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INHIBITION OF ALCOHOL DEHYDROGENASE ACTIVITY BY ACETYLENIC AND ALLYLIC ALCOHOLS: CONCORDANCE WITH IN VIVO ELECTROPHILE REACTIVITY IN FISH

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Abstract–Acetylenic and allylic alcohols have been reported to be 20 to 5,000 times more acutely toxic to fathead minnows (*Pimephales promelas*) than would be expected from a narcosis-based mechanism of action. The greater-than-expected toxicity of these alcohols has been proposed to be a result of metabolic activation to the corresponding reactive α,β-unsaturated aldehydes or allene derivatives. Using purified horse liver and rainbow trout (*Oncorhynchus mykiss*) hepatic cytosol alcohol dehydrogenase (ADH) preparations, the propensity of a series of acetylenic and allylic alcohols to inhibit enzyme activity, in both the presence and the absence of reduced glutathione, was ascertained. Those alcohols classified as reactive toxicants in acute toxicity tests were generally effective inhibitors of ADH activity, whereas those alcohols classified as narcotics were generally ineffective inhibitors. The results from this study suggest that (a) acetylenic and allylic alcohols may be metabolically activated to reactive species and (b) to compare and ultimately predict the toxicity of these unsaturated alcohols, their rates of metabolic activation and the reactivity of the subsequently produced aldehydes must be quantified.

Keywords–Acetylenic alcohols Allylic alcohols Alcohol dehydrogenase activity Rainbow trout

INTRODUCTION

Veith et al. [1] demonstrated that a series of propargylic alcohols were 100 to 5,000 times more acutely toxic to fathead minnows (*Pimephales promelas*) than would be expected from a narcosis-based quantitative structure–activity relationship (QSAR) [2]. Allylic alcohols were also reported to be about 20 to 30 times more toxic than what would be estimated from the narcosis QSAR. Signs of intoxication with these alcohols included lordosis, scoliosis, edema, and tetany. In contrast, tertiary propargylic alcohols and unconjugated alken-ols elicited signs of intoxication consistent with narcosis, and measured 96-h LC50 values were within a factor of one to those estimated from the narcosis QSAR.

Veith et al. [1] and Lipnick et al. [3] have proposed that the greater-than-predicted toxicity of the identified allylic and primary and secondary propargylic alcohols is due to metabolic activation, via alcohol dehydrogenase (ADH), to the corresponding α,β-unsaturated aldehydes and ketones. The reactivity of homopropargylic alcohols is hypothesized to be the result of enzymatic oxidation to the corresponding aldehydes, followed by enolization and tautomerization to electrophilic allene derivatives. To further assess the likelihood that enzymatically mediated activation of specific acetylenic and allylic alcohols is a critical component in their acute toxicity to fish, a representative set of compounds was studied to quantify their ability to inhibit ADH activity. ADH was selected to simultaneously serve as a model-activating enzyme system and a nucleophile trap for the produced electrophiles. These studies were undertaken with both purified horse liver ADH and rainbow trout (*Oncorhynchus mykiss*) hepatic cytosol preparations.

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MATERIALS AND METHODS

General

The alcohols used in this study were obtained from either Aldrich Chemical Co. (Milwaukee, WI) or Farchan Laboratories (Gainesville, FL) and were of 95% purity or greater. Horse liver ADH, reduced glutathione (GSH), oxidized nicotinamide adenine dinucleotide (NAD), and reduced nicotin-

amide adenine dinucleotide (NADH) were obtained from Sigma Chemical Co. (St. Louis, MO).

Juvenile rainbow trout (100–200 g) were obtained from a local hatchery (Seven Pines Trout Hatchery, Lewis, WI) and held in flow-through tanks (sand-filtered Lake Superior water) maintained at 11°C. Trout were fed three times weekly and held on a 16:8-h light:dark cycle. ADH activity was monitored in the 105,000-*g* hepatic supernatant. Livers from three to five fish were rapidly excised and placed into cold 1.15% KCl, washed, blotted, and weighed. The tissue was minced in four volumes KCl, homogenized with a mechanically driven Teflon® pestle, and centrifuged at 10,000 *g* for 20 min at 4°C in a Beckman (Irvine, CA) L5-50 ultracentrifuge. The resulting supernatant was centrifuged at 105,000 *g* for 60 min. The postmicrosomal supernatant was then used to ascertain ADH activity.

ADH activity was determined by the methods of Vallee and Hoch [4]. The optimum reaction mixture (2.5 ml) consisted of 50 mM sodium pyrophosphate (pH 8.0), appropriate volumes of either horse liver ADH or trout hepatic cytosol solutions, and the alcohol of concern. The reaction was initiated by adding NAD (2.0 mM). Typically used protein concentrations, as determined by the biuret method [5], were 0.2 and 6.0 mg/ml, respectively, for the horse and trout preparations. ADH activity was based on the background-corrected rate of reduction of NAD monitored at 340 nm with a Beckman DU-7 spectrophotometer. All experiments were performed at 25°C.

ADH inhibition—Experiment I

The time course of ADH inhibition by the test alcohols was estimated following the general approach of Rando [6]. Incubations of 5 ml total reaction volume (50 mM pyrophosphate buffer at pH 8.0) were prepared that consisted of either horse (0.2 mg/ml) or trout (6.0 mg/ml) protein and the respective substrates (100 mM). Reactions were initiated by adding NAD (2.0 mM). At 0.25, 1, 24, 48, 72, and 96 h, 100-μl aliquots were withdrawn and assayed for ADH activity with the standard protocol with ethanol (100 mM) as the substrate. The percent remaining activity in alcohol incubations was based on a comparison to a control preparation that contained no alcohol substrate. ADH activity in controls of this nature was essentially unchanged through 96 h. Additional control incubations containing the test alcohols, but no NAD, showed no loss of ADH activity at 96 h.

ADH activity—Experiment II

To assist in determining whether or not ADH preparations were producing electrophilic products, GSH was employed as a nucleophilic trap [7,8]. Using ethanol as a substrate, initial optimization studies indicated that GSH concentrations up to 100 mM did not alter ADH activity. Therefore, to maximize the ability to detect electrophilic products, the effect of GSH on ADH activity was determined by monitoring the rate of NAD reduction in preparations containing 100 mM substrate and either 0 or 100 mM GSH. Preliminary studies established that at substrate concentrations of 100 mM maximal ADH activity was attained for each alcohol under investigation.

RESULTS AND DISCUSSION

ADH was used in the present study as a model enzyme that could potentially activate the selected alcohols in vivo. ADH also simultaneously served as a model nucleophile to ascertain aldehyde reactivity; i.e., the loss of ADH activity is a composite measure of both metabolic activation and aldehyde reactivity. Obviously, the in vivo toxicity of the selected alcohols should not be ascribed to ADH inhibition, rather the studies were designed to assess the hypothesis that acetylenic and allylic alcohols are activated to electrophiles capable of reacting with biologically relevant nucleophiles.

General support for the activation hypothesis proposed by Veith et al. [1] can be found in studies where ADH and alcohol oxidase activity from horse liver and yeast preparations, respectively, were reported to be irreversibly inhibited by 3-butyn-1-ol, 2-propyn-1-ol, and 2-butyn-1,4-diol [7,8]. These studies further suggested that the above alcohols do not act as suicide inhibitors, but rather that the reactive aldehyde products are released from the enzyme and subsequently react with nucleophiles in the active site. To establish additional support for the hypothesis that the acute toxicity of primary, secondary, and homopropargylic alcohols and allylic alcohols is due to metabolic activation products, a larger and more diverse set of compounds were screened for their ability to inhibit ADH activity.

ADH inhibition—Experiment I

In the first series of experiments, the inhibition of ADH activity in horse and trout preparations was examined through 96 h. The results of these experiments at 48 h are listed in Table 1; percentage activities remaining at 72 and 96 h were essentially

Table 1. Acute toxicity (96-h fathead minnow LC50 values), ADH inhibition, and ADH activity for a series of unsaturated alcohols

Alcohol	LC50 (mg/L)[b]	ADH inhibition—Experiment I: Percentage ADH activity remaining after 48 h incubation[a]		ADH activity (nmol/min/mg)[a]—Experiment II			
				Horse liver		Rainbow trout liver	
		Horse liver	Rainbow trout liver	No GSH	100 mM GSH	No GSH	100 mM GSH
Primary propargylic							
2-Propyn-1-ol	1.50 (P)	16.3 ± 1.14[c]	96.5 ± 12.0	12.1 ± 4.35	39.2 ± 5.30[d]	5.50 ± 0.267	9.67 ± 0.483[d]
2-Butyn-1-ol	10.1 (P)	75.9 ± 7.40	89.4 ± 26.0	61.9 ± 8.45	159 ± 33.0[d]	3.48 ± 0.300	17.2 ± 1.53[d]
2-Butyn-1,4-diol	53.6 (P)	57.2 ± 3.22	99.0 ± 17.6	216 ± 24.0	397 ± 10.0[d]	14.0 ± 0.907	18.7 ± 2.82[e]
Secondary propargylic							
1-Octyn-3-ol	0.413 (P)	2.24 ± 2.80	30.9 ± 16.9	15.6 ± 10.0	0.850 ± 1.45[d]	0.898 ± 0.988	2.02 ± 1.88
Tertiary propargylic							
2-Methyl-3-butyn-2-ol	3,290 (N)	108 ± 12.9	111 ± 13.4	12.6 ± 11.1	1.90 ± 1.65	0.132 ± 0.183	1.13 ± 0.163[d]
3-Methyl-1-pentyn-3-ol	1,270 (N)	98.5 ± 12.0	112 ± 11.3	16.4 ± 5.50	11.2 ± 11.7	0.338 ± 0.477	1.91 ± 0.817
Homopropargylic							
3-Butyn-1-ol	36.1 (P)	0.830 ± 1.44	20.8 ± 5.42	434 ± 44.5	408 ± 14.0	24.0 ± 14.5	29.2 ± 6.02
4-Pentyn-2-ol	35.1 (P)	2.52 ± 2.49	14.4 ± 15.5	77.5 ± 9.30	67.0 ± 3.20	2.73 ± 1.52	7.62 ± 0.933[e]
Alken-ols							
1,5-Hexadien-3-ol	38.1 (P)	15.4 ± 7.38	89.9 ± 21.1	281 ± 39.0	341 ± 15.0[e]	5.18 ± 2.63	18.0 ± 1.83[d]
cis-3-Hexen-1-ol	381 (N)	73.1 ± 4.30	29.7 ± 5.56	490 ± 33.5	463 ± 30.0	6.05 ± 1.48	10.6 ± 0.783[e]
trans-3-Hexen-1-ol	271 (N)	71.2 ± 9.70	18.8 ± 4.86	435 ± 15.0	428 ± 76.5	2.07 ± 0.933	7.57 ± 1.27[d]

[a]See Materials and Methods section for experimental details.
[b]Data from Veith et al. [1]; (P) or (N) indicates whether the associated alcohol was classified as either a proelectrophile or a narcotic, respectively.
[c]Mean ± SD based on three to four replicate enzyme preparations.
[d]ADH activity in the presence of 100 mM GSH significantly different from activity in the appropriate no-GSH treatment (two-sample t test, $p = 0.05$).
[e]ADH activity in the presence of 100 mM GSH significantly different from activity in the appropriate no-GSH treatment (two-sample t test, $p = 0.10$).

the same as that noted at 48 h. In the horse liver preparations, those alcohols identified as proelectrophiles [1] were generally quite effective in inhibiting ADH activity. With the homopropargylic alcohols, 1-octyn-3-ol (the secondary propargylic alcohol), and 1,5-hexadien-3-ol (an allylic alcohol), horse liver ADH activity was inhibited by approximately 20 to 80% after 15 min, and by 48 h 0.83 to 15.4% activity remained. In the case of the primary propargylic alcohols, the greatest inhibition was noted with propyn-1-ol. At 15 min approximately 40% activity remained; ADH activity steadily decreased to 16.3% at 48 h. The other two primary propargylic alcohols were moderately effective in inhibiting ADH activity (57.2–75.9% activity remaining at 48 h). Horse liver ADH activity was essentially unchanged by the tertiary propargylic alcohols and moderately inhibited when in the presence of the 3-hexen-1-ol isomers (71.2–73.1% activity remaining at 48 h). These latter four alcohols were classified as narcotics by Veith et al. [1].

As noted in the horse liver ADH experiments, the homopropargylic alcohols and 1-octyn-3-ol were effective in inhibiting trout ADH activity (14.4–30.9% activity remaining at 48 h). Interestingly, all three primary propargylic alcohols and 1,5-hexadiene-3-ol did not inhibit trout ADH activity. Of the alcohols classified as narcotics by Veith et al. [1], the tertiary propargylic alcohols did not inhibit trout ADH activity, whereas the isomers of 3-hexen-1-ol were effective in inhibiting activity (18.8–29.7% activity remaining at 48 h).

Alston et al. [7] established that both 2-propyn-1-ol and 3-butyn-1-ol effectively inactivated horse liver ADH; however, 3-butyn-1-ol inactivation was more rapid and extensive, which is consistent with the current results for both horse and trout ADH activity. Nichols and Cromartie [8] established that 2-propyn-1-ol and 2-butyn-1,4-diol inactivated alcohol oxidase from *Candida boidini* in a similar manner. The results with horse liver ADH from the current study provide preliminary evidence of a larger substrate domain for those inhibitors that may be self-generated by this enzyme. The horse liver inhibition studies are also generally consistent with the findings of Veith et al. [1], i.e., alcohols classified as proelectrophiles were capable of inhibiting horse liver ADH. The results based on trout liver ADH indicate that the secondary propargylic and homopropargylic alcohols were also capable of inhibiting enzyme activity; however, the primary propargylic alcohols and the allylic alcohols were generally ineffective inhibitors. Tertiary propargylic alcohols, which are classified as narcotics [1], did not inhibit horse or trout liver ADH activity. Curiously, the 3-hexen-1-ol isomers were moderate to potent inhibitors, even though they were reported to act as narcotics in vivo [1].

The inhibition experiments were adapted from studies designed to specifically ascertain whether or not ADH inhibitors could be self-generated by the target enzyme [6–8]. As such, the results of these assays are a composite measure of substrate (alcohol) and product (aldehyde/inhibitor) specificity for ADH as well as the reactivity of the generated α,β-unsaturated aldehydes or allene intermediates. The level of ADH inhibition is also critically related to the extent by which the activated products competitively react with nucleophiles in solution and in the active site of the enzyme. The differences noted in enzyme inhibition between the trout and horse liver preparations could be due to ADH variation in substrate/inhibitor specificity. Although differences in coenzyme and substrate specificity do exist, the ADH catalytic domain is generally considered quite similar across vertebrate species [9]. Tsai et al. [10] have also reported that purified horse liver and trout ADH have similar specificity for NAD and similar relative substrate specificity for several primary and secondary alcohols. Certainly, additional studies with purified trout ADH would be required to rigorously quantify substrate specificity for the alcohols studied here; however, as described below, the species differences in ADH inhibition are more likely due to the presence of additional soluble nucleophiles in the trout cytosol preparation.

ADH activity—Experiment II

In a second series of assays, horse and trout ADH activity associated with each of the unsaturated alcohols was determined in the presence and absence of GSH. These studies were undertaken to quantify ADH activity as a function of alcohol substrate and to determine whether a model nucleophile (GSH) could improve activity, under the assumption that it would trap reactive products in solution. In the absence of GSH, horse and trout liver ADH activity ranged from 12.1 to 490 and 0.132 to 24.0 nmol/min/mg protein, respectively (Table 1). In the presence of GSH, both horse and trout ADH activity increased significantly when the primary propargylic alcohols and 1,5-hexadiene-3-ol were used as substrates. In the trout preparation, activity also increased significantly with one of the homopropargylic alcohols (4-pentyn-2-ol). In the case of the secondary propargylic alcohol, 1-octyn-3-ol, GSH did not increase ADH activity

with either preparation. For those alcohols classified as narcotics by Veith et al. [1], horse liver ADH activity was not significantly affected by GSH. With the trout liver preparation, ADH activity significantly increased with one of the tertiary propargylic alcohols (2-methyl-3-butyn-2-ol); however, this result should be viewed with caution due to the overall extremely low activity. Interestingly, trout liver ADH activity, in the presence of GSH, significantly increased with the 3-hexen-ol isomers as well.

Increased ADH activity in the presence of GSH is consistent with the assumption that reactive aldehyde products are released from the enzyme and react with the added nucleophile, perhaps via a Michael addition reaction [8], thereby protecting nucleophiles within the active site. Thus, the presence of multiple nucleophiles could influence the extent of competing Michael addition reactions. Of course, ADH inhibition is also influenced by the substrate specificity of the reactive aldehyde. For the primary propargylic alcohols and 1,5-hexadiene-3-ol, classified as proelectrophiles by Veith et al. [1], the presence of GSH was usually associated with increased ADH activity. It would seem that in the trout liver ADH inhibition experiments, nucleophiles in solution were effective in trapping the reactive aldehydes produced from these alcohols. In the horse liver ADH inhibition studies, the less extensive and/or different pool of available nucleophiles were presumably somewhat effective in protecting enzyme activity from the aldehydes derived from 2-butyn-1-ol, 2-butyn-1,4-diol, and 1,5-hexadiene-3-ol, but not from 2-propyn-1-ol. The secondary propargylic alcohol, 1-octyn-3-ol, effectively inhibited both trout and horse liver ADH activity; however, in the presence of GSH, enzyme activity was not increased. These results could indicate that 1-octyn-3-one either preferentially reacts with nucleophiles in the ADH active site or that the resultant aldehyde does not leave the enzyme. This aldehyde has an estimated log octanol/water partition coefficient of approximately 2.0 [11], and it is conceivable that it may remain associated with the enzyme.

The homopropargylic alcohols were both effective in inhibiting trout and horse liver ADH activity; however, GSH was seemingly ineffective in protecting the enzyme. Under the conditions of this study, these results taken together seem to indicate that either the activated metabolites preferentially react with nucleophiles in the enzyme active site or the metabolites do not diffuse from the protein.

The 3-hexen-1-ol isomers were moderately effective inhibitors of horse liver ADH activity and comparatively potent inhibitors of trout liver ADH activity, although the parent alcohols seem to act as narcotic toxicants in vivo [1]. GSH was also significantly effective in protecting trout liver ADH activity. These results may be an artifact of the in vitro conditions whereby the alcohols have ready access to ADH. Although oxidation of these alcohols would not directly lead to an electrophilic Michael acceptor, the resulting 3-hexen-1-als could form 2-hexen-1-als via enolization and tautomerization, which would act as Michael acceptor electrophiles.

Veith et al. [1] proposed that to predict the toxicity of small α,β-unsaturated alcohols, their rate of metabolic activation would be more important than the reactivity of the corresponding electrophilic aldehydes. For the primary propargylic alcohol substrates studied here, horse liver ADH activity in the presence of GSH (Table 1) was greatest with 2-butyn-1,4-diol (397 nmol/min/mg), followed by 2-butyn-1-ol (159 nmol/min/mg) and 2-propyn-1-ol (39.2 nmol/min/mg). Alston et al. [7] also reported that 2-propyn-1-ol was a relatively poor substrate for horse liver ADH. The trend in ADH activity (i.e., the rate of metabolic activation) is opposite to the trend observed for the acute toxicity (96-h LC50 values) of these alcohols to fathead minnows (Table 1), for which 2-propyn-1-ol is the most toxic (1.50 mg/L), followed by 2-butyn-1-ol (10.1 mg/L) and 2-butyn-1,4-diol (53.6 mg/L). The results from this study suggest that when comparing, and ultimately predicting, the toxicity of α,β-unsaturated alcohols the reactivity of the enzymatically produced aldehydes, as well as their rates of metabolic activation, may be important. Future studies are planned to quantify ADH activity based on the rate of aldehyde production and to directly measure the reactivity of these Michael acceptor electrophiles.

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