STEREOSPECIFICITY OF ETHANOL OXIDATION

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Summary

The stereospecificity of ethanol oxidation via alcohol dehydrogenase, the microsomal ethanol oxidizing system (MEOS) and catalase was determined using stereospecific ethanol-1-^{3}H. All systems showed the same stereospecificity towards ethanol. All pathways displayed an isotope effect, but the effect with MEOS and catalase was greater than with alcohol dehydrogenase.

In the liver ethanol is metabolized by several pathways. Alcohol dehydrogenase (ADH), an enzyme located in the cytosol, oxidizes the major portion of ingested ethanol (1). ADH is a stereospecific enzyme (2); the 1-C atom of ethanol acts as a potential asymmetric C-atom. Only one position is acted upon by the enzyme in both the forward and reverse reactions. This reaction is the following: $CH_3CHH^*OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH^* + H^+$. The microsomal ethanol oxidizing system (MEOS), characterized by Lieber and De Carli (3), is reported to account for a lesser proportion of ethanol oxidation. The reaction of MEOS is: $CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow{MEOS} CH_3CHO + NADP^+ + 2\ H_2O$. A third pathway, the catalase-peroxide system (4), proceeds as follows: $CH_3CH_2OH + H_2O_2 \xrightarrow{catalase} CH_3CHO + 2H_2O$. Nicholls and Schonbaum (5) mention that the oxidation of ethanol by catalase is also stereospecific, but do not specify in which way. We therefore compared the stereospecificity of these three enzyme systems

Materials and Methods

Since acetaldehyde is the metabolite common to all ethanol metabolizing systems, the study of the stereospecificity of the enzyme systems acting on

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* denotes stereospecific H.

ethanol involved the determination of radioactivity in acetaldehyde, using stereospecifically labeled ethanol-1-^{3}H. Stereospecifically labeled ethanol was prepared from racemically labeled ethanol-1-^{3}H according to Loewus et al (4), by the following reactions:

$$CH_3C^3H_2OH + NAD^+ \xrightarrow{ADH} CH_3C^3HO + NAD^3H^* + H^+ \quad (pH\ 9.2)$$

$$CH_3CHO + NAD^3H + H^+ \xrightarrow{ADH} CH_3C^3H^*HDH + NAD^+ \quad (pH\ 7.4)$$

Racemically labeled ethanol-1-^{3}H (New England Nuclear Corp., 91μCi per μmole) was diluted with unlabeled 95% ethanol to a final activity of 4 X 10^6cpm per μmole. 1.5 gm NAD^+, 2 mg yeast ADH (Boehringer Mannheim) and 33 mmoles racemically labeled ethanol-1-^{3}H were added to 40 ml of 50 mM pyrophosphate buffer, pH 9.2, containing 50 mM semicarbazide HCl. The reaction was allowed to proceed to completion at 37°, as indicated by the absorption at 340 nm. The reaction mixture was then placed in dry ice. Ethanol, which had been kept in dry ice, was added until incipient turbidity developed. The pH was then adjusted to 7.35, and more chilled ethanol added to a final volume of 430 ml. The mixture was left overnight, and the precipitate, which contained NAD^3H, was filtered, washed with chilled ethanol and dried in vacuo. A typical yield was about 1 gm NAD^3H.

The second step in the synthesis was carried out as follows: 2.8 mmoles NAD^3H, 2.8 mmoles acetaldehyde and 5 mg ADH were added to 50 ml 0.5 M phosphate buffer, pH 7.4, and 45 ml H_2O. The reaction proceeded to 95-100 percent completion, as indicated by the disappearance of the absorption at 340 nm. 10 mM pyrazole (final concentration), an inhibitor of ADH, was added to prevent the back reaction, and 300 mM semicarbazide was added to bind residual acetaldehyde. Ethanol was then distilled from the reaction mixture in a 120°C paraffin bath, and the concentration of ethanol in the distillate was determined according to Bonnichsen (6). The concentration of distilled ethanol was 200 μmoles per ml. No measurable acetaldehyde was present in the distillate, as measured by gas chromatography or spectrophotometrically after the addition of semicarbazide. The enzymatic reactions with either racemically or stereo-

specifically labeled ethanol were carried out under the following conditions: ADH: 30 mg NAD^+, 0.15 mg yeast ADH, 1 ml 150 mM Tris-HCl buffer, pH 9.6, and 100 µmoles ethanol-1-^3H (final concentration, 80 mM). MEOS: 0.36 ml 1 M phosphate buffer, pH 7.4, 0.23 ml 100 mM $MgCl_2$, 0.45 ml 3 mM $NADP^+$, 0.45 ml 100 mM isocitrate, 0.45 ml 100 mM pyrophosphate, 5.4 units isocitrate dehydrogenase (Sigma), 150 µmoles ethanol-1-^3H (final concentration 30 mM) and microsomes prepared from 700 mg liver, in a final volume of 5 ml; the reaction was stopped with 2 ml of 0.6 N trichloracetic acid (TCA), and the mixture centrifuged; Catalase: 0.36 ml 1 M phosphate buffer, pH 7.4, 0.23 ml 100 mM $MgCl_2$, 0.45 ml 100 mM pyrophosphate, 0.45 ml 30 mM hypoxanthine, 0.84 units xanthine oxidase (Sigma), 6000 units catalase (Sigma) and 150 µmoles ethanol-1-^3H (final concentration 30 mM), in a total volume of 5 ml.

All reactions were carried out at 37° and terminated after 2 hours. 1-2 ml aliquots of the reaction mixtures or of the MEOS supernatant were added to 10 ml of cold dimedone buffer (0.1 N sodium acetate-acetic acid buffer, pH 4.25, containing 4 mg of dimedone ((Eastman) per ml)(7,8). The tubes were then vigorously shaken until slightly turbid. Precipitation was completed by allowing the tubes to stand at room temperature overnight. The dimedone-acetaldehyde complex was then collected by centrifugation and washed 3 times with either cold water or 0.1 N sodium acetate-acetic acid buffer, pH 4.25. The precipitate was finally dissolved in methanol. The washes and the dissolved precipitate were scanned in the UV region, and aliquots counted in a liquid scintillation counter.

Results

In the preparation of NAD^3H some difficulties were initially encountered using 0.6 M Tris-HCl buffer (2). The precipitated NAD^3H was viscous, and the yield poor. With pyrophosphate as the buffer, both the quality of the precipitate and the yield were improved. 50 mM semicarbazide had to be added to the reaction to obtain a complete reduction of NAD^+. To assess the radioactivity of the acetaldehyde produced by the enzymatic reactions, we initially used

TABLE I

SPECIFIC ACTIVITY OF ACETALDEHYDE GENERATED BY ALCOHOL DEHYDROGENASE (ADH), THE MICROSOMAL ETHANOL OXIDIZING SYSTEM (MEOS) AND CATALASE. The reactions were carried out as described in Methods. Values represent cpm/μmole acetaldehyde.

Ethanol-1-^{3}H	ADH	MEOS	Catalase
Racemically labeled (21,700 cpm/μmole)	9100	5500	5300
Stereospecifically labeled (800 cpm/μmole)	0	0	0

semicarbazide, in a center-well flask, to trap the acetaldehyde. In a typical experiment of this series, using racemically labeled ethanol-1-^{3}H, the ADH and MEOS reactions, the blanks in which TCA was added at zero time, and the ADH reaction with enzyme omitted, all yielded the same number of counts in the center well. The optical density at 224 nm (where the semicarbazide-aldehyde complex has its maximum absorption (3)), gave readings of 2.8 and 1.3 with the enzymes added, but only 0.26-0.30 for the zero time controls. Therefore, although the reactions proceeded effectively, diffusion of labeled ethanol into the center well masked counts derived from acetaldehyde.

We therefore decided to precipitate acetaldehyde with dimedone. Initially we distilled the acetaldehyde into cold dimedone buffer (8), but the yields of the aldehyde-dimedone complex were very low. Dimedone was, therefore, added directly to the incubation mixture. The dimedone-aldehyde complex thus obtained had a maximum absorption at 262 nm, and the absorption co-efficient for the complex was found to be $E^{mM}_{262} = 8.0$. Acetaldehyde-dimedone was present in most of the washes, as well as in the methanol solution. The dimedone itself had an absorption maximum at 280 nm, and did not interfere with the determination of the complex.

Theoretically, since ADH is stereospecific, 50% of the initial specific activity of racemically labeled ethanol-1-^{3}H should be found in the resulting acetaldehyde. However, the specific activity actually found in the acetalde-

hyde was only 42% of that in the labeled ethanol (Table I). This is consistent with the isotope effect which has been demonstrated for the ADH catalyzed oxidation of methanol (9,10). Similarly, the specific activity of the acetaldehyde generated by the MEOS and catalase reactions was 25% of that of racemically labeled ethanol, which points to an even more pronounced isotope effect. In the experiments with stereospecific ethanol-1-^{3}H, no radioactivity was found in the acetaldehyde-dimedone complex with any of the three reactions, (Table I).

Discussion

The results of this study indicate that all three ethanol oxidizing systems exhibit the same stereospecificity towards ethanol, since no counts were found in the acetaldehyde produced by these systems, when stereospecifically labeled ethanol was used. Apparently, these systems all proceed by pathways which have the same steric requirements. The active sites of these enzymes probably orient the ethanol molecule, so that the same hydrogen atom is available for transfer to the acceptor; for ADH, NAD^+, for MEOS, molecular oxygen, and for catalase, H_2O_2, (via catalase compound I (5)). The differences in the specific radioactivity of acetaldehyde obtained using racemically labeled ethanol probably reflects an isotope effect. The specific activity of acetaldehyde generated by yeast ADH is close to the theoretical value, indicating a small isotope effect. The yeast enzyme was reported to exhibit a larger isotope effect than horse liver ADH, showing an effect comparable to the one found in this study (9,10). However, the isotope effect of yeast ADH was studied using semicarbazide, which produced a pronounced increase in the isotope effect of horse liver ADH. Without semicarbazide yeast ADH might show a smaller isotope effect. The reactions with MEOS and catalase display a greater isotope effect. It may be that the heme group, which is common to both systems, has some influence on this effect.

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