Tritium Effect in Peroxidation of Ethanol by Liver Catalase

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1. Simultaneous determination of the rate of appearance of ^3H in water from [(1*R*)-1- $^3\text{H}_1$]-ethanol and the rate of acetaldehyde formation in the presence of rat or ox liver catalase under conditions of steady-state generation of H_2O_2 allowed calculation of the ^3H isotope effect. The mean value of 2.52 obtained for rat liver catalase at 37°C and pH 6.3–7.7 was independent of both ethanol concentration and the rate of H_2O_2 generation over a wide range. At 25°C a slightly lower mean value of 2.40 was obtained with the ox liver catalase. 2. Neither the product, acetaldehyde, nor 4-methylpyrazole influenced the two rates measured in the assay. 3. Relating the value obtained for the ^3H isotope effect to a known value for the ^2H isotope effect strongly supports the view that both values are close to the true isotope effect with the respective substituted compounds on the rate constant in the catalytic step involving scission of the C–H bond. 4. The constancy of the isotope effect under various conditions makes it possible to use it for interpretations *in vivo*. 5. It was established that β -D-galactose dehydrogenase exhibits B-specificity towards the nicotinamide ring in NAD.

The use of [1- ^3H]ethanol as a tracer for hydrogen in mammalian liver metabolism has provided much important information (Rognstad & Clark, 1974; Cronholm, 1974; Hoberman & Carnicero, 1974; Grunnet *et al.*, 1976).

Quite often the primary effect of isotopic substitution on reaction velocities is an important parameter in these studies. Under physiological conditions, however, the exact value of this is usually not known in advance, but has to be evaluated from results that are too complex to allow safe estimates. At least two enzyme systems present in the liver catalyse the oxidation of ethanol to acetaldehyde *in vitro*. The first of these is alcohol dehydrogenase, which, when saturated with ethanol, operates in a reversible manner under physiological conditions and therefore exhibits a relatively low ^3H isotope effect (Damgaard, 1977). The second enzyme is catalase. This enzyme is generally thought to be quantitatively less important *in situ* owing to lack of the second substrate, H_2O_2 . However, as the residual ethanol oxidation in liver after complete inhibition of alcohol dehydrogenase with pyrazole or 4-methylpyrazole may at least in part be attributed to catalase (Rognstad, 1974), it has become important to determine the ^3H isotope effect in this reaction under conditions that can be compared with the physiological ones. Apart from this, determination of ^3H isotope effects in catalase-catalysed oxidation of ethanol may yield information about the catalytic mechanism of the enzyme system.

Part of this work has been presented in a preliminary communication at the Second International

Symposium on Alcohol and Aldehyde Metabolizing Systems in Philadelphia, October 15–17, 1976 (Damgaard, 1977).

Materials and Methods

Enzymes

Alcohol dehydrogenase (EC 1.1.1.1) from yeast and horse liver, peroxidase (EC 1.11.1.7) from horseradish (grade I), catalase (EC 1.11.1.6) from ox liver, glucose oxidase (EC 1.1.3.4) of fungal origin, β -galactose dehydrogenase (EC 1.1.1.48) from *Pseudomonas fluorescens*, hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from yeast were from Boehringer, Mannheim, Germany.

Before use the liver alcohol dehydrogenase was dialysed for 1 h against 400 vol. of 0.1 M-potassium phosphate buffer, pH 7.0, at 3°C. This dialysis was repeated three times to remove ethanol. Liver catalase from female albino Wistar rats (weighing about 200 g) was prepared and the purity measured by the method of Price *et al.* (1962). The final preparation was 98% pure. This preparation was stored in 30% (v/v) glycerol at 3°C and found to be stable for at least 6 months.

Isotopes

[1,1- $^3\text{H}_2$]Ethanol (specific radioactivity about 100 mCi/mmol) was originally from New England Nuclear Corp., Boston, MA, U.S.A. Since that product on several occasions was found to exchange

more than 90% of its radioactivity with water, a more reliable preparation was obtained from ICN, Irvine, CA, U.S.A.

The procedures described for synthesis of stereo-specifically labelled ethanol utilize the fact that alcohol dehydrogenase reacts stereospecifically with both position-4 of the nicotinamide ring of NAD⁺ and position-1 of the other substrate (see review by Goodwin, 1973).

Preparation of [(1R)-1-³H₁]ethanol. D-[1-³H]Galactose (specific radioactivity 5.2 Ci/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.) was evaporated to dryness, redissolved in water and used for preparation of [(1R)-1-³H₁]ethanol as follows. A solution (110 μl) containing 0.05 mg of galactose dehydrogenase, 0.05 mg of dialysed liver alcohol dehydrogenase, 0.015 mg of yeast alcohol dehydrogenase and 0.5 μmol of NAD⁺ was added to a quartz spectrophotometer cuvette (1.00 cm light-path) containing 3 ml of 0.1 M-potassium phosphate, pH 7.5, at room temperature (22°C). The reactions were followed by recording the A₃₄₀. Addition of 0.35 μmol of D-[1-³H]galactose resulted in the transfer of ³H to the accumulating NADH. When this reaction was complete, 0.38 μmol of acetaldehyde was added, which caused a rapid reoxidation of the accumulated NADH. After this the ³H was found exclusively in NAD⁺, indicating that the β-D-galactose dehydrogenase has B-specificity towards NAD⁺. The reduction of NAD⁺ by addition of D-[1-³H]galactose and reoxidation of NADH by addition of acetaldehyde was repeated three times. Then unlabelled D-galactose was added in excess to obtain complete reduction of NAD⁺. When this was completed, NADH was reoxidized by addition of sufficient acetaldehyde. This procedure was chosen as it allowed each step to be followed in the spectrophotometer. After the last reaction was completed, 4 μmol of AgNO₃ was added in 400 μl of water to inactivate the enzymes, and about 1 mmol of carrier ethanol was added together with 10 ml of water. The latter was used to facilitate quantitative transfer of the reaction mixture into a 50 ml distillation flask. The yield of labelled ethanol after three consecutive redistillations from water was 76%. The specific radioactivity was about 4 × 10⁶ c.p.m./μmol and the concentration was 400 mm-ethanol in about 2 ml of water.

Preparation of [(1S)-1-³H₁]ethanol. [1,1-³H₂]Ethanol (100 μl, 8 M) in water was added to 400 μl of potassium dichromate in H₂SO₄ (Henry *et al.*, 1948) contained in a 10 ml flask. The flask was fitted with a bent glass tube leading into 3 ml of water contained in a small test tube cooled in ice. After 5 min, 3 ml of water and two pumice stones were added, and on gentle heating of the mixture to boiling acetaldehyde was collected together with 0.5 ml of water in the 3 ml of water in the test tube. This acetaldehyde was

further purified by carrying it on a gentle stream of N₂ through four consecutive tubes each containing 5 ml of water at 32°C. The product was finally collected in a series of three tubes in ice each containing 2 ml of water. The yield of radioactivity was 40%. The specific radioactivity of the acetaldehyde was half that of the ethanol and it did not exchange its ³H with water even at 95°C. This [1-³H]acetaldehyde was reduced with NADH and alcohol dehydrogenase to give [(1S)-1-³H]ethanol.

Before use, each preparation of ethanol was purified to give an approx. 1:10 (w/w) ethanol/water mixture in which the total amount of radioactivity in or exchangeable with water was in the range 0.05–0.005% of the total radioactivity. This mixture was obtained by repeated distillations through a 1.5 cm × 15 cm glass column filled with 5 mm glass rings. In each distillation the small amount of ethanol was isolated almost quantitatively in an equal or smaller volume of pure water after refluxing through the column. The amount of radioactivity contained in water was determined after the radioactive ethanol had been 'chased' out of the water by a standard procedure, which is described below.

[1-¹⁴C]Ethanol (specific radioactivity 9 mCi/mmol) was from New England Nuclear Corp. and purified by fractionated distillations.

o-Dianisidine of minimum purity 98% was from BDH Chemicals, Poole, Dorset, U.K., and was recrystallized from 25% (v/v) acetone in water containing Acid Washed Activated Charcoal (Sigma, St. Louis, U.S.A.) and 10% (w/w) of *o*-dianisidine, and converted into the hydrochloride. Benzene was dried over NaOH. Other chemicals were of analytical grade. 4-Methylpyrazole was a gift from Dr. S. E. Hansen from this Department.

Isotope-effect determination

Two assays were used. Both were based on the simultaneous determination of the rate of ³H appearance in water and the rate of acetaldehyde production during oxidation of [(1R)-1-³H]ethanol by catalase.

Standard assay. Glucose oxidase (0.0005–0.025 mg), 0.15 mg of yeast alcohol dehydrogenase, 0.05 mg of horse liver alcohol dehydrogenase and an amount of catalase corresponding to between 178 and 1250 pmol of haem were mixed in a small cellophan bag containing 2.5–4.0 ml of 0.1 M-potassium phosphate, pH 7.0 (unless otherwise specified). Catalase haem was measured as the cyanide complex by the method of Oshino *et al.* (1973). The contents of the cellophan bag were dialysed twice for 1 h at 3°C against 200 ml of the same buffer to decrease the content of glycerol and NH₄⁺ ions, and thymol, which is present in commercial ox liver catalase.

The contents of the bag were transferred to a glass

spectrophotometer cuvette with a light-path of 2.00 cm mounted in a Zeiss PMQ II spectrophotometer equipped with automatic recording devices. The temperature in the cuvette was kept at 25°C or 37–38°C during the course of reactions. To the enzyme mixture in the cuvette was added 1.5 mg of NADH dried *in vacuo*, which caused the A_{340} to increase to about 0.9. Then the ^3H -labelled ethanol preparation was added. At this time the first sample was withdrawn. Each sample was of 0.20–0.50 ml, and the protein was immediately precipitated by adding an equal volume of ice-cold 1 M-HClO₄. The supernatant after centrifugation (10⁴g for 10 min) was neutralized with 2 M-KOH at 0°C. In each experiment the ethanol concentration was determined in two or more of the neutral supernatants. In the same supernatants total ^3H radioactivity was counted to obtain the specific radioactivity of ethanol. Before determination of $^3\text{H}_2\text{O}$ each neutral supernatant was diluted 5–10-fold with water, distilled *in vacuo* at 60°C and the radioactive ethanol removed by 'chasing' as described below. Between 5 and 30 min later the second sample was withdrawn. Immediately thereafter the reaction was initiated by addition of glucose to give a desired concentration between 0.05 and 7.5 mM, corresponding to H₂O₂ production rates of respectively 0.13 and 10 nmol of H₂O₂/min per μg of glucose oxidase (Fig. 1).

In some experiments, the enzyme mixture was divided equally between two spectrophotometer cuvettes of 1.00 cm light-path after the ^3H -labelled ethanol had been mixed in. In the subsequent steps these two cuvettes were treated in the same manner, except that one did not receive glucose.

The oxidation of NADH was followed at 340 nm and taken as a measure of the peroxidation of ethanol to which it was coupled in the presence of alcohol dehydrogenase. At suitable times after glucose addition, the third and subsequent samples were withdrawn.

In control experiments alcohol dehydrogenase and NADH were not added to the reaction mixture. Acetaldehyde was thus allowed to accumulate during the reaction. To assess the degree of reaction [$1\text{-}^{14}\text{C}$, (1R)-1- $^3\text{H}_1$]ethanol was used as substrate. Samples for determination of $^3\text{H}_2\text{O}$ were treated as described above. Samples (1.0 ml) for acetaldehyde determination were taken in closed vessels containing 0.50 ml of 1 M-HClO₄ cooled in ice. After neutralization with 2 M-KOH in the cold, 250 μmol of semicarbazide was added to 1.00 ml of the clear supernatant, and after 24 h the labelled ethanol and water were removed by evaporation to dryness, followed by three cycles of dissolving the residue in 0.5 ml of 95% (v/v) ethanol and evaporation to dryness under N₂. The residual radioactivity did not decrease after repeated evaporations of ethanol and

was taken as a measure of [$1\text{-}^{14}\text{C}$]acetaldehyde semicarbazone. The specific radioactivity of [$1\text{-}^{14}\text{C}$]ethanol (260 000 c.p.m./ μmol) was used to calculate the amount of acetaldehyde formed. Recovery of [$1\text{-}^{14}\text{C}$]acetaldehyde was 95–100%.

Assay with direct H₂O₂ infusion. The reaction took place at 25°C in a glass cuvette (2.0 cm light-path) equipped with efficient magnetic stirring. The reaction medium was as described above for determination of the catalase isotope effect, except for the omission of glucose and glucose oxidase. Sampling for determination of $^3\text{H}_2\text{O}$ was the same as described above. 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 between 3.75 and 25 $\mu\text{l}/\text{min}$ controlled by a Unita IIB piston pump (B. Braun, Melshungen, Germany).

The rate of ethanol peroxidation to acetaldehyde was recorded at 340 nm as described above. Before H₂O₂ was infused, the NADH concentration was constant, as reflected by a stable A_{340} value.

Determination of the rate of H₂O₂ generation in the reaction mixture

Method 1. In the Zeiss PMQ II spectrophotometer the increase in A_{436} was followed at 25°C or 37–38°C in cuvettes of 1.00 or 2.00 cm light-path containing 0.02 mg of peroxidase, 0.16 mg of glucose oxidase and 0.10 mg of *o*-dianisidine in 2.00 ml of 0.10 M-potassium phosphate, pH 7.0.

The reactions were started by addition of 50 μl of various concentrations of glucose to give concentrations in the reaction mixture between 0.0 and 0.1 M. After 10 min the reactions were completed. The standard curves were identical straight lines at both temperatures, with an absorption coefficient of 10³ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. This value was used to convert the initial reaction rates in similar assays at lower concentrations of glucose oxidase and higher glucose concentrations into μmol of H₂O₂ produced/min (see Fig. 1). The rate of direct H₂O₂ infusion in the catalase-isotope-effect assay without glucose oxidase was determined by the *o*-dianisidine-plus-peroxidase method. The content of catalase in both peroxidase and glucose oxidase was negligible as judged from the 90% recovery of A_{436} when *o*-dianisidine was not added until 30 min after addition of glucose.

Method 2. The rate of disappearance of O₂ was recorded by means of an oxygen electrode (no. 451-5352; Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) at 37°C in solutions of 2.5 ml of 0.10 M-potassium phosphate, pH 7.0, containing various concentrations of glucose and glucose oxidase in the presence or absence of 50 mM-ethanol. The electrode signal was calibrated by addition of sodium dithionite in small amounts to remove O₂ completely

Fig. 1. Determination of the rate of H_2O_2 generation by glucose oxidase under the conditions of the catalase assay. Rate of H_2O_2 production in nmol/min per μg of glucose oxidase at 37°C in 0.10M-potassium phosphate, pH 7.0, was assayed at various concentrations of D-glucose: Δ , with *o*-dianisidine and peroxidase; Δ , same, in the presence of 50 mM-ethanol; $\circ$, assayed with the oxygen electrode; $\bullet$, same, in the presence of 50 mM-ethanol. Each point represents the mean of three determinations at 0.5, 1.25 and 2.5 μg of glucose oxidase. The range was too small to be shown except in one case.

from the reaction mixture. It was assumed that the signal was linearly related to O_2 concentration. This was in agreement with the linear relationship obtained between electrode signal and time during the enzyme assays. Results are shown in Fig. 1.

Removal of ethanol from water samples

The equipment is shown in Fig. 2. The water sample was heated to between 65 and 75°C while a stream of dry air mixed with either benzene or ethanol vapour was passed through it. Benzene causes removal of ethanol owing to azeotrope (b.p. 64.6°C) formation. The ethanol vapour dilutes the radioactivity of the labelled ethanol. Every 7 min the organic component in the dry-air stream was changed. After eight changes the sample no longer contained any radioactivity from ethanol. The air condenser is a glass tube of 6 mm diameter and 1500 mm high.

During treatment of the sample in the 'chasing' apparatus, two effects tend to change the specific radioactivity of the water (Fig. 3). The isotope effect on evaporation of water tends to increase the specific radioactivity, whereas the dilution of water hydrogen atoms with hydrogen atoms from the hydroxyl group of ethanol tends to lower the specific radioactivity. Both effects are relatively small, since usually not more than 30% of the sample water is removed, and

Fig. 2. Apparatus for removing labelled ethanol from water samples before determination of $^3\text{H}_2\text{O}$.

The sample is heated in the flask (A) to keep the temperature between 65 and 75°C in the lower part of the air condenser (B) as read by the thermometer (C), which is in close contact with the condenser inside the insulation (D). Mild constant suction from the top of the air condenser causes a stream of air to pass through the sample carrying with it either dry ethanol vapour or dry benzene vapour according to the position of the stopcock-equipped arm (E), which according to its position will take in air through either the tube with ethanol (F) or the tube with dry benzene (G). The arm (E) automatically changes its position every 7 min.

the amount of ethanol passed through the sample is small (10–15%). As a routine, the reliability of the apparatus was controlled by sampling during the 'chasing' procedure. In six controls the recovery of standards was $99.4 \pm 2.5\%$ (s.d.).

Counting of radioactivity

Aqueous samples (1 ml) in 10 ml of scintillation liquid prepared as described by Anderson & McClure (1973) were counted for ^3H with an efficiency of 40% and for ^{14}C with an efficiency of 70% in a liquid-scintillation spectrometer. In general, the radioactivities of samples were counted long enough to ensure an s.d. of 0.1%. For the lowest radioactivities, however, s.d. values of 2–5% were accepted.

Fig. 3. ^3H isotope effect in the 'chasing' procedure. Samples containing 4 ml of water and $300\mu\text{l}$ of $^3\text{H}_2\text{O}$ standard were 'chased' in the set-up described in Fig. 2, but without changes in the composition of the air stream. In the first experiment (○), the sample was 'chased' with benzene vapour in air and in the second experiment (△) with ethanol in the air. Samples were counted for radioactivity directly before the start and when the amount of sample had decreased as indicated on the abscissa. ●, Samples from a routine control 'chasing' of a standard $^3\text{H}_2\text{O}$ sample with altering composition of the air stream.

Enzymic assays

Glucose was determined with hexokinase and glucose 6-phosphate dehydrogenase as described by Slein (1962). Ethanol was determined as described by Bonnichsen (1962). Acetaldehyde was determined as described by Bergmeyer (1962).

Results

Production of $^3\text{H}_2\text{O}$

The appearance of ^3H in water during the rat liver catalase-catalysed peroxidation of [(1*R*)-1- $^3\text{H}_1$]ethanol at 37°C proceeds linearly for 40 min (Fig. 4). At the end of the incubation the acetaldehyde concentration had reached 0.18 mM without affecting the reaction rate. At concentrations of ethanol as low as 0.13 mM the production of $^3\text{H}_2\text{O}$ appeared to be linear up to 25% of total reaction. It may be concluded that

Fig. 4. Production of $^3\text{H}_2\text{O}$ from [(1*R*)-1- ^3H]ethanol during its peroxidation to acetaldehyde catalysed by rat liver catalase

Two independent experiments carried out under similar conditions are shown. Both proceeded in test tubes at 37°C in 2.50 ml of 0.10 M-potassium phosphate, pH 7.0, containing 0.178 nmol of catalase haem, $1.78\mu\text{g}$ of glucose oxidase and 40 mM-[(1*R*)-1- $^3\text{H}_1$]ethanol. Each point represents one sample withdrawn from the reaction medium. After the second sample, the peroxidation of ethanol was initiated by addition of $2.50\mu\text{mol}$ of D-glucose as indicated by the arrows (1 and 2). In the second experiment (△), 4-methylpyrazole ($2\mu\text{mol}$) was added after 21 min, as indicated by the arrow (3). ▲, Samples withdrawn after addition of 4-methylpyrazole.

the rate of glucose oxidase reaction is insensitive to changes in O_2 concentration under these conditions where glucose concentration apparently is limiting (Gibson *et al.*, 1964; Bright & Gibson, 1967).

^3H isotope effect in the rat liver catalase reaction

The ratio of the rate of oxidation of unlabelled to that of ^3H -labelled ethanol molecules is the ^3H effect. This ratio is equal to the ratio between the specific radioactivity of the substrate and the product, which carries the ^3H . It is to be understood that only one ^3H atom is considered at a time when more than one

Fig. 5. Recorded A_{340} during the standard assay of the isotope effect

The experiments used three cuvettes of 1.00 cm light-path adjusted to zero extinction when they contained only water. The experiment was started with two empty cuvettes and one containing 2.50 ml of 0.10 M-potassium phosphate, pH 7.0, 0.25 nmol of catalase haem, 2 μ g of glucose oxidase, 0.05 mg of liver alcohol dehydrogenase and 0.15 mg of yeast alcohol dehydrogenase. As indicated by the two first arrows, addition of 50 μ l and 10 μ l of NADH solution increased the A_{340} to 0.9. As indicated by the third arrow, addition of 150 μ l of ethanol diluted the reaction medium to a lower A_{340} (0.78). Concentration of ethanol in the assay was 4.0 mM. After withdrawal of the two first samples (samples 1 and 1') the reaction medium was divided equally between the two empty cuvettes; this event is marked (*). For the rest of the experiment the A_{340} was recorded alternately from these two cuvettes. At the time indicated by the fourth arrow 25 μ l of glucose was added to one of the two cuvettes to give a concentration of 7.5 mM. Subsequent samples were withdrawn as indicated. Samples with even numbers were withdrawn from the cuvette containing glucose, and the odd numbers represent samples from the control cuvette. ^3H in water in the samples was determined as described in the Materials and Methods section.

is present in the substrate. When [(1R)-1- $^3\text{H}_1$]-ethanol is substrate, water is the product that carries the ^3H . The specific radioactivity of this product can only be determined indirectly as the rate of appearance of ^3H in water divided by the rate of acetaldehyde production. These two rates were determined simultaneously as described in the Materials and Methods section. The trace of one such experiment is shown in Fig. 5. The A_{340} of the reaction

mixture minus glucose (control) appears to decrease. This instability was not seen in the assay with direct infusion H_2O_2 in the absence of glucose oxidase, nor did it release any ^3H to the water from any of the two positions in [1,1- $^3\text{H}_2$]ethanol. In the calculations the rate of NADH disappearance was corrected by means of this control rate to give the rate of acetaldehyde production.

Fig. 6 presents two experiments carried out under similar conditions except for the position of ^3H label at C-1 in ethanol. In agreement with earlier results (Gang *et al.*, 1973; Corral *et al.*, 1974) peroxidation of ethanol catalysed by catalase involves breaking of the C-H bond to the pro-*R* hydrogen, which appears in water, while the pro-*S* hydrogen remains with acetaldehyde. With rat liver catalase in the standard assay at 37°C and pH 7.0 with glucose oxidase an isotope effect with [(1R)-1- $^3\text{H}_1$]ethanol of 2.52 ± 0.21 (S.E.M.) was obtained in nine experiments. This value was unchanged when the actual reaction rate in the assay was changed by a factor of 50. Further, it did not depend on the kinetics of the catalase reaction over a wide range of ethanol concentrations and ratios of H_2O_2 -generation rates to catalase haem concentration (Table 1).

Effect of pH

Small changes of pH in the standard assay buffer had no significant effect on the ^3H isotope effect. At pH 6.3 and 7.7, the isotope effect was 2.64 and 2.54 respectively.

Effect of 4-methylpyrazole

It appears from Fig. 4 that the addition of 4-methylpyrazole up to a concentration of 1.6 mM does not alter the rate of $^3\text{H}_2\text{O}$ production. When 4-methylpyrazole was present in the standard assay at a concentration of 0.73 mM a ^3H isotope effect of 2.36 was obtained. To circumvent the effect of inhibition of liver alcohol dehydrogenase by 4-methylpyrazole in this assay, the enzyme was omitted and instead the yeast enzyme was added in double amount. In assays in which acetaldehyde was measured there was no effect of 4-methylpyrazole on peroxidative reaction rates with ethanol.

^3H isotope effect in ox liver catalase reaction

All the experiments with the ox liver enzyme were carried out with H_2O_2 -generation rates per catalase haem concentration in the range between 10 and 100 min^{-1} . When the [(1R)-1- $^3\text{H}_1$]ethanol concentration was 25–30 mM in the standard assay with glucose oxidase at 25°C the ^3H isotope effect was 2.40 ± 0.13 (S.E.M.) in nine experiments.

Table 1. ³H isotope effect in relation to the kinetics of the reaction

With the catalase from rat liver in the standard assay (see the Materials and Methods section) at 37°C and pH7.0, the ratio of H₂O₂ generation (dx/dt) to haem concentration (e) was varied by a factor of 10³, while the ethanol concentration was varied as indicated.

$\frac{1}{e} \cdot \frac{dx}{dt}$ (min ⁻¹)	e (nmol)	[Ethanol] (mM)	Reaction velocities (nmol/min)		Peroxidation (%)	³ H isotope effect
			NADH	³ H ₂ O		
0.96	1.25	0.6	1.05	0.42	88	2.50
1.04	1.25	85	1.33	0.58	102	2.30
2.24	1.25	54	2.78	1.12	99	2.48
18	1.25	7	22.48	8.49	100	2.65
55	0.25	0.13	2.62	1.03	19	2.55
52	0.25	38	12.27	5.00	94	2.45
70	0.18	39	9.31	3.60	75	2.59
300	0.25	39	31.07	12.43	41	2.50
1095	0.25	37	52.31	20.12	19	2.60

Fig. 6. Isotope effect with [(1R)-1-³H₁]ethanol and [(1S)-1-³H₁]ethanol in the standard assay for catalase peroxidation Both experiments were designed as described in the legend to Fig. 5 except for the concentrations of ethanol and glucose oxidase. Expt. (a) was carried out in presence of 40 mM-[(1R)-1-³H₁]ethanol and 5 μg of glucose oxidase, and experiment (b) was carried out in the presence of 4 mM-[(1S)-1-³H₁]ethanol and 2 μg of glucose oxidase. Expt. (b) is identical with that described in Fig. 5. ----, Aldehyde production calculated from the corrected decrease in NADH extinction. ○, ³H in water in the samples from the reaction mixture in absence of glucose; ●, ³H in water in the samples in the presence of glucose. The time of glucose addition is indicated by the arrows.

In assays with direct infusion of H₂O₂ at room temperature, the ³H effect was 2.2 and 2.4 with [(1R)-1-³H₁]ethanol, 2.3 and 2.8 with [1,1-³H₂]-ethanol, and with [(1S)-1-³H₁]ethanol only insignificant radioactivity appeared in water. A secondary isotope effect with [(1S)-1-³H₁]ethanol should not be

discernible, since such an effect is completely absent with [(1S)-1-²H₁]ethanol (Schonbaum & Chance, 1976). In the experiments with direct infusion of H₂O₂ the ethanol concentrations were between 16 and 19 mM.

Two independent control experiments with

[1- ^{14}C , (1*R*)-1- ^3H]₁ethanol (34mm) were carried out at 37°C. The ^3H effects obtained in this assay were 2.44 and 2.59.

Discussion

In the standard assay used in this study, the haem concentration, the ethanol concentration and the rate of H_2O_2 generation could be varied to cause a 50-fold change in the rate of peroxidation without affecting the isotope effect obtained. The control experiments with [1- ^{14}C , (1*R*)-1- ^3H]₁ethanol, as well as the results from the assay with direct infusion of H_2O_2 , support the conclusion that the obtained value for the isotope effect is a parameter specific to liver catalase. To reduce the acetaldehyde formed in the standard assay a mixture of horse liver and yeast alcohol dehydrogenases was used. The yeast enzyme was chosen to ensure a fast reduction of acetaldehyde, and the presence of the liver enzyme with its comparative lower molecular activity appears superfluous in this regard.

As the ratio of H_2O_2 generation to haem concentration is changed, this alters the relative fluxes between the peroxidation of ethanol and the catalytic reaction, represented in Scheme 1 by k_{+1} , k_{+2} , k_{+3} , k_{+4} and k_{+8} , k_{+9} , k_{+10} respectively. Since the latter pathway is a by-pass, isolated from ethanol oxidation, it is not surprising that the primary isotope effect obtained with [(1*R*)-1- ^3H]₁ethanol (listed in Table 1) does not vary with $(dx/dt) \times (1/e)$ (e , haem concentration). The applicability of the obtained isotope effect for interpretation of results *in vivo* is thus dependent on the following features: (1) the source

of the enzyme; (2) the temperature; (3) the pH; (4) possible effectors. In this study only liver catalase was used. Catalases from rat and ox liver did not differ significantly in their discrimination against ^3H -labelled ethanol. The lower mean value obtained with ox liver enzyme may well be attributed to the lower temperature in these assays. With the control assay with [1- ^{14}C , (1*R*)-1- ^3H]₁ethanol at 37°C the ox liver enzyme apparently exhibited a slightly higher ^3H effect than at 25°C, close to the value for the rat liver enzyme at 37°C. Catalases from other sources have been shown to exhibit ^2H isotope effects that differ from that of the ox liver enzyme. Thus the ^2H isotope effects were 2.2, 1.9, and 1.5 in peroxidation of [1,1- $^2\text{H}_2$]₁ethanol by horse erythrocyte catalase, ox liver catalase and catalase from *Micrococcus lysodeikticus* respectively (Schonbaum & Chance, 1976). Variation in pH within the physiological range (6.3–7.7) apparently does not change the ^3H isotope effect. This is not very surprising, since the rate of peroxidation of ethanol by catalase is almost insensitive to pH between 4 and 12 (Chance, 1952). From this it seems sensible to assume that the value for the isotope effect obtained *in vitro* reflects the discrimination against ^3H -labelled ethanol in the catalase-catalysed peroxidation in liver tissue or hepatocytes. An effect of acetaldehyde, one product of the reaction, on the ^3H effect can be ruled out, since neither the rate of $^3\text{H}_2\text{O}$ formation nor the rate of acetaldehyde production was influenced by its concentration. The effect of 4-methylpyrazole has been tested, since this compound is widely used to inhibit liver alcohol dehydrogenase in studies of ethanol metabolism. Both the rate of ethanol peroxidation and the rate of

Scheme 1. Sequence of reactions in catalysis by catalase of H_2O_2 -mediated peroxidation of ethanol. C, catalase with H_2O in the place of the exchangeable ligand to haem ion; CI (compound I), catalase that had reacted with 1 equiv. of H_2O_2 per haem; D, catalase- H_2O_2 complex; F, complex between ethanol and compound I; G, complex between H_2O_2 and compound I; X and Y, central complexes between which the pro-*R* carbon-hydrogen bond at position C-1 in ethanol is split; Z, the central complex in the catalytic reaction. k_{+1} , k_{+2} , ..., k_{+10} and k_{-1} , k_{-2} , ..., k_{-10} are rate constants. Positive subscripts indicate reactions proceeding from left to right and negative subscripts indicate reactions in the opposite direction.

$^3\text{H}_2\text{O}$ formation when [(1*R*)-1- $^3\text{H}_1$]ethanol was substrate were insensitive to this compound in concentrations that usually result in nearly complete inhibition of liver alcohol dehydrogenase. Pyrazole itself has been reported to inhibit catalase activity after administration *in vivo*, but did not inhibit catalase *in vitro* at a concentration of 4mM (Lieber & DeCarli, 1970).

Catalytic mechanism of catalase

The present knowledge about the catalytic mechanism of the catalase reaction is remarkably complete. As discussed in the review by Schonbaum & Chance (1976), certain points are, however, in need of further elucidation. Among these the difference in ^2H isotope effect between [1,1- $^2\text{H}_2$]ethanol and [$^2\text{H}_3$]methanol is subject to renewed analysis in the light of the present results with [(1*R*)-1- $^3\text{H}_1$]ethanol. Scheme 1 describes the most likely sequence in the catalysis by catalase of H_2O_2 -mediated peroxidation of ethanol.

An isotope effect of 1.9 with [1,1- $^2\text{H}_2$]ethanol at pH 7.0 and 25°C in 10mM-potassium phosphate has been reported by Schonbaum & Chance (1976); they did not find any secondary isotope effect with [(1*S*)-1- $^2\text{H}_1$]ethanol. The isotope effect obtained with ^2H is a V effect, whereas the ^3H isotope effect obtained in the present study is a V/K effect (Cleveland, 1975; Northrop, 1975). Provided that the isotope effects are negligible, the two different isotope effects depend on the individual rate constants as described by Northrop (1975):

$$V_{\text{H}}/V_{3\text{H}} = \frac{k_{\text{bH}}/k_{\text{b}3\text{H}} + (k_{\text{b}}/k_{\text{c}})_{\text{H}}}{(k_{\text{b}}/k_{\text{c}})_{\text{H}} + 1}$$

$$\frac{(V/K)_{\text{H}}}{(V/K)_{3\text{H}}} = \frac{(k_{\text{bH}}/k_{\text{b}3\text{H}})/(k_{\text{aH}}/k_{\text{a}3\text{H}}) + (k_{\text{b}}/k_{\text{a}})_{\text{H}}}{(k_{\text{b}}/k_{\text{a}})_{\text{H}} + 1}$$

where V is the maximal reaction velocity per unit of enzyme (haem) at infinite substrate concentration, and V/K is the apparent first-order rate constant of the reaction with respect to ethanol per unit of enzyme. Subscripts H, ^2H and ^3H refer to the substrate to which the constant corresponds and denote ethanol, [(1*R*)-1- $^2\text{H}_1$]ethanol and [(1*R*)-1- $^3\text{H}_1$]ethanol respectively. k_{a} , k_{b} and k_{c} relate to Scheme 1 thus:

$$k_{\text{a}} = \frac{k_{-1}}{[k_{+2}/(k_{+2} + k_{+3})] + 1}$$

$$k_{\text{b}} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{-2} + k_{+3}}$$

$$k_{\text{c}} = \frac{k_{+4}k'_5[\text{H}_2\text{O}_2]}{k_{+4} + k_{-4}[\text{A}] + k'_5[\text{H}_2\text{O}_2]}$$

Since the flux rate through the catalytic pathway, complexes G and Z in Scheme 1, had no influence on

the isotope effect, it is not considered in the following discussion. $[\text{H}_2\text{O}_2]$ and $[\text{A}]$ are the steady-state concentrations of H_2O_2 and acetaldehyde respectively. The value of k_7 has been determined as $2.5 \times 10^5 \text{ s}^{-1}$ for proton exchange (Schonbaum & Chance, 1976). This rate is sufficient in comparison with the apparent catalytic rate constant discussed below to account for equilibration of $^3\text{H}_2\text{O}$ with the medium and thus render the values of k_{+5} , k_{-5} , k_{+6} and k_{-6} unimportant for the measured isotope effect. This will then only depend on the reaction sequence from CI to C (see Scheme 1), which are both in steady state during the experimental conditions. Therefore it suffices to describe the remaining part of the catalytic cycle by a single rate constant k'_5 :

$$k'_5 = \frac{k_{+5}k_{+6} - k_{-5}k_{-6} \left(\frac{[\text{CI}][\text{H}_2\text{O}]}{[\text{C}][\text{H}_2\text{O}_2]} \right)}{k_{+6} + k_{-5}}$$

k_{-3} is assumed to be negligible compared with k_{+3} and k_{+4} . This assumption is based on the sizeable thermodynamic equilibrium constant:

$$10^{50} \text{ M} = \frac{[\text{Acetaldehyde}][\text{H}_2\text{O}][\text{H}_2\text{O}]}{[\text{ethanol}][\text{H}_2\text{O}_2]}$$

calculated from the standard reduction potentials of the involved redox systems at pH 7 and 25°C given by Clark (1972) and Mahler & Cordes (1971), and the following observations. (1) The reaction velocity, as well as the rate of ^3H release, was independent of the acetaldehyde concentration. (2) Small concentrations of ethanol are sufficient to cause a profound decrease in the $[\text{CI}]/[\text{C}]$ ratio during steady-state conditions (Chance *et al.*, 1974). (3) When 16 nmol of catalase haem was incubated with 7 μmol of ethanol and 75 μmol of acetaldehyde at pH 7 and 25°C in 4 ml of 50mM-potassium phosphate in $^3\text{H}_2\text{O}$ ($2 \times 10^3 \text{ c.p.m.}/\mu\text{mol}$ of water), isotope equilibrium was not attained within 43 h. This rate is negligible in comparison with the apparent rate constant of 10^3 s^{-1} in the reverse direction (Schonbaum & Chance, 1976). For the reasons discussed above $k_{-4} \ll k_{+4}$.

From the expressions of the V and V/K isotope effects in terms of the individual rate constants it may be deduced that the degree to which the true isotope effect on k_{b} itself is manifested in the observed isotope effects is independent of k_{a} for the former and independent of k_{c} for the latter. $k_{\text{aH}}/k_{\text{a}3\text{H}}$ may normally be expected to be close to unit. In this case that assumption is supported by the absence of a secondary isotope effect. Swain *et al.* (1958) have found the relation between the primary isotope effect of ^2H and ^3H to be:

$$\frac{k_{\text{H}}}{k_{3\text{H}}} = \left(\frac{k_{\text{H}}}{k_{2\text{H}}} \right)^{1.442}$$

When the V isotope effect with $[1,1\text{-}^2\text{H}_2]$ ethanol of 1.9 is used to calculate the corresponding ^3H effect, a value of 2.52 is obtained. This strongly suggests that neither of the two experimentally determined isotope effects depends on k_a or k_c . This then implies that they both represent an isotope effect on k_b alone. Furthermore, since the net rate constant in the catalytic reaction is 4×10^3 times the net rate constant of the peroxidation of ethanol or methanol, it follows that the net rate of dissociation of water from the enzyme is a rapid process compared with the rate of ethanol peroxidation. Therefore k_{+2} should be much larger than k_{-2} . Scission of the C-H bond at C-1 in ethanol must be a slow step in ethanol peroxidation by compound I, since primary isotope effects are seen on both V/K and V , while both solvent and secondary isotope effects are absent (Schonbaum & Chance, 1976). Therefore it follows that k_{+2} must be considerably larger than k_{+3} and thus k_b can be expected to have a value nearly identical with k_{+3} . It may then not be inferred, as has been done by Schonbaum & Chance (1976), that the unusual low isotope effect with ethanol, as compared with methanol (^2H effect of 5.5), is due to a higher commitment to catalysis for ethanol than for methanol. Such a conclusion may not apply even for the extremely low primary ^2H isotope effect obtained with formate, as there cannot be any commitment to catalysis with V isotope effects. In absence of a kinetic explanation for the small primary isotope effect, it seems relevant to discuss other explanations briefly. Dissymmetry of the transient activated complex as well as a possible deviation from linearity in the arrangement of the atoms in this complex are important factors in conservation of the C-H bond energy during formation of this complex (Jencks, 1969). The degree to which this energy is conserved determines the degree to which the primary isotope effect is decreased from the theoretical value calculated from the stretching frequency of the C-H bond undergoing scission. This rationale yields a qualitative explanation of the difference in isotope effect between $[^2\text{H}_3]$ methanol and $[1,1\text{-}^2\text{H}_2]$ ethanol. Methanol will form a more symmetrical transient complex, causing a lower conservation of C-H bond energy, in comparison with ethanol. Further, the potential dissymmetry in the ethanol molecule, which is a condition for a stereospecific oxidation at the active site of catalase, does not exist in methanol. It appears likely that the transient complex with ethanol has a non-linear arrangement, causing a lower isotope effect.

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