Rapid Papers

The Effect of Some Analogues of Disulfiram on the Aldehyde Dehydrogenases of Sheep Liver

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The effect of some close structural analogues of disulfiram on the activity of the cytoplasmic and mitochondrial aldehyde dehydrogenases of sheep liver was studied. Several thiuram disulphides are equally potent inhibitors of the cytoplasmic enzyme, but in all cases the mitochondrial enzyme is much less strongly affected. Tetramethylthiuram monosulphide decreases the activity of the cytoplasmic enzyme in a process apparently involving a covalent interaction.

The term 'disulfiram-ethanol reaction' refers to the unpleasant symptoms experienced on drinking alcoholic beverages after having previously ingested disulfiram (tetraethylthiuram disulphide, Antabuse), a drug used in the management of chronic alcoholism. The prime cause of the syndrome is inhibition of aldehyde dehydrogenase by disulfiram, followed by the accumulation of toxic concentrations of acetaldehyde (Truitt & Walsh, 1971). In a previous paper (Kitson, 1975), the mechanism of action of disulfiram at pH 9.3 on the aldehyde dehydrogenases of sheep liver was discussed; it was concluded that the compound exerts an immediate inhibitory effect on the enzymes, and that this is followed by a further gradual inactivation, probably as the result of a disulphide-interchange reaction with essential thiol groups (Neims *et al.*, 1966*a,b*). It was further noted that the cytoplasmic enzyme is considerably more sensitive to disulfiram than is the mitochondrial form, an observation also made with the enzymes from horse liver (Eckfeldt *et al.*, 1975). The present series of experiments was undertaken to determine if disulfiram-like activity is exhibited *in vitro* by thiuram disulphides in general, and, if so, whether the greater susceptibility of the cytoplasmic enzyme is a general phenomenon. Certain other disulfiram analogues [tetramethylthiuram monosulphide, methyl *NN*-diethyldithiocarbamate and methylene bis-(*NN*-diethyldithiocarbamate)] were also tested as inhibitors of aldehyde dehydrogenase.

Materials and Methods

The following compounds were obtained from the sources indicated: NAD⁺ and tetramethylthiuram disulphide, BDH Chemicals (Poole, Dorset, U.K.); acetaldehyde and tetramethylthiuram monosulphide, Fluka A.G. (Buchs, Switzerland); disulfiram, Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Thiuram disulphides were prepared by a simple

standard procedure as follows. Equimolar amounts of NaOH, carbon disulphide and the requisite secondary amine were stirred in water for 1 h. (Two equivalents of NaOH were used in the preparation of the thiuram disulphide from *L*-proline.) The resulting substituted dithiocarbamate was then oxidized to the corresponding thiuram disulphide by the dropwise addition of the calculated amount of iodine dissolved in KI solution. The product was filtered off and purified by recrystallization. The purity of each thiuram disulphide was checked by t.l.c. and microanalysis. Bis-(1-pyrrolidinylthiocarbonyl) disulphide had m.p. 140–142°C. Hoffmeister & Tarbell (1967) gave m.p. 143–144°C (Found: C, 40.9; H, 5.3; N, 9.5; S, 43.6. Calc. for C₁₀H₁₆N₂S₄: C, 41.1; H, 5.5; N, 9.6; S, 43.9%). Bis-(1-morpholinylthiocarbonyl) disulphide had m.p. 145–147°C. Hoffmeister & Tarbell (1967) give m.p. 144.5–145.5°C (Found: C, 37.0; H, 5.1; N, 8.6; S, 39.7. Calc. for C₁₀H₁₆N₂O₂S₄: C, 37.0; H, 5.0; N, 8.7; S, 39.5%). Bis-[(4-methyl-1-piperazinyl)-thiocarbonyl] disulphide had m.p. 150–151°C (Found: C, 41.3; H, 6.3; N, 15.7; S, 36.8. C₁₂N₂₂N₄S₄ requires: C, 41.1; H, 6.3; N, 16.0; S, 36.6%). Bis-(1-*L*-prolylthiocarbonyl) disulphide had m.p. 140–150°C (decomp.) (Found: C, 37.7; H, 4.1; N, 7.4; S, 34.2. C₁₂H₁₆N₂O₄S₄ requires: C, 37.9; H, 4.2; N, 7.4; S, 33.7%). Methyl *NN*-diethyldithiocarbamate was prepared by the method of Gessner & Jakubowski (1972). Its identity was confirmed by mass spectrometry (measured mass = 163.0491; calc. for C₆H₁₃NS₂, 163.0489). Methylene bis-(*NN*-diethyldithiocarbamate) was prepared in a similar way (measured mass = 310.0671; C₁₁H₂₂N₂S₄ requires 310.0665). It had m.p. 71–72°C. Melting points are uncorrected.

The cytoplasmic and mitochondrial aldehyde dehydrogenases of sheep liver were prepared separately as previously described (Crow *et al.*, 1974). Enzyme activity was assayed as before (Kitson, 1975), in either 33 mM-sodium pyrophosphate buffer,

pH 9.3, or 33 mM-potassium phosphate buffer, pH 7.3.

In the experiment to determine if the inhibition caused by tetramethylthiuram monosulphide is reversed by dialysis, reaction mixtures were made up as follows: 0.9 ml of water, 0.5 ml of cytoplasmic enzyme solution, 0.4 ml of 0.1 M-pyrophosphate buffer, pH 9.3, 0.1 ml of 2-mercaptoethanol solution (final concentration 0.2 mM), 0.1 ml of tetramethylthiuram monosulphide in ethanol (final concentration 0.5 mM). The control solutions contained ethanol, but no inhibitor. After 5 h at 0–4°C, 0.1 ml portions were taken from the reaction mixtures and assayed for aldehyde dehydrogenase activity. 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, and subsequently re-assayed.

Solubilities of thiuram disulphides in water were estimated as follows. A suspension of the finely powdered material in water was vigorously stirred for several days at room temperature (20°C). After filtration, the u.v. spectrum of the diluted solution was compared quantitatively with that of standard solutions.

Results and Discussion

Table 1 records the inhibitory effect of certain disulfiram analogues on the aldehyde dehydrogenases of sheep liver at physiological pH, and in

Table 1. *Effect of some analogues of disulfiram on the aldehyde dehydrogenases of sheep liver*

The activity of the enzymes in the presence of various concentrations of the inhibitors listed was assayed as before (Kitson, 1975). The initial rate of the enzyme reaction in the presence of an inhibitor is expressed as a percentage of the rate in its absence. The results for disulfiram at pH 9.3 are taken from Kitson (1975).

Inhibitor	Concentration (M)	Initial activity (% of control rate)			
		Cytoplasmic enzyme		Mitochondrial enzyme	
		pH 7.3	pH 9.3	pH 7.3	pH 9.3
$[(C_2H_5)_2N-CS-S]_2$	10^{-4}	—	0	92, 92	81, 81, 91
	10^{-5}	3.4	5, 5	95, 100	96
	10^{-6}	5.5, 5.7	9	—	—
	10^{-7}	78, 79	—	—	—
$[(CH_3)_2N-CS-S]_2$	10^{-4}	—	—	84, 84	—
	10^{-5}	3.7	—	91	—
	10^{-6}	9.1, 10	—	—	—
	10^{-7}	80	—	—	—
10^{-5}	6.3	—	95	—
	10^{-6}	9.7, 10	—	—	—
	10^{-7}	89, 89	—	—	—
10^{-5}	4.4, 6.3	—	84	—
	10^{-6}	6.5, 7.9	—	—	—
	10^{-7}	61, 70	—	—	—
10^{-5}	8.4, 7.6	25, 24	88	—
	10^{-6}	7.5, 8.3	21, 22	—	—
	10^{-7}	87, 80	66, 81	—	—
10^{-4}	16	—	—	—
	10^{-5}	75, 72	—	97	—
$[(C_2H_5)_2N-CS-S]_2CH_2^*$	10^{-5}	36, 41, 42	35, 34, 31	101	—

* The effect of this inhibitor was determined in the presence of 0.2 mM-2-mercaptoethanol.

Table 2. *Effect of tetramethylthiuram monosulphide on sheep liver aldehyde dehydrogenases*

The activity of the enzymes in the presence of 0.5 mM-tetramethylthiuram monosulphide, with and without 2-mercaptoethanol, was assayed as before (Kitson, 1975). The initial rate of the enzyme reaction in the presence of the inhibitor is expressed as a percentage of the rate in its absence. Also listed is the loss of enzyme activity over a period of 15 min, expressed as a percentage of the initial activity.

	pH	2-Mercaptoethanol (mM)	Initial activity (% of control rate)	Loss of activity in 15 min (%)
Cytoplasmic	9.3	0	8.3, 7.0, 8.0	47, 57, 60
aldehyde	9.3	0.2	54, 51, 43	45, 32, 35
dehydrogenase	7.3	0.2	58, 56	35, 24
Mitochondrial	9.3	0	70	12
aldehyde	9.3	0.2	89, 96	0
dehydrogenase	7.3	0.2	100	0

some cases, pH 9.3, in the presence of 1.67 mM-NAD⁺ and 1.67 mM-acetaldehyde. At pH 7.3, under these conditions, the subsequent further loss of activity brought about by the thiuram disulphides (Kitson, 1975) is very slow. As is evident from Table 1, several thiuram disulphides, including disulfiram, are equally potent inhibitors of the cytoplasmic enzyme. In all cases, however, the mitochondrial form of aldehyde dehydrogenase is very much less sensitive to the inhibitory action of these compounds.

Bis-(1-L-prolylthiocarbonyl) disulphide is a relatively poor inhibitor of the cytoplasmic enzyme. This may be a steric effect, but is more likely due to the negative charge carried by this compound at physiological pH; Deitrich (1967) reported that uncharged molecules such as *p*-chloromercuribenzoic acid are more efficient as inhibitors of aldehyde dehydrogenase than are the related anions. Bis-[(4-methyl-1-piperazinyl)thiocarbonyl] disulphide, which is positively charged at physiological pH, is similar in effectiveness to the neutral thiuram disulphides.

Disulfiram is often administered therapeutically as a subcutaneous implant. The efficacy of this procedure is doubtful, owing to the low water-solubility of the compound and its consequent negligible steady-state concentration *in vivo* after implantation (Malcolm *et al.*, 1974). In this respect, the physiological properties of some of the aldehyde dehydrogenase inhibitors used in the present study may warrant examination. For instance, bis-(1-morpholinylthiocarbonyl) disulphide is approximately five times as soluble in water as disulfiram. (The two compounds were estimated to have solubilities of 185 and 35 μ M respectively by the method described above.) The protonated form of bis-[(4-methyl-1-piperazinyl)thiocarbonyl] disulphide is of course very water-soluble. The action of this compound *in vivo* will be particularly interesting to ascertain; it has quite different electrical and solubility properties from disulfiram and yet behaves in a very similar way towards aldehyde dehydrogenase *in vitro*.

Methyl *NN*-diethyldithiocarbamate, which has been shown to be a metabolite of disulfiram (Gessner & Jakubowski, 1972), has no effect on either of the aldehyde dehydrogenases, even at a concentration of 1 mM. Methylene bis-(*NN*-diethyldithiocarbamate), which is a close structural analogue of disulfiram lacking the latter compound's reactive disulphide bond, is quite an effective inhibitor of cytoplasmic aldehyde dehydrogenase, but not of the mitochondrial form (see Table 1). In view of the known chemical unreactivity of dithiocarbamate esters (Thorn & Ludwig, 1962), it is expected that this compound would not interact covalently with the enzyme, and indeed, unlike the case of disulfiram itself (Kitson, 1975), the initial inhibitory effect is not followed by a further slow loss of activity at either pH value used.

Tetramethylthiuram monosulphide, which also lacks a disulphide bond, nevertheless causes a progressive inactivation of the cytoplasmic enzyme (Table 2). Part of this effect is spurious and due to contamination with the corresponding disulphide, which can be detected in the commercial material by mass spectrometry. In the presence of 2-mercaptoethanol, which destroys thiuram disulphides (Kitson, 1975), however, there is still a time-dependent loss of activity which must be caused by the monosulphide itself. After 5 h in the presence of 0.5 mM-tetramethylthiuram monosulphide at pH 9.3 the activity of the cytoplasmic enzyme was 14% of the control; after exhaustive dialysis the activity was found to be 24% of the control. Removal of the inhibitor, therefore, does little to restore the activity of the enzyme, suggesting the occurrence of a covalent interaction between tetramethylthiuram monosulphide and the protein. A possibility for such a reaction, by analogy with that between disulfiram and protein thiol groups (Neims *et al.*, 1966a) is:

Tetramethylthiuram monosulphide is effective in producing hyperacetaldehydaemia after ethanol ingestion (Hald *et al.*, 1952). This effect may be due to the direct action of the compound on aldehyde dehydrogenase as studied here. An alternative mechanism could involve the production of dimethyldithiocarbamate from the monosulphide *in vivo*, perhaps by reaction with thiol groups; subsequent oxidation of dimethyldithiocarbamate to the disulphide would then provide a means for inactivation of aldehyde dehydrogenase in a similar way to the process thought to occur with disulfiram itself (Deitrich & Erwin, 1971; Kitson, 1975). The first product of the reaction between tetramethylthiuram monosulphide and H_2S at $140^\circ C$ is dimethyldithiocarbamic acid (Craig *et al.*, 1951). However, no formation of dimethyldithiocarbamate [which has a distinctive u.v. spectrum (Koch, 1949)] could be detected during the present work in solutions containing the monosulphide and 2-mercapto-ethanol left overnight at room temperature and pH 7.3. It would be interesting to determine if tetramethylthiuram monosulphide is an inhibitor of dopamine β -hydroxylase (3,4-dihydroxyphenethylamine-oxygen oxidoreductase) *in vivo*. Disulfiram inhibits this metalloenzyme [an effect which has been implicated in the pharmacogenesis of the disulfiram-ethanol reaction (Truitt & Walsh, 1971)], but the inhibition is mediated through the metabolite, diethyldithiocarbamate, a powerful metal ion chelator (Goldstein *et al.*, 1964).

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