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Further studies of the action of disulfiram and 2,2′-dithiodipyridine on the dehydrogenase and esterase activities of sheep liver cytoplasmic aldehyde dehydrogenase

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1. Pre-modification of cytoplasmic aldehyde dehydrogenase by disulfiram results in the same extent of inactivation when the enzyme is subsequently assayed as a dehydrogenase or as an esterase. 2. 4-Nitrophenyl acetate protects the enzyme against inactivation by disulfiram, particularly well in the absence of NAD^+. Some protection is also provided by chloral hydrate and indol-3-ylacetaldehyde (in the absence of NAD^+). 3. When disulfiram is prevented from reacting at its usual site by the presence of 4-nitrophenyl acetate, it reacts elsewhere on the enzyme molecule without causing inactivation. 4. Enzyme in the presence of aldehyde and NAD^+ is not at all protected against disulfiram. It is proposed that, under these circumstances, disulfiram reacts with the enzyme–NADH complex formed in the enzyme-catalysed reaction. 5. Modification by disulfiram results in a decrease in the amplitude of the burst of NADH formation during the dehydrogenase reaction, as well as a decrease in the steady-state rate. 6. 2,2′-Dithiodipyridine reacts with the enzyme both in the absence and presence of NAD^+. Under the former circumstances the activity of the enzyme is little affected, but when the reaction is conducted in the presence of NAD^+ the enzyme is activated by approximately 2-fold and is then relatively insensitive to the inactivatory effect of disulfiram. 7. Enzyme activated by 2,2′-dithiodipyridine loses most of its activity when stored over a period of a few days at 4°C, or within 30 min when treated with sodium diethyldithiocarbamate. 8. Points for and against the proposal that the disulfiram-sensitive groups are catalytically essential are discussed.

The cytoplasmic aldehyde dehydrogenase of sheep liver catalyses the hydrolysis of 4-nitrophenyl acetate as well as the oxidation of a variety of aldehydes by NAD^+. In previous work (Kitson, 1978) it was reported that the two activities of the enzyme were differently affected by disulfiram, with the dehydrogenase activity being much more severely decreased by stoicheiometric concentrations of the modifier than was the esterase activity. [Disulfiram, or tetraethylthioperoxydicarbonic diamide, is a drug used in the treatment of chronic alcoholics (Kitson, 1977).] MacGibbon *et al.* (1978) observed that a high concentration of NAD^+ prevents 4-nitrophenyl acetate from binding to the enzyme but does not inhibit the dehydrogenase reaction. On the basis of this and the differential effect of disulfiram, they concluded that the binding sites for ester and aldehyde are distinct. On the other hand, Duncan (1979) showed than an aldehyde dehydrogenase from rabbit liver catalyses the reaction between 4-nitrophenyl acetate and NADH to give acetaldehyde and NAD^+ (as well as hydrolysing the ester), which is compelling evidence that ester hydrolysis occurs at the usual active site of the enzyme. He suggested that the results obtained by Kitson (1978) and by MacGibbon *et al.* (1978) might be misleading because of contamination of their cytoplasmic enzyme samples with mitochondrial aldehyde dehydrogenase (which is relatively insensitive to disulfiram; Kitson, 1975, 1976).

There have recently been developed three methods for removing traces of mitochondrial contamination from otherwise pure sheep liver cytoplasmic aldehyde dehydrogenase (Dickinson & Berrieman, 1979; Dickinson *et al.*, 1981; Kitson, 1981*a*). It therefore became possible and indeed necessary to re-investi-

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gate the action of disulfiram with the use of uncontaminated cytoplasmic enzyme. Accordingly, a detailed study of the effect of disulfiram on the dehydrogenase and esterase activities is presented below.

In previous work, several thiuram disulphides were found to inactivate the enzyme in a similar way to disulfiram (Kitson, 1976), but rather surprisingly the effect of 2,2′-dithiodipyridine is to activate cytoplasmic aldehyde dehydrogenase (Kitson, 1978, 1979). Furthermore, enzyme modified by 2,2′-dithiodipyridine is relatively insensitive to the inactivatory effect of disulfiram. As stated before (Kitson, 1979), if disulfiram and 2,2′-dithiodipyridine react with the same thiol groups then these cannot be essential to the catalytic functioning of the enzyme (otherwise both reagents would cause inactivation). The present work includes further studies that have a bearing on this important point.

Experimental

Materials

All chemicals were analytical-reagent grade whenever available and were purchased from Boehringer Corp. (London), London W.5, U.K., Sigma (London) Chemical Co., London S.W.6, U.K., or BDH Chemicals, Poole, Dorset, U.K. [1-^{14}C]Disulfiram was the same as used before (Kitson, 1978). Solutions of acetaldehyde were made up daily from 1 M stock solutions (kept frozen), which were prepared from freshly distilled acetaldehyde. Sheep livers were obtained from the local slaughterhouse, and enzyme isolation was started as soon as possible after the death of the animal.

Protein concentrations

For purified cytoplasmic aldehyde dehydrogenase a specific absorption coefficient at 280nm of $A^{1\%}_{1cm} = 11.3$ was used (Dickinson *et al.*, 1981). The molecular weight of the enzyme was taken to be 212000 (MacGibbon *et al.*, 1979).

Preparation of cytoplasmic aldehyde dehydrogenase

This enzyme was isolated by the method of Dickinson *et al.* (1981), which is based on that of Crow *et al.* (1974). In all cases the final enzyme preparation was subjected to two or three $(NH_4)_2SO_4$ fractionations in accordance with the method of Dickinson & Berrieman (1979) to minimize contamination by mitochondrial aldehyde dehydrogenase. Where mentioned in the text other procedures for removal of mitochondrial enzyme were also used [pH-gradient ion-exchange chromatography (Dickinson *et al.*, 1981); covalent chromatography on reduced thiopropyl-Sepharose 6B (Kitson, 1981*a*)]. The enzyme was always thoroughly dialysed before use to remove dithiothreitol.

Mitochondrial aldehyde dehydrogenase

A sample of this enzyme prepared by the method of Hart & Dickinson (1977) was generously given by Dr. G. J. Hart.

Enzyme assay (as a dehydrogenase)

This was performed fluorimetrically as described by Hart & Dickinson (1977). The final volume of the assay solution was 4ml. Assays were performed at 25°C in 33mM-sodium phosphate buffer, pH7.4. The concentrations of NAD^+ and acetaldehyde were each 1mM. The bisulphite addition compound of indol-3-ylacetaldehyde was dissolved in water, and sufficient 0.1M-HCl was added to liberate the free aldehyde; 0.1ml of the resulting solution was added to assay mixtures to give a final concentration of 100μM.

Enzyme assay (as an esterase)

This was performed at 25°C with 4-nitrophenyl acetate as substrate by following the production of 4-nitrophenoxide ion at 400nm in a Zeiss PMQ II spectrophotometer equipped with a Vitatron UR 40 chart recorder. 4-Nitrophenyl acetate was added in 0.1ml of ethanol/water (1:9, v/v) to give a final concentration of 100μM.

Enzyme modification

Ethanol/water (1:9, v/v) was used as the solvent for the addition of disulfiram; 0.1ml of this solvent without disulfiram was added to control assays. The constituents of the assay mixture in the presence of disulfiram were added in various orders; either disulfiram was added last to a pre-mixed enzyme assay, or the enzyme was treated with disulfiram and after 1min the substrates were added to initiate the enzyme reaction. In all cases the inactivatory effect of disulfiram was evident within the time of mixing, and the resultant rate of the enzyme-catalysed reaction was linear thereafter. [Other workers have also found the reaction between disulfiram and aldehyde dehydrogenase to be very rapid. Eckfeldt *et al.* (1976) state that it is complete within 1min, Hempel *et al.* (1980) within approx. 45s. In previous work (Dickinson *et al.*, 1981), we have found that the fully inhibited rate is established immediately after the burst.] 2,2′-Dithiodipyridine was dissolved in ethanol/water (1:9, v/v); 0.1ml of this solution was added to enzyme in 33mM-sodium phosphate buffer, pH7.4, either in the absence or in the presence of NAD^+ (1mM in the dehydrogenase assays, 100μM in the esterase assays). After 1min, addition of the remaining substrate(s) initiated the enzyme-catalysed reaction. The solvent used for 6,6′-dithionicotinic acid and 5,5′-dithiobis-(2-nitro-

benzoic acid) was 33mM-sodium phosphate buffer, pH7.4 (0.1ml).

Reaction of enzyme with 2,2'-dithiodipyridine

This was investigated at 25°C by following the production of 2-thiopyridone at 343nm by using a Cary 14 u.v. recording spectrophotometer. 2,2'-Dithiodipyridine at a final concentration of 4μM or 10μM was added to the enzyme (final concn. 1.94μM) in 33mM-sodium phosphate buffer, pH7.4, either with or without 1mM-NAD^+. An absorption coefficient for 2-thiopyridone of $7.4 \times 10^3 M^{-1} \cdot cm^{-1}$ was determined and used in calculating the progress of the reaction.

Determination of burst amplitude

This was done by using a recording filter fluorimeter of the type described by Dalziel (1962). Buffer (sodium phosphate, pH7.4, 33mM), NAD^+ (final concn. 1mM), enzyme and various amounts of disulfiram were mixed at 25°C, and the base-line fluorescence level was recorded. Acetaldehyde (final concn. 1mM) was then added as rapidly as possible as 10μl of an aqueous solution on a glass nail; the paper-feed of the chart recorder was started simultaneously with the addition of acetaldehyde. The recorded steady-state increase in fluorescence was projected backwards the few seconds to the time of mixing, giving an estimate of the amplitude of the burst of NADH production.

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

Reaction mixtures were made up as follows, with order of mixing as described in Table 2: 2.0ml of sodium phosphate buffer, pH7.4 (33mM), 50μl of enzyme solution (1μM), 50μl of 4-nitrophenyl acetate (0.5mM) in ethanol and 5μl of [1-^{14}C]-disulfiram (1μM) in ethanol. (Concentrations are final values after mixing of all components.) After $\frac{1}{2}$ or 3min, the reaction mixtures were extracted with 2ml of hexane by vortex-mixing for 15s, centrifuged at approx. 400*g* for 1min to separate the phases, and then 0.5ml of the hexane layer was added to 5ml of scintillation solvent for determination of radioactivity. [This solvent was made from 2,5-diphenyloxazole (1.75g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.06g) in toluene (350ml) and ethanol (150ml).]

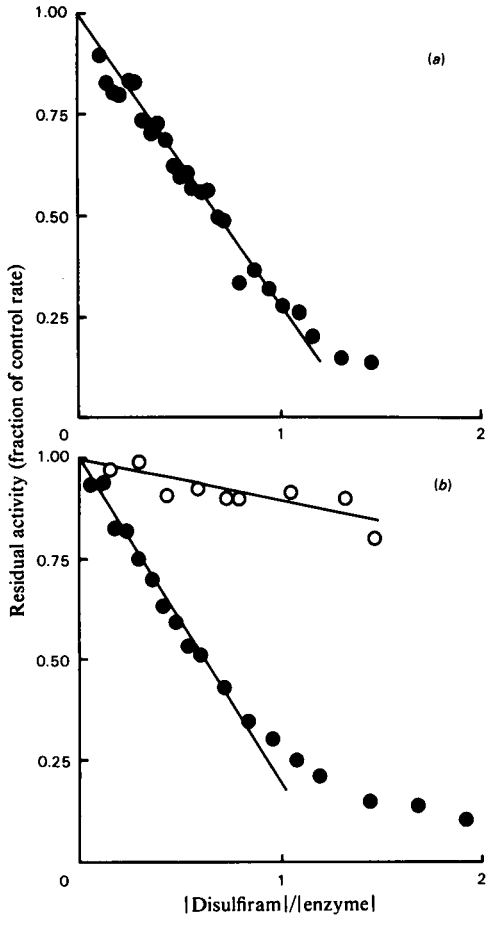


Fig. 1. *Disulfiram inactivation profile for cytoplasmic aldehyde dehydrogenase: comparison of the effects of disulfiram on the dehydrogenase and esterase activities of the enzyme*

The activity of the enzyme at pH7.4 (as a fraction of the control rate) is plotted against the ratio of the concentration of added disulfiram to enzyme concentration. The enzyme was pretreated with various amounts of disulfiram and then assayed as a dehydrogenase (*a*) or an esterase (*b*, ●). In (*b*) the ○ symbols record the effect of adding disulfiram to the enzyme already in the presence of its substrate, 4-nitrophenyl acetate. Enzyme concentration was 0.14μM (dehydrogenase experiment) or 0.8μM (esterase experiments).

Results

Figs. 1(*a*) and 1(*b*) depict the effect of disulfiram on the dehydrogenase and esterase activities respectively of cytoplasmic aldehyde dehydrogenase. When enzyme treated with disulfiram (in the absence of substrates) is subsequently assayed as a dehydrogenase or as an esterase, the results are clearly the same. In Fig. 1(*b*), however, it is seen that enzyme already in the presence of 4-nitrophenyl acetate is relatively inert to the inactivatory effect of disulfiram.

The titration profiles in Fig. 1 can be seen to tail off at higher disulfiram concentration. Even at 10μM-disulfiram (approx. 50-fold excess over the

enzyme concentration) the activity (dehydrogenase and esterase) is not completely decreased to zero. This is partly due to contamination with mitochondrial aldehyde dehydrogenase (Dickinson & Berrieman, 1979; Dickinson *et al.*, 1981). However, during the present work it was found that after the use of all available techniques to remove mitochondrial contamination [i.e. $(NH_4)_2SO_4$ precipitation (Dickinson & Berrieman, 1979), pH-gradient ion-exchange chromatography (Dickinson *et al.*, 1981), covalent chromatography on reduced thiopropyl-Sepharose 6B (Kitson, 1981*a*), and various repetitions and combinations of these methods] there always remained some (approx. 2.5%) residual disulfiram-insensitive activity.

Previous work (Kitson, 1978) showed that the effect of disulfiram on the dehydrogenase reaction is consistent with covalent modification of thiol groups, and not reversible competitive inhibition as had sometimes been claimed; the Lineweaver–Burk plot of Fig. 2 of the effect of disulfiram on the esterase activity supports the same conclusion. The value of K_m (10 μM) for 4-nitrophenyl acetate is not significantly altered by treating the enzyme with disulfiram.

Assays in which disulfiram was added to the enzyme either before or after the ester substrate were linear. This is reflected in the results of Expts. A and B of Table 1, where it is evident that 4-nitrophenyl acetate protects the enzyme almost completely against disulfiram for a period of at least 10 min. It was thought that this might mean that disulfiram, in circumstances where its usual reactive site on the enzyme is blocked, reacts instead with other enzymic thiol groups (of which there are plenty; MacGibbon *et al.*, 1979) without any consequent inactivation. Information on this point was sought by using ^{14}C-labelled disulfiram as described above and in Table 2. Expt. A in Table 2 is a control, which shows

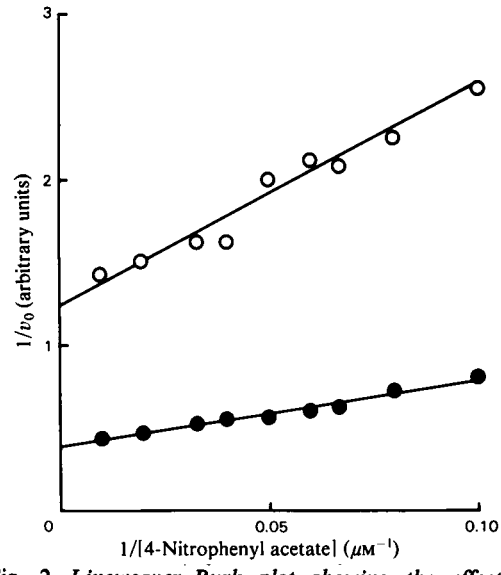


Fig. 2. *Lineweaver–Burk plot showing the effect of disulfiram on the esterase activity of cytoplasmic aldehyde dehydrogenase*

The activity of the enzyme was measured at various concentrations of 4-nitrophenyl acetate with the native enzyme (●) or with enzyme pre-modified with 0.8 μM-disulfiram (○), as described in the Experimental section. The enzyme concentration was 0.84 μM.

Table 1. *Effect of disulfiram on the esterase activity of cytoplasmic aldehyde dehydrogenase under various conditions and with various orders of mixing*

The enzyme activity was measured as described in the Experimental section after the reactants had been mixed in the order shown. The ester concentration was 100 μM-4-nitrophenyl acetate and the chloral hydrate concentration was 0.5 mM. In Expts. A and B the enzyme concentration was 0.17 μM; in the remainder of the experiments it was 0.15 μM. The numbers of values that were averaged to give the results shown are shown in parentheses.

Expt.	Order of mixing	Activity (% of control rate*)
A	Enzyme, disulfiram (0.1 μM), ester	
	Initial rate	60.3 (2)
	Rate after 10 min	59.6 (2)
B	Enzyme, ester, disulfiram (0.1 μM)	
	Initial rate	100 (2)
	Rate after 10 min	97.4 (2)
C	Enzyme, disulfiram (0.2 μM), ester, chloral hydrate	46.3 (3)
D	Enzyme, chloral hydrate, disulfiram (0.2 μM), ester	77.5 (4)
E	Enzyme, disulfiram (0.2 μM), ester	30.7 (2)
F	Enzyme, NADH (10 μM), disulfiram (0.2 μM), ester	23.2 (2)
G	Enzyme, NADH (100 μM), disulfiram (0.2 μM), ester	17.5 (2)

* For Expts. A, B and E the control rate was the true blank rate (i.e. in the absence of disulfiram and any other modifier). For Expts. C and D the control rate was that in the presence of 0.5 μM-chloral hydrate, and for Expts. F and G that in the presence of 10 μM- and 100 μM-NADH respectively.

Table 2. *Effect of 4-nitrophenyl acetate on the binding of [1-^{14}C]disulfiram to cytoplasmic aldehyde dehydrogenase*
Reaction mixtures were made up as described in the Experimental section, the order of mixing shown being used. The final concentrations after mixing were sodium phosphate buffer, pH 7.4 (33 mM), enzyme (1 μM), 4-nitrophenyl acetate (0.5 mM) and disulfiram (1 μM). After $\frac{1}{2}$min or 3 min the reaction mixtures were extracted with hexane, and the recovery of radioactivity in the organic phase is expressed as a percentage of that originally present in the reaction mixture ± S.E.M. (calculated from four to six separate determinations).

Expt.	Order of mixing	Recovery of radioactivity (%) $\frac{1}{2}$min	3 min
A	Buffer, disulfiram	98 ± 1.4	—
B	Buffer, disulfiram, dithiothreitol (1 mg), enzyme	5 ± 1.0	—
C	Buffer, enzyme, disulfiram	21 ± 1.5	—
D	Buffer, enzyme, ester, disulfiram	51 ± 3.6	31 ± 4.7

that the technique used was completely efficient in removing unreacted disulfiram. In control Expt. B, all the disulfiram should have been reduced to hexane-insoluble diethyldithiocarbamate by reaction with excess dithiothreitol; the small amount of recovered radioactivity in the organic fraction presumably represents radioactive impurities or traces of occluded aqueous fraction, or possibly the atmospheric re-oxidation of diethyldithiocarbamate to disulfiram during the extraction procedure. This result suggests that all subsequent data in Table 2 are slight overestimates, but it does not affect the broad conclusions that may be drawn. Expt. C indicates that the bulk of the disulfiram reacts rapidly with the enzyme, as expected. In the presence of 4-nitrophenyl acetate, however (Expt. D), the reaction with disulfiram is slowed but by no means stopped. Even in 30 s half the disulfiram has reacted, and in 3 min appreciably more, and yet, as already described above, in such circumstances very little inactivation of the enzyme is brought about. This result substantiates the conclusion that disulfiram reacts at alternative sites on the enzyme when its usual target is blocked by the presence of the ester substrate.

Fig. 3(*a*) shows that there is no discernible difference in the extent of inactivation when disulfiram is added to the enzyme already in the presence of NAD^+ and acetaldehyde or when the enzyme is pretreated with disulfiram and the substrates added later. The same applies when the bulkier substrate indol-3-ylacetaldehyde is used (Fig. 3*b*). Thus, although the substrate for the esterase reaction can protect the enzyme against disulfiram, the dehydrogenase substrates do not, at least when NAD^+ is present. On the other hand, Fig. 3(*b*) also shows that, when disulfiram is allowed to react with enzyme previously mixed with indol-3-ylacetaldehyde, with NAD^+ not being added until later, there is an appreciable degree of protection evident.

The ability of chloral hydrate to protect the enzyme against disulfiram was also investigated. Expts. C and D in Table 1 compare the effect of treating the enzyme with disulfiram in the absence and in the presence of chloral hydrate respectively. Since chloral hydrate is a competitive inhibitor of the esterase reaction, these experiments necessitated the measurement of rather low rates; however, the results were reproducible and show that chloral hydrate exerts a significant protective effect against disulfiram.

The final entries in Table 1 concern experiments designed to test whether NADH protects cytoplasmic aldehyde dehydrogenase against disulfiram. Adding disulfiram to enzyme in the presence of NADH (Expts. F and G) is clearly no less effective in bringing about inactivation than in the absence of NADH (Expt. E). In fact, with NADH present, the loss of activity is apparently greater, but this is probably due to inhibition by NADH of the residual activity after disulfiram modification. NADH at 100 μM lowered the control rate (no disulfiram) to 88% of the value without NADH.

The effect of disulfiram on the size of the burst in the dehydrogenase reaction was investigated. The results are recorded in Table 3, where it is evident that, broadly speaking, increasing amounts of disulfiram lower the amplitude of the burst of NADH fluorescence to much the same extent as they lower the steady-state rate of reaction.

It has been reported before (Kitson, 1979) that 2,2′-dithiodipyridine produces activation of sheep liver aldehyde dehydrogenase only in the presence of NAD^+. This point is demonstrated more fully by the results in Fig. 4. Adding increasing amounts of 2,2′-dithiodipyridine to the enzyme followed later by the addition of NAD^+ and acetaldehyde gives no significant loss or gain in enzymic activity. However, when 2,2′-dithiodipyridine is added to the enzyme in the presence of NAD^+, and then the enzyme-catalysed reaction is initiated by the addition of acetaldehyde, a substantial degree of activation results. By contrast, small amounts of

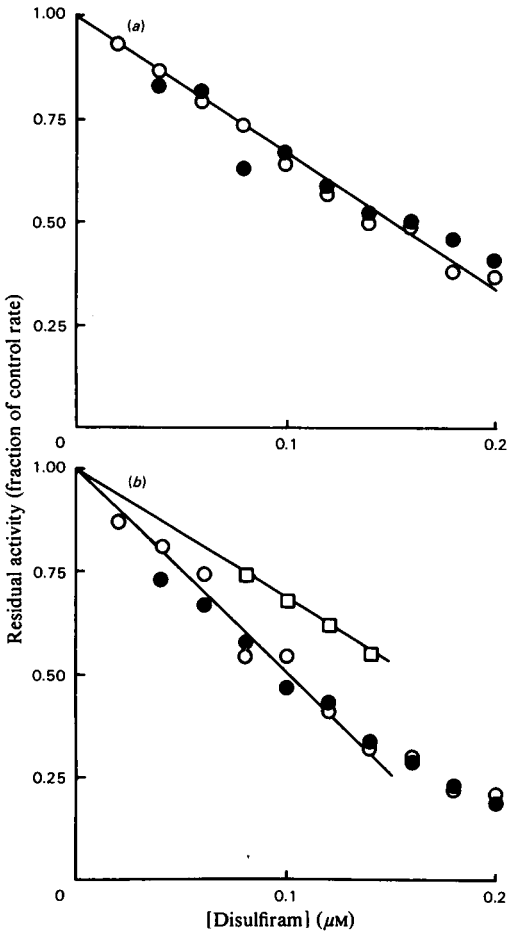


Fig. 3. *Effect of disulfiram on the dehydrogenase activity of cytoplasmic aldehyde dehydrogenase with different orders of mixing*

Enzyme was pretreated with disulfiram and then assayed with acetaldehyde (● in *a*) or with indol-3-ylacetaldehyde (● in *b*). Alternatively, disulfiram was added to the enzyme already in the presence of NAD^+ and acetaldehyde (○ in *a*) or NAD^+ and indol-3-ylacetaldehyde (○ in *b*). The □ symbols in (*b*) show the result of adding disulfiram to the enzyme in the presence of indol-3-ylacetaldehyde and then adding NAD^+ 3 min later. Concentrations were: acetaldehyde (1 mM), NAD^+ (1 mM), indol-3-ylacetaldehyde (100 μM) and enzyme [0.28 μM in (*a*) and 0.145 μM in (*b*)].

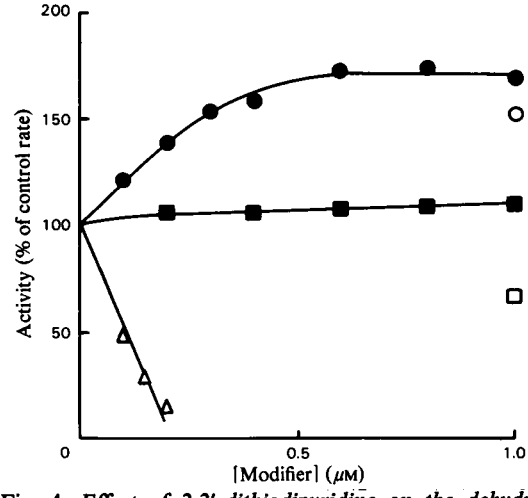


Fig. 4. *Effect of 2,2′-dithiodipyridine on the dehydrogenase activity of sheep liver cytoplasmic aldehyde dehydrogenase*

The activity of the enzyme at pH 7.4 (as a percentage of the control rate) is plotted against the concentration of added modifier. 2,2′-Dithiodipyridine was added to the enzyme, and then NAD^+ and acetaldehyde subsequently were added (■), or the modifier was added to the enzyme in the presence of 1 mM-NAD^+ and then acetaldehyde subsequently added (●). The inactivation of the enzyme by disulfiram is also shown as a contrast (△). The ○ point shows the effect of adding 0.2 μM-disulfiram to enzyme pretreated (in the presence of NAD^+) with 1.0 μM-2,2′-dithiodipyridine, and the □ point shows the effect of adding 0.2 μM-disulfiram to enzyme pretreated (in the absence of NAD^+) with 1.0 μM-2,2′-dithiodipyridine. The enzyme concentration was 0.125 μM.

disulfiram severely diminish the activity of the enzyme. Furthermore, Fig. 4 shows that enzyme pretreated with 2,2′-dithiodipyridine (particularly when this pretreatment is performed in the presence of NAD^+) is relatively resistant to the inactivatory effect of disulfiram.

The above result in the absence of NAD^+ means that under these conditions either 2,2′-dithiodipyridine and the enzyme do not react or that they do react but without much affecting the enzyme's activity. This point was examined by following the production of 2-thiopyridone when 2,2′-dithiodipyridine was added to the enzyme. With 1.94 μM enzyme and 4 μM modifier approx. 1 molecule of 2-thiopyridone per enzyme tetramer was released immediately (i.e. within the time of mixing) and all the 2,2′-dithiodipyridine reacted within approx. 3 min. With the same concentration of enzyme and 10 μM modifier, approx. 2 groups per enzyme tetramer reacted immediately and the reaction went to completion within approx. 5 min. The presence of 1 mM-NAD^+ had little if any effect on the rate of production of 2-thiopyridone. Obviously, 2,2′-dithiodipyridine does react readily with the enzyme in the absence of NAD^+, but in its presence the reaction must be somehow different such that activation of the enzyme is caused. (Whatever process is responsible for the activation is rapid, since the higher rate was evident as soon as an

Table 3. *Effect of disulfiram on the burst of NADH production catalysed by cytoplasmic aldehyde dehydrogenase*
The amplitude of the burst of NADH fluorescence after modification of the enzyme with various amounts of disulfiram was measured as described in the Experimental section. The results are compared with the decrease in the steady-state rate. The enzyme concentration was 0.17 μM. Each value is the average of seven to nine separate determinations; the extreme values are shown in parentheses.

Concn. of disulfiram (μM)	Burst amplitude (% of control value without disulfiram)	Steady-state rate (% of control value without disulfiram)
0.04	64 (77–52)	71 (75–68)
0.08	49 (62–43)	42 (43–41)
0.12	41 (51–35)	31 (32–30)
0.16	38 (42–35)	24 (25–23)
0.20	29 (34–22)	18 (19–17)

Table 4. *Effect of diethyldithiocarbamate on 2,2'-dithiodipyridine-treated cytoplasmic aldehyde dehydrogenase*
Native enzyme or enzyme pretreated with 2,2'-dithiodipyridine in the presence of 1 mM-NAD^+ was treated with sodium diethyldithiocarbamate or disulfiram. The activity was measured immediately, or after standing for up to 30 min, and is expressed as a percentage of the appropriate blank (for either the native or the modified enzyme). The enzyme concentration was 0.156 μM. Results are the averages of two separate determinations.

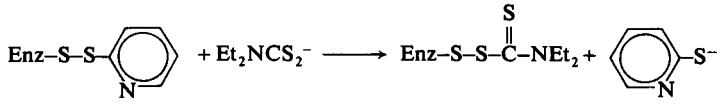
Expt.	Concn. of 2,2'-dithiodipyridine (μM)	Concn. of diethyldithiocarbamate (μM)	Time (min)	Activity (% of control)
A	1	100	0	98.4
	1	100	3	75.6
	1	100	30	8.5
B	0	100	0	56.5
	0	100	30	22.3
C*	0	100	0	91.4
	0	100	30	68.7
		Concn. of disulfiram (μM)		
D	0	0.2	0	14.5
E	1	0.2	0	81.0
	1	0.2	30	68.1

* The reaction mixtures in this experiment also contained 10 μM-dithiothreitol.

enzyme assay could be followed; the rate then remained constant during the assay. However, the enzyme and modifier were as a routine left to stand for 1 min before the enzyme-catalysed reaction was initiated.)

The simplest explanation for the protection against disulfiram afforded by 2,2'-dithiodipyridine is if both modifiers react with the same enzymic thiol group. If this is so then it might be possible for the diethyldithiocarbamate ion to displace 2-thiopyridone from the 2,2'-dithiodipyridine-modified enzyme, giving rise to essentially the same enzyme species as that produced when disulfiram reacts with the enzyme, and effectively turning activated enzyme into inactivated enzyme (see eqn. 1).

The results of experiments designed to test this possibility are presented in Table 4. The most striking result here is that treating 2,2'-dithiodipyridine-modified enzyme with 100 μM-sodium diethyldithiocarbamate does indeed lead, within 30 min, to loss of most of the enzyme's activity (Expt. A). However, native enzyme also loses activity when treated with diethyldithiocarbamate (Expt. B). This is explained as follows. Only a small amount of disulfiram contamination of the 100 μM-diethyldithiocarbamate solution used would account for the initial decrease in activity in Expt. B (considerably less than 0.2 μM-disulfiram, as shown by Expt. D), and over 30 min atmospheric oxidation of diethyldithiocarbamate to disulfiram would ac-



(1)

count for the further fall in activity. This suggestion is supported by the fact that 10 μM-dithiothreitol, which of course would inhibit the oxidation of diethyldithiocarbamate, cuts down the extent of loss of activity of the native enzyme in the presence of 100 μM-diethyldithiocarbamate (Expt. C). [It is perhaps surprising that there should be any loss of activity under these conditions, but, as reported by Kitson (1981*b*), cytoplasmic aldehyde dehydrogenase can compete very well for traces of disulfiram against high concentrations of dithiothreitol or glutathione.] Adding disulfiram to 2,2'-dithiodipyridine-treated enzyme results in slight inactivation (Expt. E, and as found before, see Fig. 1), but over 30 min not much further loss of activity is observed. Thus, although with the native enzyme the loss of activity in the presence of 100 μM-diethyldithiocarbamate can be explained by contamination with disulfiram, the same explanation cannot account for the diethyldithiocarbamate-caused inactivation of 2,2'-dithiodipyridine-modified enzyme, and instead the results are most simply explained in terms of the reaction in eqn. (1). (This point is discussed again below.)

Pre-modification of cytoplasmic aldehyde dehydrogenase with disulfiram decreases the dehydrogenase and esterase activities of the enzyme in tandem (see above). However, Fig. 5 shows that modification with increasing amounts of 2,2'-dithiodipyridine (with or without NAD^+) results in gradual loss of the esterase activity; unlike the dehydrogenase reaction, no activation is observed. Since the rate-determining step of the dehydrogenase reaction (MacGibbon *et al.*, 1977*a*) is thought to be dissociation of the enzyme–NADH complex (a process not involved in the esterase reaction), it is not necessarily surprising that 2,2'-dithiodipyridine-modification should affect the two activities differently.

During this work it was found that the 2,2'-dithiodipyridine-modified enzyme loses activity with time. This point is demonstrated by the results in Fig. 6. Over a period of 5 days at 4°C the native enzyme is relatively stable. (The reason for the slight apparent rise in activity during the first two days is not known.) However, 2,2'-dithiodipyridine-treated enzyme loses activity comparatively rapidly; re-plotting the data as a first-order decay process gives a half-life of 0.83 day. (Unless otherwise stated all results with the modified enzyme quoted in this paper were obtained within a few minutes of the modification reaction.)

The effect of some other thiol-group-modifying

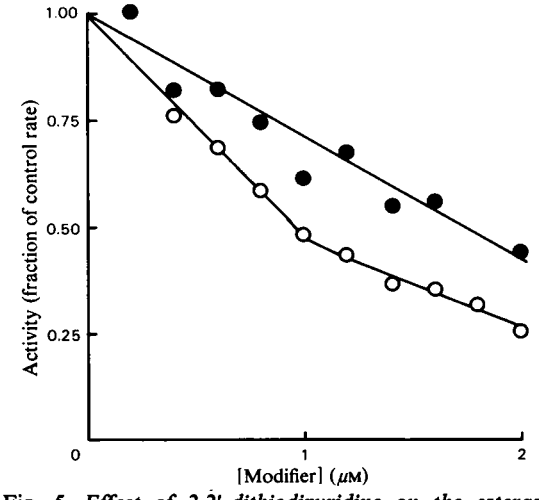


Fig. 5. *Effect of 2,2'-dithiodipyridine on the esterase activity of sheep liver cytoplasmic aldehyde dehydrogenase*

The activity of the enzyme with 100 μM-4-nitrophenyl acetate as substrate is plotted (as a fraction of the control rate) against the concentration of added modifier. The enzyme was treated with modifier either in the presence (○) or in the absence (●) of 100 μM-NAD^+ before 4-nitrophenyl acetate was added. The enzyme concentration was 0.16 μM.

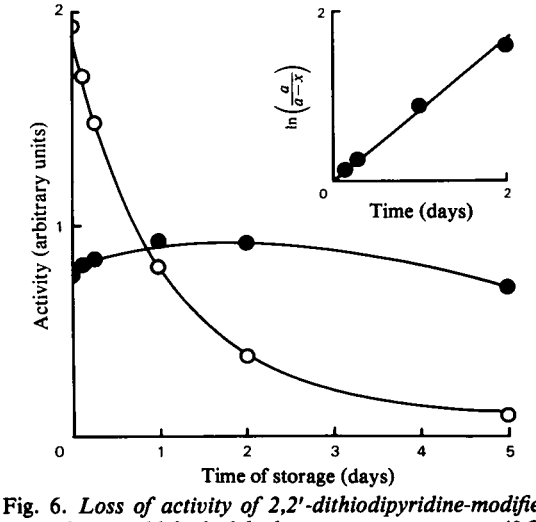


Fig. 6. *Loss of activity of 2,2'-dithiodipyridine-modified cytoplasmic aldehyde dehydrogenase on storage at 4°C*

The enzyme (31 μM) was treated with 2,2'-dithiodipyridine (150 μM) in the presence of NAD^+ (1 mM) and stored at 4°C. Samples were taken for assay at pH 7.4 (with 1 mM-NAD^+ and 1 mM-acetaldehyde) at intervals over a period of 5 days, giving the results shown as ○. Unmodified enzyme treated in the same way gave the results shown as ●. The inset shows the result of re-plotting the data for the modified enzyme as a first-order decay process, where a is the initial activity at time zero, and $a-x$ is the activity at time t.

Table 5. *Effects of 5,5'-dithiobis-(2-nitrobenzoic acid) and 6,6'-dithionicotinic acid on the activity of cytoplasmic aldehyde dehydrogenase and on the inactivation of the enzyme by disulfiram*

The enzyme (0.19 μM) was incubated for various times with the modifiers (1 μM) either in the presence or in the absence of NAD^+ (1 mM) and then assayed as described in the Experimental section. The subsequent loss of activity on adding disulfiram (0.2 μM), 1 min after starting the assay, is also recorded. Results are the averages of two determinations.

Modifier	Time of incubation (min)	Activity (% of control)	Loss of activity on adding disulfiram (%)
5,5'-Dithiobis-(2-nitrobenzoic acid), NAD^+ present	5	98	96
	10	75	95
5,5'-Dithiobis-(2-nitrobenzoic acid), NAD^+ absent	10	75	94
6,6'-Dithionicotinic acid, NAD^+ present	10	83	92
6,6'-Dithionicotinic acid, NAD^+ absent	10	87	94
No modifier	—	(100)	92

agents was examined. Table 5 shows that 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and 6,6'-dithionicotinic acid (which has the same structure as 2,2'-dithiodipyridine except for the presence of two carboxyl groups) both cause a small amount of inactivation of cytoplasmic aldehyde dehydrogenase (regardless of the presence of NAD^+). Neither gives any activation or protection against disulfiram.

Discussion

The effect of disulfiram on the esterase activity of cytoplasmic aldehyde dehydrogenase has been shown to depend on the order of mixing of enzyme, substrate and modifier (Fig. 1). The presence of 4-nitrophenyl acetate protects the enzyme against disulfiram, but pretreatment of the enzyme with disulfiram results in essentially the same extent of inactivation of the esterase activity as that observed when the enzyme is assayed as a dehydrogenase. In previous work (Kitson, 1978) the critical importance of this order of mixing was not appreciated, and hence the results were interpreted as possibly suggesting that esterase and dehydrogenase activities occur at different sites on the enzyme. The present study removes this misapprehension, and the results are now in full agreement with those obtained by Duncan (1979), who showed that iodoacetamide affects the two activities of rabbit liver aldehyde dehydrogenase to the same extent, and that the enzyme catalyses the reaction between 4-nitrophenyl acetate and NADH to give acetaldehyde and NAD^+, and who therefore concluded that ester hydrolysis occurs at the usual active site of the enzyme.

The results detailed above show that under various circumstances cytoplasmic aldehyde dehydrogenase is protected to a greater or smaller degree against the inactivatory action of disulfiram. The presence of indol-3-ylacetaldehyde (in the absence of NAD^+), chloral hydrate (which is also a competitive inhibitor of both esterase and dehydrogenase activities) or particularly 4-nitrophenyl acetate will all diminish the extent to which disulfiram inactivates the enzyme. In the presence of NAD^+, 4-nitrophenyl acetate is less effective; this was shown to be because a higher concentration of the ester is needed to saturate the enzyme under these conditions. It is clear from these results that disulfiram reacts with the enzyme at or near to its active site.

If 4-nitrophenyl acetate and disulfiram compete for the same site on the enzyme, then it might be expected that adding disulfiram to the enzyme in the presence of the ester would result in a gradual progressive loss of activity. That is, the substrate might slow the inactivation, but not stop it completely. This is not the case; as shown above, the protection by the ester is apparently long-lasting. Experiments involving radioactively labelled disulfiram show that this is because under these circumstances the disulfiram does react quite rapidly with the enzyme, but presumably by modifying thiol groups other than the usual ones, and thus without any significant effect on the activity. [Cytoplasmic aldehyde dehydrogenase of sheep liver contains 36 thiol groups per (tetrameric) molecule; approx. 20 of these are accessible to Ellman's reagent in the native enzyme (MacGibbon *et al.*, 1979).]

4-Nitrophenyl acetate protects the enzyme against disulfiram, but the substrates for the dehydrogenase reaction are much less effective. Thus indol-3-ylacetaldehyde in the absence of NAD^+ significantly decreases the extent of inactivation (see Fig. 3*b*), but when the enzyme is actually functioning as a dehydrogenase (i.e. in the presence of indol-3-ylacetaldehyde and NAD^+, or acetaldehyde and NAD^+) the addition of disulfiram results in very rapid inactivation (within the time of mixing) to the same extent as when disulfiram is added to enzyme in the absence of its substrates. On the surface, it seems very surprising that high concentrations of aldehyde (100 μM–1 mM, many times higher than

their Michaelis constants) are not more effective in blocking the active site to the action of a low concentration (typically considerably less than 1 μM) of disulfiram. It is proposed that the solution to this difficulty is as follows. When the enzyme is acting as an esterase, then in the presence of saturating concentrations of 4-nitrophenyl acetate the enzyme is always either in the form of the enzyme–substrate complex or the putative acyl-enzyme (see below), and thus the active site is very effectively shielded against disulfiram. During the dehydrogenase reaction, however, a considerable fraction of the enzyme is in the form of the enzyme–NADH complex, since the dissociation of this complex, the last step of the pathway, is the rate-determining step of the enzyme-catalysed reaction (MacGibbon *et al.*, 1977*a*). There is apparently no evidence for the existence of an enzyme–NADH–aldehyde complex (MacGibbon *et al.*, 1977*b*), and, as shown by the present experiments, disulfiram rapidly inactivates the enzyme in the presence of NADH. Presumably therefore it is reaction between disulfiram and the enzyme–NADH complex that occurs when disulfiram is added to the enzyme functioning as a dehydrogenase.

The effect of modification of the enzyme by disulfiram on the amplitude of the burst observed in the dehydrogenase reaction was examined. The burst of NADH formation is taken as showing that the rate-determining step of the enzyme-catalysed reaction is dissociation of NADH from the enzyme–NADH complex, as mentioned above. One way in which a modifier could affect the enzyme activity is by changing the rate of this process. Were disulfiram to act by rendering this process much slower, then the steady-state rate would be lowered without any effect on the size of the burst. However, the results in Table 3 show that, although there is appreciable experimental error in measuring the burst size by the method used, broadly speaking increasing amounts of disulfiram lower the steady-state rate and the burst amplitude in tandem. The same observation has been made by other workers using stopped-flow techniques (L. F. Blackwell, personal communication).

All the data discussed so far are consistent with ester hydrolysis, aldehyde oxidation and modification by disulfiram occurring at the same site on the enzyme. Moreover, the form of Fig. 2 (i.e. the lack of significant change in K_m for 4-nitrophenyl acetate on enzyme modification) suggests that treatment with a limited amount of disulfiram results in a mixed population of native enzyme and inactive enzyme. The simplest explanation of these results is that the thiol groups that disulfiram modifies are catalytically essential in the reactions mediated by the enzyme. Indeed, the inactivation of aldehyde dehydrogenase by thiol-modifying reagents such as disulfiram and iodoacetamide (Hempel & Pietruszko, 1980) is one of the main pieces of evidence on which a mechanism for the enzyme-catalysed reaction has been proposed (see Li, 1977). This envisages attack of the essential thiolate ion on the carbonyl group of the aldehyde to give a thiohemiacetal, from which is transferred a hydride ion (to NAD^+) in the oxidation–reduction step. The resultant acyl-enzyme (which can also be produced by attack of the enzymic thiolate group on 4-nitrophenyl acetate with the displacement of 4-nitrophenoxide) is then hydrolysed to give the acid product, and lastly NADH is released. The results certainly do not prove, however, that the disulfiram-sensitive groups are catalytically essential in this way, and the following points are less easy to reconcile with this notion.

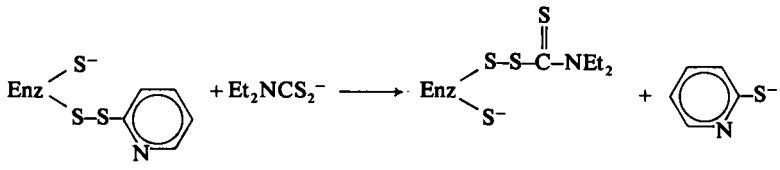
(1) Excess of disulfiram does not abolish all the activity. This may indicate that modification of the enzyme by disulfiram results in loss of activity by some indirect mechanism, such as the steric blocking of access of the substrates to the active site. This has been shown to be the case, for example, in the action of several thiol-group-modifying agents on aspartate aminotransferase (Birchmeier *et al.*, 1973) and on an isocitrate dehydrogenase (Chung *et al.*, 1971). However, the results are also consistent with the cytoplasmic aldehyde dehydrogenase samples used in the present work containing a small amount of a disulfiram-insensitive isoenzyme that tenaciously follows the main enzyme through all the separatory methods employed (see above). Alternatively, it is possible that cytoplasmic aldehyde dehydrogenase displays 'internal heterogeneity', i.e. the four apparently equal-sized subunits of which the enzyme molecule is composed (MacGibbon *et al.*, 1979) may be of two types: I, which is responsible for most of the enzymic activity and which is completely inactivated by disulfiram by modification of its essential groups, and II, which is not sensitive to disulfiram, and which has a very small though finite activity. It would be modification of the two subunits I that is reflected in the observed stoicheiometry of the disulfiram reaction, i.e. between 1 and 2 molecules of disulfiram remove most of the activity of the enzyme molecule, and not 4 as might be expected from the tetrameric structure of the enzyme (Dickinson *et al.*, 1981). [A somewhat similar proposal has been made for the mitochondrial aldehyde dehydrogenase of horse liver; this evidently has only two functioning active sites in the tetrameric form (Takahashi *et al.*, 1980).]

(2) The protection against disulfiram afforded the enzyme by 2,2′-dithiodipyridine is most simply explained by the two modifiers reacting with the same enzymic thiol groups. It has been shown (Kitson, 1981*b*) that the enzymic groups that disulfiram modifies are very reactive, and it would

seem surprising if such reactive groups did not also react rapidly with 2,2′-dithiodipyridine. The two modifiers are not of greatly differing size, and in any case 2,2′-dithiodipyridine is the smaller of the two, so there should be no steric reason why it could not react with the disulfiram-sensitive groups. Furthermore, the two modifiers are of similar reactivity, at least in the sense that there is little difference in their reduction potentials (Bishop *et al.*, 1981).

If we conclude that disulfiram and 2,2′-dithiodipyridine react with the same groups, then such groups are precluded from a direct involvement in the enzyme's catalytic mechanism, otherwise any chemical modification would lead to inactivation of the enzyme. However, the argument above that it would be surprising if disulfiram and 2,2′-dithiodipyridine did not react with the same groups is perhaps superficial. After all, it is no more surprising than the idea of the two modifiers reacting with the same enzymic groups and yet with completely different effects on the enzymic activity, and one or other of these situations must apply. Moreover, Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] reacts with cytoplasmic aldehyde dehydrogenase (MacGibbon *et al.*, 1979), but, as shown above, it causes little inactivation and affords no protection against disulfiram. The same applies to 6,6′-dithionicotinic acid, which, like Ellman's reagent, is negatively charged at pH 7.4. Thus, notwithstanding the enhanced reactivity of the disulfiram-sensitive groups, they certainly do not react with certain thiol-modifying reagents, and so we need not necessarily expect them to react with 2,2′-dithiodipyridine either. If they do not, then the protection against disulfiram afforded the enzyme by modification with 2,2′-dithiodipyridine would be through some indirect mechanism, such as by sterically interfering with the access of disulfiram to its usual target, or perhaps through alteration in the reactivity of the disulfiram-sensitive groups by a change in the enzyme's three-dimensional structure.

(3) The inactivation of 2,2′-dithiodipyridine-treated enzyme by incubation with sodium diethyldithiocarbamate argues most simply for the operation of the reaction shown in eqn. (1) and thus for the identity of the groups that react with 2,2′-dithiodipyridine and disulfiram. To preserve the idea of the disulfiram-sensitive groups being catalytically essential would require an alternative explanation of the data. For example, eqn. (2) postulates the oxidative coupling of a diethyldithiocarbamate ion to an essential thiol group, brought about by a neighbouring disulphide moiety (not involving an essential group), which was produced in the 2,2′-dithiodipyridine-modification reaction:



(2)

Likewise it is quite possible that, during the slow fall in activity of 2,2′-dithiodipyridine-treated enzyme (see Fig. 6), the 2-thiopyridine moiety is being transferred from its original non-essential thiol group to a neighbouring catalytically essential group, or that enzymic disulphide bonds (involving essential groups) are being formed with the release of 2-thiopyridone.

The completely unequivocal identification of the disulfiram-sensitive groups as catalytically essential could only come through detailed knowledge of the primary and tertiary structure of the enzyme gained from sequencing and X-ray-crystallography studies. Such work is in its infancy as regards aldehyde dehydrogenase. The sequence around the cysteine residue in human aldehyde dehydrogenase that reacts with iodoacetamide is being investigated (Hempel & Pietruszko, 1980). Recently a peptide sequence with a reactive cysteine residue close to the coenzyme-binding site of the horse liver enzyme was characterized (von Bahr-Lindström *et al.*, 1981). However, in neither of these cases is it established if the cysteine residue is the disulfiram-sensitive one or if it is involved in acyl-enzyme formation during catalysis.

From the results presented in the present paper it would seem that the mechanistic proposal for cytoplasmic aldehyde dehydrogenase involving a catalytic thiol group that is modified by disulfiram is a reasonable working hypothesis. However, the reservations to this idea discussed above should be borne in mind by workers with this enzyme. Recently it was stated that 'this suggestion [of the mechanism] is supported by the fact that aldehyde dehydrogenase has ... ... a reactive cysteine residue susceptible to sulfhydryl reagents' (von Bahr-Lindström *et al.*, 1981). The present work shows that the nature of the enzyme is not as clear-cut as this assertion would suggest.

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