The inactivation of aldehyde dehydrogenase by disulfiram in the presence of glutathione

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It is shown that *in vitro* glutathione provides little protection of cytoplasmic aldehyde dehydrogenase against the inactivatory action of disulfiram. This observation provides support for the current explanation of how disulfiram acts *in vivo*. The results show that the disulfiram-sensitive thiol groups of aldehyde dehydrogenase have an unusually high reactivity; possible mechanisms by which this might arise are discussed.

The 'disulfiram-ethanol reaction' is the unpleasant consequence of drinking alcohol after having taken the drug disulfiram (Kitson, 1977). The causes of the various symptoms are not completely understood, but the main factor is the inactivation of hepatic aldehyde dehydrogenase and the subsequent accumulation of acetaldehyde in the bloodstream.

The action of disulfiram on aldehyde dehydrogenase *in vitro* has been shown to be consistent with a covalent modification of enzymic thiol groups by a disulphide-interchange mechanism (Kitson, 1975, 1978). The occurrence of the same reaction *in vivo* is somewhat less well established, however, since upon absorption into the bloodstream disulfiram is rapidly and virtually quantitatively reduced to diethyldithiocarbamate by reaction with glutathione (Cobby *et al.*, 1977). Diethyldithiocarbamate itself has little direct action on aldehyde dehydrogenase (Kitson, 1975), but administration of diethyldithiocarbamate to rats causes inactivation of the enzyme to a similar extent as does disulfiram (Deitrich & Erwin, 1971). In order to explain this inactivation of the enzyme *in vivo*, and consequently to help understand the disulfiram-ethanol reaction, it became necessary to suppose (Kitson, 1977) that diethyldithiocarbamate is capable of being oxidized back to the active species, disulfiram, which then modifies hepatic aldehyde dehydrogenase in the same way as it does *in vitro*. Several pathways exist for the re-oxidation of diethyldithiocarbamate; for example, methaemoglobin (Stromme, 1963), xanthine oxidase (Fried, 1976) and cytochrome *c* (DuBois *et al.*, 1961) can all bring about the reaction. Nevertheless, any molecule of disulfiram which survives its journey to the liver, or is produced there by re-oxidation of diethyldithiocarbamate, finds itself in a reducing environment, rich in glutathione. Inactivation of

aldehyde dehydrogenase could therefore only occur if the rate of reaction of the enzyme with disulfiram is considerably greater than the rate at which disulfiram can be destroyed by glutathione. Clearly it is necessary to try to substantiate this suggestion, since if it is false and glutathione does provide efficient protection of the enzyme against disulfiram, then the above explanation of how disulfiram acts *in vivo* could no longer apply. This argument provided the rationale for the experiments described below.

Materials and methods

Glutathione, dithiothreitol and disulfiram were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Cytoplasmic aldehyde dehydrogenase was prepared from sheep liver essentially as described by Crow *et al.* (1974), and before use was thoroughly dialysed to remove 2-mercaptoethanol or dithiothreitol. The enzyme was assayed fluorimetrically as described by Hart & Dickinson (1977). A typical reaction mixture for assay was made up as follows (concentrations are the final values after mixing all constituents): 0.2 ml of NAD⁺ in water (1 mM), 0.2 ml of acetaldehyde in water (1 mM), 0.1 ml of glutathione in water, 10 μ l of aldehyde dehydrogenase (0.1–0.2 μ M), 20 μ l of disulfiram in ethanol (0.2 μ M), and sodium phosphate buffer (33 mM), pH 7.4, to a total volume of 4 ml. The order of mixing was varied as described in the text. In similar mixtures at pH 9.9, disulfiram was added immediately after the glutathione, since at high pH glutathione is oxidized on standing in the presence of air.

Results and discussion

Previous work (Kitson, 1978) has shown that *in vitro* disulfiram reacts with cytoplasmic aldehyde dehydrogenase in a rapid, covalent and essentially

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irreversible process with a consequent loss of enzymic activity, according to eqn. (1):

Disulfiram is also reduced by low-molecular-weight thiols such as 2-mercaptoethanol or glutathione (Johnston, 1953; Deitrich & Hellerman, 1963; Kitson, 1975) according to eqn. (2). [This is analogous to the well-known reaction between thiols and 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent).]

Again this reaction goes virtually to completion, since diethyldithiocarbamate is a much better leaving group than the thiolate anion. [The pK_a of diethyldithiocarbamic acid is 4.04 (Thorn & Ludwig, 1962), whereas that of the thiol group in glutathione is 8.72 (Reuben & Bruice, 1976).]

If disulfiram is added, therefore, to a mixture of aldehyde dehydrogenase and glutathione, then the two reactions above will compete with each other. The outcome will depend on the relative rates of the reactions and on the concentrations of enzyme and glutathione. One can envisage two extremes (either that the disulfiram reacts predominantly with enzyme, with a concomitant large loss of enzymic activity, or predominantly with glutathione, without much affecting the enzyme) or some case intermediate between the extremes.

Fig. 1 shows the result of titrating cytoplasmic aldehyde dehydrogenase (in the absence of other thiols) with increasing amounts of disulfiram. There is clearly a linear relationship between amount of disulfiram added and remaining enzymic activity. This means that in the experiments discussed below we can deduce how much disulfiram has reacted with the enzyme (and thus how much has been destroyed by glutathione) by noting the amount of enzymic activity remaining.

Fig. 2 shows the results of experiments in which the effect of disulfiram on cytoplasmic aldehyde dehydrogenase was ascertained in the presence of relatively high concentrations of competing thiol. The disulfiram was added to the enzyme either in the presence of buffer, glutathione, NAD⁺ and acet-aldehyde, or in the presence only of buffer and glutathione, with the substrates added subsequently. The extent of inactivation of the enzyme was reached within the time of mixing and the residual activity then remained constant over the period of assay. It is evident that under these conditions

Fig. 1. Titration of cytoplasmic aldehyde dehydrogenase with disulfiram

Enzyme activity was measured as described in the Materials and methods section at pH 7.4 and is plotted against the molar ratio of the concentration of added disulfiram to the enzyme concentration (which was $0.14 \mu\text{M}$).

Fig. 2. Protective effect of glutathione against the inactivation of cytoplasmic aldehyde dehydrogenase by disulfiram at pH 7.4

The remaining activity of the enzyme [$0.185 \mu\text{M}$, expressed in terms of the tetrameric species of mol.wt. 212000 (MacGibbon *et al.*, 1979)] was measured after the addition of disulfiram ($0.2 \mu\text{M}$). ●, Disulfiram was added to the enzyme in the presence of its substrates and the concentration of glutathione shown; ○, disulfiram was added to the enzyme in the presence of the concentration of glutathione shown, and the enzyme's substrates were then added; ■, disulfiram was added to glutathione 1 min before the enzyme and its substrates were added.

glutathione provides only limited protection against the inactivatory effect of disulfiram. For instance, in Fig. 2 we can note the point at which the extent of inactivation of the enzyme is half as much as that observed in the absence of glutathione. (Here, as explained above, half the added disulfiram has reacted with aldehyde dehydrogenase and half with glutathione.) This shows that the concentration of glutathione required to compete equally with 0.185 μM -aldehyde dehydrogenase is approx. 40 μM in the absence of NAD^+ and 100 μM in its presence. In other words, at physiological pH, even a 200-fold excess (without NAD^+) or a 500-fold excess (with NAD^+) of glutathione over enzyme decreases the inactivatory effect of disulfiram by only 50%. Alternatively one could say that with equal concentrations of enzyme and glutathione the reaction in eqn. (1) is clearly very much faster than that of eqn. (2).

(The results in Fig. 2 confirm that glutathione largely or completely eliminates the inactivatory effect of disulfiram if glutathione and modifier are mixed before the enzyme is added.)

The stimulatory effect of NAD^+ on the reaction of cytoplasmic aldehyde dehydrogenase with disulfiram is not surprising. There is evidence, for example, that NAD^+ induces a conformational change in the enzyme and that the aldehyde substrate only binds well to the enzyme after this change (MacGibbon *et al.*, 1977). Likewise, the esterase activity of the enzyme is accelerated by NAD^+ in moderate concentration (MacGibbon *et al.*, 1978). Glyceraldehyde 3-phosphate dehydrogenase behaves similarly in that NAD^+ promotes the rate of alkylation of a critical thiol group by iodoacetate (Harris & Waters, 1976).

As discussed in the introduction, the inactivation of aldehyde dehydrogenase by disulfiram *in vivo* argues for an unusually high rate of reaction between this enzyme and the modifier. Were this not so, the high glutathione concentration in the liver presumably would completely protect the enzyme against inactivation; on the other hand, if all enzymic thiol groups were equally reactive to disulfiram, then the drug might be expected to have a much more devastating effect on an individual's metabolic processes than is in fact the case. The high reactivity of cytoplasmic aldehyde dehydrogenase to disulfiram reported here is therefore not unexpected; nevertheless, the experiments do provide the first direct demonstration of the relative insensitivity to glutathione of disulfiram modification of aldehyde dehydrogenase and give a semi-quantitative estimate of the enhanced reactivity of the enzymic thiol groups. The normal concentration of glutathione in the liver of various animals is approx. 4 mM (Boyland & Chasseaud, 1969); MacGibbon (1976) has estimated the concentration of cytoplasmic aldehyde

dehydrogenase in sheep liver as 70 μM . There is thus *in vivo* an approximately 60-fold excess of glutathione over enzyme, and therefore (from the results *in vitro* discussed above) disulfiram should readily be able to cause a marked inactivation of the enzyme under these conditions. There is a close similarity between the enzymes from sheep and from humans (with respect to subcellular distribution, sensitivity to disulfiram etc.; Greenfield & Pietruszko, 1977), and thus this conclusion probably also applies to the alcoholic patient on disulfiram therapy. The description given in the introduction of how disulfiram acts on aldehyde dehydrogenase *in vivo* is now more firmly based.

What factor augments the reactivity of the disulfiram-sensitive thiol groups of aldehyde dehydrogenase compared with a simple thiol such as glutathione? One possibility would be if the pK_a of the enzymic thiol groups is much lower than that of glutathione, such that at pH 7.4 a larger proportion of the enzymic groups is in the (more nucleophilic) form of $-\text{S}^-$ rather than $-\text{SH}$. Such an idea is widely held to explain the pronounced reactivity of thiol groups of several other enzymes; for example, in glyceraldehyde 3-phosphate dehydrogenase (Harris & Waters, 1976) and in papain and thiolsubtilisin (Polgar, 1974) enzymic thiolate anions are thought to be stabilized in that form by ion-pair interaction with neighbouring imidazolium (histidinium) groups. This proposal is apparently not sufficient in the present case, however, since at pH 9.9, where glutathione itself is predominantly ionized [the pK_a according to Reuben & Bruce (1976) being 8.72], the thiol still does not protect the enzyme very efficiently against disulfiram (see Fig. 3).

(In Fig. 3, dithiothreitol apparently competes with the enzyme for disulfiram better than does glutathione, but when consideration is given to the fact that dithiothreitol has two thiol groups per molecule, this difference largely disappears. That is, the effect of, say, 25 mM-dithiothreitol is not much different from that of 50 mM-glutathione, to which it is equivalent in terms of concentration of thiol groups. Thus the enhanced reactivity of the enzymic thiol groups over low-molecular-weight thiols appears general.)

An alternative factor which may be important in promoting the reactivity of the thiol groups under discussion is their extent of solvation. According to Noller (1965) 'solvation of the nucleophile is of paramount importance' in displacement reactions. A relatively hydrophobic region of the enzyme molecule would give an environment in which the degree of solvation of a thiol group would be less than in the bulk aqueous solvent (surrounding, for example, the glutathione molecule), and consequently the enzymic group would be considerably more nucleophilic. In a similar way a non-polar environment is thought to increase the nucleo-

Fig. 3. Protective effect of glutathione and dithiothreitol against the inactivation of cytoplasmic aldehyde dehydrogenase by disulfiram at pH 9.9

The remaining activity of the enzyme ($0.17 \mu\text{M}$) was measured after the addition of disulfiram ($0.2 \mu\text{M}$). Disulfiram was added to the enzyme in the presence of its substrates and the various concentrations shown of glutathione (●) or dithiothreitol (○).

philicity of the essential carboxylate anion in the mechanism of action of carboxypeptidase A (Roberts & Caserio, 1977).

It is conceivable that both the reaction of the enzymic thiol groups with disulfiram and their attack on the aldehyde substrate during the enzymic reaction could be catalysed by an acidic group on the enzyme. This would, in the first reaction, protonate the incipient leaving group (diethyldithiocarbamate), and in the second, protonate the aldehydic carbonyl group, rendering it more susceptible to nucleophilic attack by the thiol group. However, at present there is no direct evidence for the participation of any such acidic group in the reactions of aldehyde dehydrogenase.

Implicit in most of the above discussion has been the assumption that the disulfiram-sensitive thiol groups are identical with those that are thought to be directly involved in the enzyme's catalytic mechanism (Li, 1977). On this basis, of course, the high reactivity of the disulfiram-sensitive groups observed here is readily understandable. However, it should be pointed out that there are several observations which argue against this identity (and are discussed in the references indicated). Thus (1) highly purified cytoplasmic aldehyde dehydrogenase still retains a small activity in the presence of a great excess of disulfiram (Dickinson *et al.*, 1981), (2) the enzyme is not protected against disulfiram by its substrates (Sanny & Weiner, 1979) (a conclusion also borne out by the results discussed above), and

(3) another thiol-modifying agent, 2,2'-dithiodipyridine, gives activation of cytoplasmic aldehyde dehydrogenase (Kitson, 1979). At present, therefore, it is impossible to say definitely whether or not disulfiram modifies catalytically essential groups on aldehyde dehydrogenase.

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