The Effect of Disulfiram on the Aldehyde Dehydrogenases of Sheep Liver

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1. The effect of disulfiram on the activity of the cytoplasmic and mitochondrial aldehyde dehydrogenases of sheep liver was studied. 2. Disulfiram causes an immediate inhibition of the enzyme reaction. The effect on the cytoplasmic enzyme is much greater than on the mitochondrial enzyme. 3. In both cases, the initial partial inhibition is followed by a gradual irreversible loss of activity. 4. The pH-rate profile of the inactivation of the mitochondrial enzyme by disulfiram and the pH-dependence of the maximum velocity of the enzyme-catalysed reaction are both consistent with the involvement of a thiol group. 5. Excess of 2-mercaptoethanol or GSH abolishes the effect of disulfiram. However, equimolar amounts of either of these reagents and disulfiram cause an effect greater than does disulfiram alone. It was shown that the mixed disulphide, $\text{Et}_2\text{N-CS-SS-CH}_2\text{-CH}_2\text{OH}$, strongly inhibits aldehyde dehydrogenase. 6. The inhibitory effect of diethyldithiocarbamate *in vitro* is due mainly to contamination by disulfiram.

Disulfiram (tetraethylthiuram disulphide, Antabuse) is used in the treatment of alcoholism by 'aversion therapy', since the ingestion of alcohol after the administration of disulfiram results in very unpleasant symptoms (Hald *et al.*, 1948). This action is attributed to the inhibition of aldehyde dehydrogenase and subsequent accumulation of acetaldehyde (Hald & Jacobsen, 1948; Truitt & Duritz, 1967). Previous reports (Graham, 1951; Deitrich & Hellerman, 1963) have claimed that disulfiram is a competitive inhibitor of aldehyde dehydrogenase with respect to NAD^+ , and either non-competitive or uncompetitive with respect to the aldehyde substrate. Stromme (1963), however, has discussed the inactivation of hexokinase and other glycolytic enzymes by disulfiram in terms of the blocking of essential thiol groups through mixed-disulphide formation. This mode of action was proved with D-amino acid oxidase (Neims *et al.*, 1966b), and radioactively labelled disulfiram has been introduced as a sensitive, specific and convenient reagent for the determination of protein thiol groups (Neims *et al.*, 1966a).

It is probable that aldehyde dehydrogenase also has an essential thiol group (Blair & Bodley, 1969; Rutledge & Deitrich, 1971; Duncan & Tipton, 1971). It appeared therefore that the action of disulfiram on this enzyme warranted further study. The present paper describes the effect of disulfiram on the cytoplasmic and mitochondrial aldehyde dehydrogenases which have been isolated from sheep liver in this laboratory (Crow *et al.*, 1974). The effect of diethyldithiocarbamate, to which disulfiram is rapidly and completely reduced *in vivo* (Stromme, 1965), was also investigated.

Experimental

Materials

The following compounds were obtained from the sources indicated. NAD^+ and 2-chloroethanol, BDH Chemicals (Poole, Dorset, U.K.); acetaldehyde and 2-mercaptoethanol, Fluka A.G. (Buchs, Switzerland); disulfiram and GSH, Sigma Chemical Co. (St Louis, Mo., U.S.A.); sodium diethyldithiocarbamate, May and Baker (Dagenham, Essex, U.K.).

Preparation of $\text{Et}_2\text{N-CS-SS-CH}_2\text{-CH}_2\text{OH}$, the mixed disulphide of diethyldithiocarbamate and 2-mercaptoethanol. The synthesis of this compound from disulfiram and 2-mercaptoethanol was attempted but a mixture of products (including $\text{Et}_2\text{N-CS-SS-CH}_2\text{-CH}_2\text{OH}$, $\text{HOCH}_2\text{-CH}_2\text{-SS-CH}_2\text{-CH}_2\text{OH}$ and unchanged disulfiram) was obtained. An alternative route utilized these reactions:

2-Chloroethanol (8.05 g, 0.1 mol) was mixed with sodium thiosulphate pentahydrate (24.8 g, 0.1 mol) dissolved in water (65 ml). The reaction mixture was heated under reflux for 1 h. At this stage a sample of the reaction mixture gave no precipitate of sulphur when acidified. An aqueous solution of sodium diethyl dithiocarbamate (22.5 g, 0.1 mol) was added and the mixture was heated under reflux for 1 h. The reaction mixture was then extracted with ethyl acetate, the organic layer was dried over MgSO_4 and evaporated to dryness, yielding a small quantity (1-2 g) of viscous brown oil. This material was shown by

mass spectrometry to be $\text{Et}_2\text{N-CS-SS-CH}_2\text{-CH}_2\text{OH}$ (measured mass = 225.0318, $\text{C}_7\text{H}_{15}\text{NOS}_3$ requires 225.0315). For the purposes of the present work, no attempt was made to optimize the yield of this synthesis.

Methods

The cytoplasmic and mitochondrial aldehyde dehydrogenases of sheep liver were prepared separately as previously described (Crow *et al.*, 1974). Before use the enzyme solutions were thoroughly dialysed to remove 2-mercaptoethanol. Enzyme activity was assayed by continuously following the increase in E_{340} caused by the production of NADH for up to 20 min at 20°C. Final concentrations in the assay system were 33 mM-sodium pyrophosphate buffer, pH 9.3, 1.67 mM-NAD⁺ and 1.67 mM-acetaldehyde. When desired, 2-mercaptoethanol, GSH and diethyldithiocarbamate (all in water) and disulfiram (in ethanol) were added as 0.1 ml portions; 0.1 ml of water or ethanol was added to the corresponding control assays. The final volume was 3 ml. The enzyme (a 0.1 ml portion) was added last to the assay mixture. Activity is expressed in international units (μmol of NADH produced/min).

In the experiment to determine if the inhibition caused by disulfiram is reversed by dialysis, reaction mixtures were made up as follows: 1 ml of water, 0.4 ml of 0.1 M-pyrophosphate buffer, pH 9.3, 0.5 ml of mitochondrial enzyme, 0.1 ml of disulfiram in propylene glycol such that the final concentration of disulfiram was 0.1 mM. The control solutions contained propylene glycol, but no disulfiram. After 4 h at 0–4°C, 0.1 ml portions were taken from the reaction mixtures and assayed for aldehyde dehydrogenase activity as described above. The reaction mixtures were then separately dialysed in the cold against four changes of a large volume of 5 mM-potassium phosphate buffer, pH 7.3, 1% (v/v) in propylene glycol, and subsequently re-assayed.

The pH-dependence of the inactivation of mitochondrial aldehyde dehydrogenase by disulfiram was determined by treating the enzyme with disulfiram (0.2 mM) at 25°C over a range of pH values for a period of 20–30 min. Barbitol (veronal) buffers (50 mM) were used and were prepared by standard procedures (Gomori, 1955). Samples (0.1 ml) of these reaction mixtures were taken every 3 or 4 min and assayed for activity in the usual way. The logarithm of the remaining activity was plotted against time, and the slopes of the resulting straight lines were plotted against pH. (The data were corrected for the small spontaneous loss of activity which occurred under similar conditions in the absence of disulfiram.)

The pH-dependence of the enzyme-catalysed reaction was determined as follows. Lineweaver–

Burk plots were constructed showing the variation of rate of reaction with NAD⁺ concentration at a constant saturating concentration of acetaldehyde (0.833 mM) over a range of pH values at 25°C. Values of maximum velocity were determined from these graphs and plotted against pH. Similar experiments were performed at constant NAD⁺ concentration (1.67 mM) by varying the concentration of DL-glyceraldehyde as substrate. (Acetaldehyde itself has an inconveniently low K_m for this purpose.)

Standard deviations were calculated from an unweighted least-squares analysis of the data (Youden, 1951).

Results and Discussion

Effect of disulfiram on mitochondrial aldehyde dehydrogenase

The inhibition of sheep liver mitochondrial aldehyde dehydrogenase by disulfiram in the presence of various concentrations of 2-mercaptoethanol and GSH is shown by the data presented in Table 1. For each experiment in the presence of disulfiram, the initial rate (measured as described above) is expressed as a percentage of the rate of the control assay. In the presence of disulfiram, and when 2-mercaptoethanol was not in excess, the rate of production of NADH was not constant, but decreased with time. This is illustrated in Table 1

Table 1. *Effect of disulfiram on sheep liver mitochondrial aldehyde dehydrogenase*

The activity of the enzyme at various concentrations of disulfiram and 2-mercaptoethanol (or GSH) was assayed as described under 'Methods'. The initial rate of the enzyme reaction in the presence of disulfiram is expressed as a percentage of the rate in its absence. Also listed is the loss of enzyme activity over a period of 20 min, expressed as a percentage of the initial activity.

Disulfiram (mM)	2-Mercapto-ethanol (mM)	Initial activity (% of control rate)	Loss of activity in 20 min (%)
0.01	0	96	17, 16
0.01	0.01	55	48
0.1	0	81, 81, 91	39, 53, 48
0.1	0.01	36	100
0.1	0.1	21, 17, 29, 26	100
0.1	0.2	100	0
0.1	1.0	98	0
	GSH (mM)		
0.1	0.1	71	65
0.1	0.2	95	0

Table 2. Irreversibility by dialysis of the inactivation of mitochondrial aldehyde dehydrogenase by disulfiram

Duplicate reaction mixtures (A) with disulfiram and (B) without disulfiram were prepared as described under 'Methods'. The enzyme was assayed after a period of 4 h. After exhaustive dialysis the enzyme activity in each reaction mixture was assayed again. Results are recorded as units/ml as defined under 'Methods'. The relative activity of the A samples is also expressed as a percentage of the activity of the B samples.

	Activity (unit/ml)		Relative activity (%)
Before dialysis:	A1 0.0802	0.0853	58
	A2 0.0903		
	B1 0.144	0.146	100
	B2 0.147		
After dialysis:	A1 0.0314	0.0331	36
	A2 0.0347		
	B1 0.0812	0.0926	100
	B2 0.104		

by recording the decrease in rate over a period of 20 min, expressed as a percentage of the initial inhibited rate. (The rate of the control assays remained constant for at least 20 min.) Thus, for example, in the presence of 0.1 mM-disulfiram and in the absence of 2-mercaptoethanol, the initial rate of the enzyme reaction is 81–91% of the control containing no disulfiram, and this rate decreases to approximately one-half its original value over a period of 20 min. Similar but quantitatively smaller effects are observed with 0.01 mM-disulfiram. These results suggest that a chemical reaction is occurring between disulfiram and the enzyme, since any simple non-covalent inhibitory action would be expected to be instantaneous. (The latter process would of course explain the small initial effect on the rate of reaction.)

To determine the reversibility of the inhibitory process, the dialysis experiment described under 'Methods' was performed. In contrast with a similar experiment by Deitrich & Hellerman (1963), in which large losses of total enzyme activity obviated any definite conclusions, the present experiment (see Table 2) shows quite clearly that removal of disulfiram by dialysis does not restore the activity of the enzyme. (Indeed, the activity of the inhibited enzyme samples is lower after dialysis than before, presumably owing to the continued action of significant concentrations of disulfiram during the first stages of dialysis.) This supports the conclusion that a covalent interaction is involved in the lowering of enzyme activity and suggests that aldehyde dehydrogenase, like the other enzymes referred to in the introduction (Stromme, 1963; Neims *et al.*,

Fig. 1. pH-dependence of the inactivation of mitochondrial aldehyde dehydrogenase by disulfiram (0.2 mM) at 25°C

Inactivation is expressed as pseudo-first-order rate constants. The values are means $\pm$ S.E.M. The line is the theoretical curve for an ionizing group of pK_a 8.7.

Fig. 2. pH-dependence of the maximum velocity of the reaction catalysed by mitochondrial aldehyde dehydrogenase

Each point represents the vertical intercept of a Lineweaver-Burk plot obtained with constant acetaldehyde concentration (0.833 mM) and various NAD^+ concentrations at 25°C. The values are means $\pm$ S.E.M. The line is the theoretical curve for an ionizing group of pK_a 8.7.

1966a,b), is inactivated by disulfiram through mixed-disulphide formation:

If this is the case, the rate of inactivation might be expected to vary over the pH range in which a thiol group ionizes [as, for example, in the action of

5,5'-dithiobis-(2-nitrobenzoate) on isocitrate dehydrogenase (Fan & Plaut, 1974)]. Fig. 1 shows that the pH-dependence of the pseudo-first-order rate constant for the inactivation of mitochondrial aldehyde dehydrogenase by disulfiram shows a point of inflexion at pH 8.7, which is close to the range (8.8–9.1) expected for the pK_a of a protein thiol group (Bernhard, 1968). The curve is closely similar to that obtained for the pH-dependence of the maximum velocity of the enzyme-catalysed reaction (Fig. 2). Consequently, it is tempting to suggest that both the catalytic activity of the enzyme and the inactivation by disulfiram involve the same thiol group at the enzyme's active site. However, other factors (such as conformational changes of the enzyme) cannot be discounted as possible causes of the observed pH-dependence.

Early in this work it was discovered that the presence of traces of 2-mercaptoethanol (added as a routine to the enzyme preparations as a stabilizer against oxidation) caused anomalous results. Accordingly, all results reported here were obtained with enzyme which had been thoroughly dialysed beforehand to remove 2-mercaptoethanol. It is known that GSH is capable of reducing disulfiram completely to diethyldithiocarbamate (Johnston, 1953; Deitrich & Hellerman, 1963) and the present results show that 2-mercaptoethanol also has this property. Immediately after mixing solutions of disulfiram and 2-mercaptoethanol, the characteristic double peak in the u.v. spectrum of diethyldithiocarbamate (Koch, 1949) may be observed (λ_{max} , 257 and 281 nm). Further, the presence of a twofold, or greater, molar excess of 2-mercaptoethanol over disulfiram completely eliminates the ability of the latter compound to inhibit aldehyde dehydrogenase (see Table 1). These data also indirectly show that the products of the reaction, diethyldithiocarbamate and $HOCH_2-CH_2-SS-CH_2-CH_2OH$, have no effect on the mitochondrial enzyme.

It was decided to determine the effect of an equimolar proportion of 2-mercaptoethanol on the inhibitory effect of disulfiram, since under these conditions one of the following situations would obtain: (1) the conversion of one-half of the disulfiram present completely into diethyldithiocarbamate; (2) the conversion of all the disulfiram into equimolar amounts of diethyldithiocarbamate and the mixed-disulphide compound, $Et_2N-CS-SS-CH_2-CH_2OH$; (3) some situation intermediate between the previous two extremes. The first possibility may be excluded, since this cannot explain the much greater effect of a given concentration of disulfiram in the presence of an equimolar concentration of 2-mercaptoethanol, compared with the effect in the absence of 2-mercaptoethanol (Table 1). For instance, the initial rate in the presence of 0.1 mM-disulfiram–0.1 mM-2-mercapto-

ethanol is 17–29% of the control, whereas with 0.1 mM-sulfiram alone it is 81–91%. Hence, under these conditions, at least some of the mixed disulphide must be formed. An authentic sample of this compound was synthesized and indeed proved to have a powerful inhibitory action (Table 3). As expected, its effect is abolished by excess of 2-mercaptoethanol.

The effect of GSH on the inhibition produced by disulfiram parallels that of 2-mercaptoethanol (Table 1). A twofold excess of GSH destroys the disulfiram, whereas an equimolar concentration results in an inhibitory effect greater than in the presence of disulfiram alone, though not as great as that caused by equimolar amounts of 2-mercaptoethanol and disulfiram.

Effect of disulfiram on cytoplasmic aldehyde dehydrogenase

Comparison of the data in Tables 1 and 4 shows that a given concentration of disulfiram causes a much larger initial diminution in the activity of the cytoplasmic enzyme than in that of the mitochondrial enzyme. Where tested, the cytoplasmic enzyme also shows a time-dependent loss of activity, similar to that discussed above for the mitochondrial enzyme. It appears therefore that disulfiram may initially bind to and inhibit the cytoplasmic enzyme much more

Table 3. *Effect of $Et_2N-CS-SS-CH_2-CH_2OH$ on sheep liver mitochondrial aldehyde dehydrogenase*

Conditions were as described in Table 1.

Inactivator (mM)	2-Mercapto-ethanol (mM)	Initial activity (% of control rate)	Loss of activity in 20 min (%)
0.01	0	39, 30	39, 50
0.01	0.1	100	0
0.1	0	0	

Table 4. *Effect of disulfiram on sheep liver cytoplasmic aldehyde dehydrogenase*

Conditions were as described in Table 1.

Disulfiram (mM)	2-Mercapto-ethanol (mM)	Initial activity (% of control rate)	Loss of activity in 20 min (%)
0.001	0	9	Not measured
0.01	0	5, 5	33, 34
0.01	0.01	5	47
0.1	0	0	
0.1	0.1	0	
0.1	0.2	48, 32	0
0.1	1.0	84	0

readily than it does to the mitochondrial enzyme, but that both enzymes are subsequently inactivated by the disulphide-interchange capabilities of disulfiram.

This conclusion is important in view of previous reports (Graham, 1951; Deitrich & Hellerman, 1963) that disulfiram acts merely as a reversible inhibitor of aldehyde dehydrogenase, competitive with NAD^+ . If, in the studies referred to, the effect of disulfiram was followed in each experiment for only a few minutes, the gradual loss of activity over a relatively long period of time would be missed. This would be especially likely if the enzyme preparations used by Graham (1951) and Deitrich & Hellerman (1963) [the intracellular origins of which were not determined] resembled the cytoplasmic enzyme studied here, with which the initial inhibitory effect is particularly pronounced compared with the subsequent inactivating process. It is noteworthy that in their investigation of the effect of disulfiram on D-amino acid oxidase, Neims *et al.* (1966b) observe that 'although the inhibitory process is irreversible, curves reminiscent of those obtained with reversible competitive inhibitors were derived when the preincubation time was short'.

As shown in Table 4, the rate in the presence of disulfiram and excess of 2-mercaptoethanol is lower than the blank rate, suggesting that diethyldithiocarbamate may inhibit the cytoplasmic enzyme.

Effect of diethyldithiocarbamate on cytoplasmic and mitochondrial aldehyde dehydrogenases

The results in Table 5 show that diethyldithiocarbamate does indeed inhibit the cytoplasmic enzyme, but that the effect largely disappears in the presence of 2-mercaptoethanol. This is suggestive of

Table 5. *Effect of diethyldithiocarbamate on cytoplasmic and mitochondrial aldehyde dehydrogenases*

The activity of the enzymes in the presence of various concentrations of diethyldithiocarbamate and 2-mercaptoethanol is expressed as a percentage of the activity of the corresponding control.

	Diethyldithio- carbamate (mM)	2-Mercapto- ethanol (mM)	Activity (% of control rate)
Cytoplasmic aldehyde dehydrogenase	0.1	0	24
	0.5	0	10
	0.1	0.1	89
	0.25	0.1	86
	0.5	0.1	81
Mitochondrial aldehyde dehydrogenase	0.1	0	101
	0.5	0	104

contamination of the diethyldithiocarbamate used in these experiments by its oxidation product, disulfiram. Mass-spectral analysis showed this to be the case.

An alternative explanation (to inhibition by diethyldithiocarbamate) for the low activity of the cytoplasmic enzyme in the presence of a twofold excess of 2-mercaptoethanol over disulfiram (Table 4) would be provided by the molar ratio of these compounds being, by experimental error, slightly less than 2:1. A very small residual concentration of disulfiram would then be sufficient to account for the inhibition observed.

Diethyldithiocarbamate has a slight inhibitory effect on the cytoplasmic enzyme even in the presence of 2-mercaptoethanol (Table 5). One might speculate that this is due to the metal-chelating properties of diethyldithiocarbamate; a Zn^{2+} ion has been reported to be present in aldehyde dehydrogenase (Stoppani *et al.*, 1966).

Table 5 shows that the mitochondrial enzyme is not affected by diethyldithiocarbamate, a result already deduced indirectly from the effect of disulfiram and excess of 2-mercaptoethanol (Table 1). As expected, the amount of disulfiram impurity in diethyldithiocarbamate (estimated to be less than 1% from its effect on the cytoplasmic enzyme) is too low to affect the relatively insensitive mitochondrial enzyme.

Action of disulfiram in vivo

The precise mechanism by which disulfiram affects aldehyde dehydrogenase and other enzymes *in vivo* is still unclear. It is known that disulfiram is reduced to diethyldithiocarbamate in the blood immediately on absorption (Stromme, 1965), presumably by reaction with GSH (Johnston, 1953) and other thiols. Administration of disulfiram or diethyldithiocarbamate to rats lowers the aldehyde dehydrogenase activity in the liver (Deitrich & Erwin, 1971). The inhibition was found to be irreversible, and return of activity was dependent on protein synthesis. Thus if the inhibition is due to the disulphide-interchange reaction of disulfiram, there must be some means of reoxidation of diethyldithiocarbamate to disulfiram, as discussed by Deitrich & Erwin (1971). For human erythrocytes, Stromme (1963) has shown that diethyldithiocarbamate catalyses the autoxidation of haemoglobin, and that methaemoglobin in turn serves as the immediate electron acceptor in the conversion of diethyldithiocarbamate into disulfiram. It may be that similar processes give rise to the re-formation of disulfiram in the liver. The present work shows that production of disulfiram itself is not necessary; co-oxidation of diethyldithiocarbamate and some other thiol (e.g. GSH) to give a mixed-disulphide compound would suffice to cause inactivation of aldehyde dehydrogenase.

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