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Contribution of liver alcohol dehydrogenase to metabolism of alcohols in rats

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ABSTRACT

The kinetics of oxidation of various alcohols by purified rat liver alcohol dehydrogenase (ADH) were compared with the kinetics of elimination of the alcohols in rats in order to investigate the roles of ADH and other factors that contribute to the rates of metabolism of alcohols. Primary alcohols (ethanol, 1-propanol, 1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol) and diols (1,3-propanediol, 1,3-butanediol, 1,4-butanediol, 1,5-pentanediol) were eliminated in rats with zero-order kinetics at doses of 5–20 mmol/kg. Ethanol was eliminated most rapidly, at 7.9 mmol/kg h. Secondary alcohols (2-propanol-d_7, 2-propanol, 2-butanol, 3-pentanol, cyclopentanol, cyclohexanol) were eliminated with first order kinetics at doses of 5–10 mmol/kg, and the corresponding ketones were formed and slowly eliminated with zero or first order kinetics. The rates of elimination of various alcohols were inhibited on average 73% (55% for 2-propanol to 90% for ethanol) by 1 mmol/kg of 4-methylpyrazole, a good inhibitor of ADH, indicating a major role for ADH in the metabolism of the alcohols. The Michaelis kinetic constants from *in vitro* studies (pH 7.3, 37 °C) with isolated rat liver enzyme were used to calculate the expected relative rates of metabolism in rats. The rates of elimination generally increased with increased activity of ADH, but a maximum rate of 6 ± 1 mmol/kg h was observed for the best substrates, suggesting that ADH activity is not solely rate-limiting. Because secondary alcohols only require one NAD^+ for the conversion to ketones whereas primary alcohols require two equivalents of NAD^+ for oxidation to the carboxylic acids, it appears that the rate of oxidation of NADH to NAD^+ is not a major limiting factor for metabolism of these alcohols, but the rate-limiting factors are yet to be identified.

1. Introduction

Alcohol dehydrogenases (ADH) are ubiquitous in higher organisms and participate in metabolizing a wide variety of alcohols and aldehydes, as an important "detoxification" mechanism [1]. ADHs catalyze the first step in alcohol oxidation, using NAD^+ as a cofactor and producing NADH and the corresponding carbonyl compound. Various studies have suggested that ADH activity is a major rate-limiting factor in ethanol metabolism, because the amount of liver ADH is approximately sufficient to account for the rate of elimination in animals, and inhibitors of ADH act equivalently *in vitro* and *in vivo* in rats [2–8]. Fed rats eliminate ethanol at about 8 mmol/kg h, and the total liver ADH activity could provide about a 1.4-fold higher rate, but the concentrations of coenzymes and acetaldehyde *in vivo* could limit the rate of ethanol metabolism to the observed value [7,8]. Humans eliminate ethanol at about 2.2 mmol/kg h, but the total ADH activity and the mass of the liver relative to body weight are each about one-half of that found in the rat, supporting the conclusion that ADH activity is a major rate limiting factor for ethanol metabolism in humans [2].

Kinetic simulation with estimated rate constants for alcohol and aldehyde dehydrogenases can approximately describe ethanol and acetaldehyde metabolism in humans, and it is significant that the rate of elimination of ethanol is directly related to ADH activity, whereas the steady-state level of acetaldehyde (almost a constant blood concentration) depends on levels of both alcohol and aldehyde dehydrogenases [9]. Although metabolism of ethanol in

Abbreviations: ADH, alcohol dehydrogenase; MP, 4-methylpyrazole; IBA, isobutyramide; AT, 3-amino-1,2,4-triazole.

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humans is complicated because humans have five different ADHs that can contribute to ethanol metabolism and the kinetic constants and the concentrations for these enzymes should be considered [10,11], it is remarkable that the rate of metabolism can be described by a single set of kinetic constants [9]. However, in the steady-state of metabolism, it is likely that several steps, such as aldehyde dehydrogenase activity, transport of reducing equivalents from NADH into the mitochondria, and reoxidation of NADH in oxidative phosphorylation contribute to controlling the overall rate of metabolism, and more complete quantitative descriptions are required [2,12]. Other enzymes, such as catalase and cytochrome P450 2E1, can also contribute to the oxidation of alcohols.

The specificities of ADHs for various alcohols and the kinetics of metabolism (elimination) in animals are also of fundamental interest because it is clear that ethanol is not the only substrate, and metabolism of ethanol can affect the metabolism of other alcohols and aldehydes, such as retinoids [13,14]. Identification of endogenous substrates that may have physiological roles is a continuing challenge. The rat is a good model for these studies because of extensive prior use of this animal for studies of alcohol metabolism. Moreover, metabolism of various alcohols in rats should be studied as a prelude to any studies with humans.

Rats produce only four different active ADHs (see Ref. [15] for ADH nomenclature), but the single liver class 1 enzyme (ADH1, UniProt P06757) is the major ADH responsible for metabolism of common alcohols [16,17]. The rodent ADH2 (UniProt Q64563) is much less active than the other ADHs [18]. The substrate specificities of three rat enzymes have been surveyed, and ADH3 (UniProt P12711) has no detectable activity on ethanol and butanol [19]. The "stomach" enzyme, ADH4 (UniProt P41682), has much lower catalytic efficiencies than ADH1, but may contribute to metabolism of high concentrations of alcohols and in metabolism of retinoids and lipid peroxidation products [16,20–22]. ADH5 (UniProt Q5X195) is not expressed in an active form [23]. Having one major ADH makes it simpler to study the correlation of *in vitro* and *in vivo* activities. Studies with the rat are relevant for understanding metabolism of alcohols in humans, even though humans produce three class I ADHs with quantitatively-different substrate specificities, because the specificities of class I ADHs overlap and the metabolic pathways (chemical transformations) are probably similar in rats and humans. This work presents results on the specificities of rat liver ADH for alcohols, the kinetics of elimination of various alcohols in rats, and the correlation of *in vitro* ADH activities with the rates of metabolism.

2. Experimental procedures

2.1. Substrate specificity of rat liver alcohol dehydrogenase

Rat liver alcohol dehydrogenase was partially purified by precipitation with $(NH_4)_2SO_4$ (35–75% saturation), passage through DEAE-cellulose, and chromatography on Sephadex G-100 [24]. The specific activity was about 0.2 unit/mg, as assayed at 37 °C in a standard enzyme assay [25]. The enzyme was stabilized by adding 5% ethanol during the purification, but preparations gradually lost about 50% of the original activity during storage at 5 °C for a week. The insertion of Cys-112 may account for the lability [26]. (Adding NAD^+ also stabilized the enzyme, but after a few days of storage, the kinetics did not fit simple Michaelis–Menten kinetics, as K_m values for ethanol increased substantially, and it appeared that two forms of enzyme were present.) The ethanol was removed by gel filtration before kinetics studies with various alcohols, and a small reaction rate observed in the absence of added alcohol was subtracted from the rates with varied concentrations of alcohols. The reactions were also almost totally inhibited by 10 mM pyrazole, confirming the ADH activity. The kinetics of the ADH were studied in 83 mM potassium phosphate and 40 mM KCl buffer, pH 7.3, at 37 °C, with 0.5 mM NAD^+, which is thought to resemble the conditions found *in vivo* [4]. The initial velocity data were fitted to the Michaelis–Menten equation [27]. Crystalline horse liver alcohol dehydrogenase, EE isoenzyme (ADH1E, UniProt P003327), was purchased from Boehringer Mannheim Co.

2.2. Elimination of alcohols in rats and effects of inhibitors

Male Sprague–Dawley rats (180–280 g) were fed certified rodent diet (equivalent to Harlan 8728C; 24% protein, 5% fat, 40% carbohydrate; tekladinfo@harlan.com) *ad libitum*, except for those that were fasted for 24 h. Lights were on from 5 a.m. to 5 p.m. during Central Standard Time and 6 a.m. to 6 p.m. during Daylight Savings Time. Experiments were begun mid-morning. Alcohols were diluted in physiological saline to 1 M or lower and administered intraperitoneally at sub-lethal doses. The doses were (usually) chosen to be within the concentration range used in the *in vitro* studies and to give measurable levels in the blood. Blood samples (10 μL) were drawn from the tail vein at regular intervals, such as 10 min, as shown in the figures, and mixed with an internal standard (an alcohol chosen to have a retention time close to the alcohol under investigation), and deproteinized with $Ba(OH)_2$ and $ZnSO_4$. (For 2,3-butanediol, methanol, and *tert*-butanol, which are slowly-eliminated, blood samples were taken about every 30 min.) The concentrations of ethanol determined in the tail vein are approximately the same as those in arterial blood [28]. The supernatant was analyzed on a Varian 3740 gas chromatograph with five different columns (usually 6 ft × 2 mm) and varied oven temperatures chosen to provide base-line resolution for the alcohols and their metabolites. Most primary and secondary alcohols and ketones were separated on 60/80 mesh Carbopack B/5% Carbowax PEG 20 M or 60/80 mesh Carbopack C/0.2% Carbowax 1500 developed at 20 ml N_2/min, with fixed column temperatures ranging from 60 to 130 °C. 1,2-Ethanediol, 1,5-pentanediol, and δ-valerolactone were separated on the Carbowax 20 M column at 140 or 180 °C. Ethanol, 1-butanol, and 1,2-ethanediol were separated on 80/100 Chromasorb 102 at 30 ml N_2/min, at 150, 200 and 200 °C. Ethanol and 1,2-ethanediol were also separated on 80/100 Porapak-S at 30 ml N_2/min at 150 and 200 °C. The diols, benzyl alcohol and benzaldehyde were separated on 80/100 Carbopack C/0.8% THEED (3 ft × 2 mm ID) at 40 ml N_2/min at 100 or 125 °C. The concentrations were calculated with a Hewlett–Packard 3388A integrator calibrated with standard curves.

The elimination data were fitted to the equations appropriate for the observed kinetics using the non-linear least squares Fortran program, NONLIN (C.M. Metzler, The Upjohn Co.). For zero-order (straight line dependence on time) elimination, the data were fitted to the equation $[A]_t = k_1 t + [A]_o$, and the rate constant was calculated by dividing the dose administered by the time required for complete elimination as determined by the linear extrapolation to zero concentration (mathematically, $-[A]_o/k_1$). The rate constants for three or more animals were averaged and the standard deviation calculated. The results for two animals were averaged and the range of values is reported. For non-linear (exponential) elimination, various equations were tested to determine the best fits. For the simplest reaction, A → B (A the alcohol and B an unidentified product), the equation $d[A]/dt = -k_1[A]$ was used. For sequential A → B → C reactions (B usually a ketone and C not identified), data for [A] and [B] were fitted simultaneously with various kinetic descriptions to find the best fit: irreversible, sequential first order, $d[A]/dt = -k_1[A]$ and $d[B]/dt = k_1[A] - k_2[B]$; sequential first order with reversible first step and irreversible second step, $d[A]/dt = -k_1[A] + k_2[B]$ and $d[B]/dt = k_1[A] - (k_{-1} + k_2)[B]$; sequential, irreversible, with Michaelis–Menten kinetics for first step followed