Redox Interactions between Catalase and Alcohol Dehydrogenase Pathways of Ethanol Metabolism in the Perfused Rat Liver*

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Alcohol metabolism via alcohol dehydrogenase (ADH) and catalase was studied in perfused rat livers by measuring the oxidation of methanol and butanol, selective substrates for catalase and ADH, respectively. In livers from fasted rats, basal rates of methanol uptake of 15 ± 1 μmol/g/h were decreased significantly to 8 ± 2 μmol/g/h by addition of butanol. Concomitantly, pyridine nucleotide fluorescence detected from the liver surface was increased by butanol but not methanol. Both effects of butanol were blocked by an inhibitor of ADH, 4-methylpyrazole, consistent with the hypothesis that elevation of the NADH redox state by butanol inhibited H2O2 production via NAD+ requiring peroxisomal β-oxidation. Thus, catalase-H2O2 generation leading to diminished rates of catalase-dependent methanol uptake. In support of this idea, both butanol and butyraldehyde inhibited H2O2 generation. The NADII redox state was also elevated by xylitol, causing a 75% decrease in rates of methanol uptake by livers from fasted rats. This effect was not observed in livers from fed rats unless malate-aspartate shuttle activity was reduced by infusion of the transaminase inhibitor aminotriazole (7, 8). Three recent experiments (9-11) demonstrated that there are problems with these arguments. First, in perfused livers from fasted rats, rates of H2O2 production were increased significantly from 10 to about 80 μmol/g/h by the addition of fatty acid-albumin complexes (palmitate and oleate) which are substrates for H2O2 generation via the peroxisomal β-oxidation system (9). Thus, with the addition of appropriate physiological substrates, rates of H2O2 generation in the liver can be quite high. Albumin, which is critical for fatty acid uptake by liver cells, was either omitted or present in unphysiologically low concentrations in older work, accounting for low rates of H2O2 generation observed (9). Second, in carefully controlled studies in deermice lacking ADH, catalase was inhibited by treatment with aminotriazole and rates of ethanol elimination in vivo were decreased by about 75% (10). Thus, catalase can be a significant pathway of ethanol metabolism in vivo. Third, it was demonstrated recently in perfused livers from fasted rats that methanol uptake could be stimulated 4-fold by fatty acids (11). Taken together, these three results indicate that conditions exist under which catalase-H2O2 is a major pathway of alcohol metabolism.

Alcohol dehydrogenase will oxidize ethanol, propanol, and butanol at relatively higher velocities as the chain length is increased, but will not metabolize methanol (12). Conversely, catalase will metabolize methanol but not butanol (13). Thus, methanol and butanol are selective substrates for catalase and ADH, respectively. In a study which employed selective substrates, it was demonstrated that catalase-H2O2 was the predominant pathway of total alcohol metabolism in the fasted state in the presence of the physiological fatty acid oleate (11). While peroxidation of methanol by catalase-H2O2 was stimulated by oleate due to increased H2O2 generation from peroxisomal β-oxidation of fatty acyl-CoA compounds, rates of butanol uptake actually decreased in the presence of the fatty acids (11). Both alcohol oxidation via ADH and H2O2 generation via peroxisomal β-oxidation are NAD+ requiring reactions. Accordingly, the purpose of the present study was to investigate whether or not interactions between the ADH and catalase pathways of alcohol oxidation occur. The data indicate that increases in the NADH/NAD+ ratio caused by alcohol oxidation via ADH can inhibit peroxidation via catalase-H2O2 by decreasing rates of H2O2 generation.

MATERIALS AND METHODS

Liver Perfusion—Livers from fed or fasted (24 h) Sprague-Dawley rats were perfused in recirculating (65 ml volume) or nonrecirculating...
perfusion systems as described previously (14, 15). The concentration of oxygen in the effluent perfusate was monitored continuously with a Teflon-shielded, Clark-type O2 electrode.

Concentrations of butanol in the perfusate were determined enzymatically (10). Methanol in perfusate was determined either enzymatically (9) or by gas chromatography. Samples (1.0 ml) of effluent perfusate were placed in capped 25-ml Erlenmeyer flasks and incubated at 37 °C for 30 min. One ml of the headspace was sampled with a gas-tight syringe and injected onto a Carbobox 60/80 column. Operating conditions were: oven, 110 °C; detector, 250 °C; and carrier gas flow, 40 ml/min. Methanol was detected with a flame ionization detector and was quantitated by comparison with external methanol standards in a manner similar to the quantitation of ethanol described in detail elsewhere (17). Rates were calculated from the influent minus effluent concentration differences, the flow rate, and the liver wet weight.

**Determination of Pyridine Nucleotide Fluorescence and the Steady-state Level of Catalase H2O2**—The steady-state level of catalase-H2O2 was determined spectrophotometrically (660-640 nm) through a layer of the liver as described by Sies and Chance (18) with a dual-channel, air-driven spectrophotometer (19). Pyridine nucleotide fluorescence from the surface of perfused livers was determined with a large tipped, bifurcated, fiber-optic light guide. One end of the light guide was connected to a lamp-watt mercury arc lamp fitted with a 366-nm transmittance filter, and the other end was connected to a photomultiplier filtered to detect 450 nm light. The output from the photomultiplier was amplified and recorded as described elsewhere.

**Oxidation of Methanol and Butanol by Catalase and ADH in Vitro**—Livers from fed rats were perfused for 1-2 min with Krebs-Henseleit buffer, pH 7.4. Homogenates were centrifuged for 2 min at 2,000 g for 20 min. The pellets (mitochondrial/peroxisomal fraction containing catalase) were washed twice with 0.15 M KCl, pH 7.4, and resuspended in 0.15 M KCl. Supernatants were centrifuged at 100,000 × g for 60 min, and the high-speed supernatant containing ADH (cytosol) was collected.

The oxidation of butanol by supernatants containing ADH was measured by following the reduction of NAD' to NADH at 366 nm. Reaction mixtures containing 5 mg of cytosolic protein were incubated at 25 °C in a 1-ml volume containing 500 mM glucose, 1 mM NAD', and 1 mM hydrazine hydrate, pH 9.0. Reactions were initiated by the addition of butanol (0-15 mM), and the reduction of NAD' was measured spectrophotometrically and recorded for 3-5 min. Methanol (final concentration, 30 mM) was added subsequently, and reactions were incubated for 3 additional minutes.

The oxidation of catalase in mitochondrial/peroxisomal fractions was determined by measuring the formation of formaldehyde colorimetrically. Five mg of protein was incubated in 1 ml of 0.1 M phosphate buffer, pH 7.0, containing 100 mM semicarbazide and 10 mM glucose. Reactions were initiated by the addition of 10 units of glucose oxidase and were terminated by the addition of 100 μl of 0.3 M glacial acetic acid. Formaldehyde was measured colorimetrically using the method of Nash (20).

**Statistics**—Comparison between groups were made using Student's t test (21).

## Results

**Validation of the Selectivity of Methanol and Butanol for Catalase and ADH**—To verify that methanol was a selective substrate for catalase, and that butanol was selective for ADH in rat liver, the oxidation of methanol and butanol was measured in subcellular fractions (Fig. 1). Rates of formation of formaldehyde from the peroxidation of methanol by 10,000 × g pellets incubated with an H2O2-generating system increased as the concentration of methanol was elevated up to 100 mM (Fig. 1A). Rates were half-maximal with about 35 mM methanol. Butanol (15 mM), which was not metabolized by the 10,000 × g pellets, had no effect on peroxidation of methanol (Fig. 1A).

In the presence of NAD' and cytosol of rat liver homogenates, rates of NADH generation from the oxidation of butanol by ADH increased as the concentration of butanol was elevated (Fig. 1B). Maximal rates of NADH formation of about 4.5 nmol/min/mg protein were observed with about 5 mM butanol; rates were half-maximal with concentrations of butanol around 2 mM (Fig. 1B). As expected, the oxidation of butanol by cytosol was not affected by methanol (30 mM, Fig. 1B), and NADH was not formed by methanol alone (data not shown).

Methanol and butanol are both metabolized by isolated microsomes (22). In perfused rat liver, however, basal rates of methanol uptake were not affected by an inhibitor of mixed-function oxidation, 4-methylpyrazole (see below, Fig. 3). p-Nitrophenol is a good substrate for the isozyme of cytochrome P-450 (II.E.1) which is induced by ethanol and has a high affinity for butanol (23). It was oxidized at rates about 2 orders of magnitude lower than butanol (9), indicating that cytochrome P-450 plays only a minor role in oxidation of methanol and butanol in perfused liver. Taken together, these data verify that methanol and butanol are selective substrates for catalase and ADH, respectively.

**Oxidation of Methanol and Butanol by Perfused Rat Liver**—The results of a typical experiment employing the experimental design used in these studies is shown in Fig. 2. In a perfused liver from a fasted rat methanol (30 mM) did not affect basal rates of oxygen uptake (about 100 μmol/g/h) or steady-state levels of NADH fluorescence; however, the steady-state level of catalase-H2O2 measured spectrophotometrically (660-640 nm) was decreased sharply. The addition of butanol (0-15 mM) as described under "Materials and Methods." The formation of NADH from the oxidation of butanol by ADH was measured spectrophotometrically at 366 nm. Methanol alone (solid line), butanol alone (dashed line), and butanol plus 30 mM methanol (solid line) Data represent means of measurements from four different liver preparations.

**Fig. 1. Oxidation of methanol and butanol by subcellular fractions.** A, Pellets from a 10,000 × g centrifugation of rat liver homogenates were resuspended and incubated with increasing concentrations of methanol (0-150 mM) in the presence and absence of butanol (15 mM) with an H2O2 generating system described under "Materials and Methods." Formaldehyde formed from the peroxidation of methanol via catalase-H2O2 was trapped as the semicarbazone and quantitated as described previously (14). , methanol alone (solid line); , methanol plus butanol (dashed line). B, Rat liver cytosol was incubated with NAD' and butanol (0-15 mM) as described under "Materials and Methods." The formation of NADH from the oxidation of butanol by ADH was measured spectrophotometrically at 366 nm. , butanol alone (dashed line); , butanol plus 30 mM methanol (solid line). Data represent means of measurements from four different liver preparations.
peroxisomal metabolism of fatty acids. At present, however, there are a number of possible explanations for this phenomenon, including uptake, activation, and oxidation. Branden et al. (24). There are a number of possible explanations for this phenomenon, including uptake, activation, and oxidation. Branden et al. (24).

The subsequent addition of oleate (1 mm) increased rates of methanol uptake significantly to 38 ± 4 μmol/g/h (p < 0.05) but diminished rates of butanol uptake to about 70 μmol/g/h. Thus, it is concluded that ADH is the predominant pathway of alcohol oxidation in the fed state, as expected.

In the present study, rates of methanol uptake, which reflect rates of H₂O₂ generation by catalase and ADH, livers from fed rats were perfused as in Fig. 2. Basal rates of uptake of methanol and butanol in the presence of both alcohols in livers from fed rats were 5 ± 1 and 95 ± 2 μmol/g/h, respectively (n = 4).

The addition of oleate (1 mm) increased rates of methanol uptake significantly by a factor of 2 (p < 0.05) but diminished rates of butanol uptake to about 26 μmol/g/h. Thus, it is concluded that ADH is the predominant pathway of alcohol oxidation in the fed state, as expected.

In the present study, rates of methanol uptake, which reflect rates of H₂O₂ generation, were 3–4 times greater in the fasted than in the fed state in the presence of oleate (Table I). Similar results were obtained in mice in vivo by van den Branden et al. (24). There are a number of possible explanations for this phenomenon, including uptake, activation, and peroxisomal metabolism of fatty acids. At present, however, it is not clear which of these factors is responsible for the effect of nutritional state on rates of peroxisomal H₂O₂ generation.

To quantify alcohol oxidation by catalase and ADH in the absence of inhibitors, rates of uptake of methanol and butanol were measured simultaneously in perfused liver. Basal rates of methanol and butanol uptake in the presence of both alcohols were 8 ± 1 and 77 ± 15 μmol/g/h, respectively (Table I); total rates of alcohol oxidation were around 85 μmol/g/h. The subsequent addition of oleate (1 mm) diminished overall rates of alcohol uptake to about 65 μmol/g/h (Table I). Rates of methanol uptake increased significantly to 38 ± 4 μmol/g/h, while butanol uptake was diminished to about 26 μmol/g/h. When fructose (10 mm) was infused, overall rates of alcohol uptake were diminished about 20%. Under these conditions, rates of methanol uptake were diminished to 5 ± 2 μmol/g/h while rates of butanol uptake were increased significantly to 101 ± 4 μmol/g/h (Table I). Fructose decreased rates of H₂O₂ generation measured spectrophotometrically in livers from fasted rats by 9 ± 2 ± 4 ± 1 μmol/g/h (n = 4), consistent with observations in perfused livers from deer mice genetically deficient in ADH (25). Peroxidation of alcohols is limited by the supply of H₂O₂ in rat liver (26); thus the data are consistent with the hypothesis that methanol uptake was mediated via catalase. In support of this hypothesis, methanol uptake was decreased below the limits of detection (<2 μmol/g/h) in livers from rats pretreated for 1.5 h with the catalase inhibitor aminotriazole (1.5 g/kg, intraperitoneally; data not shown), confirming that methanol uptake was mediated via catalase in intact cells.

**Redox Inhibition of Methanol Oxidation by Butanol.** Rates of methanol uptake of 15 ± 2 μmol/g/h in perfused livers from fasted rats (Fig. 3, left) were diminished to about 8 μmol/g/h by butanol (p < 0.05; Fig. 3, center). Under these conditions, rates of butanol uptake were about 75 μmol/g/h. When
an inhibitor of ADH, 4-methylpyrazole (4 mM), was added subsequently, rates of methanol uptake returned to basal values (Fig. 3, right). Concomitantly, rates of butanol uptake were below the limits of detection and pyridine nucleotide fluorescence, which was increased initially by about 20% when butanol was added (Fig. 2), decreased to basal levels (data not shown).

Since butanol did not affect the peroxidation of methanol by catalase from rat liver in vitro (Fig. 1), and because the supply of H$_2$O$_2$ is rate-limiting for the peroxidation of methanol and ethanol by catalase (26), one possible explanation for the observed inhibition of methanol uptake is that butanol oxidation decreased H$_2$O$_2$ generation. To test this hypothesis, rates of H$_2$O$_2$ generation were measured in livers from fasted rats perfused in a nonrecirculating system in the presence and absence of butanol and butyraldehyde plus 4-methylpyrazole. Butanol and butyraldehyde plus 4-methylpyrazole both increased pyridine nucleotide fluorescence by about 20% and diminished rates of H$_2$O$_2$ generation from about 8 μmol/g/h to between 2 and 3 μmol/g/h (Table III).

**Effect of Xylitol on Methanol Uptake and Pyridine Nucleotide Fluorescence**—The fact that the inhibition of H$_2$O$_2$ generation observed with butanol and butyraldehyde, which are metabolized via NAD$^+$-linked dehydrogenases, was associated with an increase in pyridine nucleotide fluorescence (Table III) suggested that H$_2$O$_2$ generation could be inhibited by increasing the NADH/NAD$^+$ ratio. To test this hypothesis, livers from fasted rats were perfused in a nonrecirculating system and methanol (2 mM) was infused in the presence and absence of xylitol (5 mM), a substrate for the cytosolic NAD$^+$-linked xylitol dehydrogenase. Upon infusion of methanol, basal rates of O$_2$ uptake of 100–110 μmol/g/h were unaffected (Fig. 4, upper panel), while pyridine nucleotide fluorescence increased slightly (110% of basal), due most likely to dehydrogenase-dependent metabolism of formaldehyde (Fig. 4, center panel). Under these conditions, rates of methanol uptake were about 8 μmol/g/h (Fig. 4, bottom). Upon infusion of xylitol, pyridine nucleotide fluorescence increased about 35% and rates of O$_2$ uptake increased to 130 μmol/g/h. Concomitantly, rates of methanol uptake declined to values around 3 μmol/g/h (Fig. 4). When xylitol infusion was terminated, all parameters tended to return to basal values.

In livers from fed rats, methanol infusion increased pyridine nucleotide fluorescence to about 110% of basal values without affecting oxygen consumption. Basal rates of methanol uptake were about 3 μmol/g/h (Fig. 5). Infusion of xylitol (5 mM) increased rates of O$_2$ uptake from about 95 to 115 μmol/g/h (Fig. 5) and pyridine nucleotide fluorescence to about 130% of basal levels, but did not alter rates of methanol uptake

![Fig. 4. Effect of methanol and xylitol on oxygen and methanol uptake and NADH fluorescence in a perfused liver from a fasted rat.](image)

**TABLE II**

| Addition            | H$_2$O$_2$ generation μmol/g/h | Pyridine nucleotide fluorescence % of basal |
|---------------------|--------------------------------|--------------------------------------------|
| None                | 7.1 ± 0.8                      | 100                                        |
| Butanol             | 2.0 ± 0.3$^*$                  | 119 ± 5$^*$                                |
| Butyraldehyde       | 1.7 ± 0.6$^*$                  | 122 ± 6$^*$                                |
| + 4-methylpyrazole  |                                |                                            |

$p < 0.01$ as compared to no addition control values.

![Fig. 5. Effect of methanol, xylitol, and aminoxyacetate on oxygen and methanol uptake and NADH fluorescence in a perfused liver from a fed rat.](image)
basal values, and methanol uptake decreased to about 1 μmol/g/h (Fig. 6).

**DISCUSSION**

**Catalase Is a Major Pathway of Ethanol Metabolism in the Fasted State**—As noted in the Introduction, the lack of specificity of inhibitors of pathways of ethanol metabolism makes interpretation of results difficult. This problem was circumvented by employing selective substrates for the major enzymes involved in ethanol oxidation. In the present study, methanol and butanol were verified as selective substrates for rodent catalase and ADH, respectively.

Rates of butanol uptake by livers from fed rats were about an order of magnitude greater than rates of methanol uptake. These data support the well accepted notion that ADH is the predominant pathway of alcohol metabolism in the fed state (27). In the fasted state, increasing concentrations of an albumin-oleate complex, a physiological substrate for peroxisomal β-oxidation, elevated rates of H₂O₂ generation by 400% (9). In the present study rates of methanol oxidation via catalase-H₂O₂ accounted for about 60% of total alcohol oxidation in the presence of fatty acids. Thus, in the fasted state where concentrations of fatty acids in the circulation and liver are elevated, rates of alcohol oxidation are decreased and oxidation is mediated predominantly via catalase. High concentrations of fatty acids, which are found in the fasted state in vivo, are necessary for maximal rates of catalase-dependent alcohol oxidation (9). Thus, in vivo one would predict a pattern of increasing and decreasing rates of alcohol metabolism resulting from changes in the contributions of catalase and ADH. Rhythmic increases and decreases in rates of ethanol metabolism have been observed by Sturtevant and Garber (29); nutritionally mediated alterations in the contributions of ADH and catalase-H₂O₂ may explain why these rhythms occur.

**Redox Interactions between ADH and Catalase**—The β-oxidation of fatty acyl-CoA compounds in peroxisomes proceeds via an acyl-CoA oxidase which consumes O₂ and produces H₂O₂ and a dehydrogenase which requires NAD⁺ (Fig. 6). In fact, cyanide-insensitive reduction of NAD⁺ in the presence of fatty acyl-CoA compounds is used routinely to measure peroxisomal β-oxidation in vitro (30). In the perfused liver, increases in the NADH/NAD⁺ ratio caused by the metabolism of butanol, butyraldehyde, or xylitol inhibited H₂O₂ generation (Figs. 3-5, Table II). This inhibition of methanol uptake by butanol was reversed when oxidation of butanol via ADH was inhibited (Fig. 3). Thus, it is concluded that H₂O₂ generation via peroxisomal β-oxidation and methanol uptake were inhibited by elevation of the NADH/NAD⁺ ratio by these compounds, presumably at the NAD⁺-requiring β-hydroxyacyl-CoA dehydrogenase (Fig. 6). Based on these data, it is also concluded that interactions between the ADH and catalase pathways occur and are mediated via elevation of the pyridine nucleotide redox state.

Although it is known that peroxisomes are permeable to NAD⁺ (31), the mechanism(s) by which reducing equivalents generated via peroxisomal β-oxidation move into and out of peroxisomes is not clear. Redox interactions between catalase and ADH suggest that in intact cells the pyridine nucleotide redox state within the peroxisome and the cytosol are in equilibrium. It is generally accepted that reducing equivalents generated in the cytosol must be moved into the mitochondria to be combusted by the mitochondrial electron transport...
chain. A major route of transport of reducing equivalents into and out of mitochondria is the malate-aspartate shuttle (Fig. 6). Reducing equivalents generated by the oxidation of both butanol and xylitol are transferred predominately via this route (32). Since butanol and xylitol both inhibited \( \text{H}_2\text{O}_2 \) generation in the fasted state resulting in lower rates of methanol uptake, it is possible that reducing equivalents formed from the oxidation of butanol or xylitol compete with reducing equivalents generated via peroxisomal \( \beta \)-oxidation of fatty acyl-CoA compounds for transport into the mitochondria via the malate-aspartate shuttle. In support of this hypothesis, xylitol alone did not affect rates of methanol uptake in livers from fed rats (Table II), where the capacity of the malate-aspartate shuttle is involved in the reoxidation of reducing equivalents generated via peroxisomal \( \beta \)-oxidation (32). Since butanol and xylitol both inhibited \( \text{H}_2\text{O}_2 \) generation in the fasted state resulting in lower rates of methanol uptake, it is possible that reducing equivalents generated via peroxisomal \( \beta \)-oxidation of fatty acyl-CoA compounds for transport into the mitochondria via the malate-aspartate shuttle. In support of this hypothesis, xylitol alone did not affect rates of methanol uptake in livers from fed rats (Table II), where the capacity of the malate-aspartate shuttle is involved in the reoxidation of reducing equivalents generated via peroxisomal \( \beta \)-oxidation (32).

Taken together, the data indicate that significant interactions exist between the ADH and catalase pathways of ethanol metabolism. The redox effects of ethanol metabolism via ADH on peroxidation via catalase may represent an important physiological form of regulation of metabolism. The redox effects of ethanol metabolism via ADH on peroxidation via catalase may represent an important physiological form of regulation of metabolism. The redox effects of ethanol metabolism via ADH on peroxidation via catalase may represent an important physiological form of regulation of metabolism.