# Some Properties of Aldehyde Dehydrogenase from Sheep Liver Mitochondria

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Aldehyde dehydrogenase from sheep liver mitochondria was purified to homogeneity as judged by electrophoresis on polyacrylamide gels, and by sedimentation-equilibrium experiments in the analytical ultracentrifuge. The enzyme has a molecular weight of 198000 and a subunit size of 48000, indicating that the molecule is a tetramer. Fluorescence and spectrophotometric titrations indicate that each subunit can bind 1 molecule of NADH. Enzymic activity is completely blocked by reaction of 4mol of 5,5'-dithiobis-(2-nitrobenzoate)/mol of enzyme. Excess of disulfiram or iodoacetamide decreases activity to only 50% of the control value, and only two thiol groups per molecule are apparently modified by these reagents.

The oxidation of ethanol to acetate by mammalian liver was shown by Racker (1949) to be catalysed by two separable NAD-linked dehydrogenases. The first of these enzymes, alcohol dehydrogenase, has been extensively studied, but the second, aldehyde dehydrogenase, has been less well studied. Aldehyde dehydrogenases from several species have been investigated. Two isoenzymes from horse liver have been purified to homogeneity, and shown to have molecular weights in the region of 200000–250000 (Eckfeldt *et al.*, 1976). The F2 isoenzyme was provisionally identified as that studied by Feldman & Weiner (1972), which was shown to be a tetramer, with a subunit weight of 57000 and a molecular weight of 200000–260000. Eckfeldt & Yonetani (1976), using equilibrium titration, found that the F1 isoenzyme from horse liver binds approx. 2mol of NADH/mol of enzyme, but caution should be used in interpreting this finding because of the extreme sensitivity of this isoenzyme to oxidation during the preparation. Blair & Bodley (1969) partially purified an enzyme from human liver, and estimated its molecular weight to be 200000. Kraemer & Deitrich (1968) isolated a human liver enzyme with basically the same kinetic properties, but found the molecular weight to be 90000.

Crow *et al.* (1974) studied the subcellular distribution of aldehyde dehydrogenase activity in sheep liver, and found activity to be present in both the mitochondrial and the cytoplasmic fractions of the cell. Preliminary experiments on partially purified enzymes suggested that the activities in the two compartments were due to different enzymes. In previous studies workers have often failed to establish the subcellular origin of the enzyme that they have studied. There is thus a need for work on aldehyde dehydrogenase isolated from known cellular compartments.

It has been argued for rat liver that acetaldehyde is metabolized predominantly in the mitochondria (Parrilla *et al.*, 1974; Corrall *et al.*, 1976). Marjanen (1972) has suggested that in the presence of ethanol, the amount of NAD$^+$ in the cytoplasm may become limited, so that most of the aldehyde oxidation would take place in the mitochondria. In view of these reports it seemed important to try to obtain detailed information about the properties of mitochondrial aldehyde dehydrogenase. Sheep liver was chosen as the source of the mitochondria because of its ready availability.

## Experimental

### Materials

NAD$^+$ (grade II) and NADH (grade I) were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. 5,5'-Dithiobis-(2-nitrobenzoic acid) and disulfiram (tetraethylthiuram disulphide) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. All other chemicals were analytical-reagent grade wherever available, purchased from Fisons Chemicals, Loughborough, Leics., U.K. or BDH Chemicals, Poole, Dorset, U.K. Iodoacetamide was recrystallized from aq. 50% (v/v) ethanol. Whatman DE-11 DEAE-cellulose and CM-32 CM-cellulose were precycled according to the manufacturers' directions before being equilibrated to the appropriate pH and ionic strength. Solutions of acetaldehyde were generally made up daily from 1M stock solutions, which were prepared by using freshly redistilled acetaldehyde, and were stored at 0–4°C. Stock solutions were replaced every 5–7 days. Sheep livers were obtained from the local slaughterhouse, and mitochondria were prepared within a few hours of the death of the animal,

*Methods*

All dialysis tubing was boiled in 10mM-EDTA, pH7.0, and washed well with water before use. All solutions were prepared in glass-distilled water.

*Protein concentrations.* In the early stages of the enzyme-purification procedure, protein concentrations were determined spectrophotometrically by assuming $A^{1\%}_{1cm} = 10$ at 280nm. For purified enzyme solutions, a specific extinction coefficient at 280nm of $A^{1\%}_{1cm} = 9.9$ was used, determined from dry-weight measurements.

*Enzyme assay.* Assays were performed at 25°C by using a recording filter fluorimeter of the type described by Dalziel (1962). The assay mixture contained NAD+ (4μmol) and acetaldehyde (4μmol) in 4ml of sodium phosphate buffer, pH8.0, $I = 0.1$. The reaction was initiated by the addition of enzyme. One unit of enzyme activity was defined as that amount of enzyme producing 1μmol of NADH/min in the above assay. Assays were found to exhibit a slight lag phase for about 30s after the addition of enzyme. However, after this period the progress curves became linear and remained so for at least 3min. The rates obtained after 30s were used in calculating rates of reaction. Such behaviour has not previously been reported for any aldehyde dehydrogenase studied. This may be due to the fact that most workers have used spectrophotometric rather than fluorimetric methods, and the former are generally much less sensitive. At present we do not know the cause of this non-linearity in the assay. It is not, however, a result of product activation.

*Preparation of aldehyde dehydrogenase.* The preparation of the enzyme was based on the procedure of Crow *et al.* (1974). Aldehyde dehydrogenase activity was found in both the cytoplasmic and the mitochondrial fractions of the cell, with about 45% of the mitochondrial plus cytoplasmic activity being found in the mitochondria. This finding is in good agreement with the distribution obtained by Crow *et al.* (1974).

All operations were performed at 0–4°C. Unless otherwise stated buffers contained 0.1% (v/v) 2-mercaptoethanol.

Portions (250g) from 1.5–1.8kg of chopped liver were homogenized for 45s in an MSE Ato-Mix blender with 800ml of 5mM-sodium phosphate buffer, pH7.3, containing 0.25M-sucrose. The homogenates were combined, centrifuged at 500g for 10min and the supernatant was then re-centrifuged at 20000g for 25min to sediment the mitochondria. The mitochondria were washed with 0.25M-sucrose, resuspended in 500ml of 0.1M-sodium citrate buffer, pH5.0, and disrupted either by freezing and thawing (two cycles, by using solid $CO_2$/ethanol as the freezing mixture), or by sonication, in 200ml batches, for 2×45s with a Dawe Soniprobe type 1130/1A. Both

methods release approximately the same amount of aldehyde dehydrogenase activity, but freezing and thawing gives an extract with a 10–15% higher specific activity than does sonication. However, freezing and thawing is a time-consuming process, and in view of the relative instability of the enzyme in the early stages of the preparation, the more rapid sonication procedure may seem preferable. Either method, however, yields an extract from which homogeneous enzyme is obtained by the following procedure.

After disruption of the mitochondria, the pH of the suspension was readjusted to pH5.0 with 0.5M-citric acid, and after 30min mitochondrial debris and other insoluble material was removed by centrifugation at 20000g for 10min. $(NH_4)_2SO_4$ (209g/litre of extract) was added, and the precipitate formed after 20min was removed by centrifugation (20000g/10min). More $(NH_4)_2SO_4$ (200g/litre of extract) was then added, and after standing for 30min the precipitate, which contained the aldehyde dehydrogenase activity, was collected by centrifugation at 20000g for 10min, dissolved in 5mM-sodium phosphate buffer, pH7.3, and dialysed against 3×4 litre changes of the same buffer. During the addition of $(NH_4)_2SO_4$ the pH of the solution was kept between 6.8 and 7.3 by the addition of 4M-$NH_3$.

After dialysis, insoluble material was removed by centrifugation, and the protein solution was applied to a column (3cm×50cm) of DEAE-cellulose, previously equilibrated with 5mM-phosphate buffer, pH7.3. The column was washed with this buffer until the $A_{280}$ of the effluent in 1cm-path-length cells was less than 0.35. Aldehyde dehydrogenase activity was then eluted with a gradient made by running 400ml of 70mM-phosphate buffer, pH7.3, into a constant-volume reservoir which initially contained 200ml of 5mM-phosphate buffer, pH7.3. The enzyme was eluted in 30–35mM buffer. The protein in these fractions was precipitated by dialysis overnight against 40mM-phosphate buffer, pH7.3, containing sufficient $(NH_4)_2SO_4$ to give a final concentration both inside and outside the bag of 450g/litre. The precipitate was dissolved in a minimum volume of 5mM-phosphate buffer, pH7.0, dialysed against the same buffer to remove $(NH_4)_2SO_4$, and then against 5mM-phosphate buffer, pH6.0. Insoluble material was removed by centrifugation, and the enzyme was passed through a column (1.5cm×15cm) of CM-cellulose, equilibrated with 5mM-phosphate buffer, pH6.0. The aldehyde dehydrogenase activity was eluted immediately after the void volume.

The active fractions from the CM-cellulose column were dialysed against $(NH_4)_2SO_4$ (450g/litre) in 40mM-phosphate buffer, pH7.3, and the precipitate was dissolved in the minimum volume of sodium phosphate buffer, pH7.0, $I = 0.1$, containing 100μM-dithiothreitol. After dialysis against a 200-fold excess

of the same buffer, the protein mixture was fractionated on a column (1 cm × 75 cm) of Sephadex G-200 that was equilibrated and eluted with this buffer. The protein in the enzyme-bearing fractions with a specific activity of 0.185 unit/mg or better was precipitated by dialysis against $(NH_4)_2SO_4$ as above.

Enzyme solutions for experiment were prepared from the $(NH_4)_2SO_4$ precipitate by dialysis against buffer of the appropriate pH and ionic strength before use.

*Polyacrylamide-gel electrophoresis.* Polyacrylamide-gel electrophoresis was performed in 9% gels by the method of Clarke (1964) at 4°C for 5 h at a current of 1.5 mA/gel. The buffer used was 5 mM-Tris/50 mM-glycine, adjusted to pH 8.4 with HCl. Gels were stained for protein by using 1% Naphthalene Black in 7% (v/v) acetic acid, and destained electrophoretically with 7% acetic acid, and for activity by the production of formazan from Nitro Blue Tetrazolium in the presence of $NAD^+$, acetaldehyde and phenazine methosulphate. The activity stain was freshly made up before use by mixing 40 ml of solution A (40 mg of $NAD^+$, 10 mg of Nitro Blue Tetrazolium, 1 ml of 90 mM-acetaldehyde in 40 ml of 0.1 M-phosphate buffer, pH 8.0) with 0.4 ml of solution B (aqueous phenazine methosulphate, 1 mg/ml). Gels were stained overnight at 4°C.

*Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.* This was performed by the method of Weber & Osborn (1969). For the determination of the subunit weight of aldehyde dehydrogenase, standards with accurately known subunit weights were used. These were: bovine serum albumin, 68000 (Tanford *et al.*, 1967); ox liver catalase, 60000 (Sund *et al.*, 1967); horse liver alcohol dehydrogenase, 41000 (Castellino & Barker, 1968); bovine trypsin, 23300 (calculated from the amino acid sequence given in Dayhoff & Eck, 1969); and egg-white lysozyme, 14300 (Canfield, 1963).

*Molecular-weight determination.* Short-column sedimentation-equilibrium experiments were performed at 10°C with enzyme solutions in 44 mM-sodium phosphate buffer, pH 7.0, containing 10 μM-dithiothreitol, in an MSE analytical ultracentrifuge equipped with u.v. scanner. Rotor speeds of 9000 rev./min were used, with an initial enzyme concentration of 0.5 mg/ml. Aldehyde dehydrogenase was completely stable for the duration of the experiment under the conditions used. The partial specific volume of the enzyme was determined pycnometrically at 10°C as described by Schachman (1957).

Molecular weight was also estimated by gel filtration on a column (0.9 cm × 50 cm) of Sephadex G-200 equilibrated at 0–4°C with sodium phosphate buffer, pH 7.0, $I = 0.1$, containing 10 μM-dithiothreitol. The standards used, with their molecular weights, were: jack-bean urease, 432000 (Reithel *et al.*, 1964); ox liver catalase, 250000 (Sund *et al.*,

1967); crystalline yeast alcohol dehydrogenase, 141000 (Bühner & Sund, 1969); ovalbumin, 45000 (Warner, 1954); egg-white lysozyme, 14300 (Canfield, 1963). The void volume ($V_0$) of the column was obtained by using Blue Dextran 2000 (Pharmacia, Uppsala, Sweden). Fractions (1.5 ml) were collected and protein concentrations were monitored by $A_{280}$ measurements. The elution volume ($V_e$) was determined as the peak volume from the elution profile, and the molecular weight of aldehyde dehydrogenase was estimated by interpolation by using a plot of log (molecular weight) against $V_e/V_0$ (Andrews, 1964).

*Dry-weight measurement.* A solution of aldehyde dehydrogenase was dialysed against 5 mM-phosphate buffer, pH 7.0, and centrifuged to remove traces of insoluble material. The $A_{200}^{1cm}$ of the enzyme solution was determined and samples of enzyme (containing about 15 mg) were pipetted into preweighed vials. The samples were dried *in vacuo* and maintained under vacuum over $CaCl_2$ until at constant weight.

*Zinc analysis.* All glassware used for zinc analysis was soaked overnight in 10% (v/v) $HNO_3$ before use. A solution of aldehyde dehydrogenase, dialysed against 5 mM-phosphate buffer, pH 7.0, containing 20 mM-EDTA, was dried at 90°C. The sample was wet-ashed in 2 ml of concentrated $HNO_3$ plus 0.3 ml of 36% (v/v) $HClO_4$ and made up to 10 ml with water. A buffer blank was similarly dried and ashed. The $Zn^{2+}$ content was estimated by using a Pye–Unicam SP.1950 atomic absorption spectrophotometer.

*Coenzyme binding.* The binding of NADH to aldehyde dehydrogenase was monitored fluorimetrically, in a recording filter fluorimeter of the type described by Dalziel (1962), and spectrophotometrically with a Zeiss PMQ II spectrophotometer.

In the fluorimeter the standard glass and $NaNO_2$ filters were replaced by a narrow-band-pass filter obtained from Grubb Parsons, Newcastle upon Tyne, U.K. The filter showed peak transmission at 428 nm with a bandwidth of 7 nm. Determinations were made in sodium phosphate buffer, pH 7.0, $I = 0.1$, at 25°C. The enzyme was stable for the duration of the titration, and blank oxidation of NADH was not detected.

The absorption spectrum of enzyme-bound NADH was determined in a Cary 14 u.v. recording spectrophotometer, and the uncorrected fluorescence emission spectrum of the enzyme–NADH complex was determined in a Farrand Mark I spectrofluorimeter, by using an excitation wavelength of 355 nm and 10 nm slits.

*Inhibition studies.* For these experiments dithiothreitol was removed from enzyme solutions by dialysis against appropriate buffers for 12–16 h. Enzyme solutions were incubated at 25°C with various concentrations of disulfiram or iodo-acetamide in sodium phosphate buffer, $I = 0.1$. Experiments were performed at pH 7.0 and 8.0. Control

samples of enzyme and buffer were similarly incubated at 25°C and the activities of the controls were completely stable for the duration of the experiments.

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) at 25°C was monitored by measuring the change in $A_{412}$ after the addition of a portion of aldehyde dehydrogenase solution to the cuvette containing 5,5'-dithiobis-(2-nitrobenzoic acid) (6mM) in 0.04M-phosphate buffer, pH8.0. Samples were withdrawn at intervals and assayed for enzymic activity. A molar absorption coefficient at 412nm of 13600 for the thionitrobenzoate anion at pH8.0 was used (Ellman, 1959).

## Results and Discussion

### Purification and criteria of purity

Results of a typical preparation are summarized in Table 1. The final product is stable for several weeks if stored at 0–4°C as a 5mg/ml solution in 44mM-phosphate buffer, pH7.0, containing 100 $\mu$M-dithiothreitol. Electrophoresis of 10–100 $\mu$g of purified enzyme on 9% polyacrylamide at pH8.4 yielded gels showing one sharp band halfway into the gel (3.5cm) when either a protein or an activity stain was used. There was no evidence of any inactive protein material. The high degree of homogeneity of the preparation is confirmed by the strict linearity of the plot of log concentration versus (radius)$^2$ shown in Fig. 1, which is obtained from a sedimentation-equilibrium experiment in the analytical ultra-centrifuge. The above criteria suggest that the final product is pure and suitable for detailed study.

The specific activity of 0.2 unit/mg for the purified enzyme is equivalent to a specific activity of 0.73 unit/mg measured by the assay method of Crow et al. (1974). These authors measured protein concentrations by the method of Lowry et al. (1951) and assayed enzyme activity under somewhat different conditions from ours. In our hands the method of Lowry et al.

(1951) gave estimates of purified enzyme concentration about 15% lower than those based on dry-weight measurements, and at 25°C the rate of reaction in the assay of Crow et al. (1974) was some 3.1-fold faster than at pH8.0. The specific activity of the purified mitochondrial aldehyde dehydrogenase of Crow et al. (1974) is 0.157 unit/mg if the unit of activity is defined as above, which suggests that their material is either substantially less active or less pure than ours. Evidence of some inhomogeneity was observed by Crow et al. (1974) on polyacrylamide-gel electrophoresis.

A straightforward comparison with the preparation of Crow et al. (1974) is not possible, however, because the temperature at which assays were performed was not stated. However, two points can be made. The present procedure is a modification of that used by Crow et al. (1974) and the modifications introduced would be expected to improve the method. Thus a CM-cellulose column step is included, which yields a significant purification, and gradient elution from the DEAE-cellulose column seems to give much better resolution than the stepwise method used by Crow et al. (1974). The 170-fold overall purification obtained here is substantially better than the 12-fold obtained by Crow et al. (1974) and the yields of activity are about the same.

### Molecular properties

The partial specific volume of the enzyme was estimated to be 0.733ml/g at 10°C, and this value together with the slope of Fig. 1 may be used to calculate a value of 198000 for the molecular weight of the enzyme. Gel-filtration experiments on Sephadex G-200 at pH7.0 indicate a molecular weight in the region of 190000. Polyacrylamide-gel electrophoresis of the enzyme in the presence of 0.1% sodium dodecyl sulphate by the method of Weber & Osborn (1969) yielded gels showing only one protein-staining band. The mobility of the band relative to Bromophenol Blue was 0.286, indicating a subunit weight

Table 1. *Purification of mitochondrial aldehyde dehydrogenase from 1.5 kg of sheep liver*
The yields given represent the fraction of the original activity carried over to the next stage of the purification. The overall yield for each stage was generally at least 80%, but fractions with low specific activities were discarded.

| Step | Volume (ml) | Activity (units) | Protein (mg) | Specific activity (unit/mg) | Yield (%) | Purification |
|---|---|---|---|---|---|---|
| Mitochondrial extract | 400 | 26.5 | 22000 | 0.0012 | 100 | 1 |
| (NH$_4$)$_2$SO$_4$ fraction after dialysis | 80 | 23 | 5700 | 0.004 | 87 | 3.3 |
| Eluate from DEAE-cellulose column after concentration and dialysis | 5 | 13 | 105 | 0.12 | 49 | 100 |
| Eluate from CM-cellulose column after concentration and dialysis | 2.0 | 9 | 56 | 0.16 | 34 | 133 |
| Eluate from Sephadex G-200 column | 8.0 | 5 | 25 | 0.2 | 19 | 170 |
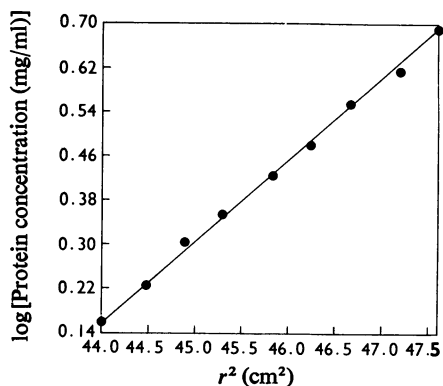
Fig. 1. *Plot of data from a sedimentation-equilibrium experiment with purified aldehyde dehydrogenase*
Ultracentrifuge experiments were performed at 10°C in sodium phosphate buffer, pH7.0, $I = 0.1$, containing $10\mu$M-dithiothreitol, at a rotor speed of 9000 rev./min. Absorbance measurements were determined throughout the cell with the use of the u.v. scanner, which monitored $A_{280}$. Protein concentrations were calculated by using $A_{1cm}^{1\%} = 9.9$.



Fig. 2. *Fluorimetric and spectrophotometric titrations of aldehyde dehydrogenase with NADH in sodium phosphate buffer, pH7.0, $I = 0.1$, at 25°C*
Fluorimetric titrations (●) were done with an enzyme concentration of 0.31 mg/ml (1.55 $\mu$M), and spectrophotometric titrations (○, $\Delta A_{340}$) were done with an enzyme concentration of 3.00 mg/ml (15.1 $\mu$M).

of 48000. The presence of only one band in these experiments confirms the homogeneity of the preparation and shows that the enzyme is not contaminated with significant amounts of proteinase activity. Zinc analysis on the purified enzyme indicates a maximum of 0.05 atom of zinc/molecule, which is clearly of no catalytic or structural importance. These studies suggest that the mitochondrial enzyme is a tetramer made from four identical or very similar subunits. A similar conclusion has been reached for the $F_2$ isoenzyme from horse liver (Feldman & Weiner, 1972; Eckfeldt et al., 1976).

The u.v.-absorption spectrum of the enzyme is typical of that for a normal protein. The absorption maximum is at 280nm, with $A_{1cm}^{1\%} = 9.9$ ($\varepsilon = 1.97 \times 10^5 M^{-1} \cdot cm^{-1}$) based on dry-weight measurements. The ratio $A_{280}/A_{260} = 1.70$ does not suggest the presence of bound nucleotide. The binding of NADH to the enzyme is accompanied by changes in the absorption and fluorescence spectrum of the co-enzyme. Spectrophotometric and fluorimetric titrations of the enzyme are therefore suitable techniques for the estimation of the number of coenzyme-binding sites. The results of two such titrations are shown in Fig. 2, and it is evident from the sharpness of the titrations that the binding of NADH is very tight indeed. Detailed experiments based on fluorescence measurements indicate $K_{E\cdot NADH} = 0.05\mu$M approx. at pH7.0, 25°C. Both titrations in Fig. 2 indicate 3.8–3.9 NADH-binding sites/molecule of enzyme. This is in agreement with the proposed tetrameric structure of the enzyme and suggests one binding site and prob-
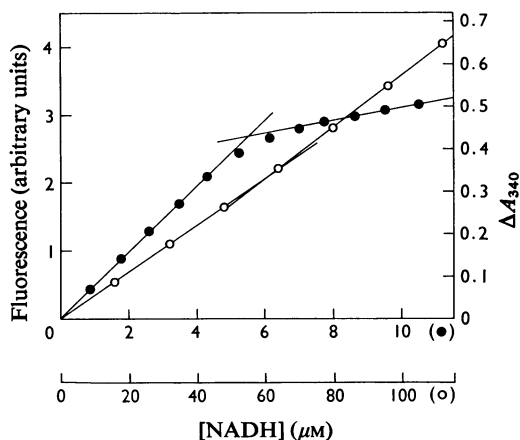
ably one active site/subunit. The fluorescence titration is clearly the preferred method because of the substantial enhancement of fluorescence on binding. Under the conditions relevant to Fig. 2 with the filter instrument the enhancement was 6.5-fold. In the Farrand mark I spectrofluorimeter with 10nm slits, an enhancement on binding of NADH of 11-fold was observed at 445nm, with the peak of maximum emitted fluorescence shifted from 470 to 445nm.

The spectrophotometric titration in Fig. 2 shows that the absorption coefficient of the bound co-enzyme at 340nm is $\varepsilon = 5.5 \times 10^3$ litre·mol$^{-1}$·cm$^{-1}$. There is thus a hypochromic effect on binding, as for several other dehydrogenases, for example glycerol 3-phosphate dehydrogenase (Bentley et al., 1973). The absorption spectrum of the bound coenzyme is similar in form to that for glycerol 3-phosphate dehydrogenase. The same disturbance of the spectrum at 285nm is seen, which is probably due to perturbation of tryptophan residues in the enzyme. The main absorption bands show maxima at 250nm and 335nm. At 250nm the absorbance of the bound NADH is characterized by a value of $\varepsilon = 10.7 \times 10^3$ litre·mol$^{-1}$·cm$^{-1}$.

*Inhibition studies*

The progress curve for inhibition by 5,5'-dithiobis-(2-nitrobenzoate) at 25°C and pH8.0 is shown in Fig. 3. It is clear that the loss of activity and extent of
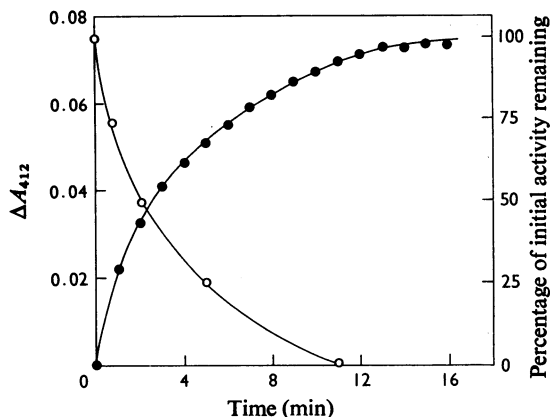
Fig. 3. *Progress curve for the inhibition of aldehyde dehydrogenase by 5,5'-dithiobis-(2-nitrobenzoate)*
The increase in $A_{412}$ (●), and the loss of enzymic activity (○) was monitored after the addition of a portion of enzyme solution to a cuvette containing 6mM-5,5'-dithiobis-(2-nitrobenzoate) in 0.04M-sodium phosphate buffer, pH 8.0. The final enzyme concentration was 0.26mg/ml (1.30 $\mu$M).
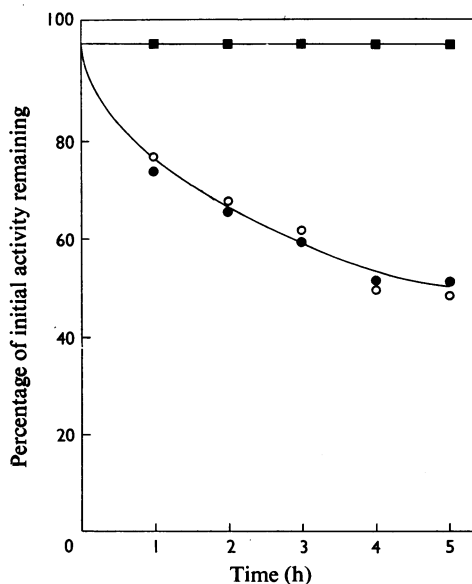


Fig. 4. *Progress curves for the inhibition of aldehyde dehydrogenase by iodoacetamide, disulfiram and iodoacetate*
Aldehyde dehydrogenase at a final concentration of 1mg/ml (5 $\mu$M) was incubated at 25°C with 10-fold molar excesses of iodoacetamide (●), disulfiram (○) or iodoacetate (■). Samples were withdrawn at intervals and assayed for enzymic activity. Experiments were performed at both pH 7.0 and 8.0 in sodium phosphate buffers, $I = 0.1$. The results at the two pH values were identical.
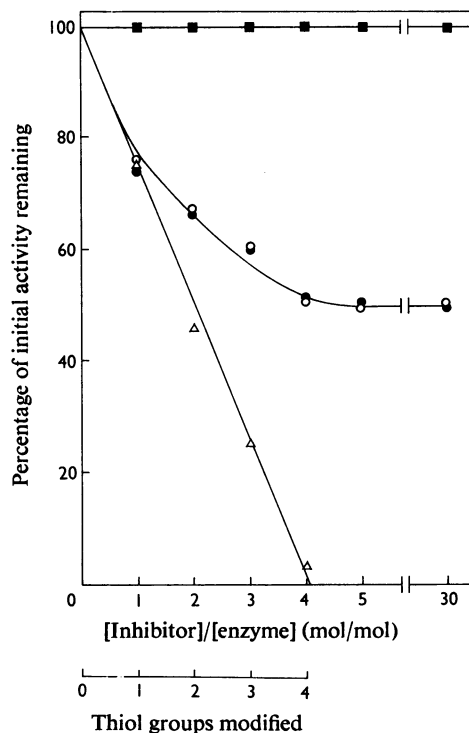


Fig. 5. *Activity of aldehyde dehydrogenase as a function of inhibitor concentration, and thiol groups modified*
Aldehyde dehydrogenase, at a final concentration of 1 mg/ml, was incubated at 25°C in sodium phosphate buffer, $I = 0.1$, with various molar excesses of iodoacetamide (●), disulfiram (○) and iodoacetate (■). After 5h incubation, samples were withdrawn and assayed for enzymic activity. Experiments were performed at pH 7.0 and 8.0, identical results being obtained at each pH value. The Figure also shows the relationship between activity and number of thiol groups modified by 5,5'-dithiobis (2-nitrobenzoate) at pH 8.0 (△).

reaction as measured by $\Delta A_{412}$ proceed in parallel. Release of 1, 2, 3 and 4mol of thionitrobenzoate/mol of enzyme results in the loss of about 25%, 50%, 75% and 100% of the initial activity (see also Fig. 5). Analysis of the progress curve shown in Fig. 3 by the method of Guggenheim (1926) suggests that the reaction between 5,5'-dithiobis-(2-nitrobenzoate) and aldehyde dehydrogenase may be biphasic, indicating that the four essential thiol groups of the enzyme are not identical. A more rapid initial phase (apparent rate constant 0.26min⁻¹), during which something over 2 thiol groups/mol become modified, is followed by a less rapid phase (apparent rate constant 0.14min⁻¹) in which the remaining thiol groups react. Dialysis of inactive enzyme against 0.1M-

phosphate buffer, pH7.0, containing 0.1% 2-mercaptoethanol or 2mM-dithiothreitol results in the complete recovery of activity. It is noteworthy that the reaction with 5,5'-dithiobis-(2-nitrobenzoate) does not proceed readily beyond this modification of four groups/molecule. When the reaction is carried out in 8M-urea we observe eight reactive groups/molecule.

Results with other thiol reagents are rather different. The enzyme at a concentration of 1 mg/ml (5 $\mu$M) is quite insensitive to 50 $\mu$M-iodoacetate. On the other hand, as the progress curves in Fig. 4 show, the enzyme is inhibited by reaction with disulfiram and iodoacetamide. The reactions are complete in about 5h. When the enzyme is incubated with various initial concentrations of disulfiram or iodoacetamide at either pH7.0 or pH8.0 and the residual activity at 5h is measured, curves as in Fig. 5 result. It is evident that enzyme treated with an excess of either of these reagents retains about 50% of its initial activity. This could be due to the fact that only two of the four essential thiol groups have reacted. When enzyme treated with excess of disulfiram or iodoacetamide is subsequently treated with 5,5'-dithiobis-(2-nitrobenzoate), only 2mol of thionitrobenzoate/mol of enzyme is released and the residual activity is lost.

The results with disulfiram and iodoacetamide indicate that the four thiol groups which react with 5,5'-dithiobis-(2-nitrobenzoate) may be separated into two groups. Whether this is because of slight differences in the amino acid sequence of the poly-peptide chains, or because of differences in the con-formation of the subunits within the tetrameric enzyme, is unknown. The results, however, suggest the four active centres on the enzyme may not be identical. The same observation might be true for the $F_2$ isoenzyme of aldehyde dehydrogenase from horse liver. Eckfeldt et al. (1976) reported that the enzyme is completely inactivated by p-chloromercuri-benzoate but is only 45% inhibited by disulfiram.

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