The absence of a V isotope effect strongly supports their view. The insensitivity of the V isotope effect to increases in the H_2O_2 concentration suggests that the intra-enzymic reaction leading to compound I, rather than the association of H_2O_2 , is rate limiting.

Comparison of the V/K 2H isotope effect obtained in this study with the 3H effect obtained previously (Damgaard, 1977) reveals a complete agreement with the Swain relationship which links primary 2H and 3H isotope effects to each other on a quantum-mechanical basis (Swain *et al.*, 1958). When enzyme kinetic isotope effects obey this relation it means that the intrinsic primary isotope effect is fully expressed in the kinetic isotope effect due to absence of interfering rate-limiting steps. The intrinsic primary 2H isotope effect of 1.9 determined in this way is significantly lower than the theoretical one of 6–7 (Jencks, 1969; Buddenbaum & Shiner, 1977). This indicates conservation of some of the zero-point energy of the bond to the 1-*pro-R* hydrogen atom in ethanol in the transient reaction complex at the 'bond-breaking step'. This can occur when the reaction complex is non-linear (Jencks, 1969; More O'Ferrall, 1975). The 2H isotope effect of 5.5 for the catalase-mediated peroxidation of methanol studied by Schonbaum & Chance (1976) may then indicate a more linear transient reaction complex with the smaller substrate molecule. In contrast, the very small isotope effect (1.1) for catalase-mediated peroxidation of formic acid reported by the same authors is more likely to have a kinetic explanation, i.e. a V isotope effect. For this reaction Frei & Aebi (1958) and Aebi (1964) found isotope effects increasing from 1 to 1.5 when the catalase haem concentration was decreased. This change in isotope effect can be interpreted as a shift between a V and a V/K isotope effect upon increasing the rate of H_2O_2 generation per catalase haem. Such a change is known to decrease the affinity to the hydrogen donor, formic acid or ethanol (Keilin & Hartree, 1955; Oshino *et al.*, 1973). A similar change in the apparent isotope effect upon peroxidation of ethanol has been predicted on a theoretical basis by Rognstad (1974). The fact that the V/K 2H isotope effect of 1.5 for peroxidation of formate could only be reproduced *in vivo* when dilution of the labelled

compound with endogenous formate could occur (Aebi *et al.*, 1957) supports this view, as such dilution prevents the appearance of *V* isotope effects (Simon & Palm, 1966).

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