Effect of some thiocarbamate compounds on aldehyde dehydrogenase and implications for the disulfiram ethanol reaction

Trevor M. KITSON

Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand

The effects of *S*-methyl diethyldithiocarbamate, *S*-methyl diethylmonothiocarbamate and bis(diethylcarbamoyl) disulphide on sheep liver cytoplasmic aldehyde dehydrogenase were investigated *in vitro*. The first compound has negligible effect. The second one is a weak inhibitor of the esterase activity of the enzyme and a weaker inhibitor of the dehydrogenase activity. A very low concentration of the third compound, however, acts as a potent inactivator of aldehyde dehydrogenase, similar in this respect to disulfiram, although somewhat slower to react. The possible involvement of these compounds in the physiological phenomenon known as the disulfiram ethanol reaction is discussed.

INTRODUCTION

Disulfiram, or Antabuse as it is known medically, is used in the treatment of chronic alcoholism since it causes an unpleasant aversive reaction to alcohol. It works by inactivating hepatic aldehyde dehydrogenase, leading to a pronounced rise in acetaldehyde concentration when ethanol is metabolized (Kitson, 1977). Notwithstanding the fact that disulfiram is an extremely potent and rapid inactivator of aldehyde dehydrogenase *in vitro* (Kitson, 1982, 1983, 1985), some workers have postulated that it only works *in vivo* after metabolism to some other compound. This is because disulfiram itself is virtually undetectable *in vivo*, being rapidly reduced to the diethyldithiocarbamate ion and subsequently further metabolized (Cobby *et al.*, 1977). One major metabolite is *S*-methyl diethyldithiocarbamate (MDD). Administration of this compound to rats results in loss of aldehyde dehydrogenase activity, and it was proposed that the 'disulfiram ethanol reaction' would be more correctly termed the 'diethyldithiocarbamate methyl ester-ethanol reaction' (Yourick & Faitman, 1987, 1989). However, it was briefly reported earlier that MDD has no effect on aldehyde dehydrogenase *in vitro* (Kitson, 1976). More recently, *S*-methyl diethylmonothiocarbamate (MDM) has also been shown to be a metabolite of disulfiram and an inactivator of aldehyde dehydrogenase *in vivo*. Furthermore, it was also claimed to be a potent and irreversible inactivator *in vitro* (Johansson *et al.*, 1989; Helander & Johansson, 1989) and was suggested to be the active metabolite of disulfiram (Petersen, 1989). On the other hand, Hart *et al.* (1990) reported that MDM is ineffective as an inhibitor *in vitro*.

In view of these conflicting reports it was thought desirable to make a careful detailed examination of the effects of MDD and MDM on aldehyde dehydrogenase *in vitro*, with the use of the sheep liver cytoplasmic enzyme for which there is a large body of previous information available about the action of disulfiram and other compounds (Kitson, 1989, and references cited therein). In addition, bis(diethylcarbamoyl) disulphide, which is structurally related to both disulfiram and MDM and for which

the convenient name 'dioxiram' is coined, was also investigated for its effect on aldehyde dehydrogenase *in vitro*.

The structures and abbreviations of compounds used in this study are shown in Fig. 1.

EXPERIMENTAL

Materials

***S*-Methyl diethylmonothiocarbamate (MDM).** Diethylamine (10 ml) was dissolved in ethanol (50 ml), and an equimolar amount of NaOH was added. Carbonyl sulphide was bubbled slowly through the mixture with stirring until all the NaOH had dissolved. The solution was cooled in ice and a slight excess of iodomethane was added. The mixture was left at room temperature overnight and then evaporated to dryness. The residue was extracted with chloroform and water; the chloroform layer was dried over $MgSO_4$ and evaporated to give a brownish-yellow mobile liquid. This was distilled under reduced pressure three times, giving finally a perfectly colourless liquid (b.p. 90 °C at 12 mmHg). The identity was verified by electron-impact m.s. [M^+ = 147.071785968, calc. for $C_6H_{13}NOS$, 147.0717860; m/z (relative intensity) 147 (39), 100 (100) and 72 (79)] and 1H n.m.r. [δ (p.p.m.) 3.41 (4H, q) 2.32 (3H, s) and 1.17 (6H, t)].

***S*-Methyl diethyldithiocarbamate (MDD).** Sodium diethyldithiocarbamate trihydrate (10 g) was dissolved in ethanol (50 ml), and a slight excess of iodomethane was added. The mixture was left at room temperature overnight. The product was purified as for the previous compound, b.p. 129–131 °C at 12 mmHg. The identity was confirmed by electron-impact m.s. [M^+ = 163.048943128, calc. for $C_6H_{13}NS_2$, 163.0489431; m/z (relative intensity) 163 (100), 116 (51) and 88 (46)].

Bis(diethylcarbamoyl) disulphide (dioxiram). This was prepared according to the method that Gregg (1952) used for the methyl analogue. The resultant pale-yellow oil was stored in the freezer, where after a few days it crystallized. The identity was verified by fast-atom-bombardment m.s. [MH^+ = 265.1071, calc. for $C_{10}H_{21}N_2O_2S_2$, 265.1044467; m/z (relative intensity) 265 (100),

Disulfiram

Bis(diethylcarbamoyl) disulphide, dioxiram

S-Methyl diethyldithiocarbamate, MDD

S-Methyl diethylmonothiocarbamate, MDM

Fig. 1. Structures and abbreviations of compounds used in this study

Abbreviations used: MDM, *S*-methyl diethylmonothiocarbamate; MDD, *S*-methyl diethyldithiocarbamate.

100 (100) and 72 (19)] and ^1H n.m.r. [δ (p.p.m.) 3.48 (4H, q) and 1.22 (6H, t)].

Methods

Purification and assay of aldehyde dehydrogenase. Cytoplasmic aldehyde dehydrogenase from sheep liver was purified and assayed as previously described (Dickinson *et al.*, 1981).

Effects of modifiers. The activity of aldehyde dehydrogenase was measured at 25 °C in 50 mM-sodium phosphate buffer, pH 7.4, with the use of an Aminco DW-2a spectrophotometer. Dehydrogenase assays utilized 1 mM- NAD^+ and 1 mM-acetaldehyde; esterase assays used 100 μM -4-nitrophenyl acetate. The esterase rates were corrected for the small spontaneous rate of hydrolysis. Modifiers were added as 15 μl or 25 μl of a solution in ethanol; the same volume of ethanol was added to control assays (total volume 3 ml). With dioxiram, enzyme and modifier were routinely left for a period of 15 min before addition of substrate. With other modifiers, substrates were added within less than 1 min unless otherwise stated. In all cases, at least two or three identical assays were performed and the results were averaged to give the points in the Figures.

Test of reversibility of effect of methyl diethylmonothiocarbamate (MDM). Enzyme (24 or 6 μM) was incubated with MDM (544 μM or 1.09 mM respectively) for 15 min in 0.5 ml of 50 mM-sodium phosphate buffer, pH 7.4, at room temperature. The mixture was then passed down a small column of Bio-Gel P-6 (12 cm $\times$ 0.9 cm), with the same buffer being used for elution and 1 ml being collected immediately after the void volume. This sample was then assayed for enzymic activity with 4-nitrophenyl acetate in the usual way, and the protein concentration was estimated from the A_{280} . The specific activity was compared with that from a control experiment carried out in exactly the same way but with no MDM added.

Test of solubility of disulfiram and of dioxiram. A large excess of disulfiram or dioxiram was stirred vigorously in 10 ml of 50 mM-sodium phosphate buffer, pH 7.4, at room temperature for 2 days. After filtration, small samples of the resulting clear aqueous solution of disulfiram were added to assays of aldehyde dehydrogenase with 4-nitrophenyl acetate in the usual way. In the case of dioxiram, the saturated solution was first diluted 1000-fold, and then small samples were mixed with aldehyde dehydrogenase with a wait of 15 min before the substrate was added. The concentration of disulfiram or dioxiram in these assays was estimated from the known effect of stoichiometric concentrations of these compounds on a given concentration of enzyme (see Kitson, 1982, and Fig. 4).

Reaction of disulfiram and dioxiram with 2-thiopyridone. Solutions of disulfiram or dioxiram in ethanol (25 μl) and 2-thiopyridone in ethanol (25 μl) were mixed in 2.95 ml of 50 mM-sodium phosphate buffer, pH 7.4. Immediately after mixing the concentration of disulfiram or dioxiram was 50 μM and that of 2-thiopyridone was 100 μM . The A_{343} (due to 2-thiopyridone) was then monitored as the disulphide interchange reaction approached equilibrium.

RESULTS

Effects of *S*-methyl diethyldithiocarbamate (MDD) and *S*-methyl diethylmonothiocarbamate (MDM)

Fig. 2 records the effects of MDD and of MDM on the dehydrogenase activity of sheep liver cytoplasmic aldehyde dehydrogenase *in vitro*. The former compound is not an inhibitor, confirming an earlier brief report (Kitson, 1976). In fact, approx. 1 mM-MDD seems to have a very small but consistent activatory effect. Increasing concentrations of MDM, however, inhibit the

Fig. 2. Effects of *S*-methyl diethyldithiocarbamate (MDD) and *S*-methyl diethylmonothiocarbamate (MDM) on the dehydrogenase activity of cytoplasmic aldehyde dehydrogenase *in vitro*

Various concentrations of MDD (■) or MDM (●) were added, in the presence of NAD^+ , to enzyme (1.1 μM), which was then assayed by the addition of acetaldehyde as described in the Experimental section. Alternatively, MDD (□) or MDM (○) was added to the enzyme, with NAD^+ and acetaldehyde being added subsequently. The symbol Δ shows the effect of 10 μM -disulfiram.

enzyme, and the decrease in activity is greater when the modifier is added to the enzyme in the absence of NAD^+ . The points in Fig. 2 were obtained by adding remaining substrates (acetaldehyde, or acetaldehyde and NAD^+) within less than 1 min of adding MDM. No difference in results was found, however, if a wait of 1 h was included before adding substrates. Thus these high concentrations of MDM do not result in any further time-dependent loss of activity (either in the presence or in the absence of NAD^+). Fig. 2 also shows that disulfiram is in a completely different class to MDM when it comes to inactivating aldehyde dehydrogenase.

The effects of MDD and MDM on the esterase activity of aldehyde dehydrogenase are shown in Fig. 3. A slight inhibition is shown at very high concentrations of MDD and an appreciably greater effect is shown by MDM, but again at very low concentration disulfiram is far more effective at destroying enzyme activity.

The effect of MDM was tested for reversibility as described in the Experimental section. With a ratio of concentrations of modifier to enzyme of 23:1 the treated enzyme exhibited 77% and 85% of the activity of the control before and after gel filtration respectively, and with an initial ratio of modifier to enzyme of 181:1 the residual activity was 20% and 49% of the control before and after gel filtration respectively. There is, therefore, some recovery of activity on removal of excess reagent, but it is clear that the effect of MDM is not totally reversible, at least not by a simple rapid passage through Bio-Gel. Johansson *et al.* (1989) reported that the inhibition also could not be reversed by treatment with GSH or 2-mercaptoethanol.

Effect of bis(diethylcarbamoyl) disulphide (dioxiram)

Fig. 4 shows the inactivation profiles obtained with both the dehydrogenase and esterase activities of aldehyde dehydrogenase when it is treated with dioxiram. Note that the concentration range here is orders of magnitude less than that used with MDD and MDM in Figs. 2 and 3. These profiles are very reminiscent of those previously obtained with disulfiram (Kitson, 1982). The two activities are abolished in tandem by disulfiram, and this is

Fig. 3. Effects of *S*-methyl diethyldithiocarbamate (MDD) and *S*-methyl diethylmonothiocarbamate (MDM) on the esterase activity of cytoplasmic aldehyde dehydrogenase *in vitro*

Various concentrations of MDD (■) or MDM (●) were added to enzyme (1.1 μM), which was then assayed by the addition of 4-nitrophenyl acetate as described in the Experimental section. The symbol $\triangle$ shows the effect of 10 μM -disulfiram.

Fig. 4. Effect of bis(diethylcarbamoyl) disulphide (dioxiram) on cytoplasmic aldehyde dehydrogenase *in vitro*

Various concentrations of dioxiram were added to enzyme, and after 15 min the activity was assayed by the addition of substrates as described in the Experimental section. (a) Esterase activity, [enzyme] = 0.65 μM ; (b) dehydrogenase activity, [enzyme] = 1.1 μM . The symbol $\triangle$ shows the effect of 2 μM -disulfiram.

true also for dioxiram, bearing in mind that Figs. 4(a) and 4(b) were obtained with different enzyme concentrations. [Considerable evidence suggests that the same catalytic enzyme group is involved in both dehydrogenase and esterase activities and is sensitive to disulfiram (see, e.g., Kitson *et al.*, 1991).]

It was found that it takes several minutes for dioxiram to react with aldehyde dehydrogenase (unlike disulfiram, which reacts 'within the time of mixing'), and this is why modifier and enzyme were routinely left together for 15 min before the addition of substrate to initiate the assays used in obtaining Fig. 4. The difference in rates of reaction can be conveniently demonstrated by adding modifier to an on-going assay, as shown in Fig. 5. With disulfiram, the diminished rate is evident immediately; with dioxiram, the rate declines over a period of minutes. The decline in rate is not substantially slower than that observed when the

Fig. 5. Rate of inactivation of cytoplasmic aldehyde dehydrogenase by bis(diethylcarbamoyl) disulphide (dioxiram)

The Figure shows spectrophotometer traces obtained with 1 mM- NAD^+ , 1 mM-acetaldehyde and 1.1 μM -enzyme. Trace A, no further addition; traces B and C (duplicates), addition of 2 μM -dioxiram at the break in the trace; trace D, addition of 2 μM -disulfiram at the break in the trace. The vertical bar represents $A_{340} = 0.05$ and the horizontal bar represents 1 min.

Fig. 6. Rate of reaction of disulfiram and bis(diethylcarbamoyl) disulphide (dioxiram) with 2-thiopyridone

The Figure shows the spectrophotometer traces of absorbance of 2-thiopyridone (100 μM) in 50 mM-sodium phosphate buffer, pH 7.4. Trace A, with no further addition; trace B, following the addition of dioxiram (50 μM); trace C, following the addition of disulfiram (50 μM).

modifier is added to the enzyme before substrates, showing that NAD^+ and acetaldehyde at 1 mM concentrations have little protective effect against dioxiram. Under similar conditions it has previously been shown that aldehyde dehydrogenase is not protected against disulfiram either (Kitson, 1985).

In Fig. 6 we see that dioxiram is also much slower than disulfiram to react with a low-molecular-mass thiol. (2-Thiopyridone was chosen as it is a convenient chromophore.) The initial rate of reaction in this experiment was 20 times faster with disulfiram than with dioxiram.

Finally, the concentrations of disulfiram and of dioxiram in presumably saturated solutions were estimated from the effect of known concentrations of these modifiers (as found in Kitson, 1982, and in Fig. 4). The results were 35 μM and 19 mM respectively, a difference of over 500-fold.

DISCUSSION

Many drugs act as enzyme inhibitors only after bioactivation, i.e. the administered form of the drug (the 'pro-drug') is inactive

but some metabolite of it is responsible for modifying the enzyme. With disulfiram, however, the drug itself is perfectly capable of inactivating aldehyde dehydrogenase. Indeed, since disulfiram reacts so quickly and is effective at concentrations stoichiometric with the enzyme, it is difficult to imagine how it could be any more potent. Nevertheless, because of the difficulty of detecting any disulfiram *in vivo*, it has been proposed that a metabolite of it must be actually responsible for loss of enzyme activity in the body [see, e.g., Johansson *et al.* (1989) and Hart *et al.* (1990)]. The results reported above show that two recently proposed candidates for the active metabolite, MDD (Yourick & Faiman, 1987, 1989) and MDM (Johansson *et al.*, 1989), must be rejected. The former does not decrease the dehydrogenase activity of aldehyde dehydrogenase *in vitro* and has only a slight effect on the esterase activity at very high concentrations (Figs. 2 and 3). The latter is somewhat more effective at decreasing the esterase activity, but not very good at inhibiting the dehydrogenase activity, particularly when added in the presence of NAD⁺ (which would be the case *in vivo*) (Figs. 2 and 3). A telling comparison can be made from the results in Fig. 2; 10 μ M-disulfiram abolishes over 97% of the enzyme activity, but 10 μ M-MDM would result in less than 2% inhibition. A similarly small effect of high concentrations of MDM is evident from the results obtained *in vitro* by Johansson (1989). It seems that to describe MDM as a 'potent' inactivator *in vitro* (Petersen, 1989) is an exaggeration.

The explanation for the small effect that MDM does have is not clear. If the compound binds non-covalently to aldehyde dehydrogenase, one would expect the inhibition to be reversible on gel filtration, and this is not the case. If it reacts covalently one might expect that a progressive loss of activity would be observed as enzyme stands in the presence of excess MDM, and again this is not the case. The loss of activity might be due to traces of a highly reactive impurity, but this is unlikely as the MDM was carefully purified by triple distillation [and, unlike the sample prepared by Hart *et al.* (1990), was colourless]. To speculate, as Johansson (1989) has done, on the precise nature of the chemical interaction between MDM and aldehyde dehydrogenase is of academic interest only. The important point is that an appreciable loss of activity only occurs when an overwhelming excess of MDM over enzyme is used (see the Results section), and this is unlikely to have any physiological significance.

There still remains the problem of what the active species is when disulfiram, the diethyldithiocarbamate ion, MDD or MDM is administered to animals. It may be some as yet unidentified further metabolite, as suggested by Hart *et al.* (1990), but on the other hand, for the first three of the compounds listed, we may not have to look any further than disulfiram itself. It has been proposed before (Kitson, 1983) that there is a redox equilibrium between disulfiram and diethyldithiocarbamate (mechanisms for oxidation of the latter *in vivo* are well established), and that, although difficult to detect, there may be sufficient disulfiram present in the liver to inactivate aldehyde dehydrogenase, since it

is highly effective at very low concentration. This would also explain the effect of MDD *in vivo* if we assume that the methylation of diethyldithiocarbamate is a reversible process.

Analogously, I propose that after administration of MDM it too is demethylated to some extent and the resulting diethylmonothiocarbamate ion is oxidized to dioxiram, but again perhaps in undetectably low concentration. Alternatively, the ion may be co-oxidized with, for example, methanethiol, to give a mixed disulphide, as proposed by MacKerell *et al.* (1985) for the case of disulfiram. Dioxiram, like disulfiram but unlike MDM, is certainly a highly potent inactivator of aldehyde dehydrogenase *in vitro*. It reacts somewhat more slowly than disulfiram (Fig. 5), but with similar stoichiometric effectiveness (Fig. 4). Dioxiram differs from disulfiram in being less rapidly reduced by thiols (Fig. 6) and in being very much more soluble in water. These characteristics of dioxiram make it an attractive substance to investigate as a possible alternative to disulfiram in alcoholism therapy. The greater solubility is a reflection of increased polarity associated with the more electronegative oxygen atoms (as ethanol, say, is completely soluble in water and ethanethiol is not). The slower reaction with thiols is probably because diethylmonothiocarbamate is a poorer leaving group than diethyldithiocarbamate, the latter being a symmetrical resonance-stabilized structure.

In conclusion, of all the metabolites or suggested metabolites of disulfiram so far investigated, only disulfiram itself or similar reactive disulphide compounds have the potential to be inactivators of aldehyde dehydrogenase.

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