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Studies on the Interaction between Disulfiram and Sheep Liver Cytoplasmic Aldehyde Dehydrogenase

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The effect of disulfiram, [1-^{14}C]disulfiram and some other thiol reagents on the activity of cytoplasmic aldehyde dehydrogenase from sheep liver was studied. The results are consistent with a rapid covalent interaction between disulfiram and the enzyme, and inconsistent with the notion that disulfiram is a reversible competitive inhibitor of cytoplasmic aldehyde dehydrogenase. There is a non-linear relationship between loss of about 90% of the enzyme activity and amount of disulfiram added; possible reasons for this are discussed. The remaining approx. 10% of activity is relatively insensitive to disulfiram. It is found that modification of only a small number of groups (one to two) per tetrameric enzyme molecule is responsible for the observed loss of activity. The dehydrogenase activity of the enzyme is affected more severely by disulfiram than is the esterase activity. Negatively charged thiol reagents have little or no effect on cytoplasmic aldehyde dehydrogenase. 2,2′-Dithiodipyridine is an activator of the enzyme.

Disulfiram (tetraethylthiuram disulphide, Antabuse) has been widely used for the purpose of reducing alcohol consumption amongst alcoholics. Fear of the unpleasant consequences (the 'disulfiram–ethanol reaction') which will ensue in a patient on disulfiram, if he drinks, is often a valuable contribution towards his attempt at sobriety. The biochemical basis of the disulfiram–ethanol reaction has been the subject of much research and has been reviewed by Kitson (1977*a*). Briefly, it is believed that disulfiram acts mainly through the inhibition of aldehyde dehydrogenase, resulting, during the metabolism of ethanol, in the accumulation of toxic concentrations of acetaldehyde. At the same time, dopamine β-hydroxylase (3,4-dihydroxyphenethylamine–oxygen oxidoreductase) is inhibited by diethyldithiocarbamate (a metabolite of disulfiram), resulting in a blockade of the synthesis of noradrenaline and explaining the observed hypotension (Truitt & Walsh, 1971).

A full understanding of how disulfiram works at the molecular level would be of great significance to the use of this compound both as a therapeutic drug and as an experimental laboratory tool in the study of alcohol metabolism. Thus the present extensive investigation of the action of disulfiram on sheep liver cytoplasmic aldehyde dehydrogenase was initiated. Previous work has shown that the cytoplasmic aldehyde dehydrogenases from sheep (Kitson, 1975, 1976), ox (Sugimoto *et al.*, 1976), horse (Eckfeldt *et al.*, 1976) and human liver (Greenfield & Pietruszko, 1977) are much more sensitive to disulfiram than the corresponding mitochondrial enzymes.

Experimental

Materials

The following compounds were obtained from the sources indicated. Disulfiram, 5,5′dithiobis-(2-nitrobenzoic acid), 2,2′-dithiodipyridine, *N*-acetyl-L-cysteine and Sephadex G-25-80, Sigma Chemical Co. (St. Louis, MO, U.S.A.); acetaldehyde and 2-mercaptoethanol, Fluka A.G. (Buchs, Switzerland); NAD^+ and sodium diethyldithiocarbamate, BDH Chemicals (Poole, Dorset, U.K.); 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene, Koch–Light Laboratories (Colnbrook, Bucks., U.K.); 4-nitrophenyl acetate, Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); di[1-^{14}C]ethylamine hydrochloride (28 Ci/mol), The Radiochemical Centre (Amersham, Bucks., U.K.).

Bis-[(4-methyl-1-piperazinyl)thiocarbonyl] disulphide and bis-(1-L-prolylthiocarbonyl) disulphide were prepared as before (Kitson, 1976).

4-Nitrophenyl *NN*-dimethyldithiocarbamate was prepared by the method of Clifford & Lichty (1932) [Found: C, 45.2; H, 4.0; N, 11.3; S, 26.2. Calc. for $C_9H_{10}N_2O_2S_2$: C, 44.6; H, 4.2; N, 11.6; S, 26.5%. *m/e* 242 (M^+), 198 and 88 (base)].

[1-^{14}C]Disulfiram was prepared on a small scale (approx. 50mg) by the standard method of synthesis of thiuram disulphides (Kitson, 1976). The recrystal-

lized material was identical with an authentic sample of disulfiram and had a specific radioactivity of 4.15×10^{11} c.p.m./mol under the conditions used.

Methods

Cytoplasmic aldehyde dehydrogenase from sheep liver was purified substantially as previously described (Crow *et al.*, 1974). Minor modifications to the isolation procedure (including the use of ultrafiltration through a Diaflo XM100 membrane instead of $(NH_4)_2SO_4$ precipitation for concentration of dilute enzyme samples, gel filtration through Bio-Gel A 0.5M in place of Sephadex G-200, and the use of Whatman DE-52 DEAE-cellulose instead of DE-32) enable the enzyme to be obtained more quickly and in a virtually homogeneous state (Crow, 1975; MacGibbon, 1976). Before use the enzyme solutions were thoroughly dialysed to remove 2-mercaptoethanol. The final dialysis buffer was checked for the absence of 2-mercaptoethanol by the use of 5,5′-dithiobis-(2-nitrobenzoic acid).

Enzyme activity was assayed as before (Kitson, 1975) at pH7.3 unless otherwise stated. The effect of disulfiram and the other compounds studied on the enzyme activity was determined in two ways: (A) by incorporating disulfiram as a 0.1ml ethanol solution directly in the assay system (final volume 3ml), or (B) by premixing a solution of disulfiram in ethanol with the stock enzyme solution and subsequently taking 0.1ml portions for assay in the usual way. [It has been shown that the report of a chemical reaction between disulfiram and ethanol is fallacious (Kitson, 1977*b*).]

The rate of hydrolysis of 4-nitrophenyl acetate was determined spectrophotometrically at 25°C and pH7.3 by following the increase in A_{400} due to the production of the 4-nitrophenoxide ion.

The extent of reaction of aldehyde dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoic acid) was determined spectrophotometrically at 25°C and pH7.3 by measuring the increase in A_{412} due to the production of the 3-carboxy-4-nitrothiophenoxide ion.

Non-covalently bound radioactivity was removed from enzyme samples either by dialysis against four changes of a large volume of 0.01M-potassium phosphate buffer, pH7.3, at 4°C, or by passage through a column (0.8cm×11cm) of Sephadex G-25 at 4°C. (The efficiency of gel filtration of large and small molecules was checked visually by using 4-nitrophenol and Blue Dextran, and confirmed with separate elutions of aldehyde dehydrogenase and [1-^{14}C]disulfiram.)

Liquid-scintillation counting was done on a Packard (model 2002) instrument with a solvent made from 2,5-diphenyloxazole (1.75g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.06g) in toluene (350ml) and ethanol (150ml). To 5ml of this solvent was added 0.1ml of the aqueous solution to be tested.

Polyacrylamide-gel electrophoresis was performed at pH8.9 in 7% gels with a loading of 10μg of protein. Gels were stained with Coomassie Brilliant Blue.

Protein concentrations were determined by the method of Bradford (1976). Enzyme concentration in the assay system was 0.4–0.6μM.

Results and Discussion

Reversibility of the interaction between disulfiram and cytoplasmic aldehyde dehydrogenase

The effect of disulfiram on aldehyde dehydrogenase has been explained in two ways, either that the compound acts as a reversible inhibitor, competitive with NAD^+ (Graham, 1951; Deitrich & Hellerman, 1963), or that it reacts covalently through mixed-disulphide formation with enzymic thiol groups:

$$E\text{–}S^- + Et_2N\text{–}CS\text{–}SS\text{–}CS\text{–}NEt_2 \rightarrow E\text{–}SS\text{–}CS\text{–}NEt_2 + Et_2NCS_2^- \quad (1)$$

As regards the mitochondrial sheep liver enzyme (which shows a relatively slow progressive loss of activity in the presence of disulfiram), the latter explanation is well substantiated (Kitson, 1975; Hart & Dickinson, 1977). However, it is the more sensitive cytoplasmic enzyme which is probably of importance from the point of view of the disulfiram–ethanol reaction. Recently, a claim was made that disulfiram is a competitive inhibitor (towards NAD^+) of the human cytoplasmic enzyme (Greenfield & Pietruszko, 1977), although other workers (Sanny & Weiner, 1977; Eckfeldt *et al.*, 1976) have discussed the effect of disulfiram on the horse cytoplasmic enzyme in terms of the covalent process mentioned above.

Fig. 1 shows that for the cytoplasmic sheep liver enzyme disulfiram is certainly not a competitive inhibitor towards NAD^+. (This experiment was performed at pH9.3, since at pH7.3 the K_m for NAD^+ is inconveniently low.) Instead, the Lineweaver–Burk plot approximates to that expected for the irreversible inactivation of the enzyme (in which the lines would intersect on the horizontal axis). Likewise, the results presented in Table 1 suggest that the binding of disulfiram to the enzyme is extremely tight or possibly covalent. A low concentration of disulfiram (similar to the enzyme concentration) causes a very substantial loss of activity; this is complete within the time of mixing. The subsequent addition of a small to moderate excess of a thiol (2-mercaptoethanol or *N*-acetyl-L-cysteine) does not relieve this inhibition, although pre-mixing of the thiol and disulfiram abolishes most of the loss of activity, as found before (Kitson, 1975). This shows that a rapid reversible association between the enzyme and disulfiram (as would be necessary for the system to show competitive kinetics) is not occurring.

Only when a massive excess of 2-mercaptoethanol (0.4M) is used can the inactivatory process be reversed. This agrees with the results of Eckfeldt *et al.* (1976) with the horse enzyme. [Neims *et al.* (1966) found with D-amino acid oxidase that, although excess glutathione released the bound diethyldithiocarbamate moiety from the enzyme, the activity did not reappear; they presumed this was because an irreversible conformational change had also taken place.]

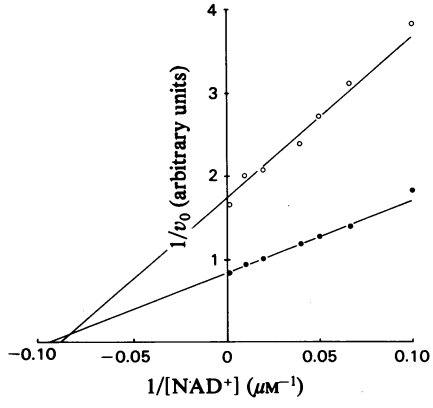


Fig. 1. *Lineweaver–Burk plot showing the effect of disulfiram on cytoplasmic aldehyde dehydrogenase*
The activity of the enzyme was measured at various concentrations of NAD^+ in the presence and absence of disulfiram (0.3 μM) as described under 'Methods' (procedure A). ●, Without disulfiram; ○, with disulfiram.

The last entry in Table 1 concerns an experiment designed to test the possibility of disulfiram reacting rapidly with aldehyde dehydrogenase only in the presence of NAD^+. [The enzyme exhibits an obligatory sequential order of binding with NAD^+ as leading substrate (MacGibbon *et al.*, 1977*a*).] It appears that this is not the case, however, since when the enzyme is treated with disulfiram, and then excess thiol is added (which largely removes unchanged disulfiram) before NAD^+ and acetaldehyde are added to initiate the enzyme reaction, the extent of loss of activity is the same as when the inactivation takes place in the presence of NAD^+.

Form of the inactivation profile for the 'titration' of cytoplasmic aldehyde dehydrogenase with disulfiram: stoicheiometry of the reaction

Since disulfiram appears to react rapidly and covalently with sheep liver cytoplasmic aldehyde

Table 1. *Effect of thiols on the interaction between disulfiram and cytoplasmic aldehyde dehydrogenase*
The activity of the enzyme at various concentrations of disulfiram and 2-mercaptoethanol or *N*-acetyl-L-cysteine (added in the order shown) was assayed as described under 'Methods' (procedure A). The initial rate of the enzyme reaction in the presence of disulfiram is expressed as a percentage of the rate in its absence.

Disulfiram (μM)	2-Mercaptoethanol (mM)	Order of mixing	Initial activity (% of control rate)
1	0	Disulfiram, buffer, enzyme, substrates	12, 10
1	0.1	Disulfiram, buffer, enzyme, substrates; thiol added 2min later	12, 10
1	0.1	Disulfiram, buffer, thiol, substrates; enzyme added 2min later	84, 85, 84
1	0.1	Disulfiram, buffer, enzyme; thiol added 2min later; substrates added after standing overnight at 4°C	33, 34
1	400	Disulfiram, buffer, enzyme, substrates; thiol added 2min later	100*
	N-Acetyl-L-cysteine (μM)		
0.5	0	Disulfiram, buffer, enzyme, substrates	27, 23, 28
0.5	2	Disulfiram, buffer, enzyme, substrates; thiol added 2min later	27
0.5	2	Disulfiram, buffer, thiol, substrates; enzyme added 1min later	69
0.5	2	Disulfiram, buffer, thiol, substrates; enzyme added 2min later	84, 78
0.5	2	Disulfiram, buffer, enzyme; thiol added 2min later; substrates added after further 2min	23, 23

* This value was achieved 4min after addition of the thiol.

dehydrogenase, it was decided to investigate the effect on the enzyme activity of a range of disulfiram concentrations. In the simplest case, assuming disulfiram to react with catalytically essential thiol groups, this would result in a linear relationship between residual enzyme activity and amount of disulfiram added, and indeed such a situation has been briefly reported for the horse enzyme (Eckfeldt *et al.*, 1976). In the present case, the profile obtained is shown in Fig. 2. The dehydrogenase activity is rapidly decreased to approx. 10% of the control rate, but this residual activity is then relatively insensitive to increasing disulfiram concentrations. Two explanations present themselves here. (1) The enzyme sample may be heterogeneous. For example, it may be contaminated with the less sensitive mitochondrial enzyme, although precautions are taken against this (Crow *et al.*, 1974), and no evidence for the existence of isoenzymes of sheep liver cytoplasmic aldehyde dehydrogenase has been obtained (MacGibbon *et al.*, 1977*b*). (2) The thiol groups with which disulfiram reacts may not be essential for catalytic activity; that is, the enzyme's active sites may only be sterically hindered or otherwise indirectly affected by modification, giving rise to an enzyme molecule with approx. 10% of the activity of the native material. (Of course, even if the activity had been completely abolished this would not prove that the groups that react with disulfiram are also directly involved in the catalytic mechanism.)

When the early part of the profile shown in Fig. 2 is expanded, curves such as those shown in Fig. 3 are obtained. These experiments were done either by incorporating various amounts of disulfiram directly in the assay mixture, or pre-mixing disulfiram and enzyme before assay (as described under 'Methods'), and as can be seen the results are similar. The titration of aldehyde dehydrogenase with disulfiram at pH9.3 is very similar to that at pH7.3, at which most of the present experiments were performed. Thus it is likely that the conclusion drawn above from Fig. 1 (obtained at pH9.3) also applies to physiological pH.

From Fig. 3 and the results of similar experiments,

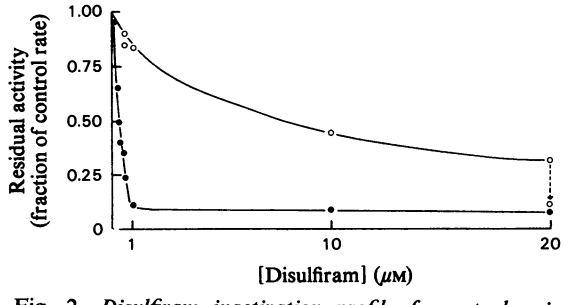


Fig. 2. *Disulfiram inactivation profile for cytoplasmic aldehyde dehydrogenase: comparison of the effects of disulfiram on the dehydrogenase and esterase activities of the enzyme*

The activity of the enzyme (as a fraction of the control rate) is plotted against the concentration of added disulfiram. Activity was measured as described under 'Methods' (procedure A) at pH7.3. ●, Dehydrogenase activity; ○, esterase activity. At high disulfiram concentrations the esterase activity was not constant; for example, the arrow shows the drop in activity over a period of 20 min in the presence of 20 μM-disulfiram.

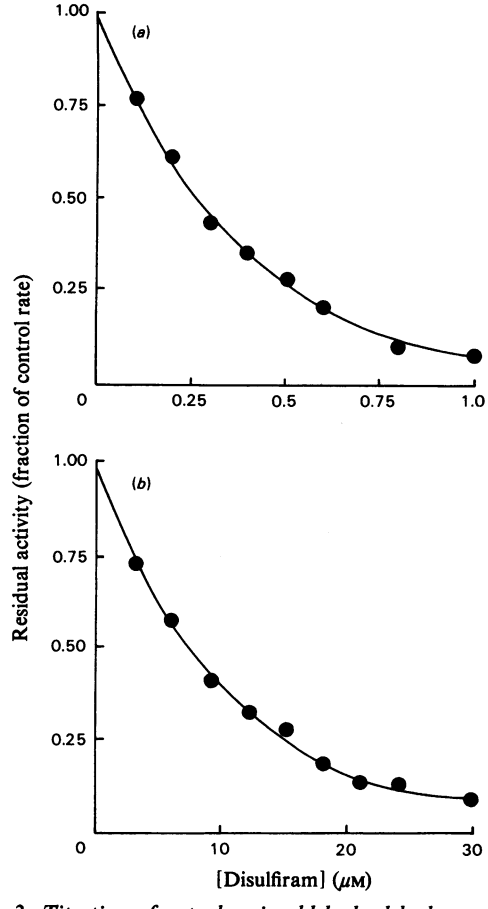


Fig. 3. *Titration of cytoplasmic aldehyde dehydrogenase with disulfiram*

(*a*) Enzyme activity was measured as described under 'Methods' (procedure A) at pH7.3 and is plotted against the concentration of disulfiram added to the assay mixture. The enzyme concentration in the assay mixture was 0.51 μM (based on mol.wt. 212000). (*b*) Enzyme activity was measured as described under 'Methods' (procedure B) at pH7.3 and is plotted against the concentration of disulfiram added to the stock enzyme solution, the concentration of which was 17.5 μM.

Table 2. *Stoicheiometry of the reaction between cytoplasmic aldehyde dehydrogenase and disulfiram*

From the results of experiments such as those illustrated in Fig. 3, the number of molecules of inactivator per tetrameric enzyme molecule necessary to decrease activity to the 10% level was calculated.

Inactivator	Experimental procedure	Molecules of inactivator/enzyme molecule
Disulfiram	A	2.0, 1.5, 2.0
Disulfiram	B	1.5, 1.4
Bis-[(4-methyl-1-piperazinyl)-thiocarbonyl] disulphide	A	1.8, 1.9

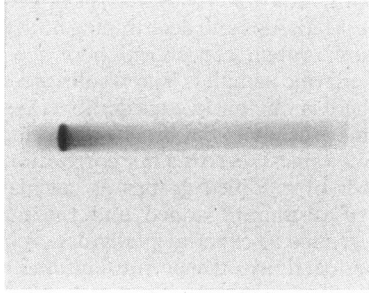


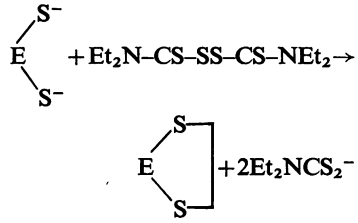
Fig. 4. *Polyacrylamide-gel electrophoresis of cytoplasmic aldehyde dehydrogenase*

Electrophoresis was performed on a 7% gel at pH 8.9 with a loading of 10 μg of protein.

it is possible to compare the concentration of disulfiram necessary to decrease cytoplasmic aldehyde dehydrogenase activity to the insensitive 10% level with the enzyme protein concentration. This gives the results recorded in Table 2.

The significance of these values depends on the veracity of certain assumptions. It is assumed that each molecule of disulfiram reacts with a single enzymic thiol group; this point is discussed in the next section. The critical assumption in all such experiments as these is that the experimentally determined protein concentration can be equated with the concentration of active enzyme. Fig. 4 shows that the procedure used here yields enzyme which is probably at least 90% pure, and the recovery of activity after dialysis (necessary to remove 2-mercaptoethanol before experiments are begun) was found to be at least 90%. Protein concentration was determined by the accurate, reproducible and convenient method of Bradford (1976). There always remains the possibility, however, that stock enzyme solutions contain significant amounts of inactive enzyme or other protein which is not separable during the purification procedure used or on gels.

There are several conceivable reasons why the graphs in Fig. 3 are found to be non-linear. For instance, the enzyme is known to be a tetramer (of mol.wt. 212000), the subunits being of equal size and charge (Crow, 1975), but it may be that they are inherently unequally responsible for the enzyme's activity, or that modification of a thiol group on one subunit indirectly affects the activity of the other subunits. Non-linearity might arise if some of the activity loss is due to two 'essential' groups sufficiently close together both to be modified by a single disulfiram molecule:



(2)

The enzyme contains approx. 36 thiol groups per tetramer (MacGibbon, 1976); obviously, since most of the activity is destroyed by modification of only a small number of groups (see Table 2), some of these thiol groups must be much more reactive to disulfiram than others. However, competition by the 'non-essential' groups for added disulfiram, especially when an appreciable fraction of the 'essential' groups has already reacted, would give rise to a curve similar in shape to that observed experimentally. If this situation applies, inspection of the initial slopes of Fig. 3 suggests that there would be approximately one essential reactive group per tetramer.

A non-linear relationship between remaining activity and amount of disulfiram added would also be obtained if the enzyme contained several 'essential' groups, modification of any one of which completely abolished activity (except in this case the residual 10% discussed above), but which still left the others free subsequently to react with disulfiram. Analysis of a situation such as this can be attempted by a procedure originated by Tsou Chen-Lu (1962) and discussed in detail by Paterson & Knowles (1972). The number of groups modified is plotted against $a^{1/i}$, where a is the fractional remaining activity and i is the number of 'essential' groups as defined above. The value of i is obtained by trial and error as that which gives the

best straight line. The data of Fig. 3(*a*) are replotted in this way in Fig. 5. It is evident that a better straight line is obtained when i is approx. 4, tempting the suggestion to be made that each subunit has a reactive thiol group and that modification of any one of these four groups results in an inactive tetramer. The procedure of Tsou Chen-Lu (1962), however, is not sufficiently precise (except at the lowest values of i) for such a conclusion to be stated with any certainty, and, as we have seen above, there are other possibilities to account for the curvature seen in Fig. 3.

In summary, the present results show that most of the activity of cytoplasmic aldehyde dehydrogenase from sheep liver is abolished by two or less molecules of disulfiram per tetramer, and that this activity loss may effectively be due to the modification of a single particularly reactive essential thiol group, or to the reaction of any one of four essential groups.

The presence of two disulfiram-reactive groups per tetrameric enzyme molecule has been reported for the horse enzyme in a preliminary communication (no experimental details) by Sanny & Weiner (1977), whereas for enzyme from the same source, Eckfeldt *et al.* (1976) found four disulfiram molecules per tetramer to be required for inactivation. Hart & Dickinson (1977) reported that only two thiol groups per molecule of the mitochondrial enzyme from sheep liver are apparently modified by disulfiram; the modified enzyme retains 50% activity and the reaction takes approx. 5h, in contrast with the cytoplasmic enzyme studied here, with which the reaction is complete within the time of mixing.

Modification of cytoplasmic aldehyde dehydrogenase with [1-^{14}C]disulfiram

In the above discussion it has been assumed that the number of enzymic groups modified can be simply determined from the amount of disulfiram added to the system, according to eqn. (1). It was decided to test this assumption by labelling enzyme samples with different amounts of radioactive disulfiram, removing any unbound radioactivity by dialysis or gel filtration (see under 'Methods') and determining both the radioactivity and protein concentration of the resulting modified enzyme samples. The results are shown in Fig. 6. There is obviously considerable experimental error in this procedure, but broadly speaking (at least at the lower values) there is a fair correlation between the number of modified groups as calculated from amount of disulfiram added and the number of modified groups as experimentally determined from bound radioactivity, which substantiates the conclusions reached above.

Fig. 6 incorporates the results from four separate experiments. On three occasions (represented by the symbols ●, ○, ■) the bound radioactivity was found to be close to or less than the expected value. Where the amount of bound radioactivity is less than cal-

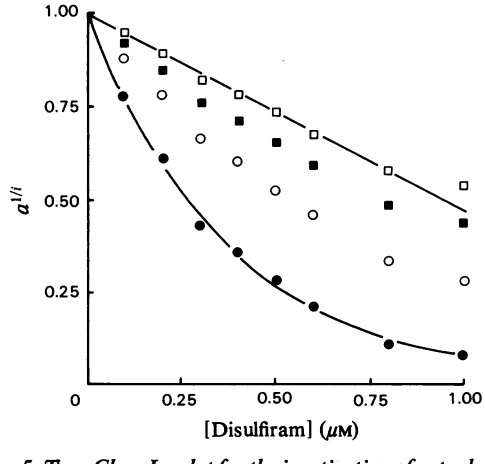


Fig. 5. *Tsou Chen-Lu plot for the inactivation of cytoplasmic aldehyde dehydrogenase by disulfiram*
Values of remaining enzyme activity (a) in the presence of various concentrations of disulfiram as recorded in Fig. 3 are replotted as $a^{1/i}$. The significance of this procedure is discussed in the text. ●, $i = 1$; ○, $i = 2$; ■, $i = 3$; □, $i = 4$.

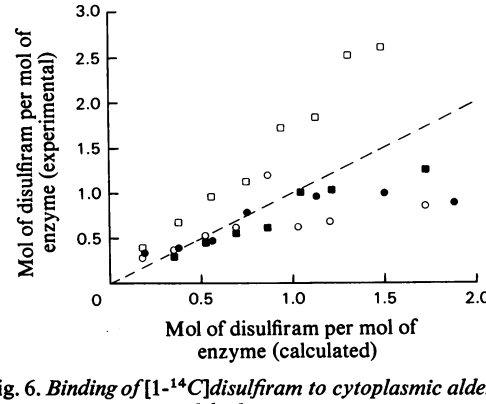


Fig. 6. *Binding of [1-^{14}C]disulfiram to cytoplasmic aldehyde dehydrogenase*
The number of molecules of [1-^{14}C]disulfiram reacting per tetrameric enzyme molecule as experimentally determined from bound radioactivity is plotted against the number calculated from the amount of added disulfiram assuming eqn. (1) to apply. The broken line shows the theoretically expected relationship. ● and ○, Two separate experiments in which unbound radioactivity was removed from the enzyme by dialysis; ■ and □, experiments in which gel filtration was used.

culated, this may be because diethyldithiocarbamate is becoming detached from the enzyme during dialysis or gel filtration. Diethyldithiocarbamate is expected to be susceptible to nucleophilic displacement from the enzyme by, for example, unmodified thiol groups. On a fourth occasion (represented by the symbol □), a surprising result was obtained in that almost twice as much radioactivity was apparently covalently bound to the enzyme as expected. This particular enzyme preparation was shown to bind radioactive diethyldithiocarbamate in the presence of 2 mM-2-mercaptoethanol, and to be inhibited 40–50% by a 2-fold molar excess of diethyldithiocarbamate under the same conditions. In the presence of 0.4 M-2-mercaptoethanol, the full activity was restored. Other enzyme preparations used in this and previous work were much less affected by diethyldithiocarbamate (see Table 1 and Kitson, 1975). It appears therefore that, under some circumstances, diethyldithiocarbamate (even in the presence of 2 mM-2-mercaptoethanol) can bind to the enzyme and cause a decrease in activity, presumably via re-oxidation to disulfiram, and this possibility must be borne in mind in interpreting experiments with these compounds. Possibly the particular enzyme preparation under discussion here contained an impurity that catalyses the oxidation of diethyldithiocarbamate to disulfiram. Cytochrome *c* (DuBois *et al.*, 1961), methaemoglobin (Strömme, 1963) and xanthine oxidase (Fried, 1976) have been shown to have this ability. If this is the case, it would parallel the situation which probably obtains *in vivo* (Kitson, 1977*a*). It is thought that disulfiram is completely reduced to diethyldithiocarbamate by reaction with glutathione on absorption into the bloodstream, but subsequently becomes re-oxidized before inactivating hepatic aldehyde dehydrogenase.

Effect of disulfiram on the esterase activity of cytoplasmic aldehyde dehydrogenase

Fig. 2 compares the effect of disulfiram on cytoplasmic aldehyde dehydrogenase acting as a dehydrogenase (NAD^+ and acetaldehyde as substrates) and an esterase (4-nitrophenyl acetate as substrate). The points represent the initial activity in the presence of a given concentration of disulfiram; for the esterase activity at high disulfiram concentrations, this is not linear but decreases with time as shown, such that both esterase and dehydrogenase activities become decreased to a similar extent. At low disulfiram concentrations, however, the rates are linear and there is a significant difference in the extent of decrease of the two activities. It has been shown above that the presence of NAD^+ is not necessary for rapid inactivation by disulfiram. Thus if the esterase and dehydrogenase reactions occur at the same site on the enzyme, disulfiram must be modifying groups not directly involved in the catalysis. On the other hand, if distinct esterase and dehydrogenase sites exist [as argued by MacGibbon *et al.* (1978) on the basis of kinetic studies], then these must be close enough for both to be affected (though to different extents) by modification of the disulfiram-sensitive thiol groups.

Action of other thiol-modifying reagents on cytoplasmic aldehyde dehydrogenase

Bis-[(4-methyl-1-piperazinyl)thiocarbonyl] disulphide (positively charged at pH 7.3) has an effect identical with that of disulfiram on cytoplasmic aldehyde dehydrogenase (see Table 2), extending the results previously obtained (Kitson, 1976). Likewise, the effect of bis-(1-L-prolylthiocarbonyl) disulphide (negatively charged at pH 7.3) was confirmed as being negligible compared with that of disulfiram. Further studies have shown that other negatively charged thiol reagents lack the inactivatory properties of disulfiram. For example, iodoacetate (1 mM) has no effect on the activity of cytoplasmic aldehyde dehydrogenase. Hart & Dickinson (1977) found the same with the mitochondrial enzyme. On the other hand, iodoacetamide does inactivate cytoplasmic aldehyde dehydrogenase, albeit slowly compared with disulfiram. Iodoacetamide (1 μM) lowers the activity to 15–16% within 20 min and to zero after about 37 min. Excess iodoacetamide lowers the activity of the mitochondrial enzyme only to a limiting extent of 50% (Hart & Dickinson, 1977).

A large excess of 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) will inactivate cytoplasmic aldehyde dehydrogenase (MacGibbon, 1976), but the present studies show that at relatively low concentrations this reagent (again negatively charged at pH 7.3) has little effect. The initial activity of cytoplasmic aldehyde dehydrogenase in the presence of 10 μM-Ellman's reagent is 92% of the blank rate. After 30 min, it is still 85%, although the increase in A_{412} shows that about 3.7 thiol groups per tetrameric enzyme molecule have become modified. These results contrast with those of Hart & Dickinson (1977) for the mitochondrial enzyme; they found that modification of 4 thiol groups by Ellman's reagent resulted in total loss of enzyme activity.

Fig. 7 illustrates the surprising activatory effect of the thiol reagent 2,2′-dithiodipyridine on cytoplasmic aldehyde dehydrogenase. Further studies need to be done to examine the nature of this interaction, particularly whether the same thiol groups are involved as react with disulfiram.

A preliminary investigation of the effect of 4-nitrophenyl *NN*-dimethyldithiocarbamate on cytoplasmic aldehyde dehydrogenase has been carried out. This compound is structurally related to the inacti-

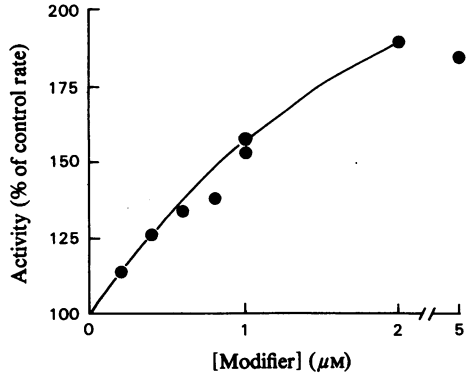


Fig. 7. *Effect of 2,2'-dithiodipyridine on cytoplasmic aldehyde dehydrogenase*
Enzyme activity was measured as described under 'Methods' (procedure A) at pH7.3 and is plotted against the concentration of modifier added to the assay mixture.

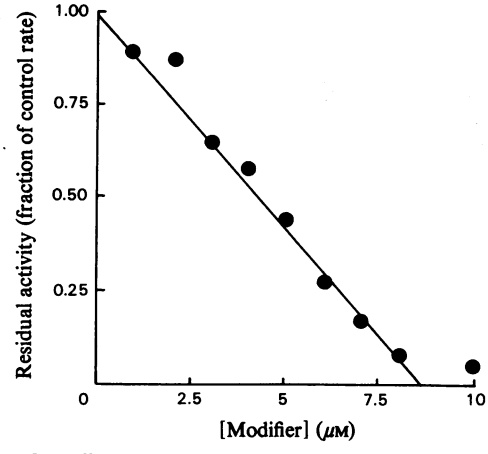


Fig. 8. *Effect of 4-nitrophenyl NN-dimethyldithiocarbamate on cytoplasmic aldehyde dehydrogenase*
Enzyme activity was measured as described under 'Methods' (procedure A) at pH7.3 and is plotted against the concentration of modifier added to the assay mixture.

vator disulfiram and to the substrate 4-nitrophenyl acetate. The result is shown in Fig. 8. The concentration of this compound necessary to cause a certain loss of activity is approximately ten times the concentration of disulfiram required for the same effect.

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