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Some properties of pig kidney-cortex aldehyde reductase

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Aldehyde reductase was purified from pig kidney cortex to homogeneity by a new procedure. The molecular weight of the enzyme was estimated by sedimentation equilibrium to be 43 700 and by gel electrophoresis in the presence of sodium dodecyl sulphate to be 41 700. The enzyme is clearly a monomer. The enzyme preparation contained no significant quantities of zinc, manganese or copper and had no essential histidine or thiol groups. Changes in the absorption and fluorescence spectra of NADPH were observed on formation of the enzyme–NADPH complexes. Large changes in the fluorescence spectra were also observed in the presence of sodium barbitone or Warfarin. These changes were used as the basis of active-site titrations, which showed that the enzyme had one active site per molecule. The dissociation constants of NADPH and $NADP^+$ from binary complexes with the enzyme were estimated in spectrophotometric titrations.

Aldehyde reductase (EC 1.1.1.2) is responsible for $NADP^+$-linked alcohol–aldehyde interconversions and has been purified from several sources. However, only the major rat liver isoenzyme and a human liver enzyme have been characterized in any detail (Wermuth *et al.*, 1977; Felsted *et al.*, 1974). Pig kidney aldehyde reductase has previously been purified by two groups (Bosron & Prairie, 1972; Flynn *et al.*, 1975; Davidson & Flynn, 1979*a*), and, though its substrate specificity (Bosron & Prairie, 1972), stereospecificity of hydrogen transfer (Flynn *et al.*, 1975) and the characteristics of an essential arginine residue (Davidson & Flynn, 1979*b*) have been investigated, many of its physicochemical properties have not been fully studied. The purpose of the present work is to describe the results of experiments concerning the molecular weight of the enzyme, its subunit structure and coenzyme-binding capacity and its reactivity towards several group-specific reagents.

Experimental

Glass-distilled water was used throughout. For experiments involving metal analysis, water was deionized by passage through an Elgastat deionizer (Elga Products, Lane End, Bucks., U.K.). $NADP^+$ and NADPH were purchased from Boehringer Corp., London W.5, U.K. $NADP^+$ was further purified by chromatography on DEAE-cellulose (Dalziel & Dickinson, 1966; Dickinson & Engel, 1977). All substrates were of the highest purity available and were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., except for pyridine-3-methanol (3-hydroxymethylpyridine), which was purchased from Aldrich Chemicals, Poole, Dorset, U.K. All other chemicals were analytical-regent grade where available and were bought from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K.

Enzyme assays

Routine assays were performed at 25°C and 340nm in 1ml of a solution containing 0.18mmol of sodium phosphate buffer, pH 7, 70nmol of NADPH and 0.6μmol of *p*-nitrobenzaldehyde. The *p*-nitrobenzaldehyde was added in 0.01ml of ethanol and the reaction was initiated by the addition of a suitable amount of enzyme. Ethanol at concentrations of less than 10% (v/v) did not affect activity determinations. One unit of enzyme activity was defined as the amount of enzyme producing 1μmol of $NADP^+$/min under the above assay conditions.

Protein concentrations

In the preparation of aldehyde reductase, protein concentration was determined spectrophotometrically by assuming a specific absorption coefficient at 280nm of $A^{1\%}_{1cm} = 10$. For pure kidney-cortex aldehyde reductase a specific absorption coefficient

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based on dry-weight measurements of $A^{1\%}_{1cm} = 11$ was used.

Determination of coenzyme concentrations

NADPH concentrations were estimated spectrophotometrically at 340nm by using a molar absorption coefficient $\varepsilon = 6.22 \times 10^3$ litre·mol^{-1}·cm^{-1}. $NADP^+$ was assayed enzymically at 25°C and 340nm with aldehyde reductase. The assay mixture (3ml) contained glycine/NaOH buffer, pH 10 (0.26mmol), glycerol (1.3mmol) and $NADP^+$ (0.15–0.3μmol).

Determination of aldehyde concentration

Aldehydes were assayed routinely at 25°C and 340nm by using aldehyde reductase. The assay mixture contained sodium phosphate buffer, pH 7 (0.26mmol), NADPH (0.2μmol) and aldehyde (0.06–0.1μmol). The reaction was allowed to reach completion and the concentration of aldehyde was found from the decrease in A_{340} of NADPH.

Purification of pig kidney-cortex aldehyde reductase

The kidneys were obtained from a local slaughterhouse and were from animals killed the same day. Kidneys were either used immediately or stored at −15°C overnight, which resulted in no loss of activity. All the following procedures were carried out at 0–4°C unless otherwise stated.

The kidney medulla was discarded and 2000–2400g of kidney cortex was blended in 400g portions for 2min each with 600ml of 0.1M-sodium phosphate buffer, pH 7.4, containing 0.2mM-EDTA.

After centrifugation the supernatant was readjusted to pH 7.0 with 2M-NaOH, and $(NH_4)_2SO_4$ (176g/litre of extract) was added. The precipitate was collected by centrifugation and discarded. More $(NH_4)_2SO_4$ (127g/l of extract) was then added, the precipitate was collected and redissolved in a minimum volume of 5mM-sodium phosphate buffer, pH 7, and was then dialysed exhaustively against the same buffer. After dialysis, protamine sulphate (5% in 5mM-sodium phosphate buffer, pH 7) was added slowly to the protein solution, which was then left for 30min. The precipitate was collected by centrifugation and discarded.

To the resulting solution, 700ml of calcium phosphate gel (30mg/ml) was slowly added and after 5min the gel was removed by centrifugation. The protein solution was then concentrated by precipitating the enzyme activity by addition of $(NH_4)_2SO_4$ (516g/litre); the precipitate was redissolved in 5mM-sodium phosphate buffer, pH 7.4, and the solution dialysed against the same buffer. After dialysis, the solution was applied to a DE-23 DEAE-cellulose column (40cm × 5cm) and enzyme activity was eluted with 30–40mM buffer by using a 2-litre linear gradient from 5mM- to 100mM-phosphate buffer, pH 7.4. Active fractions were precipitated by dialysis overnight against 50mM-sodium phosphate buffer, pH 7, containing enough $(NH_4)_2SO_4$ to give a final concentration inside and outside the dialysis bag of 516g/litre. The precipitate was redissolved in 5mM-sodium phosphate, pH 6.5, and dialysed.

The enzyme solution was then allowed to reach room temperature (15–23°C) and applied to a column of CM-32 CM-cellulose (20cm × 1.5cm) previously equilibrated at room temperature with 5mM-sodium phosphate buffer, pH 6.5. Activity was eluted by washing with 5mM-sodium phosphate buffer, pH 6.5, with the enzyme being slightly retarded by the column matrix and eluted just after the main bulk of the non-adsorbed protein. The enzyme activity was concentrated as described above and dialysed against 40mM-sodium phosphate buffer, pH 7.0. The enzyme solution was then applied to a column of Sephadex G-75 (90cm × 3cm) equilibrated and eluted with the same buffer. Active fractions were precipitated with $(NH_4)_2SO_4$ as described above and dialysed against 44mM-sodium phosphate buffer, pH 7.

Electrophoresis

Polyacrylamide-gel electrophoresis was performed by the method of Clarke (1964) in 10% gels and using 5mM-Tris/50mM-glycine buffer adjusted to pH 8.4 with HCl. Electrophoresis was carried out at 4°C and with a current of 2mA/gel for 2h. Gels were stained for protein with Coomassie Brilliant Blue R-250. Activity was revealed by incubating the gels in a stain, 10ml of which contained $NADP^+$ (10mg), Nitro Blue Tetrazolium (5mg), phenazine methosulphate (0.1mg) and glycerol (100μmol) in 10mM-glycine/NaOH buffer, pH 10.

Isoelectric focusing

Isoelectric focusing on polyacrylamide gels was carried out as described by Dickinson & Berrieman (1979).

Analytical ultracentrifugation

Short-column sedimentation-equilibrium experiments were performed at 25°C in an M.S.E. analytical ultracentrifuge equipped with a u.v. scanner. Rotor speeds of 13100rev./min were used with initial enzyme concentrations of 0.36mg/ml in 50mM-sodium phosphate buffer, pH 7, containing 10% glycerol. The density of the buffer at 25°C was found by pycnometry. Under the conditions used the enzyme was stable for the duration of the experiment.

The sedimentation coefficient of aldehyde reductase was determined at two different concentrations in sedimentation-velocity experiments at 25°C and 49000rev./min.

Results and discussion

A typical purification of the enzyme is summarized in Table 1. The overall yield and purification are quite reproducible. On some occasions there was a 30% activation of the enzyme after the protamine sulphate step. Chromatography on CM-cellulose was carried out at room temperature (15–25°C), since in the cold (0–4°C) 95% of the enzyme activity was bound so firmly to the cellulose that 0.2M-sodium phosphate buffer, pH6.5, could not elute it. The other 5% of the enzyme activity came through in the initial wash with 5mM-phosphate buffer. At room temperature (15–25°C) the enzyme was eluted from the column by 5mM-sodium phosphate buffer, pH6.5, but activity emerged after the main bulk of eluted protein. This type of behaviour has been observed with the $NADP^+$-dependent dehydrogenase, ox heart mitochondrial isocitrate dehydrogenase (MacFarlane *et al.*, 1977), but apparently not with aldehyde reductases from other sources (Kawalek & Gilbertson, 1976; Tulsiani & Touster, 1977). It might prove possible to exploit the temperature-sensitive affinity of the enzyme for CM-cellulose as the basis for a more efficient purification procedure. Davidson & Flynn (1979*a*) have described a new procedure using Blue Dextran–Sepharose 4B which gave them an 18-fold purification with no loss of activity, and Whittle & Turner (1978) have used Procion Red to great effect in aldehyde reductase purification.

The final product of the purification in Table 1 is stable for 2 or 3 weeks when stored at 0–4°C in 44mM-sodium phosphate buffer, pH7, and for 2–3 months when stored as an $(NH_4)_2SO_4$ precipitate. Aldehyde reductase has a specific activity of 21units/mg in the standard assay, based on an $A^{1\%}_{1cm} = 11$ at 280nm from dry-weight measurements. It was found from the dry-weight experiments that 1.5ml of a solution with $A^{1cm}_{280} = 9.61$ when dried in a vacuum oven at 60°C over $CaCl_2$ weighed 13.1mg at constant weight. The dry-weight value for $A^{1\%}_{1cm} = 11$ that corresponds to a molar absorption coefficient at 280nm of $48000\,M^{-1}\cdot cm^{-1}$ is to be compared with the value of $113200\,M^{-1}\cdot cm^{-1}$ found by Flynn *et al.* (1975). Wermuth *et al.* (1977), on the other hand, have found that the human liver enzyme has an absorption coefficient at 280nm of $54000\,M^{-1}\cdot cm^{-1}$, which is similar to our value for the pig kidney enzyme.

Enzyme purified by our procedure had a 5–10% higher specific activity than the preparations of Bosron & Prairie (1972) and Davidson & Flynn (1979*a*) when assayed by their methods. These differences may not be significant, but the overall yield seems to be substantially better. The present procedure was developed to allow purification of the enzyme from kilogram quantities of starting material, in contrast with that of Bosron & Prairie (1972) where, with the early use of chromatographic steps, the procedure is better adapted to smaller quantities. With the earlier procedure of Flynn *et al.* (1975), which involved larger quantities of starting material, we could only obtain enzyme with specific activities of about 50% of that obtained by Bosron & Prairie (1972). This finding, together with the differences in absorption coefficient and amino acid composition found by Morpeth (1979) between our preparation and that of Flynn *et al.* (1975), throws doubt on the quality of the earlier preparation.

The enzyme appeared to be homogeneous as judged by strict linearity of the sedimentation-equilibrium plots with data covering the whole cell (Fig. 1) and by the presence of only one protein band on polyacrylamide gels in the presence of sodium dodecyl sulphate.

On isoelectric focusing with Ampholine gels, usually only one protein and activity band (pI6.9) was seen, though occasionally a second very faint activity band (pI6.8) was visible. Crude kidney extract on gel isoelectric focusing in the presence of 10mM-pyrazole to inhibit alcohol dehydrogenase gave only one activity band. This suggests that the enzyme purified in this study is the only major isoenzyme present in kidney.

Previous workers with pig kidney aldehyde reductase have tended to base their estimates of the molecular weight on electrophoresis in the presence

Table 1. *Purification of pig kidney-cortex aldehyde reductase*

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Sp. activity (units/mg)	Yield (%)	Purification (-fold)
Extract	2000	79000	3940	0.05	100	1
Ammonium sulphate	500	37000	3740	0.1	95	2
Protamine sulphate	600	19000	4730	0.25	120	4
Calcium phosphate gel	100	7920	3900	0.6	99	12
DEAE-cellulose	12	394	2360	6	60	120
CM-cellulose	6	131	1970	15	50	300
Sephadex G-75	3	75*	1580	21	40	420

* Based on $A^{1cm}_{280,1\%} = 11.0$.

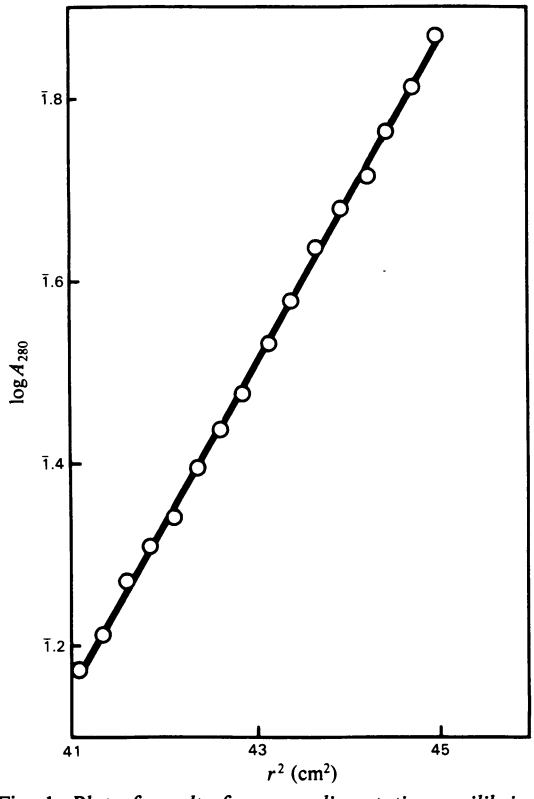

Fig. 1. *Plot of results from a sedimentation-equilibrium experiment with purified aldehyde reductase*
The experiment was performed at 25°C with enzyme (initially 0.4 mg/ml) dissolved in sodium phosphate buffer, pH 7, and with a rotor speed of 13 100 rev./min. The molecular weight was obtained from the slope of the graph as described in the text.

of sodium dodecyl sulphate and on gel-filtration experiments. Bosron & Prairie (1972) reported the results of a sedimentation-equilibrium experiment, which gave a mol.wt. of 30 200, but they did not present detailed information.

In short-column sedimentation-equilibrium experiments carried out in this study, linear plots were seen over the whole cell (Fig. 1). By using a partial specific volume of 0.73 ml/g found by pycnometry, a molecular weight of 43 700 ± 900 was obtained. There is further evidence (see below) that this is an acceptable estimate of the molecular weight. Firstly the reactions of thiol groups with 5,5′-dithiobis-(2-nitrobenzoic acid) and of histidine residues with diethyl pyrocarbonate suggest minimum mol.wts. of 43 700 and 41 200, respectively. Spectrophotometric titration suggests that there is one active site per 49 100 daltons of protein, and fluorimetric titration in the presence of sodium barbitone one active site per 46 500 daltons of protein. Since the difference in absorbance between enzyme–NADPH and NADPH is not very large (see below), spectrophotometric titration would not be expected to give a precise estimate. By the technique of Weber & Osborn (1969), of electrophoresis in the presence of sodium dodecyl sulphate, the mobility of the band corresponded to a molecular weight of 41 700. This agrees with the estimates of Bosron & Prairie (1972), Flynn *et al.* (1975) and Davidson & Flynn (1979*a*) by this method, and shows that the enzyme is a monomer.

Sedimentation-velocity experiments were also carried out at low (0.4 mg/ml) and high (4.8 mg/ml) protein concentrations. From the experiment at 0.4 mg/ml a value of $s_{20,w} = 3.9$S was found. The experiment at 4.8 mg/ml protein concentration gave $s_{20,w} = 3.4$S. Human liver aldehyde reductase and rat liver aldehyde reductase are the only other aldehyde reductases whose molecular weights have been investigated by sedimentation-equilibrium methods (Wermuth *et al.*, 1977; Felsted *et al.*, 1974), and the values found were 36 200 with an s value of 2.9 S, and 38 800 respectively. By using Sephadex G-75 and Bio-Gel P100 columns calibrated in 44 mM-sodium phosphate buffer, estimates of 28 500 and 35 000 were found for the molecular weight of our enzyme. When the Sephadex G-75 column was recalibrated in the above buffer containing 0.5 M-NaCl, the apparent mol.wt. increased to 36 000. A similar effect has been noted by Felsted *et al.* (1974) with rat liver aldehyde reductase. The results indicate interaction between enzyme and column matrix and may be connected with the high affinity of the enzyme for CM-cellulose under certain conditions. Our results suggest that 43 700 is a reliable estimate for the molecular weight of pig kidney aldehyde reductase. Lower estimates found by previous workers for pig kidney-cortex aldehyde reductase of 30 000 (Bosron & Prairie, 1972) and 33 000 (Flynn *et al.*, 1975; Davidson & Flynn, 1979*b*) on the basis of gel filtration may be in error because of interaction between the enzyme and the gel-filtration matrix.

The maximum zinc content of aldehyde reductase measured by atomic absorption spectrophotometry was 0.03 atom of zinc/molecule of enzyme. These amounts are obviously of no catalytic or structural importance to the enzyme. There was no measurable copper or manganese content.

The results are interesting in view of the fact that alcohol dehydrogenases, which catalyse a similar reaction, contain significant quantities of zinc. Horse liver alcohol dehydrogenase contains two zinc atoms per subunit, one of catalytic and one of structural importance (Bränden *et al.*, 1975). Yeast alcohol dehydrogenase contains 1–2 atoms of zinc/subunit (Bränden *et al.*, 1975; Dickinson & Berrieman, 1977), with some zinc atoms having a catalytic role.

The u.v.-absorption spectrum of aldehyde reductase is that of a normal protein, and a ratio $A_{280}/A_{260} = 2$ suggests that there is no bound nucleotide. When the enzyme binds to NADPH, however, there is a change in the nucleotide absorption spectrum similar to that reported by Wermuth *et al.* (1977) for the human liver enzyme. The absorption maximum of the coenzyme on binding to aldehyde reductase moves from 340 to 350nm, with no change in the absorption coefficient. Thus a simple red-shift of the absorption maximum has occurred. Fisher *et al.* (1969) have observed that dehydrogenases with a specificity for the 'B' hydrogen atom of the coenzyme tend to induce a red-shift on binding to the coenzyme. On the other hand, those with a specificity for the 'A' hydrogen atom induce a blue-shift. Aldehyde reductase appears to be an exception to this 'rule' since Flynn *et al.* (1975) have shown that the enzyme uses the 'A' hydrogen atom of NADPH.

The hyperchromic effect at 373nm of NADPH binding to the enzyme allows the process to be studied spectrophotometrically. Titrations of aldehyde reductase with NADPH at pH7 in sodium phosphate buffer indicate that there is 0.86 binding site for NADPH/molecule of enzyme of mol.wt. 43700 (Fig. 2). This compares favourably with the stoicheiometry for the human liver enzyme of 0.85 NADPH-binding site/molecule (Wermuth *et al.*, 1977), also found by spectrophotometric titrations. The initial slope of the titration curve indicates a molar absorption coefficient for the enzyme–NADPH complex at 375nm of $3.95 \times 10^3 M^{-1} \cdot cm^{-1}$. From this value an estimate of the dissociation constant of the enzyme–NADPH complex of $1.2 \pm 0.4 \mu M$ (S.E.M.) was found at 25°C in 44mM-sodium phosphate buffer, pH7 (Table 2). By addiing $NADP^+$ to the mixtures to compete with NADPH for the enzyme, an estimate for the dissociation constant of the enzyme–$NADP^+$ complex of $3.3 \mu M$ was obtained (Fig. 3).

When the dissociation constant ($K_{E \cdot NADPH}$) of the enzyme–NADPH complex was measured at high protein concentrations (> $100 \mu M$-enzyme), much higher results, in the order of 10–$60 \mu M$ for $K_{E \cdot NADPH}$, were found. No explanation for this phenomenon can presently be offered.

The fluorescence changes that accompany the binding of NADPH to enzyme are very small, with only a very small blue-shift of the coenzyme peak, but there is a significant change in the fluorescence

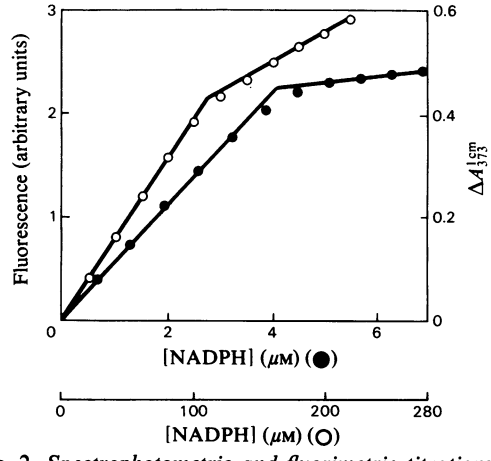

Fig. 2. *Spectrophotometric and fluorimetric titrations of aldehyde reductase with NADPH*

The titrations were performed at 25°C with enzyme solutions in sodium phosphate buffer, pH7. Spectrophotometric titrations (○) were done with 5.3mg of enzyme/ml and fluorimetric titrations (●) with 0.188mg of enzyme/ml and $100 \mu M$-sodium barbitone.

Table 2. *Spectrophotometric determination of the dissociation constant for the enzyme–NADPH binary complex*

All results are for 25°C in sodium phosphate buffer, pH7, *I* 0.1.

ΔA_{375}	[Enzyme] (μM)	[NADPH] (μM)	NADPH bound (%)	Enzyme bound (%)	K_D (μM)
0.043	7.7	16.3	40.5	85.7	1.68
0.027	7.8	8.2	69	73	0.92
0.101	15	39.5	37	96	0.93
0.065	15.4	20.2	66.7	87.5	0.96
0.09	25.9	25.1	83.3	72	1.0
0.163	29.4	47.9	48	94.6	1.15
0.128	30.1	39.5	67	89	1.5
0.175	48	46.7	91.0	88.3	0.57
0.29	53	101	51.0	97	1.54
0.23	54.2	69	72	91	1.8
0.138	55.5	35.4	97	62	0.47

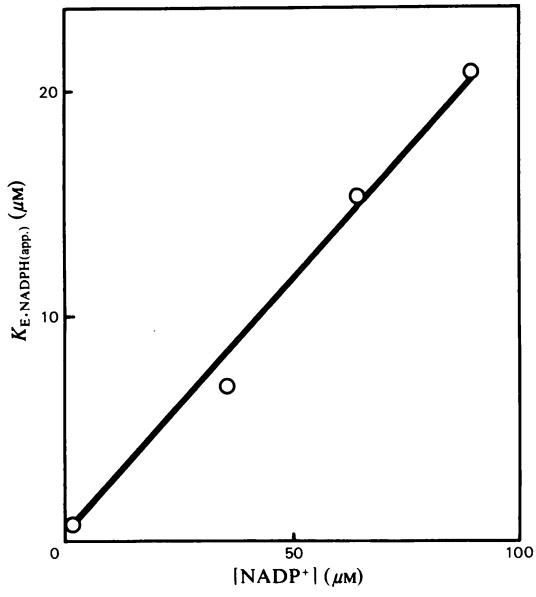

Fig. 3. *Variation of $K_{E\cdot NADPH(app.)}$ with the NADP$^+$ concentration, at 25°C and pH 7*

Values of $K_{E\cdot NADPH(app.)}$ were determined as described in the text in the presence of various concentrations of purified salt-free NADP$^+$. The enzyme concentration in all the experiments was 35.2μM and an estimate of the $K_{E\cdot NADP^+}$ was obtained from the slope ($K_{E\cdot NADPH}/K_{E\cdot NADP^+}$) and intercept ($K_{E\cdot NADPH}$) of the plot. All the experiments were done at 25°C in sodium phosphate buffer, pH 7.

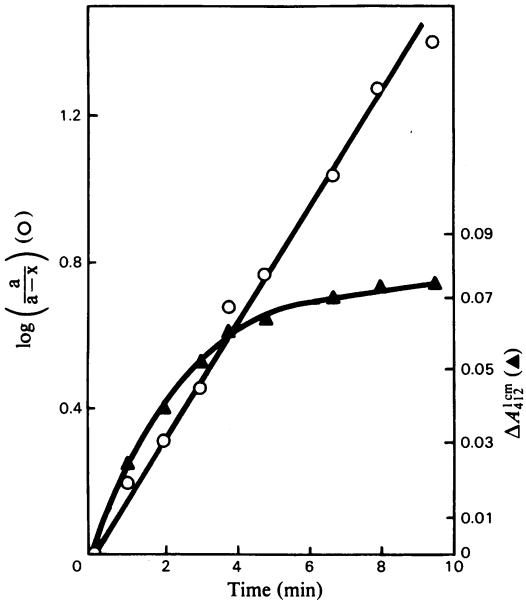

Fig. 4. *Modification of a non-essential thiol group in aldehyde reductase by 5,5′-dithiobis-(2-nitrobenzoic acid)*

The concentration of aldehyde reductase was 5.5μM and reagent 250μM. The reaction was performed at pH 8.0 in 0.44M-phosphate buffer and followed spectrophotometrically at 412nm (▲). The symbols O show the first-order plot derived from the progress curve at 412nm.

polarization on binding (Morpeth, 1979). Measurements of NADPH fluorescence were also conducted in the presence of enzyme together with 100μM-sodium barbitone, Warfarin or ethacrynic acid. These compounds are powerful inhibitors of the enzyme, but are not competitive towards NADPH (Morpeth, 1979). It was thought that they might form tight ternary complexes with enzyme and NADPH. In the presence of 100μM-sodium barbitone, the fluorescence of the enzyme–NADPH complex increases some 5-fold at the emission maximum of 450nm, indicating the formation of an enzyme–barbitone–NADPH ternary complex. Warfarin also forms a ternary complex and gives a 20% enhancement at 450nm when the high fluorescence of the compound is corrected for. No increase in the fluorescence of the enzyme–coenzyme complex was seen in the presence of 100μM-ethacrynic acid.

By using the large increase in fluorescence in the presence of sodium barbitone it is possible to perform active-site fluorescence titrations, and we found that 0.94 molecule of ternary complex per molecule of enzyme was formed, assuming a mol.wt. of 43700 (Fig. 2). This result is probably more precise than that found from the spectrophotometric coenzyme-binding titration, because there is a much larger change in signal when free coenzyme starts appearing in the mixture at the end of the titration.

None of the thiol reagents iodoacetate, iodoacetamide or disulfiram had any effect on the activity of aldehyde reductase at pH 6, 7 or 8 and 25°C in sodium phosphate buffer. The presence of 70μM-NADPH or 0.2mM-NADP$^+$ in the reaction mixture with the thiol reagents made no difference to the results. Even after 1h at 25°C and pH 7 with iodoacetate (1mM) or iodoacetamide (1mM), the enzyme retained its full native activity.

Aldehyde reductase reacted with 5,5′-dithiobis-(2-nitrobenzoate) at 25°C in 44mM-sodium phosphate buffer, pH 8. The reaction was followed by the production of the thionitrobenzoate ion by using a molar absorption coefficient at 412nm of $13.6\times10^3\,M^{-1}\cdot cm^{-1}$ (Ellman, 1959). It was estimated that one thiol group was modified per molecule of enzyme, with a second-order rate constant of $0.48\,M^{-1}\cdot s^{-1}$ (Fig. 4). In 8M-urea, with the above conditions, 3 cysteine residues/molecule reacted with the reagent.

When aldehyde reductase was modified with high concentrations of 5,5′-dithiobis-(2-nitrobenzoate) (6mM) and 20μl taken for assay with *p*-nitrobenzaldehyde, then the enzyme with approx. 1 thiol group/molecule modified apparently retained only a few per cent of its native activity. However, if the enzyme was modified with a lower concentration of reagent (0.25mM) and 5μl was taken for assay when the reaction had stopped, with again approx. 1 thiol group/molecule modified, then the enzyme apparently retained 90% of its native activity. This phenomenon is apparently due to carry-over of 5,5′-dithiobis-(2-nitrobenzoate) into assays and to this compound acting as a reversible inhibitor separately from its thiol-modifying functions, possibly owing to the presence of two carboxy groups in the molecule. Carboxy groups are a feature of good substrates and inhibitors of aldehyde reductase. In experiments where 5,5′-dithiobis-(2-nitrobenzoate) was included in assays and the reaction was initiated by addition of enzyme, it was found that the inhibition by 5,5′-dithiobis-(2-nitrobenzoate) was non-competitive towards both *p*-nitrobenzaldehyde and NADPH, with K_i approx. 27μM (Fig. 5).

Aldehyde reductase lost no activity on incubation with 0.5mM-diethyl pyrocarbonate at pH6 or 7 and in the absence or presence of reduced or oxidized coenzyme. However, by following the reaction at 240nm and observing the formation of the *N*′-carbethoxyhistidine it was seen that 1.06 histidine residues per 43700 daltons of aldehyde reductase were modified. The absorption coefficient of *N*′-carbethoxyhistidine at 240nm is $3600\,M^{-1}\cdot cm^{-1}$ (Holbrook & Ingram, 1973). Under identical conditions yeast alcohol dehydrogenase rapidly lost all its native activity and six histidine residues per subunit were modified. The second-order rate constant for the reaction between diethyl pyrocarbonate and aldehyde reductase was $1.1\,M^{-1}\cdot s^{-1}$, and was calculated from the pseudo-first-order constant and the concentration of diethyl pyrocarbonate. This rate constant is less than that for the reaction of free histidine ($k = 24\,M^{-1}\cdot s^{-1}$; Holbrook & Ingram, 1973), and much lower than that found with the essential histidine residues of yeast alcohol dehydrogenase and lactate dehydrogenase ($k = 200\,M^{-1}\cdot s^{-1}$) (Holbrook & Ingram, 1973; Dickenson & Dickinson, 1975). The rate constant is very similar to that for reaction of one of the non-essential histidine residues in lactate dehydrogenase in the presence of coenzyme.

The lack of zinc and the absence of essential thiol and histidine residues in aldehyde reductase are points of considerable interest when the enzyme is contrasted with the alcohol dehydrogenases, which catalyse very similar reactions. Both horse liver and yeast alcohol dehydrogenase contain structural and catalytic zinc moieties (Bränden *et al.*, 1975) and

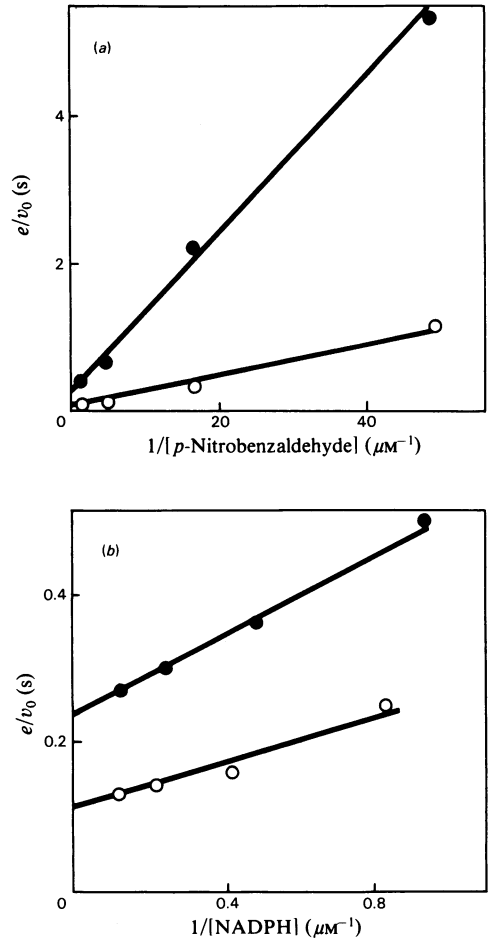

Fig. 5. *Inhibition of aldehyde reductase by 5,5′-dithiobis-(2-nitrobenzoate)*

Experiments were done in sodium phosphate buffer, pH7, *I* 0.1 at 25°C. (*a*) Activity of aldehyde reductase in the presence (●) and absence (○) of 100μM-5,5′-dithiobis-(2-nitrobenzoate) at a constant NADPH (70μM) concentration. The K_i determined from the change in slope was 26.0μM and that calculated from the change in the intercept 30.0μM. (*b*) Activity of aldehyde reductase in the presence (●) and absence (○) of 25μM-5,5′-dithiobis-(2-nitrobenzoate) at a constant *p*-nitrobenzaldehyde (1mM) concentration. The inhibition constants for the slope and intercept were calculated to be 28.4 and 25μM respectively.

essential thiol residues (Harris, 1964), and the yeast enzyme also contains one histidine residue/subunit directly implicated in the reaction mechanism (Dickenson & Dickinson, 1977). What residues might be involved in the mechanism of aldehyde reductase must be the subject of further work. A recent paper (Branlant & Biellmann, 1980) on pig liver aldehyde reductases has reported the lack of

zinc and essential thiol groups in that enzyme, and it is also shown that the liver reductase I is very similar to the kidney-cortex enzyme. One interesting point of difference, however, is that the kidney enzyme as reported here reacts with 5,5'-dithiobis-(2-nitrobenzoate) to the extent of one residue/molecule, whereas the liver reductase I apparently is inert. For both enzymes the activity is insensitive to this reagent and approx. 3 cysteine residues/molecule react under denaturing conditions.

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