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Effects of Diethylstilbestrol, 2,2′-Dithiodipyridine, and Chloral Hydrate on the Esterase Activity of Sheep Liver Cytoplasmic Aldehyde Dehydrogenase

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ABSTRACT: The binding of diethylstilbestrol (DES) to aldehyde dehydrogenase (ALDH) has a very similar effect on the dehydrogenase activity of the enzyme as has modification of the enzyme by 2,2′-dithiodipyridine [Kitson, T. M. (1982) *Biochem. J. 207*, 81-89]. The latter modification may occur at the site of the esterase activity of the enzyme [Kitson, T. M. (1985) *Biochem. J. 228*, 765-767]. This suggests that DES might be a competitive inhibitor of the esterase reaction. However, in the absence of oxidized nicotinamide adenine dinucleotide (NAD^+) or reduced nicotinamide adenine dinucleotide (NADH), and at low concentrations of substrate (4-nitrophenyl acetate, PNPA), DES is a potent partial noncompetitive inhibitor. It is concluded therefore that DES binds at a site different from the esterase active site and that the enzyme–DES complex retains some ability to act as an esterase. High concentrations of PNPA appear to displace DES from its binding site. In the presence of NAD^+, DES is a weaker inhibitor, and in the presence of NADH, DES has very little effect. Esterase activity is enhanced by NADH when PNPA concentrations are high but is inhibited when they are low. The rate of reaction of ALDH with 2,2′-dithiodipyridine is only slightly reduced by DES, suggesting that the site at which thiol modifiers react and the DES binding site are different. When ALDH is modified by 2,2′-dithiodipyridine, it has reduced esterase activity, which declines further as the modified enzyme loses its 2-thiopyridyl label. In the presence of NAD^+, chloral hydrate is a simple competitive inhibitor of the esterase reaction. The results are consistent with a single site on ALDH being responsible for the dehydrogenase and esterase activities, the binding of aldehydes at high concentration, and the reaction of the enzyme with thiol modifiers. A second site, the physiological significance of which is unknown, has the ability to bind DES and steroids.

The cytoplasmic aldehyde dehydrogenase (EC 1.2.1.3, ALDH[1]) of sheep liver has received considerable attention from a number of aspects. These include its dehydrogenase activity [e.g., MacGibbon et al. (1977a)], its esterase activity (Blackwell et al., 1983b), the effect of thiol-modifying reagents such as disulfiram and 2,2′-dithiodipyridine (Kitson, 1982a, 1984), the effect of steroid hormones and diethylstilbestrol (DES) (Kitson, 1982b; Kitson & Crow, 1982), and the observation of acyl-enzyme intermediates during its catalytic cycle (Dunn & Buckley, 1985).

On the basis of previous work [see Kitson (1985)] it has been concluded that disulfiram and 2,2′-dithiodipyridine oxidize a particular pair of enzymic thiol groups to a disulfide and that the site where this happens has the ability to bind aldehydes at high concentrations and is also probably the active site for the enzyme's esterase action. That this should also be the enzyme's dehydrogenase active site is a natural assumption [see, for example, Eckfeldt and Yonetani (1976) and Takahashi and Weiner (1981)], and evidence in support of this has been presented (Duncan, 1979). However, other

[1] Abbreviations: ALDH, aldehyde dehydrogenase; PNPA, 4-nitrophenyl acetate; DES, diethylstilbestrol; E-S-S-(2-pyridyl), aldehyde dehydrogenase covalently modified by reaction with 2,2′-dithiodipyridine; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide.

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workers (Blackwell et al., 1983a,b) have proposed, on the basis of various kinetic studies, that the dehydrogenase and esterase sites are distinct.

The activatory effect of 2,2′-dithiodipyridine on the dehydrogenase activity of ALDH is closely mimicked by DES (Kitson, 1982b), suggesting that the latter compound may also interact with the enzyme at the thiol modifier/high aldehyde/esterase site (Kitson, 1985). Accordingly, the main purpose of the work reported here was to investigate the effect of DES on the esterase activity and on the reaction of the enzyme with 2,2′-dithiodipyridine. It is shown that the prediction one might make that DES would be a competitive inhibitor of these two processes is not correct and that DES does not bind to the enzyme's active site(s).

The work led to an investigation of the esterase activity of the enzyme modified by 2,2′-dithiodipyridine and to a reexamination of the inhibition of the esterase activity by chloral hydrate in the presence of NAD^+. The latter point is of significance to the proposal that cytoplasmic ALDH may have separate esterase and dehydrogenase active sites.

Experimental Procedures

Materials. Cytoplasmic ALDH from sheep liver was purified by the method of Dickinson et al. (1981), which removes contamination by mitochondrial ALDH. It was thoroughly dialyzed before use to remove dithiothreitol. Its concentration was measured as before (Kitson, 1983).

Enzyme Assay (as a Dehydrogenase). ALDH activity was measured spectrophotometrically at 340 nm with an Aminco DW-2a instrument. The conditions were 50 mM sodium phosphate buffer, pH 7.4, 1 mM NAD^+, 1 mM acetaldehyde, 25 °C, and 3-mL volume.

Enzyme Assay (as an Esterase). The enzyme was added to 50 mM sodium phosphate buffer, pH 7.4, 25 °C, which in some cases contained NAD^+ or NADH or chloral hydrate (added as 0.1 mL of a solution in the same buffer), DES or various steroids (added as 15 μL of a solution in ethanol), or some combination of these. Control assays contained 15 μL of ethanol where appropriate. At this point the cuvette was left in the spectrophotometer for 3 min to ensure complete thermal equilibration. Then 15 μL of an ethanol solution of PNPA (of various concentrations) was added rapidly on a glass nail, and the increase in absorbance at 400 nm was recorded. The final volume of the assay solution was 3 mL. The measured rates were corrected for the spontaneous rate of hydrolysis of PNPA under similar conditions but in the absence of enzyme.

Observation of Reaction of ALDH with 2,2′-Dithiodipyridine. Stock enzyme solution (0.2 mL) was mixed with 50 mM sodium phosphate buffer, pH 7.4 (2.75 mL), containing ether 15 μL of ethanol or 15 μL of an ethanol solution of DES at various concentrations, and in some cases 1 mM NAD^+. The final enzyme concentration was 2.75 μM. After equilibration of the mixture at 25 °C, the initial absorbance at 343 nm was recorded. 2,2′-Dithiodipyridine was then added as rapidly as possible on a glass nail in 15 μL of an ethanol solution to give a concentration of 10 μM. The resulting increase in absorbance at 343 nm due to the production of 2-thiopyridone was monitored over the next 2–2$^1/_2$ min.

Isolation of ALDH Modified by 2,2′-Dithiodipyridine. Enzyme (0.4 mL), dialyzed against 50 mM sodium phosphate buffer, pH 7.4, containing 0.3 mM EDTA, was mixed with NAD^+ solution in the same buffer (0.1 mL) and 2,2′-dithiodipyridine (15 μL of an ethanol solution). The mixing took place at room temperature, and the resulting concentrations of enzyme, NAD^+, and 2,2′-dithiodipyridine were 26.8 μM, 1 mM, and 120 μM, respectively. Within 1 min of mixing, the reaction mixture was applied to a column (11 cm × 0.8 cm) of Bio-Gel P-6 and eluted with 50 mM sodium phosphate buffer, pH 7.4, containing 0.3 mM EDTA, at 4 °C. Enzyme was collected in a volume of 1 mL after the void volume. Elution was complete within ~4 min. The enzyme was then kept on ice to slow the transformation of E-S-S-(2-pyridyl) to the enzymic disulfide form, and assays of its activity were performed within 30 min. Control samples were treated in exactly the same way except for the omission of 2,2′-dithiodipyridine. Measured activities were adjusted on the basis of any slight differences in enzyme concentration after elution.

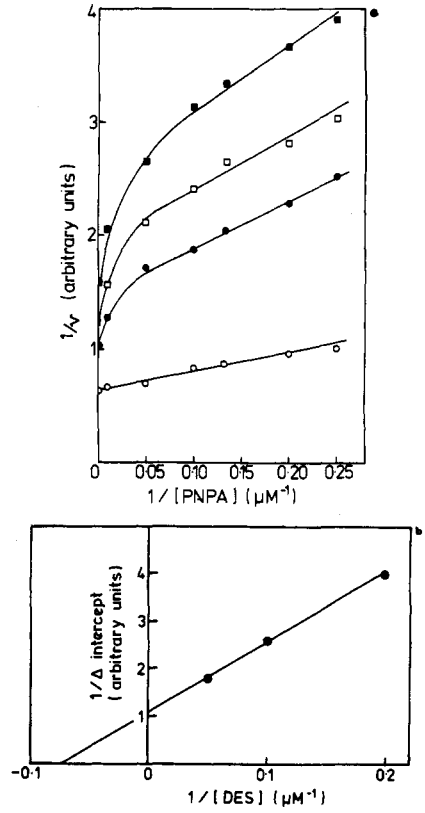
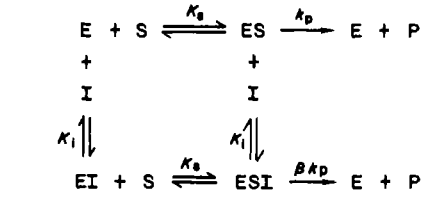


FIGURE 1: (a) Inhibition of the esterase activity of ALDH by DES. The enzyme concentration was 0.35 μM. The concentration of DES was (○) 0, (●) 5, (□) 10, and (■) 20 μM. Other details were as described under Experimental Procedures. Each point in this figure (and subsequent figures and tables) represents the average of at least two separate determinations that were always in close agreement. (b) Plot of the reciprocal of the change in intercept on the vertical axis of the projected linear portions of the plots in (a) against the reciprocal of the DES concentration.

Scheme I



Results

Effect of DES and Steroids on ALDH in the Absence of Nucleotides. Figure 1 shows the effect of DES on the esterase activity of cytoplasmic ALDH at pH 7.4 and 25 °C. At PNPA concentrations less than approximately 20 μM, the Lineweaver–Burk plots are linear and the data conform to Scheme I, defined by Segel (1975) as partial noncompetitive inhibition. From Figure 1a, K_m is estimated to be 2.4–2.8 μM.

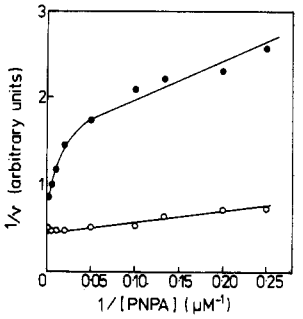


FIGURE 2: Inhibition of the esterase activity of ALDH by progesterone. The enzyme concentration was 0.34 μM and the progesterone concentration was (○) 0 and (●) 10 μM.

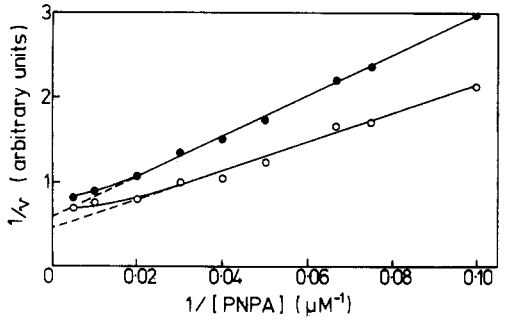


FIGURE 3: Inhibition of the esterase activity of ALDH by DES in the presence of 100 μM NAD^+. The enzyme concentration was 0.28 μM and the DES concentration was (○) 0 and (●) 10 μM.

Table I: Inhibitory Effect of DES and Some Steroids on the Esterase Activity of ALDH[a]

modifier	activity (percentage of control rate)
DES	51
progesterone	49
dehydroepiandrosterone	63
β-estradiol	82
17α-ethynylestradiol	56
estrone	27

[a] Conditions were [PNPA] = 20 μM, [modifier] = 5 μM, [enzyme] = 0.28 μM, pH 7.4, and 25 °C.

From Figure 1b, K_i = 3.6 μM and β = 0.26 (see Scheme I).

At PNPA concentrations greater than 20 μM, the Lineweaver–Burk plots in the presence of DES curve strongly downward. The same is true for the case of progesterone (Figure 2). In Figure 2 there is a suspicion of substrate inhibition in the absence of DES at the highest PNPA concentrations. (It is emphasized that all data were of course corrected for the rate of spontaneous hydrolysis of PNPA.)

The effect of a few other steroids was investigated, and the results are shown in Table I. Inhibition of the esterase activity of ALDH by steroids appears to be a general phenomenon, but there can be considerable difference in the magnitude of the inhibition by compounds of closely similar structure (e.g., estrone and β-estradiol).

Effect of DES on ALDH in the Presence of NAD^+. This was first examined in the presence of 100 μM NAD^+. It was found that under these conditions the rates (with or without DES) tail off appreciably right from the start of the assay. This curvature is evident even with high PNPA concentration so it is not due to depletion of substrate. Preincubation with NAD^+ does not eliminate the curvature. Figure 3 was obtained by drawing tangents to the curved assay traces in order to estimate the initial velocity. It reveals that in the presence of 100 μM NAD^+ (as in its absence) DES is not a competitive

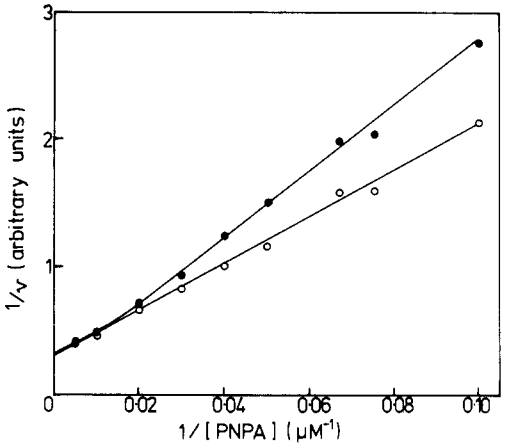


FIGURE 4: Inhibition of the esterase activity of ALDH by DES in the presence of 2 mM NAD^+. The enzyme concentration was 0.28 μM and the DES concentration was (○) 0 and (●) 10 μM.

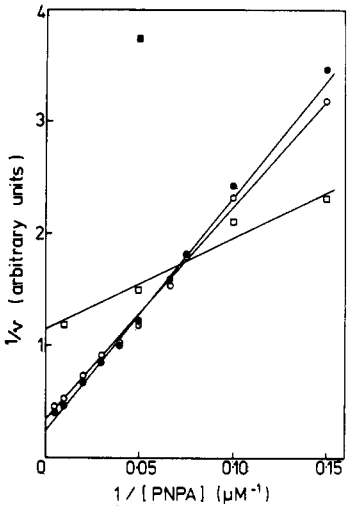


FIGURE 5: Effect of DES on the esterase activity of ALDH in the presence of 100 μM NADH. The enzyme concentration was 0.28 μM and the DES concentration was (○) 0 and (●) 10 μM. As a comparison, some points (□) were obtained in the absence of NADH and DES. A single point (■), obtained with 10 μM DES in the absence of NADH, acted as a check of what would be expected on the basis of Figure 1.

inhibitor of the esterase activity of ALDH. At high PNPA concentration there appears to be substrate inhibition.

The initial rate with 100 μM PNPA in the presence of 100 μM NAD^+ was found to be 2.64 times the rate in the absence of NAD^+. This figure compares well with a value of approximately 2.85, which can be estimated from the results of MacGibbon et al. (1978) using 118 μM PNPA. The K_m for PNPA under these conditions is 40 μM.

The effect of DES was reexamined in the presence of 2 mM NAD^+, under which conditions the rates were essentially linear for at least 4 min, and the result is shown in Figure 4. Now, DES hardly affects the rate at all with PNPA concentrations greater than 50 μM. At lower PNPA concentrations, it inhibits weakly. There is a trend to less inhibition with increasing NAD^+ concentration; thus, with 20 μM PNPA the rate (as a percentage of the control) in the presence of 10 μM DES is 33, 71, and 81, respectively, for an NAD^+ concentration of 0, 100 μM, and 2 mM. In the presence of 2 mM NAD^+ (no DES), K_m = 60 μM.

Effect of DES on ALDH in the Presence of NADH. With 100 μM NADH, 10 μM DES has little effect on the esterase activity of ALDH (Figure 5). As with NAD^+, the presence of NADH increases the size of the K_m for PNPA; with 100

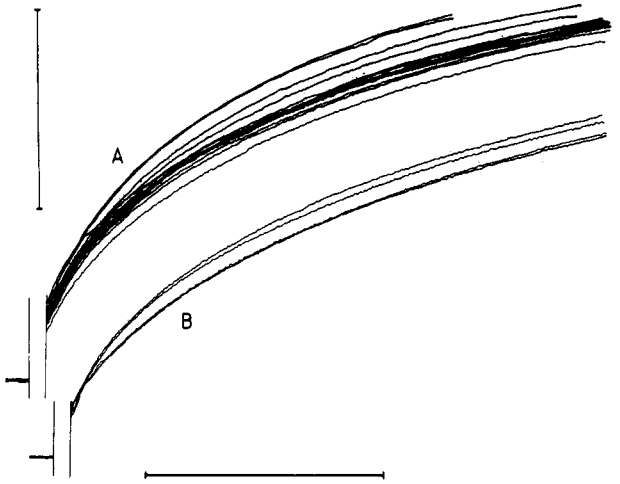


FIGURE 6: Effect of DES on the reaction of ALDH with 2,2′-dithiodipyridine. The figure shows the increase in A_{343} as 2-thiopyridone is released during the reaction of enzyme (2.76 μM) and 2,2′-dithiodipyridine (10 μM) according to the method described under Experimental Procedures. In (A), in the absence of NAD^+, the upper three curves were obtained in the absence of DES. The next curve was obtained with a DES concentration of 10 μM. Then follows a set of eight overlapping traces, which would be difficult to label separately, corresponding to DES concentrations of 10, 20, 33 (twice), 50 (twice), and 75 μM (twice). The lowest trace is a repeat at 20 μM. In (B), in the presence of 1 mM NAD^+, the upper two curves were obtained with no DES and the lower two with 50 μM DES. The vertical bar represents an A_{343} change of 0.1, and the horizontal bar represents 1 min.

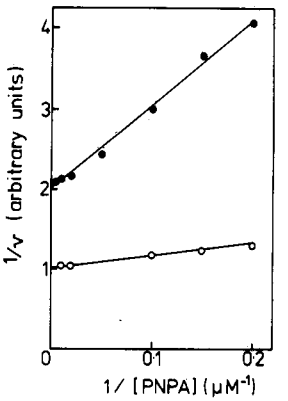


FIGURE 7: Esterase activity of ALDH modified by 2,2′-dithiodipyridine. The enzyme was isolated in the form of E-S-S-(2-pyridyl) as described under Experimental Procedures, and its esterase activity was rapidly determined (●). A control enzyme sample, treated in the same way except for the omission of 2,2′-dithiodipyridine, was also examined (○).

Table II: Change with Time of Activity of ALDH Modified by 2,2′-Dithiodipyridine

time[a]	activity (percentage of control rate)	
	dehydrogenase	esterase
<10 min	188	52
$6^1/_2$ h	32	17

[a] This is the time after the isolation by gel filtration of the E-S-S-(2-pyridyl) form of ALDH.

μM NADH (no DES) it is 57 μM.

Effect of DES on Reaction of ALDH with 2,2′-Dithiodipyridine. In Figure 6 it is seen that although in all cases the rate of reaction of ALDH with 2,2′-dithiodipyridine was less in the presence of different DES concentrations, the reduction was only small and was not a regular function of DES concentration. It is of course not possible to use DES concentrations as high as the aldehyde concentrations used in a similar previous experiment (Kitson, 1985), but nevertheless it is concluded that DES would have a much larger effect than it does have if it were binding at the same site as 2,2′-dithiodipyridine reacts. As already seen, DES at a concentration of only 10 μM (Figure 1 and Kitson, 1982b) has a marked effect on the esterase and dehydrogenase activities. On the occasion when the experiments in Figure 6 were performed, it was found that 10 μM and 20 μM DES increased the dehydrogenase activity of the enzyme to 150% and 210% of the blank rate, confirming that enzyme and DES were acting as expected from previous work (Kitson, 1982b).

Esterase Activity of ALDH Modified by 2,2′-Dithiodipyridine. ALDH was modified by 2,2′-dithiodipyridine in the presence of NAD^+. (This gives the maximum effect on the dehydrogenase activity; Kitson, 1982a.) The modified enzyme was then isolated from NAD^+ and 2-thiopyridone by rapid small-scale gel filtration, giving enzyme in the form of E-S-S-(2-pyridyl). It is known that on standing this form of the enzyme slowly loses its label to give an enzymic disulfide form (Kitson, 1984) that is inactive as a dehydrogenase. The labeled enzyme was examined as an esterase as rapidly as possible, before the loss of label could become significant, and the results are shown in Figure 7. It can clearly act as an esterase, but not as efficiently as the native enzyme. The K_m is 3-fold larger for the labeled enzyme, and the V_{max} is 50% that of the native form.

Immediately after gel filtration the dehydrogenase and esterase activities of the labeled enzyme were measured as a percentage of the corresponding controls. (The control enzyme was subjected to exactly the same treatment of gel filtration, etc., except that no 2,2′-dithiodipyridine was added.) After 6–7 h at 25 °C the activities were remeasured. The results are shown in Table II. The approximately 2-fold enhancement of dehydrogenase activity by conversion of enzyme to E-S-S-(2-pyridyl) is well established, as is the time-dependent loss of dehydrogenase activity, consequent upon the label being displaced (Kitson, 1984). It is now seen that the initial esterase activity also shows a substantial decline as the labeled enzyme changes to the disulfide form.

Inhibition of Esterase Activity of ALDH by Chloral Hydrate in the Presence of NAD^+. The results are shown in Figure 8. In the presence of 1 mM NAD^+ the inhibition pattern is simple linear competitive, with K_m = 53 μM and K_i = 42 μM. MacGibbon et al. (1977a) find that chloral hydrate is a competitive inhibitor of the dehydrogenase activity with propionaldehyde with a K_i of 19 μM (pH 7.6, 1.67 mM NAD^+). It is also a competitive inhibitor of the esterase activity in the absence of NAD^+ with a K_i of 287 μM (MacGibbon et al., 1978).

Discussion

Binding of DES and Steroids to ALDH. Previous work has shown that there is a particular site in cytoplasmic sheep liver ALDH that is responsible for several of the enzyme's characteristics (Kitson, 1985). It contains a pair of thiol groups, one of which, A, is labeled by disulfiram in a disulfide interchange reaction. The other, B, subsequently displaces the label giving rise to an enzymic disulfide form (Vallari & Pietruszko, 1982; Kitson, 1983). With 2,2′-dithiodipyridine, it appears that thiol group B is first labeled, followed by displacement of label by thiol group A (Kitson & Loomes, 1985a,b). The site is apparently where the esterase action of the enzyme occurs since PNPA protects the enzyme against disulfiram (Kitson, 1982a). The site also has the ability to

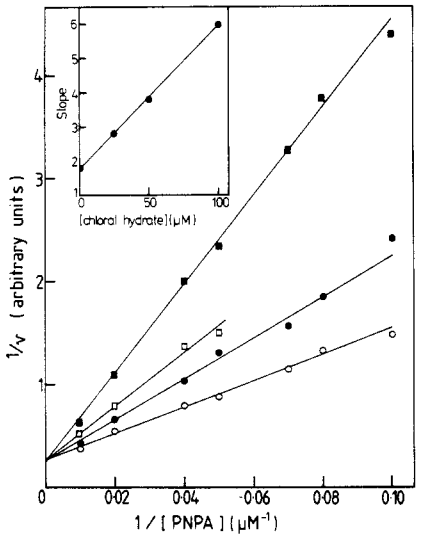


FIGURE 8: Inhibition of the esterase activity of ALDH by chloral hydrate in the presence of NAD^+. The enzyme concentration was 0.28 μM, the NAD^+ concentration was 1 mM, and the chloral hydrate concentration was (○) 0, (●) 25, (□) 50, and (■) 100 μM.

bind aldehydes at high concentration, since these slow the reaction of enzyme with disulfiram or with 2,2′-dithiodipyridine (Kitson, 1985). There is compelling evidence from recent extensive sequence studies (Hempel et al., 1984, 1985) to identify the group designated A above as Cys-302 (known to be present in the primary structure of the cytoplasmic and mitochondrial isozymes of horse and human). This group is also covalently modified by a brominated coenzyme analogue whereas a modifier of only slightly different structure labels a different thiol group (von Bahr-Lindstrom et al., 1985), possibly the same as that called B above.

When enzyme is labeled by 2,2′-dithiodipyridine in the presence of NAD^+ and assayed over a wide range of acetaldehyde concentration, a linear Lineweaver–Burk plot is obtained that contrasts with the curvature shown by the native enzyme (Kitson, 1982b). Labeled enzyme is approximately twice as active as native enzyme at 1 mM acetaldehyde, but as the acetaldehyde concentration either increases or decreases, the extent of activation gets less. Below about 30 μM acetaldehyde, the labeled enzyme becomes less active than the native form. This pattern can be very closely reproduced by replacing enzyme labeled with 2,2′-dithiodipyridine by enzyme in the presence of DES (Kitson, 1982b; Kitson & Crow, 1982). The simplest explanation for this result would be if DES also binds at the particular site referred to above, in which case it would be a competitive inhibitor of the esterase activity and of the reaction of the enzyme with 2,2′-dithiodipyridine.

The results presented here show that neither in the absence nor in the presence of NAD^+ or NADH is DES a competitive inhibitor of the esterase activity. The effect of DES is greatest in the absence of nucleotide, and at PNPA concentrations less than 20 μM the inhibition pattern is partial noncompetitive (see Figure 1). This suggests that substrate and inhibitor combine independently and reversibly to the enzyme at different sites to produce ES, EI, and ESI complexes. The ESI complex can produce product, but not as effectively as ES (see Scheme I).

The curvature seen in Figure 1 can be explained by proposing that high concentrations of PNPA displace DES from the latter's binding site, and similarly for progesterone (Figure 2). The binding of PNPA at this site may be reflected in the apparent substrate inhibition that is observed in Figures 2 and 3.

With rabbit liver ALDH, Duncan (1977) also concluded that progesterone and DES produce a new form of the enzyme with modified kinetic properties, but unlike the results shown in Figures 1 and 2, Duncan stated that the double-reciprocal plots were linear. His experiments were carried out at pH 9.0; the present ones were at pH 7.4.

The presence of NAD^+ or NADH reduces the magnitude of the inhibition by DES. This does not necessarily mean that DES binds more weakly in the presence of nucleotide; it is, of course, known to bind in the presence of NAD^+ since it has a pronounced effect on the dehydrogenase activity of the enzyme. It may be simply that the esterase activity of the enzyme is less sensitive to the binding of DES when NAD^+ or NADH is present. Conformational changes are known to be induced in the enzyme by NAD^+ or NADH (MacGibbon et al., 1977a,b; Hart & Dickinson, 1982).

Duncan (1977) reported that 20 μM NADH inhibits the esterase activity (with 5 μM PNPA) of rabbit liver ALDH by about 30–40%. On the other hand, Blackwell et al. (1983b) state that the esterase activity is stimulated by NADH by a factor of 1.2 and that their previously reported figure of 2–3 (MacGibbon et al., 1978) is incorrect, but it is unclear whether these figures refer to the same concentration of PNPA. The confusion of these conflicting results is resolved by the data in Figure 5. Clearly, NADH enhances the esterase reaction at high PNPA concentration and inhibits it at low PNPA concentration. For example, NADH (100 μM) stimulates the rate with 100 μM PNPA by a factor of 2.3 but reduces the rate with 6.67 μM PNPA by 28%.

The conclusion to be drawn from the results so far discussed is that ALDH has a binding site for DES and steroids, which is not the esterase active site but which may bind PNPA at high concentration. Regardless of whether the esterase and dehydrogenase sites are the same, it is also possible to conclude that the DES site and the aldehyde-binding site in the dehydrogenase reaction are not identical, since this would be incompatible with activation of the dehydrogenase activity by DES. Likewise, competitive binding of DES in the NAD^+ site, as has been proposed for 1,10-phenanthroline (Sidhu & Blair, 1975), would not explain the observed activation. Furthermore, the DES site is evidently not the site at which 2,2′-dithiodipyridine reacts, since even quite high concentrations of DES have only a modest effect on the rate of the 2,2′-dithiodipyridine modification reaction (see Figure 6).

Whether or not the DES/steroid binding site of ALDH has any physiological significance remains a moot point. As discussed before (Kitson & Crow, 1982), the concentrations of steroids (such as progesterone or the others in Table I) likely to occur in vivo are probably too small to affect the enzyme. However, it is intriguing that steroidal aldehydes are one of the classes of natural substrates for ALDH (Martin & Monder, 1978). Other enzymes such as glutamate dehydrogenase and glucose-6-phosphate dehydrogenase have been shown to be affected by steroids in a way similar to ALDH, but fumarase, lactate dehydrogenase, and isocitrate dehydrogenase are insensitive (Douville & Warren, 1968).

Dehydrogenase and Esterase Activities of ALDH. During the past few years there has been debate as to whether ALDH has a single active site for its dehydrogenase and esterase activities or two distinct sites. The two-site model was proposed on the basis of a number of pieces of evidence [see Blackwell et al. (1983a,b)], all of which in the opinion of Duncan (1985) are actually consistent with, and in favor of, a single type of catalytic site having both activities.

In the two-site model the dehydrogenase activity occurs at site P1 and the esterase activity at P2. The latter site can bind aldehydes when these are at high concentration. In previous work it was proposed that the site at which thiol modifiers react may also be identified with P2 (Kitson, 1985). If this is so, modification of a thiol group in site P2 by disulfiram must have an indirect, but potent, effect on the dehydrogenase activity at P1. With the related inactivator methyl diethylthiocarbamyl disulfide (Kitson & Loomes, 1985a,b), which attaches the small $-SCH_3$ label, it becomes intuitively a little difficult to accept that such minimal modification at one site should have such a large effect on activity at another site.

As an alternative, consider the following, in which it is assumed that the esterase and dehydrogenase sites are identical. First, it is suggested that the thiol group that is labeled by disulfiram (group A) is catalytically essential as a nucleophile in the manner usually proposed for ALDH (Feldman & Weiner, 1972; Li, 1977), explaining why disulfiram and methyl diethylthiocarbamyl disulfide are such effective inactivators. Modification of group B by 2,2′-dithiodipyridine gives enzyme in the form of E-S-S-(2-pyridyl), which is still active as a dehydrogenase and as an esterase (Figure 7). In this form, however, the enzyme effectively possesses its own intramolecular inactivator. Thus the proposed essential group A is slowly incorporated into a cystine disulfide linkage as the label becomes displaced, and consequently both dehydrogenase activity (Kitson, 1984) and esterase activity (Table II) decline. If this is true, it means that the thiopyridyl-labeled group is close enough to group A to react with it but is not in such a position as to sterically preclude group A from attacking the enzyme's substrates. When the enzyme's DES-binding site is occupied, both dehydrogenase and esterase activities are affected. It is possible that the labeling of the enzyme by 2,2′-dithiodipyridine and the binding of DES to the enzyme (although apparently happening at different sites) bring about a similar alteration in the enzyme (such as a conformational change), since in both cases the inhibitory effect on the esterase reaction (see Results) and the activation of the dehydrogenase reaction (Kitson, 1982b) are similar.

There remains the question why is the enzyme protected against thiol modifiers by aldehydes only when the latter are in very high concentration (of the order of 20–40 mM)? When disulfiram is added to an ALDH assay in the presence of NAD^+ and moderately high concentrations of acetaldehyde (e.g., 1 mM), there is an immediate inactivation (Kitson, 1985). If group A is catalytically essential as suggested above, it should presumably be well protected by such an aldehyde concentration, which is far higher than the K_m (MacGibbon et al., 1977a). However, it may be that in the enzyme's catalytic pathway there occurs a species that can be rapidly attacked by disulfiram but only binds aldehydes when their concentration is very high. One possibility is the E–NADH complex, and recently Dickinson (1985) has reported that this does indeed form complexes with aldehydes at very high concentrations.

One result that Blackwell et al. (1983b) used to support the two-site proposal is their finding of noncompetitive inhibition of the esterase activity by chloral hydrate in the presence of NAD^+ (101 μM). During this work it was found that rates in the presence of 100 μM NAD^+ were nonlinear and that initial rates could only be estimated by drawing tangents to the curves. Furthermore, Blackwell et al. gave results for only a narrow range of PNPA concentration, 50 μM and above. In the present work, as seen in Figure 3, deviation from linearity was found in this concentration range. Accordingly, it was thought advisable to reexamine the inhibition by chloral hydrate with a much broader range of PNPA concentration and a higher NAD^+ concentration (1 mM). It was found (Figure 8) that under these conditions the inhibition of the esterase action of cytoplasmic sheep liver ALDH by chloral hydrate is clearly competitive in nature. This result suggests that esterase and dehydrogenase sites are identical, since chloral hydrate is also a competitive inhibitor of the dehydrogenase activity (MacGibbon et al., 1977a). Sidhu and Blair (1975), working with human ALDH, also found competitive inhibition of esterase activity by chloral hydrate in the presence of NAD^+.

In conclusion, therefore, all the present results can be accommodated by the simpler and more attractive single active site model for ALDH.

Registry No. DES, 56-53-1; ALDH, 9028-86-8; NAD, 53-84-9; NADH, 58-68-4; PNPA, 830-03-5; chloral hydrate, 302-17-0; 2,2′-dithiodipyridine, 2127-03-9; progesterone, 57-83-0; dehydroepiandrosterone, 53-43-0; estradiol, 50-28-2; ethynylestradiol, 57-63-6; estrone, 53-16-7; esterase, 9013-79-0.

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Mechanism-Based Inhibition of Lactoperoxidase by Thiocarbamide Goitrogens†

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ABSTRACT: The irreversible inactivation of bovine lactoperoxidase by thiocarbamide goitrogens was measured, and the kinetics were consistent with a mechanism-based (suicide) mode. Sulfide ion inactivated, 2-mercaptobenzimidazole-inactivated, and 1-methyl-2-mercaptoimidazole-inactivated lactoperoxidases have different visible spectra, suggesting different products were formed. The results support a mechanism in which reactive intermediates are formed by S-oxygenation reactions catalyzed by lactoperoxidase compound II. It is proposed that the reaction of electron-deficient intermediates with the heme prosthetic group is responsible for the observed spectral changes and inactivation by thiocarbamides.

The inhibition of TPX-[1] and LPX-catalyzed reactions by thiocarbamides has been described (Ohtaki et al., 1985) and a relationship established with the physiological state of goiter (Gilman & Murad, 1975). A substantial body of experimental evidence suggests that modification of the iron porphyrin cofactor of these mammalian peroxidases occurs concomitant to the loss of enzymatic activity (Engler et al., 1982; Ohtaki et al., 1982; Nakamura et al., 1984). LPX was shown to be similar to TPX by several criteria, including inactivation by thiocarbamides (Ohtaki et al., 1982). The production of green, inactive derivatives of LPX by treatment of hydrogen peroxide oxidized enzyme intermediates with sodium sulfide and MMI was described (Nakamura et al., 1984); however, a mechanistic description of LPX inactivation supported by experimental results has not been presented.

A recent report from this laboratory described the catalysis of S-oxygenation reactions by LPX (Doerge, 1986). These observations provided a clue to the mechanism of inactivation of LPX by thiocarbamides and suggested the involvement of enzymatic sulfoxidation. This report describes the reactions of thiocarbamide goitrogens with LPX and provides data in support of a mechanism-based (suicide) mode of inhibition (Waley, 1985).

Materials and Methods

Bovine LPX was purchased from Sigma Chemical Co. and the activity measured by guaiacol oxidation [3×10^4 mol min^{-1} (mol of LPX)$^{-1}$] and iodide ion oxidation [3.3×10^4 mol min^{-1} (mol of LPX)$^{-1}$] at 22 °C in the presence of 0.2 mM hydrogen peroxide, 33 mM guaiacol, or 5 mM potassium iodide in 0.1 M phosphate buffer, pH 7.0, as previously described (Morrison, 1970). LPX concentration was determined spectrophotometrically (Morrison, 1970). MBI was obtained from Eastman Chemical Co. and NMBI from Aldrich Chemical Co., and both were recrystallized from aqueous ethanol. MMI was obtained from Sigma Chemical Co. Purity was checked by thin-layer or high-pressure liquid chromatography. Hydrogen proxide, obtained as a 30% solution from Sigma Chemical Co., was standardized periodically (Cotton & Dunford, 1973), and dilute solutions were prepared daily. Kinetic measurements and spectra were made with a Hewlett-Packard 8541A recording spectrophotometer.

For determination of enzymatic activity, the iodide ion oxidation method was employed because of greater reproducibility of initial rates. Assays were initiated by the addition of aliquots containing LPX at a final concentration of ca. 1 nM into a cuvette containing the reagents listed above, and initial rates were determined in the first 30 s of reaction.

The time-dependent inactivation of LPX by thiocarbamides was determined by incubation of LPX (0.25–2.0 nM) in the presence of hydrogen peroxide (0.05 mM) and inhibitor such that the ratio [LPX]/[inhibitor] was constant (Waley, 1985). The inhibitors were added as ethanolic solutions, and it was determined that addition of small amounts of ethanol had no effect on the inactivation reaction. Hydrogen peroxide was added to a mixture of LPX plus inhibitor to initiate the inactivation at 0 °C, and at various times an aliquot was removed and analyzed for enzymatic activity. The plots of enzyme inactivation vs. time were approximately first order for 50–90% inactivation, and the inactivation half-times ($t_{1/2}$) were determined from this portion of the curve where hydrogen peroxide dependent inactivation was negligible.

Stoichiometric measurements for the inactivation of LPX by thiocarbamides were made by adding excess hydrogen peroxide (0.2 mM) to a mixture of LPX (0.005 mM) plus

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; HRP, horseradish peroxidase; LPX, lactoperoxidase; MBI, 2-mercaptobenzimidazole; MMI, 1-methyl-2-mercaptoimidazole; NMBI, 5-nitro-2-mercaptobenzimidazole; TPX, thyroid peroxidase.

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