

THE EFFECTS OF DISULFIRAM ON EQUINE HEPATIC ALCOHOL DEHYDROGENASE AND ITS EFFICIENCY AGAINST ALCOHOLISM: VINEGAR EFFECT

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(Received 8 March 1995; in revised form 3 July 1995, accepted 18 August 1995)

Abstract — The effects of disulfiram, its metabolite diethyldithiocarbamate and dithiodipyridine on alcohol metabolism of equine hepatic alcohol dehydrogenase (EC.1.1.1.1.) have been investigated. They were found to form enzyme–NAD⁺–inhibitor complexes which were competitive inhibitors of alcohol metabolism with dissociation constants ($K_{E0,1}$) at pH 7.0 of 50 μ M, 1.3 mM and 260 μ M, respectively. Acetate and vinegar behaved similarly in forming an inhibitory enzyme–NAD⁺–acetate ternary complex competitive with ethanol, with at pH 7.0 essentially identical dissociation constants of 4.0 mM and 3.8 mM, respectively. Disulfiram, diethyldithiocarbamate and dithiodipyridine were also found to exhibit affinity-labelling kinetics with liver alcohol dehydrogenase. The liver enzyme is chemically modified and inactivated in a similar manner by all three reagents via binary enzyme complexes with dissociation constants of 30 μ M, 200 μ M and 50 μ M, respectively. Used as a protector against enzyme inactivation by DL- α -bromo- β -(5-imidazolyl)-propionic acid, disulfiram, diethyldithiocarbamate and dithiodipyridine were found to form competitive binary enzyme complexes by binding to the active zinc site with $K_{E,1}$ values of 30 μ M, 170 μ M and 50 μ M, respectively. The disulfiram and acetate binding to zinc results in the formation of binary and ternary complexes which inhibit alcohol metabolism at the enzyme level. Due to many unwanted side-effects and the easy removal of its anti-drinking effects by drinking vinegar (vinegar effect), disulfiram may still be questioned as an effective drug against alcoholism.

INTRODUCTION

Ethanol is one of man's oldest and most commonly used psychotropic agents. Chronic exposure to ethanol results in both physical and psychological dependence. Today, in many parts of the world, alcoholism is a major problem, costing society dearly as well as ruining the lives of the ethanol addicts. Over the years, several treatments have been suggested such as with cyanamide (Peachey and Naranjo, 1983), but with only modest success. Today, a widely used method of limiting alcoholics' drinking is to give them disulfiram [Antabuse, tetraethylthiuram disulphide or bis (*N,N'*-diethylthiocarbamoyl disulphide) (Fuller and Litten, 1995)], the structure of which is shown in Fig. 1.

The dose of disulfiram used is variable. It is generally recommended that patients initiating treatment receive 500 mg daily as a single dose for 1–2 weeks and then 250 mg as maintenance

dosage. Disulfiram is considered an aversive drug which inactivates aldehyde dehydrogenase (EC.1.2.1.3.) in the liver, with the result that toxic concentrations of acetaldehyde build up if ethanol is drunk (the disulfiram–ethanol aversive reaction). Acetaldehyde is considered the main cause of the unpleasant and, it is hoped, aversive or dissuasive symptoms that are experienced under these circumstances (Hald and Jacobsen, 1948; Kitson, 1977). The mechanism of interaction between disulfiram and aldehyde dehydrogenase has therefore been much investigated (Kitson, 1975, 1978, 1982).

Disulfiram has also been found to be an inhibitor of numerous enzymes both *in vivo* and *in vitro*. Because of its general inhibitory action, disulfiram can affect carbohydrate metabolism, mitochon-

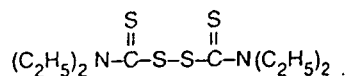


Fig. 1 Structure of disulfiram.

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drial oxidations, neurotransmissions and drug metabolism.

A number of drugs, such as calcium cyanamide (carbimide), tolbutamin, phenylbutazone, metronidazole and hypoglycaemics have been reported to produce disulfiram-like symptoms after drinking ethanol. In fact, a chemical analogue of metronidazole, flunidazole, was found to remove the disulfiram-ethanol reaction. However, from the studies carried out, it is difficult to evaluate the efficiency of such disulfiram-like drugs (Faiman, 1979).

Although the majority of disulfiram treatments are serious attempts to overcome ethanol addiction, there are those being treated who try to remove the effects of disulfiram. After interviewing eight former and present alcoholics on their drinking behaviour, we learned that among many alcoholics a well-known way of overcoming the disulfiram effect is by drinking vinegar (malt, cider or wine), which contains ~6-7% or 1 M acetic acid, prior to ethanol consumption. They were then able to consume quite large amounts of ethanol without being exposed to the usual disulfiram-ethanol aversive symptoms.

In this work, we have investigated the effect of disulfiram, diethyldithiocarbamate (DSH) and dithiodipyridine (PSSP) on equine hepatic alcohol dehydrogenase (LADH), a key enzyme of liver alcohol metabolism. In an attempt to evaluate the mechanism behind the vinegar effect, acetic acid and vinegar were also tested as reversible inhibitors of LADH.

METHODS

Enzyme and coenzyme

Equine hepatic alcohol dehydrogenase was obtained from Boehringer Mannheim, Germany. It was dialysed against three changes of 0.1 M phosphate buffer, pH 7.0. NAD⁺ was purchased from Sigma and had a purity grade of ~98%.

Other reagents

Disulfiram, diethyldithiocarbamate (DSH) and 2,2'-dithiodipyridine (PSSP) were obtained from Fluka, Switzerland. Due to low solubility in water, disulfiram used was dissolved in acetone. Vinegar from cider was obtained from Idun, Norway.

Other reagents were p.a. quality from Merck or Fluka.

All experiments were performed at 23.5°C.

Enzyme assay

Assay of the enzyme was by measurement of the initial rate of ethanol oxidation and concomitant NADH production at 340 nm as described earlier (Reynolds and McKinley-McKee, 1969). The enzyme was added to 3 ml of 62 mM glycine-NaOH buffer pH 10, containing 1 mg of NAD⁺ and 8 mM ethanol. All experiments were performed with a Phillips PU 8675 spectrophotometer and a Phillips PM 8272 recorder.

Inhibitions

Studies of the effects of reversible inhibitors on the initial rate of ethanol oxidation were carried out in quartz cuvettes of 1 cm light path. Each contained inhibitor, along with a constant (0.455 mM) concentration of NAD⁺ and a variable ethanol concentration in 3 ml 0.05 M phosphate buffer, pH 7.0. Prior to experiments with acetic acid and vinegar, the solutions were titrated to pH 7.0. The reaction was initiated by adding enzyme to a final concentration of 0.4 μM and a final volume of 3.02 ml. The initial rate of NADH production was determined at 340 nm and was taken as a measure of enzyme activity.

Inactivations

The liver enzyme was inactivated at concentrations ranging from 1.6 to 2.0 μM. A 0.1 M phosphate buffer, pH 7.0 was used. The reaction was started by adding 30 μl of enzyme to a mixture of inactivator in buffer to give a total volume of 1.0 ml. For each inactivation, a minimum of five aliquots were taken for assay measurements. Accuracy was gained by plotting the inactivation curves on semi-log paper with a one-decade ordinate, and the first order rate constants (*k'*) were determined from the slopes (*k'* = 2.3 × slope) or from the half times (*k'* = ln 2/*t*_{1/2}) (Reynolds and McKinley-McKee, 1969).

Protection against BrImPpOH inactivation

Protection against enzyme inactivation with BrImPpOH (DL- α -bromo- β -(5-imidazolyl)-propionic acid) were carried out with disulfiram, DSH, PSSP, acetone, acetate and vinegar. The inactivation cuvette contained 30 μl of LADH (con-

Table 1. Dissociation constants (mM) at pH 7.0

Reagent	$K_{EO,I}$	$K_{E,I}^*$	$K_{E,I}^\dagger$	$K_{EI,O}^\ddagger$
Disulfiram§	0.050	0.030	0.030	0.270
Diethyldithiocarbamate (DSH)	1.30	0.170	0.200	1.220
2,2-dithiodipyridine (PSSP)	0.260	0.050	0.050	0.830
Acetone	1.23	0.150	—	1.312
Acetate (vinegar)	4.00 (3.80)	85.00	—	0.008

*Found from protection against BrImPpOH inactivation of LADH; †found from LADH enzyme inactivation; ‡calculated from $K_{EI,O} = K_{EO,I} \times K_{E,O}/K_{E,I}$ ($K_{E,O} = 0.160$ mM); §values corrected for the effect of acetone; ||with cider vinegar.

centration in the cuvette $\sim 2 \mu\text{M}$), 100 μl of BrImPpOH (concentration in the cuvette between 1 and 5 mM), 100 μl protector and 770 μl of 0.1 M phosphate buffer, pH 7.0.

RESULTS

Reversible inhibition of ethanol oxidation by LADH

Table 1 summarizes the dissociation constants for the reversible inhibitors of LADH at pH 7.0. The effects of disulfiram, its metabolite DSH and PSSP (a reagent with a structural resemblance to disulfiram and often used as a model system) on the LADH catalysed reaction were studied by initial rate experiments at pH 7.0. In competition with ethanol, they were found to form ternary enzyme-NAD⁺-inhibitor (EO,I) complexes and to be strict competitive inhibitors. Disulfiram had (after correcting for the acetone contribution to inhibition) a mean dissociation constant $K_{EO,I}$ of 50 μM , while the $K_{EO,I}$ values for DSH and PSSP were 1 mM and 0.260 mM respectively (Table 1 and Fig. 2).

The experiments with acetic acid showed that this reagent is also a potent competitive inhibitor of alcohol metabolism with a $K_{EO,I}$ of 4 mM at pH 7.0 (Fig. 3). This is particularly significant in view of the considerable concentration of acetic acid that vinegar drinking results in. Experiments with vinegar (from cider, containing 7% acetic acid) using concentrations equivalent to 5 mM acetic acid gave a $K_{EO,I}$ value of 3.8 mM, confirming that acetic acid is the active component in vinegar. The vinegar effect can thus be explained according to Scheme 1. Drinking vinegar (acetic acid) prior to ethanol consumption prevents significant ethanol oxidation by strong competitive inhibition of LADH, and thereby the concomitant build up of acetaldehyde. As the

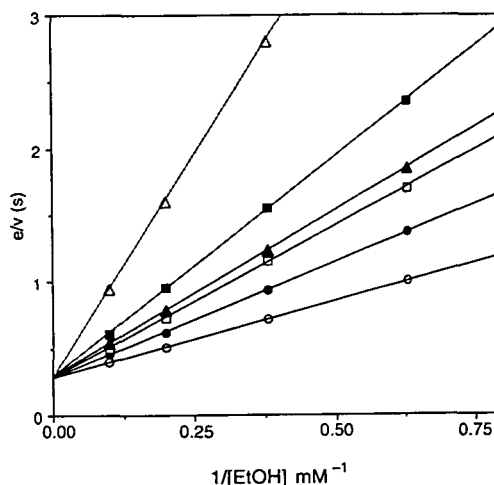


Fig. 2. Effect of different inhibitors on the initial rate of ethanol oxidation by LADH in 0.05 M phosphate buffer pH 7.0.

Plot of e/v versus the reciprocal concentration of ethanol. (The disulfiram plots are corrected for the contribution of acetone to inhibition). Inhibitors: none (○), 30 μM disulfiram (●); 50 μM disulfiram (□), 100 μM disulfiram (■); 5 mM diethyldithiocarbamate (△); 400 μM 2,2'-dithiodipyridine (▲).

build up of acetaldehyde through the inactivation of aldehyde dehydrogenase is disulfiram's primary function, its effect is partly removed by drinking vinegar.

Enzyme inactivation

Disulfiram, DSH and PSSP were all shown to inactivate LADH in a time-dependent manner in reactions which are first order with respect to enzyme. Each of the inactivation curves gives rise to a value for the apparent first-order rate constant. In Fig. 4, the variation of inactivation rate with inactivator concentration is shown in a double-reciprocal plot. There are linear relation-

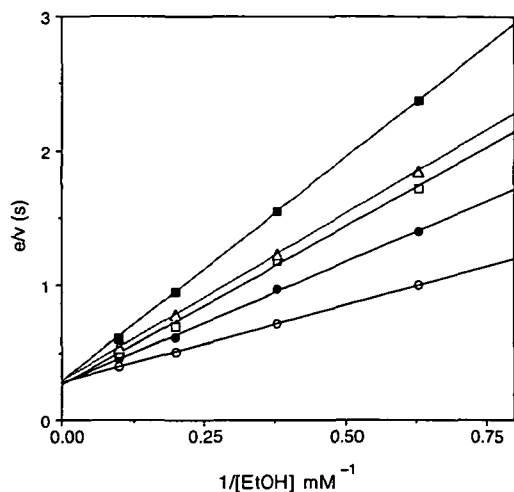


Fig. 3. Effect of acetic acid and vinegar on the initial rate of ethanol oxidation by LADH in 0.05 M phosphate buffer pH 7.0.

Plot of e/v versus the reciprocal concentration of ethanol. Inhibitors: None (○), 2 mM acetic acid (●), 4 mM acetic acid (□), 8 mM acetic acid (■), vinegar (equivalent to 5 mM acetic acid) (△).

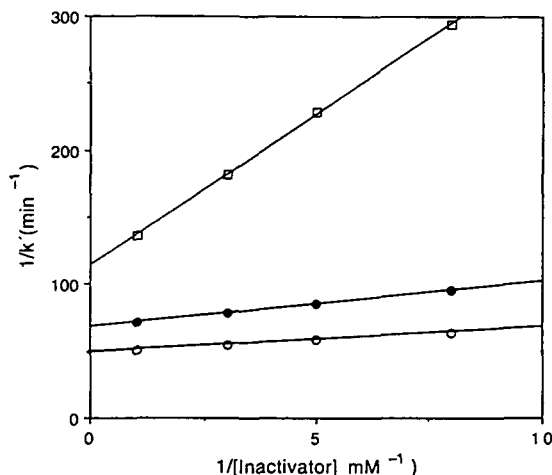
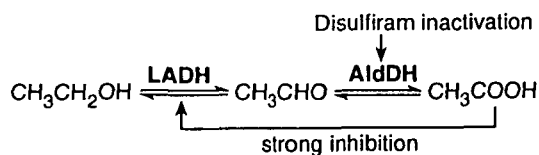


Fig. 4. Double reciprocal plots of rate constants versus different inactivator concentrations.

Disulfiram (○); diethyldithiocarbamate (□); 2,2'-dithiopyridine (●). [Enzyme] = 1.6–2.0 μ M. Buffer: 0.1 M phosphate, pH 7.0.



Scheme 1. Explanation of the vinegar effect.

ships, and dissociation constants for binary enzyme-inactivator complexes are estimated to be 0.03 mM for disulfiram, 0.2 mM for DSH and 0.05 mM for PSSP (Table 1).

Protection against BrImPpOH inactivation

In order to further establish the dissociation constants for the binary enzyme complex with the reagents, they were used as protectors against inactivation with the zinc binding affinity label BrImPpOH (Dahl and McKinley-McKee, 1977). At pH 7.0 all of the reagents tested showed competitive protection against BrImPpOH inactivation (Fig. 5), with a mean dissociation constant ($K_{E,I}$) for the binary enzyme-protector complexes as shown in Table 1. The $K_{E,I}$ values obtained for disulfiram, DSH and PSSP are in excellent agreement with the values obtained from enzyme inactivations by these reagents.

DISCUSSION

Although disulfiram has been used for over 40 years, side-effects and occasional reports of drug toxicity have made it a questionable drug with some risk. Toxic reactions for disulfiram reported are drowsiness, forgetfulness and abdominal discomfort (Silver *et al.*, 1979) as well as tiredness, need for sleep, shortness of breath and muscular pains (Christensen, 1973). Less common but far more serious side-effects are reported cases of hepatotoxicity (Wright *et al.*, 1988), peripheral neuropathy (Frisoni and DiMonda, 1989) and various cardiovascular abnormalities (for details, see Kristenson, 1995).

Because many of the clinical trials evaluating disulfiram have been uncontrolled or poorly designed (Lundwall and Baekland, 1971), its efficiency has been controversial. However, as some well-designed studies (Fuller and Roth, 1979; Brewer *et al.*, 1992) suggest that disulfiram is a useful adjunct for becoming abstinent, disulfiram is still regarded as effective and is widely used.

The inhibition experiments of ethanol metabolism with LADH at pH 7.0 showed that all the reagents tested were competitive with different ethanol concentrations in binding to zinc and forming reversible ternary enzyme-NAD⁺-

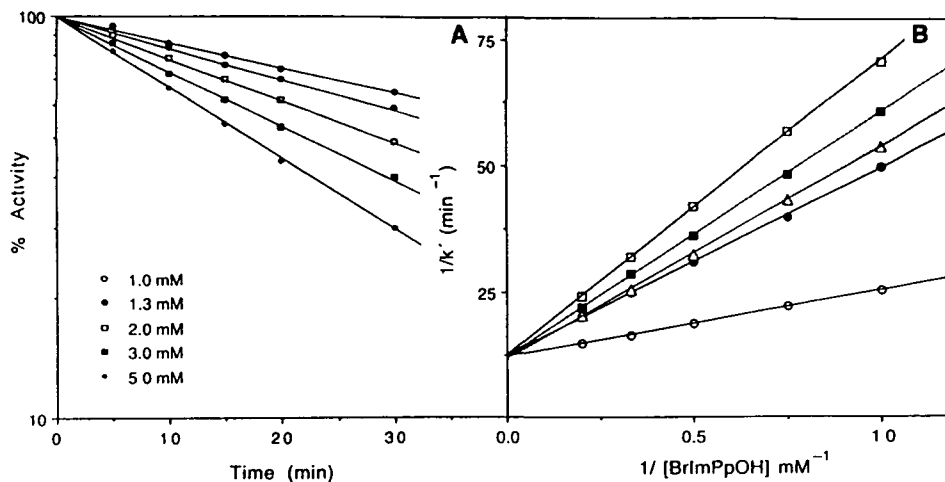


Fig. 5. Disulfiram as a protector against BrImPpOH inactivation.

(A) Semi-log plots of inactivation versus time for LADH with different BrImPpOH concentrations in the presence of 100 μM disulfiram. (B) Double reciprocal plots of rate constants versus BrImPpOH concentrations. Protectors: None (\circ); 50 μM disulfiram (\bullet); 100 μM disulfiram (\square); 500 μM diethyldithiocarbamate (\blacksquare); 100 μM dithiodipyridine (\triangle). [Enzyme] = 2.0 μM . Buffer: 0.1 M phosphate, pH 7.0.

inhibitor complexes. For disulfiram the $K_{E,O,I}$ was found to be 50 μM . By formation of an enzyme-NAD⁺-acetate complex and hence competitive inhibition of alcohol metabolism by LADH, vinegar (acetic acid) reduces the flux of ethanol to acetaldehyde and acetic acid. Thus the build up of toxic concentrations of acetaldehyde (the disulfiram-ethanol aversive reaction) by disulfiram's inactivation of aldehyde dehydrogenase is removed to various extents.

The reactions between disulfiram, DSH or PSSP and the enzyme are examples of affinity labelling kinetics. The time-dependent inactivation proceeds via a reversible complex, where one molecule of inactivator is bound at a location vital for catalytic activity. A pseudo-first-order inactivation of the enzyme is observed. The occurrence of the reversible enzyme-inactivator complex is shown in the double reciprocal plots in Fig. 4, where the straight lines cut the $1/k'$ axis. The initial rate inhibition experiments carried out with these reagents at different ethanol concentrations confirm that they form reversible complexes with the enzyme at the active site zinc which is important for the catalytic activity.

The protection experiments at pH 7.0 with different BrImPpOH concentrations support the main reversible interaction between LADH and these reagents being their binding to the active

site zinc. These three reagents, like the metal-directed affinity-labelling reagent BrImPpOH (Dahl and McKinley-McKee, 1977), thus bind to zinc, forming a reversible binary enzyme-ligand (E1) complex competitive with BrImPpOH, with dissociation constants ($K_{E,I}$) of 30 μM for disulfiram, 200 μM for DSH and 50 μM for PSSP, in excellent agreement with values from enzyme inactivation by these reagents.

The effect of disulfiram intake can thus be summarized: (1) the reduced rate of alcohol metabolism and hence the metabolic flux of ethanol to acetaldehyde and acetic acid enables the inhibition of aldehyde dehydrogenase by disulfiram to build up the acetaldehyde concentration to produce the acetaldehyde toxic and disulfiram aversive effect; (2) likewise intermediate reversible inhibition of LADH by disulfiram and DSH binding to the active site zinc facilitates a slower flux and the build up of a higher steady state acetaldehyde concentration; (3) the inhibitory effects of disulfiram on ethanol metabolism by LADH and aldehyde dehydrogenase should reduce ethanol elimination and give pharmacological effects due to increased ethanol concentrations; (4) likewise a higher acetaldehyde concentration is helped by long-term treatment with disulfiram resulting in LADH inactivation and the reduction of alcohol's metabolic flux.

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