

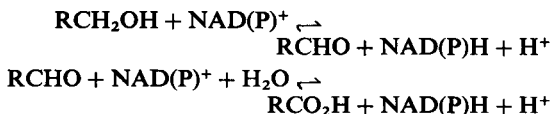
## Rapid Purification and Properties of Potassium-Activated Aldehyde Dehydrogenase from *Saccharomyces cerevisiae*

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A method for the purification of yeast  $K^+$ -activated aldehyde dehydrogenase is presented which can be completed in substantially less time than other published procedures. The enzyme has a different *N*-terminal amino acid from preparations previously reported, and other small differences in amino acid content. These differences may be the result of differential proteolytic digestion rather than a different protein *in vivo*. A purification step involves the biospecific adsorption on affinity columns containing immobilized nucleotides in the absence of the substrate aldehyde. Direct binding studies with the coenzyme in the absence of aldehyde reveal 4 NAD sites per tetrameric molecule, each with a dissociation constant of  $120 \mu M$ . These results conflict with properties of preparations previously reported and may conflict with kinetic models that have aldehyde as the leading substrate. Binding to Blue Dextran affinity columns suggests the presence of a dinucleotide fold in common with other dehydrogenases and kinases.

Ethanol metabolism involves two oxidative steps, both catalysed by nicotinamide nucleotide-dependent dehydrogenases:



In the past 30 years extensive work has been done on alcohol dehydrogenase, but comparatively little has been done on aldehyde dehydrogenase. Part of the explanation for this may be a number of difficulties associated with its purification. It was not until 1967 that a homogeneous preparation was reported with the isolation of the  $K^+$ -activated enzyme from yeast (Steinman & Jakoby, 1967). Difficulties in the purification of this enzyme are its instability in the absence of high concentrations of  $K^+$  (Sorger & Evans, 1966), its increasing instability with purification in the absence of high concentrations of polyhydric alcohol (Bradbury & Jakoby, 1972), and its modification by endogenous proteinases (Clark & Jakoby, 1970*a,b*). For these reasons we have developed a rapid method of purification for this enzyme by using an affinity-chromatographic step, which results in higher yields of enzyme activity. Differences are revealed between this preparation and those previously published (Sorger & Evans,

1966; Steinman & Jakoby, 1967; Clark & Jakoby, 1970*a,b*; Bradbury & Jakoby, 1972).

### Materials and Methods

Baker's yeast (N.G. & S.F., British Fermentation Products, London E.C.2, U.K.) was purchased from a local baker, stored at  $4^\circ C$  and used within 24h. The yeast is produced commercially from an anaerobic axenic culture by bulk fermentation (for 12h) with extensive aeration in a molasses wort supplemented with inorganic salts. In addition, yeast was purchased from a U.S.A. distributor of Anheuser-Busch, St. Louis, MO, U.S.A., and handled as above. Sepharose 6B CL 2,3-dibromopropanol cross-linked CH-Sepharose 4B (with 6 carbon spacer and free carboxyl group), 5'-AMP-Sepharose 4B, DEAE-Sephadex A-50, Sephadex G-25 and Blue Dextran were purchased from Pharmacia (G.B.), London W.5, U.K. Whatman DE-23 ion-exchange DEAE-cellulose was purchased from Whatman Biochemicals, Maidstone, Kent, U.K. NAD<sup>+</sup> (grade III), NAD<sup>+</sup> (grade AA), 5'-AMP, 2-mercaptoethanol,  $\alpha$ -thioglycerol, phenylmethanesulphonyl fluoride, phenazine methosulphate, Nitro Blue Tetrazolium, glutamate dehydrogenase, thyroglobulin, catalase (bovine), ovalbumin, cytochrome *c* (type 2), bovine serum albumin (A4378) and carbonic anhydrase were purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.  $\beta$ -Galactosidase, aldolase and muscle lactate dehydrogenase were supplied by Boehringer Corp. (London) Ltd., London W.5, U.K. Standard proteins

Abbreviations used: SDS, sodium dodecyl sulphate; Dns-, 5-dimethylaminonaphthalene-1-sulphonyl-.

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for SDS/polyacrylamide-gel electrophoresis were prepared from a Boehringer protein calibration kit, Combithek-size 1. Reagents for electrophoresis were purchased from Kodak, Kirkby, Liverpool, U.K. Reference Dns-amino acids, polyamide thin-layer sheets and AnalaR grade ethanediol were supplied by BDH Chemicals, Poole, Dorset, U.K. 3-Methyl-2-benzothiazolone hydrazone was purchased from Hopkin and Williams, Chadwell Heath, Essex, U.K. Nicotinamide- $[\text{carbonyl-}^{14}\text{C}]$ adenine dinucleotide (59.2 mCi/mmol) and nicotinamide- $[\text{U-}^{14}\text{C}]$ adenine dinucleotide (271 mCi/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Insta-Gel was from Packard Nuclear Chemical Supplies, Caversham, Berks., U.K. Acetaldehyde was redistilled and stored at 18°C as a 2M solution. All other reagents were of AnalaR grade where commercially available.

#### Enzyme assay

Enzyme activity was measured at 25°C by following the rate of reduction of NAD<sup>+</sup> by observing the increase in  $A_{340}$  in a Pye-Unicam SP.1800 spectrophotometer. A standard reaction mixture contained in a final volume of 2.5 ml: 2 mM-acetaldehyde, 0.5 mM-NAD<sup>+</sup>, 0.1 M-Tris/HCl buffer, pH 8.0, 10 mM-2-mercaptoethanol and 0.1 M-KCl. Reaction was begun by the addition of 0.1 ml or less of enzyme to an otherwise complete reaction mixture. Under these conditions a unit of enzyme activity is the amount of enzyme producing 1  $\mu\text{mol}$  of NADH/min.

#### Protein determination

Samples containing 20–100  $\mu\text{g}$  of protein were precipitated with 4 vol. of 25% (w/v) trichloroacetic acid. The precipitate was resuspended in 1 M-NaOH and protein measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

#### Aldehyde determinations

Aldehydes were measured by the colorimetric method of Sawicki *et al.* (1961). To 0.5 ml of unknown sample or standard aldehyde solution (5–100  $\mu\text{g}$ /100 ml) was added 0.5 ml of 0.4% (w/v) 3-methyl-2-benzothiazolone hydrazone in a ground-glass stoppered test tube. The solution was placed in a water bath at 98.5°C for 3 min. The mixture was cooled to 25°C in an ice-water bath, then 2.5 ml of 0.2% (w/v) FeCl<sub>3</sub> was added. After 5 min the volume was adjusted to 10 ml by the addition of 6.5 ml of acetone, and the  $A_{635}$  or  $A_{662}$  measured.

Aldehyde was also determined by its enzymic reactivity with aldehyde dehydrogenase in the presence of 0.1 M-KCl and 1 mM-NAD<sup>+</sup>. Fraction V enzyme (see purification schedule below) was

equilibrated by passage through a Sephadex G-25 column into 0.1 M-K<sub>2</sub>HPO<sub>4</sub> buffer, pH 8.0, and 1 mM-EDTA, both showing low 3-methyl-2-benzothiazolone hydrazone reactivity. The equilibrated enzyme (1 unit) was added to a solution containing 1 mM-NAD<sup>+</sup>, 0.1 M-K<sub>2</sub>HPO<sub>4</sub>, 1 mM-EDTA, with or without the sample, resulting in a final volume of 2.5 ml.  $A_{340}$  was monitored with time. Lactate dehydrogenase and pyruvic acid were then added to oxidize any NADH formed, to confirm the amount of recorded absorbance change relating to NADH production.

#### Polyacrylamide-disc-gel electrophoresis

Electrophoresis was performed by the method of Davis (1964) at 4°C, except that running buffer contained 1 mM-mercaptoethanol, and the stacking gel was omitted. Enzyme samples containing 25% (v/v) glycerol were layered directly on to the polyacrylamide gels. Enzyme samples not containing glycerol were first made 20% (w/v) sucrose by the addition of solid sucrose.

Gels were stained for protein by the method of Chrambach *et al.* (1967) and recorded by scanning with a Joyce-Loebl gel scanner. Gels were stained for activity by placing them into a standard reaction mixture supplemented to 25 mg of phenazine methosulphate/ml and 0.5 mg of Nitro Blue Tetrazolium/ml, from stocks freshly made up before use. After mixing, the gels were left to incubate at 23°C (2–5 min), washed with water and finally stored in 10% (w/v) trichloroacetic acid. Alternatively, enzyme activity in gels placed in standard reaction mixture was followed by monitoring the increase in  $A_{340}$  with a scanning accessory to a Gilford 240 spectrophotometer.

#### SDS/polyacrylamide-gel electrophoresis

Enzyme and known molecular-weight marker proteins were prepared for SDS/polyacrylamide-gel electrophoresis by dissociation in a boiling-water bath for 3 min in 0.01 M-Tris/hydrogen phosphate buffer, pH 7.0, 1% (w/v) SDS and 5% (v/v) mercaptoethanol, at a protein concentration of 1.6 mg/ml; 25–50  $\mu\text{g}$  of protein was used for loading 7% (w/v) polyacrylamide gels, and electrophoresis performed essentially by the method of Weber & Osborn (1969), except that running buffer consisted of 75 mM-Tris/hydrogen phosphate, pH 7.0, 0.1% (w/v) SDS and 0.1% (v/v) mercaptoethanol. Subunit molecular weight was estimated by calibrating the gels against bovine serum albumin dimer and monomer (mol.wt. 134000 and 67000 respectively), phosphorylase *a* (mol.wt. 92500), catalase (mol.wt. 60000), ovalbumin (mol.wt. 45000) and carbonic anhydrase (mol.wt. 30000) standards. Mobilities were repro-

ducible within 1.5% and were inversely proportional to logarithm (molecular weight) over the molecular-weight range 134000–30000.

#### *Sephacrose 6B CL chromatography*

The molecular weight of the undissociated protein was estimated on columns (1.75 cm × 53 cm) of Sepharose 6B CL equilibrated and run at 4°C in 0.1 M-KH<sub>2</sub>PO<sub>4</sub> (pH 7.4)/1 mM-mercaptoethanol/1 mM-MgSO<sub>4</sub>/0.125 M-NaCl buffer. Flow rate was 0.254 ml/min and fractions (1 ml) were collected. Samples were added in a volume of 1 ml. Elution profiles of proteins were monitored with an Isco dual-beam absorptiometer at A<sub>280</sub>. In addition, aldehyde dehydrogenase activity in the column effluent was monitored by the standard assay procedure. Columns were calibrated with Blue Dextran (mol.wt. 2000000), glutamate dehydrogenase (mol.wt. 1020000), thyroglobulin (mol.wt. 670000), β-galactosidase (mol.wt. 515000), catalase (mol.wt. 247000), aldolase (mol.wt. 145000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), carbonic anhydrase (mol.wt. 30000) and cytochrome *c* (mol.wt. 12300). Elution volumes were reproducible to ±2.5% and were inversely proportional to logarithm (molecular weight) over the molecular-weight range 10<sup>4</sup>–10<sup>6</sup>.

#### *Amino acid analysis*

Amino acid analysis was performed by g.l.c. of trifluoroacetylated amino acid methyl esters. Samples of freeze-dried fraction VI enzyme (see purification schedule below) were hydrolysed in triplicate for 24 or 48 h in 6M-HCl at 105°C. Samples of hydrolysates were converted into derivatives by the method of Darbre & Islam (1968) and quantitative identification was achieved on mixed silicone columns in a Pye series 104 chromatograph as described by Davy & Morris (1976).

#### *End-group analysis*

*N*-Terminal amino acid analysis was performed by the dansyl chloride procedure recommended for proteins by Gray (1967), with identification of the Dns-amino acids by two-dimensional chromatography on thin-layer polyamide sheets as described by Hartley (1970). Concentrated fraction VI enzyme (see purification schedule below) was prepared for dansylation by dialysis for 72 h against several changes of distilled deionized water. After freeze-drying, 1.5 mg samples containing approx. 25 nmol of *N*-terminal amino acid were taken for analysis.

#### *Equilibrium dialysis*

Equilibrium dialysis binding experiments were performed in a semi-micro dialysis apparatus con-

sisting of two Perspex blocks each with five matching chambers (15 mm diam. × 2 mm deep) equipped with small screw-capped inlet ports. The blocks were held together by ten wing-nut screws with a Visking 24/32 dialysis membrane suspended between the adjoining chambers. The membrane was prepared for use by boiling twice for 10 min in 1 mM-EDTA, thoroughly washing and then boiling for 10 min in distilled deionized water. The membrane was then rinsed and used. In chambers on one side of the dialysis membrane were placed 0.3 ml samples of a known concentration of labelled ligand in 'dialysis buffer', consisting of 0.1 M-Tris/HCl, pH 8.0, 20% (v/v) ethanediol, 1 M-KCl, 1 mM-mercaptoethanol, 2 mM-pyruvic acid and supplemented to 0.5 g/ml with lactate dehydrogenase also equilibrated against the dialysis buffer. The additions were made with 1 ml disposable syringes. Equal volumes of enzyme, equilibrated by passing through a column (1.25 cm × 6.8 cm) of Sephadex G-25 into dialysis buffer and supplemented to 0.5 mg/ml with lactate dehydrogenase in dialysis buffer, were placed in the chambers on the other side of the membrane, and the whole apparatus was coupled end-on to a Watson-Marlow pump with rotation at approx. 60 rev./min. Filling each chamber with 0.3 ml of solution left enough of an air-space to ensure adequate mixing across the membrane surface. All experiments were performed at 4°C. Lactate dehydrogenase (at 0.1% aldehyde dehydrogenase concentration) and pyruvic acid were added to buffer on both sides of the membrane to regenerate any NAD<sup>+</sup> reduced by the presence of endogenous aldehydes.

In the absence of enzyme but under otherwise similar conditions the time required for NAD<sup>+</sup> to reach equilibrium was determined as 48 h. In other tests no detectable enzyme activity was observed in the ligand chamber, cross contamination was not a problem, and recovery was consistent with the total volume of solution recovered, i.e. no adsorption of ligand to the membrane was observed.

Radioactive [*carbonyl*-<sup>14</sup>C]NAD<sup>+</sup> solutions were prepared by dissolving the freeze-dried radioactive material in 1 ml of 5 mM-non-radioactive NAD<sup>+</sup> (Sigma grade III). This solution was diluted 1:10 or more and supplemented with non-radioactive NAD<sup>+</sup> to make the appropriate starting solutions. After dialysis three weighed samples (approx. 85 μl) were removed from each chamber and measured for radioactivity with 10 ml of Insta-Gel supplemented with 4 ml of water, in a Packard Tri-Carb liquid-scintillation counter. Overall standard deviation in determining radioactivity in either chamber at equilibrium was 0.15%.

#### *Rate of dialysis*

Rate of dialysis binding studies were performed by the method of Colowick & Womack (1969) using a

dialysis flow cell previously described (Womack & Colowick, 1973), but containing a Perspex insert in the lower chamber to decrease its capacity to 2 ml. Visking 24/32 dialysis membrane was used as prepared above. Connections were made with minimal lengths of 1.5 mm inner diameter Teflon tubing constituting a dead-space volume between cell and fraction collector of 0.15 ml. The lower chamber was filled with the same buffer used for equilibrium dialysis and 1 ml of enzyme equilibrated with the same buffer by passage through a column (1.25 cm × 6.8 cm) of Sephadex G-25 was added to the upper chamber. A small volume of very high specific radioactivity [ $U\text{-}^{14}\text{C}$ ]NAD<sup>+</sup> was added to the enzyme solution and 3.25 ml fractions were collected at a flow rate of 9 ml/min. Under these conditions, rate of dialysis reached a steady state within 10 fractions, so that after every 10 fractions a sample of non-radioactive ligand was added. The total initial volume of radioactive ligand added was less than one-tenth the initial enzyme volume, and subsequent additions of non-radioactive ligand did not amount to more than 1% of the total initial volume. All experiments were performed at 4°C. That no artifacts were present in the system was observed from test experiments minus enzyme.

Radioactive NAD<sup>+</sup> effluent solution in fractions were prepared for counting in the same manner as in equilibrium dialysis, except that weighed samples of approx. 3 ml were used, and 2 ml of water was added with the 10 ml of Insta-Gel. Overall standard deviation in determining radioactivity in fractions of eluate was 0.3%.

#### *Preparation of immobilized ligands*

$\epsilon$ -Aminohexanoyl-NAD<sup>+</sup>-Sephacrose 4B was prepared by the method of Mosbach *et al.* (1972), by using 45 g of CH-Sephacrose 4B. Sigma-grade-AA NAD<sup>+</sup> was used. This gel was used to pack a column (2.5 cm × 25 cm) at 4°C, providing a gel volume of 125 ml. The column was equilibrated with 5–6 vol. of 45 mM-K<sub>2</sub>HPO<sub>4</sub> buffer, pH 8.0, containing 22% (v/v) glycerol, 1 mM-mercaptoethanol, 0.025% (w/v) phenylmethanesulphonyl fluoride (the 'affinity-chromatography buffer').

Blue Dextran-Sephacrose 4B was prepared by the CNBr method of Ryan & Vestling (1974). Columns were packed with gel in 10 mM-Tris/HCl buffer, pH 8.0, and equilibrated before use in an appropriate buffer. Column dimensions were 1.2 cm × 7 cm.

#### *Enzyme concentration*

Enzyme was concentrated by a combination of hollow-fibre ultrafiltration (Bio-Fiber 80 beaker; Bio-Rad Laboratories, Bromley, Kent, U.K.) and centrifuged at 3000 g through an Amicon PM 10

membrane supported on a porous plastic disc in a 50 ml centrifuge tube.

#### *Preparation of aldehyde dehydrogenase type A from Anheuser-Busch yeast*

Attempts at preparing aldehyde dehydrogenase A from Anheuser-Busch yeast were made by following the outline of Clark & Jacoby (1970a) as closely as possible. Unfortunately, a Manton-Gaulin homogenizer was not available for use. Alternatively, cell breakage was accomplished by the glass-bead method described in the rapid affinity-chromatographic purification (see below). Breakage was performed at room temperature with a yeast/glass-bead suspension in the buffer used by Clark & Jacoby (1970a), at a starting temperature of 4°C. Time of disruption was approximately the same in both procedures. Yeast in 0.75 kg lots was used, and subsequent steps were scaled appropriately. The crude extract was heat- and acid-precipitated (Clark & Jacoby, 1970a) and the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction, after dialysis, was loaded on a column (2.5 cm × 30 cm) of DEAE-Sephadex A-50 and chromatography conditions that allowed duplication of the chromatography times for the larger columns of Clark & Jacoby (1970a) were used. Chromatography buffer was prepared with Kodak 'spectro'-grade glycerol. A major peak of activity was eluted at the same ionic strength as described by Clark & Jacoby (1970a); these fractions were pooled, supplemented to 0.01% phenylmethanesulphonyl fluoride and stored at -80°C until further use. The hydroxyapatite chromatography described by Clark & Jacoby (1970a) was not reproducible in our hands.

#### *Rapid affinity-chromatographic enzyme purification*

At all stages of the purification phenylmethanesulphonyl fluoride was added to reagents immediately before use. The temperature was 4°C unless otherwise stated.

#### *Extraction procedure*

*Method I.* Yeast acetone-dried powder (200 g) prepared by the method of Stoppani & Milstein (1957) was resuspended in 1200 ml of 0.1 M-K<sub>2</sub>HPO<sub>4</sub>/1 mM-mercaptoethanol/0.024% (w/v) phenylmethanesulphonyl fluoride. The pH was adjusted to 8.6 with 1 M-KOH and the mixture stirred for 4 h. The pH was adjusted to 5.5 by the dropwise addition of 1 M-citric acid and this mixture centrifuged for 30 min at 23000 g. The supernatant is fraction I.

*Method II.* Packed yeast cells (0.7 kg) were suspended in 1.5 vol. of 0.1 M-K<sub>2</sub>HPO<sub>4</sub>/1 mM-mercaptoethanol/0.024% (w/v) phenylmethanesulphonyl fluoride. Acid-washed and oven-baked

0.45  $\mu\text{m}$  glass beads at  $-18^{\circ}\text{C}$  were added at a ratio of 3g/ml of cell suspension. Cells were then disrupted by shaking on an orbital shaker at 350 rev./min. Breakage was continued until it was estimated by microscopy to be 90–95% complete, which required about 15 min. The resulting slurry was passed rapidly through a glass-fibre plug and the beads were washed with extraction buffer. The combined filtrate, at a 4–5-fold dilution of the original suspension, was called fraction I.

**Heat precipitation.** Fraction I was adjusted to pH 6.5 with 1M-KOH, heated rapidly to  $54^{\circ}\text{C}$  and maintained at this temperature for 15 min with occasional stirring. It was then cooled to  $10^{\circ}\text{C}$  over liquid  $\text{N}_2$  and centrifuged for 30 min at 23000g. The supernatant is fraction II.

**Acid and second heat precipitation.** Fraction II was adjusted to pH 4.3 by the dropwise addition of 1M-citric acid and centrifuged at 23000g for 1 h. The pellet was resuspended in 250 ml of extraction buffer. After homogenization the pH was adjusted to 6.9 with 1M-HCl, the mixture heated rapidly to  $50^{\circ}\text{C}$ , and maintained at this temperature for 10 min with occasional stirring. The solution was chilled in an ice bath to  $10^{\circ}\text{C}$  and centrifuged for 30 min at 23000g. This is fraction III.

**$(\text{NH}_4)_2\text{SO}_4$  fractionation.** Fraction III was diluted to 300 ml with extraction buffer and adjusted to pH 7.0 with 1M-HCl.  $(\text{NH}_4)_2\text{SO}_4$  (63 g) was added gradually, and the mixture was stirred for 30 min. The precipitate was removed by centrifugation for 20 min at 23000g. An additional 54 g of  $(\text{NH}_4)_2\text{SO}_4$  was added to the supernatant solution and after stirring for 30 min, the precipitate collected by centrifugation for 30 min at 23000g. The pellet was resuspended in 80 ml of ion-exchange buffer consisting of 10 mM- $\text{KH}_2\text{PO}_4$ , 5 mM-Tris/HCl, 45 mM-KCl, 0.45 mM-EDTA, 0.5% (v/v)  $\alpha$ -thioglycerol and 25% (v/v) glycerol, pH 8.0 at  $4^{\circ}\text{C}$ , and if necessary stored at  $-18^{\circ}\text{C}$ . This is fraction IV.

**Ion-exchange step.** DEAE-Sephadex A-50 was equilibrated with ion-exchange buffer to within 0.01 pH unit, and the slurry supplemented with 0.024% (w/v) phenylmethanesulphonyl fluoride. Fraction IV was added in a ratio of 2.5 units/g of gel that had been allowed to filter to completion without suction. Then 1 gel volume of ion-exchange buffer was added and the slurry stirred for 50 min. A small portion of liquid was filtered off and assayed for activity. Additional gel (2–5%) was added if required and stirred for 10 min until the activity in a filtered portion of liquid was less than 0.02 unit/ml. The gel was collected on a Büchner funnel. To the gel was added 2 gel volumes of ion-exchange buffer supplemented with 52.5 mM-KCl, and the slurry was stirred for 10 min. The gel was collected on a Büchner funnel, and washed with 0.5 gel volume of ion-exchange buffer containing 52.5 mM-KCl and

the filtrate discarded. Enzyme activity was eluted with three washes of 1 gel volume of 0.15M-KCl-supplemented ion-exchange buffer followed by 0.5 gel volume of 0.21M-KCl-supplemented ion-exchange buffer. The elution filtrates were pooled and successive portions of 200 ml were diluted to 1 litre with 5% (v/v) glycerol/1 mM-mercaptoethanol, and the enzyme was collected by suction filtration on a bed of Whatman DE-23 ion-exchange cellulose containing 1 g wet wt. of gel for every 70 units of enzyme activity. The enzyme was eluted by stirring the gel for 10 min in 5–10 gel volumes of 1M- $\text{K}_2\text{HPO}_4$ /1 mM-mercaptoethanol/22% (v/v) glycerol/0.024% (w/v) phenylmethanesulphonyl fluoride, and the eluate collected by suction filtration. If necessary, the eluate may be stored at  $-18^{\circ}\text{C}$ . This is fraction V.

**Affinity chromatography.** Fraction V enzyme was equilibrated by passage through a Sephadex G-25 column into the affinity-chromatography buffer. The equilibrated enzyme, containing 500 units in a volume less than 35 ml, was loaded on the  $\text{NAD}^+$ -Sephrose 4B column equilibrated with the same buffer. Fractions (10 ml) were collected at 1.2 ml/min. The column was then washed with 1 column volume of affinity-chromatography buffer, followed by enzyme elution with 200 ml of buffer containing 10 mM- $\text{NAD}^+$ . Fractions containing activity were pooled, supplemented with solid  $\text{K}_2\text{HPO}_4$  to 0.1M, concentrated by ultrafiltration and stored at  $-18^{\circ}\text{C}$ . This is fraction VI.

## Results

Table 1 summarizes the rapid enzyme-purification procedure described above with data for preparations from N.G. & S.F.-supplied yeast. When a sample of enzyme was measured for activity in the standard assay of Steinman & Jakoby (1967) with benzaldehyde as substrate, the activity was 29.5% of that determined by the standard assay used for the present work. Yields of activity were independent of the method of extraction and independent of the source of yeast.

Under the conditions of the described purification procedure phenylmethanesulphonyl fluoride is hydrolysed with a half-life of 100 min (Pringle, 1976). When attempts were made to compensate for this hydrolysis by continuous addition of phenylmethanesulphonyl fluoride during extraction of the acetone-dried powder, and the addition of frequent small portions during subsequent procedures, activity yields were decreased. Fraction III had only 25% of the activity of the standard preparation, and subsequent fractions showed progressively poorer activities, although the mobility of the active band on polyacrylamide-gel electrophoresis remained the same.

A portion (1 mg) of freeze-dried fraction VI gave a result of 1.05 mg of protein by the method of Lowry

Table 1. Summary of aldehyde dehydrogenase purification

Results are based on the use of 200 g of acetone-dried powder derived from 0.7 kg of packed yeast cells. Fractions were obtained as given in the text. Activity was measured in standard reaction mixtures and protein was determined by the method of Lowry *et al.* (1951).

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
I	1190	23 230	—*	>0.2†
II	1140	11 172	—*	>0.3†
III	278	3075	3690‡	1.2
IV	80	1820	3640	2.0
V	215	128	3450	27.0
VI	1200	95	3210	34.0
Biofiber concentrate	75	90	3090	34.0

\* Aldehyde dehydrogenase activity masked by other NAD(H)-dependent oxidoreductase.

† On the basis of total activity demonstrated by fraction III.

‡ All alcohol dehydrogenase removed by this step.

*et al.* (1951) standardized against bovine serum albumin. The same amount of enzyme in 1 ml of 0.1 M-Tris/HCl buffer (pH 8.0)/1 mm-mercaptoethanol/0.1 M-KCl/10% (v/v) ethanediol had  $A_{260}$  values of 0.659 and  $A_{280}$  values of 1.192, giving a value of 1.35 mg of protein by the method of Warburg & Christian (1941).

Disc polyacrylamide-gel electrophoresis of N.G. & S.F.-supplied yeast aldehyde dehydrogenase preparations made in the presence or absence of phenylmethanesulphonyl fluoride is shown in Fig. 1. The phenylmethanesulphonyl fluoride preparation exhibits a single sharp coincident band of protein and activity with a mobility relative to Bromophenol Blue of 0.185. Preparations stored at  $-18^{\circ}\text{C}$  or  $4^{\circ}\text{C}$  in the absence of phenylmethanesulphonyl fluoride did not change in electrophoretic mobility. Enzyme prepared in the absence of phenylmethanesulphonyl fluoride always resulted in discrete bands, although the consequent proteolysis resulted in various electrophoretic patterns. On several occasions we have observed only single bands of activity which when tested on gels pre-run for 1 h before use result again in an electrophoretic mobility of 0.185. The same samples run on gels not pre-run demonstrated electrophoretic mobilities of 0.262. More commonly, fractions before affinity chromatography prepared in the absence of phenylmethanesulphonyl fluoride show multiple bands of activity at relative mobilities of either 0.185 and 0.230 if pre-run or 0.262, 0.290 and in addition in some preparations at 0.310 if not (Fig. 1a). When purification is continued through the affinity chromatography there is a partial

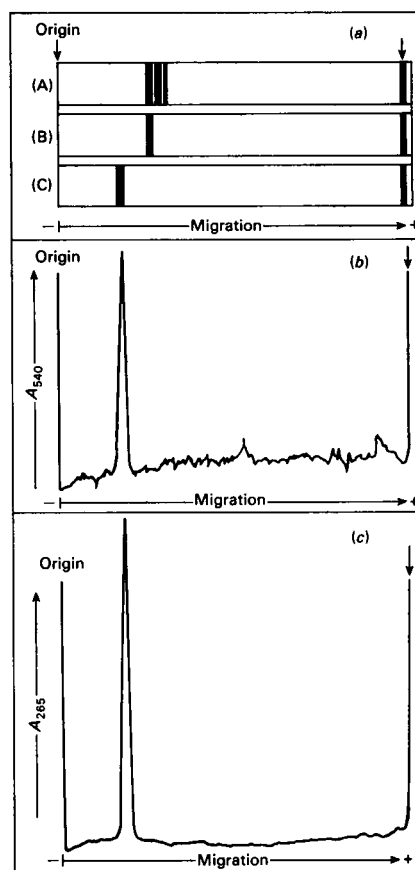


Fig. 1. Polyacrylamide-gel electrophoresis of aldehyde dehydrogenase

(a) Diagrammatic representation of gel-electrophoresis patterns obtained from fraction V prepared in the absence (gel A) and in the presence (gel B) of 0.024% phenylmethanesulphonyl fluoride, both electrophoresed on non-pre-run gels, and in the presence of 0.024% phenylmethanesulphonyl fluoride, electrophoresed on pre-run gels (gel C). Active enzyme bands were stained by enzymically reduced Nitro Blue Tetrazolium. (b) and (c), Densitometer traces of gels stained respectively for enzyme activity with Nitro Blue Tetrazolium and for protein with Coomassie Blue, by using fraction VI prepared in 0.024% (w/v) phenylmethanesulphonyl fluoride and electrophoresed on pre-run gels. Arrow indicates the marker dye Bromophenol Blue.

separation of these multiple forms, such that those with faster electrophoretic mobility tend to be eluted earlier from the affinity column. This indicates that enzyme molecules modified by proteolysis have less affinity for immobilized ligand. Enzyme purified from Anheuser-Busch yeast by either the rapid

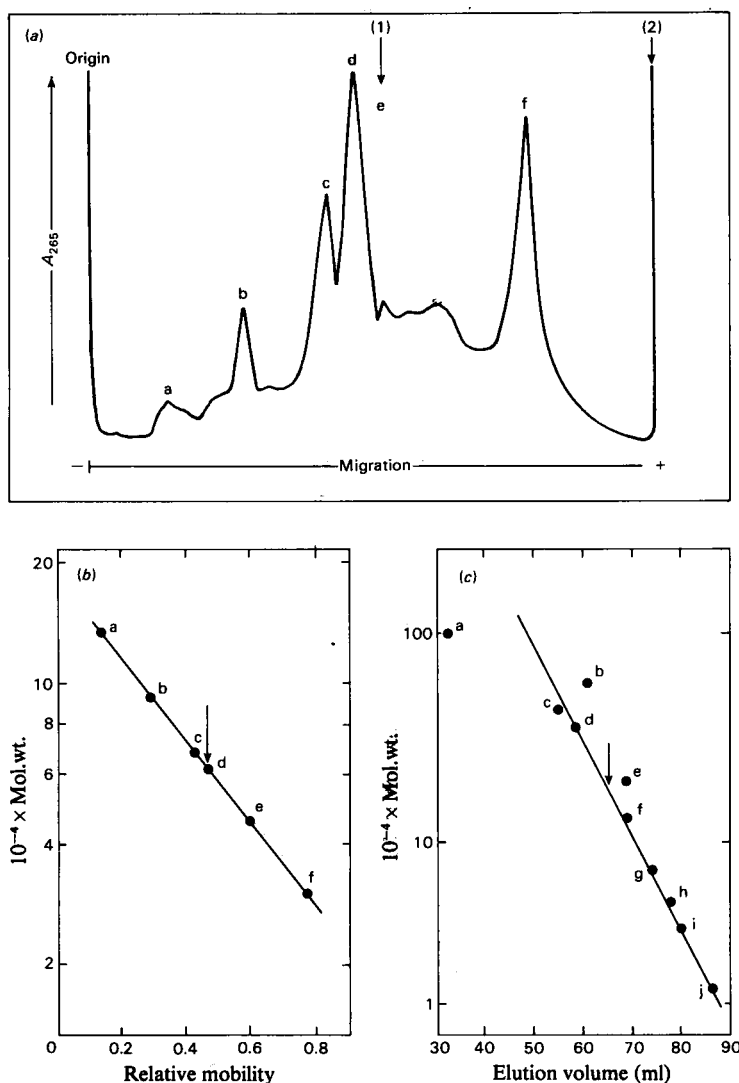


Fig. 2. SDS/polyacrylamide-gel electrophoresis and Sepharose 6B CL chromatography of aldehyde dehydrogenase and marker proteins

(a) Densitometer trace of Coomassie Blue-stained polyacrylamide gel run in 0.1% (w/v) SDS. The peaks are: a, bovine serum albumin dimer; b, aldehyde dehydrogenase dimer; c, bovine serum albumin monomer; d, aldehyde dehydrogenase monomer; e, aldehyde dehydrogenase A; f, carbonic anhydrase. Arrow (2) indicates marker dye Bromophenol Blue. (b) Molecular-weight calibration curve for SDS/polyacrylamide gels run with the proteins standards: a, bovine serum albumin dimer; b, phosphorylase A; c, bovine serum albumin monomer; d, catalase; e, ovalbumin; f, carbonic anhydrase. Arrow indicates relative mobility of aldehyde dehydrogenase. (c) Molecular-weight calibration curve for Sepharose 6B CL chromatography of the standards: a, Blue Dextran; b, glutamate dehydrogenase; c, thyroglobulin; d,  $\beta$ -galactosidase; e, catalase; f, aldolase; g, bovine serum albumin; h, ovalbumin; i, carbonic anhydrase; j, cytochrome c. Arrow indicates elution volume for aldehyde dehydrogenase. Aldehyde dehydrogenase was obtained from fraction VI prepared in the presence of 0.024% phenylmethanesulphonyl fluoride. SDS/polyacrylamide gel-electrophoresis and Sepharose 6B CL chromatography were performed and proteins were prepared as in the Materials and Methods section.

affinity-chromatographic procedure or by the dehydrogenase A-type procedure had identical gel-electrophoretic behaviour.

SDS/polyacrylamide gel-electrophoresis of fraction VI enzyme is shown in Fig. 2(a). Bovine serum albumin and carbonic anhydrase were used as markers and were run in the same gels as the enzyme sample. Arrow (1) (Fig. 2a) indicates the mobility of a yeast aldehyde dehydrogenase designated A by Clark & Jakoby (1970a) and was calculated from their data (Clark & Jakoby, 1970b) based on the same marker proteins. Under the conditions described, most of oligomeric enzymes dissociate into subunits of uniform charge and migrate relative to size on electrophoresis (Weber & Osborn, 1969). Calibration against protein standards of known molecular weight (see the Materials and Methods section) provide a monomeric molecular weight for fraction VI enzyme from N.G. & S.F.-supplied yeast of 62 500. Preparations of enzyme from Anheuser-Busch yeast made by either purification procedure provide an identical monomeric mol.wt. estimate of 62 500.

The molecular weight of intact fraction VI enzyme from N. G. & S.F.-supplied yeast was estimated by molecular exclusion chromatography on Sepharose 6B CL columns (Porath *et al.*, 1971) calibrated against globular proteins of known molecular weight (see the Materials and Methods section; Fig. 2b). A coincident band of protein and activity was eluted at a volume of 65.5 ml ( $\pm 2.5\%$ ), corresponding to a mol.wt. of 240 000.

Amino acid analysis of fraction VI enzyme prepared in phenylmethanesulphonyl fluoride is shown in Table 2. Results are expressed as integral numbers of residues per 50 000 g of protein and compared with the ion-exchange data of Clark & Jakoby (1970a). Histidine forms derivatives poorly, and this method gives irregular results with methionine and cysteine, so no data were collected for these amino acids. The overall pattern for the two determinations is similar. The reliability of such comparisons is  $\pm 17\%$  or 1 residue in 6. Only arginine and tyrosine show differences outside this error.

*N*-Terminal amino acid analysis was performed on fraction VI enzyme prepared in the presence of phenylmethanesulphonyl fluoride. For N.G. & S.F.-supplied yeast enzyme other than the reactive side groups Dns- $\epsilon$ -lysine and Dns- $\sigma$ -tyrosine, the only spot identifiable as an *N*-terminal amino acid was Dns-valine. A spot corresponding to Dns-serine, the *N*-terminus of aldehyde dehydrogenase A (Clark & Jakoby, 1970a), was not present in any of the chromatograms produced from samples prepared by several different dansylation techniques. No other spots were present in sufficient intensity to represent heterogeneity at the *N*-terminus. Enzyme preparations from Anheuser-Busch yeast made either by the

Table 2. *Amino acid analysis of aldehyde dehydrogenase*

Comparison of the amino acid composition of fraction VI enzyme and aldehyde dehydrogenase A from the data of Clark & Jakoby (1970a). G.l.c. analysis of amino acid derivatives of fraction VI prepared in the presence of 0.024% phenylmethanesulphonyl fluoride was performed as in the Materials and Methods section. Threonine, tyrosine and serine are extrapolated to zero time of hydrolysis and the remainder are averages of 24 and 48 h hydrolyses.

Amino acid	Integral no. of residues/50000 g	
	Fraction VI	Dehydrogenase A (Clark & Jakoby, 1970a)
Lysine	32	32
Arginine	12	15
Aspartic acid	44	44
Threonine	20	23
Serine	23	23
Glutamic acid	38	41
Proline	16	19
Glycine	39	39
Alanine	40	36
Valine	29	29
Isoleucine	31	35
Leucine	26	30
Tyrosine	9	12
Phenylalanine	17	17

dehydrogenase A-type procedure or by the rapid affinity-chromatographic procedure (both in phenylmethanesulphonyl fluoride) were micro-heterogeneous at the *N*-terminus, in which Dns-serine constitutes approx. 20% of the  $\alpha$ -amine derived amino acids present. Dns-valine was about equal in intensity with Dns-serine, whereas the majority of the remaining fluorescence corresponding to the  $\alpha$ -amine derived Dns-amino acids is identifiable as Dns-aspartic acid and Dns-glutamic acid.

The behaviour of fraction V enzyme from N.G. & S.F.-supplied yeast on  $\text{NAD}^+$ -Sepharose affinity columns in the absence of added aldehyde and free  $\text{NAD}^+$  is shown in Fig. 3. With the high-ionic-strength buffer used for these experiments, non-specific effects are minimized, and evidence for the biospecific retardation of enzyme is clearly seen by comparison with the elution profile of bovine serum albumin, run under identical conditions (Fig. 3). Supplementation with 5 mM- $\text{NAD}^+$  at fraction 20 resulted in elution of enzyme with the nucleotide front (Fig. 3). The sharpness of the elution profile was directly related to the concentration of nucleotide in the elution buffer.  $\text{NAD}^+$  or 5'-AMP were equally effective eluting agents. Decreasing the ionic strength of the chromatography buffer results in an increase in affinity. As the potassium phosphate concentration is decreased the front of activity occurs later



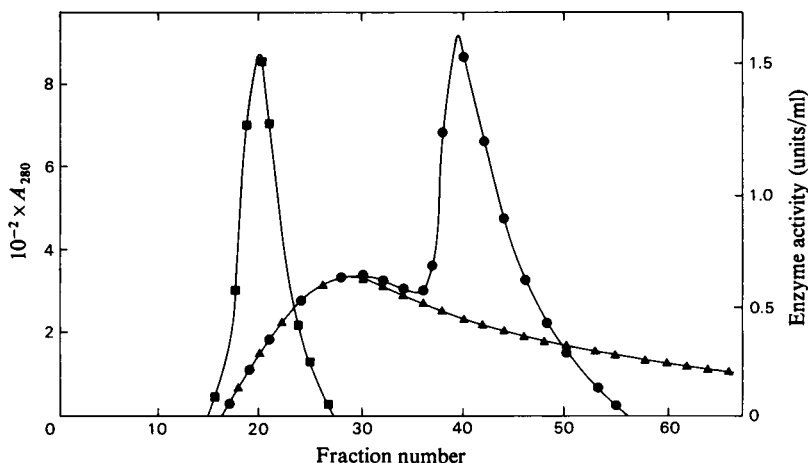


Fig. 3. Affinity chromatography of aldehyde dehydrogenase on  $NAD^+$ -Sepharose 4B. Chromatography was performed in  $0.1M$ - $K_2HPO_4$  (pH 8.0)/22% (v/v) glycerol/1 mM-mercaptoethanol/1.2 mM-phenylmethanesulphonyl fluoride buffer. ▲, Fraction V aldehyde dehydrogenase (26 units); ■, bovine serum albumin (1.3 mg); ●, 26 units of fraction V aldehyde dehydrogenase in which running buffer was supplemented with 5 mM- $NAD^+$  at fraction 20. Samples were added in a volume of 1 ml. ▲, ●, in units/ml of activity determined in the standard assay; ■,  $A_{280}$  units. Column dimensions were 1.25 cm × 16 cm. The volume of each fraction was 1.2 ml. Flow rate was 0.9 ml/min.

and is smaller than under the conditions of Fig. 3. There is a consequent increase in the amount of enzyme eluted with the front of free nucleotide. Elution of contaminating protein is only marginally effected by this decrease in ionic strength, so this effect reinforces the biospecific interaction and enables more enzyme to be processed. A 45 mM-potassium phosphate buffer, pH 8.0, allows a sufficient amount of retardation, but fulfils the  $K^+$  requirement for stability and was adopted for routine purification. Attempts at batchwise procedure were unsuccessful.

Blue Dextran binding to several enzymes has been established by molecular-sieve chromatography (Staal *et al.*, 1969, 1971), affinity chromatography (Kopperschläger *et al.*, 1971; Ryan & Vestling, 1974; Thompson *et al.*, 1975) and competitive inhibition (Thompson *et al.*, 1975). The specific binding of enzyme to immobilized Blue Dextran has been correlated with the possession of the dinucleotide fold common to several classes of enzymes (Thompson *et al.*, 1975). 5'-AMP is a competitive inhibitor of several dehydrogenases and has proved a useful immobilized ligand for affinity chromatography (Brodelius & Mosbach, 1973; Craven *et al.*, 1974). Fig. 4 shows that both 5'-AMP and Blue Dextran are inhibitors of aldehyde dehydrogenase, competitive with  $NAD^+$ . The  $K_i$  values for 5'-AMP and for Blue Dextran are  $790\mu M$  and  $1\mu M$  respectively.

The binding of enzyme to 5'-AMP affinity columns is shown in Fig. 5. Binding is similar to that on an

$NAD^+$  column, but the column used here has approx. 80% less binding capacity. 5'-AMP and  $NAD^+$  are equally effective eluting agents at equivalent concentrations. Fig. 5 also shows that inclusion throughout the chromatography of acetaldehyde at concentrations optimal for activity only marginally improves the affinity of the column for enzyme. Both Anheuser-Busch fraction-V enzyme and that produced by the dehydrogenase A-type purification bind to 5'-AMP columns with a similar or slightly greater affinity than the N.G. & S.F.-supplied yeast enzyme affinity shown in Fig. 5.

The affinity of N.G. & S.F.-supplied yeast aldehyde dehydrogenase for Blue Dextran columns in low ionic strength buffer is demonstrated in Fig. 6. Affinity is increased by the presence of acetaldehyde in the running buffer. Enzyme is eluted from the column by buffers of high ionic strength. Unlike the behaviour of other enzymes that bind specifically to Blue Dextran columns, low concentrations of free nucleotide ligand (1–10 mM) fail to accelerate elution in low-ionic-strength buffers. However, at concentrations (0.1 M) of inorganic salts producing only moderate increases in elution, the addition of 5 mM- $NAD^+$  or 5'-AMP causes a sharp specific elution. The addition of 20% (v/v) glycerol required for stability decreases enzyme affinity.

The ubiquitous contamination of endogenous aldehydes in a number of general reagents has previously been demonstrated by Bradbury & Jakob (1971) using the colorimetric method of Sawicki *et al.*

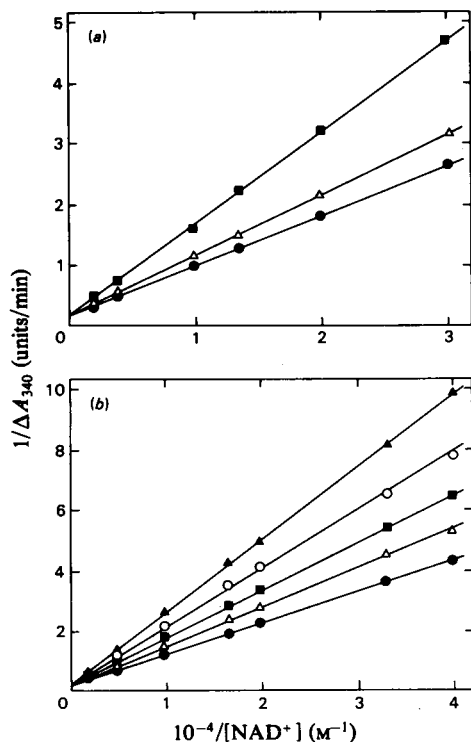


Fig. 4. Inhibition of aldehyde dehydrogenase by Blue Dextran or 5'-AMP

Double-reciprocal plots of initial velocity and  $\text{NAD}^+$  concentrations at several fixed concentrations of inhibitor. In (a) concentrations of Blue Dextran were:  $\bullet$ , 0;  $\Delta$ ,  $0.05 \mu\text{M}$ ;  $\blacksquare$ ,  $1 \mu\text{M}$ . In (b) concentrations of 5'-AMP were:  $\bullet$ , 0;  $\Delta$ , 1 mM;  $\blacksquare$ , 2 mM;  $\circ$ , 3 mM;  $\blacktriangle$ , 4 mM. Activity was measured in standard reaction mixtures, except for varied  $\text{NAD}^+$  concentrations and the presence of Blue Dextran or 5'-AMP as shown.

(1961). An assessment of contamination in the reagents used here for affinity chromatography was therefore made both by the colorimetric method and by enzymic reactivity with aldehyde dehydrogenase. Tris/HCl, mercaptoethanol,  $\alpha$ -thioglycerol and possibly glycerol interfered with the colorimetric reaction.

By the 3-methyl-2-benzothiazolone hydrazone method, polyhydric alcohol and secondly  $\text{NAD}^+$  are the most highly contaminated constituents in the affinity-chromatography buffer; 100% glycerol gave an aldehyde concentration of  $0.3 \text{ mM}$  and aldehyde was 2.4% of the concentration of  $\text{NAD}^+$ . Contamination by enzymically reactive aldehyde in the aldehyde dehydrogenase reaction mixture supplemented to 25% (v/v) with glycerol was estimated at  $40 \mu\text{M}$ . Chemical analyses using 3-methyl-2-benzothiazolone hydrazone performed on samples

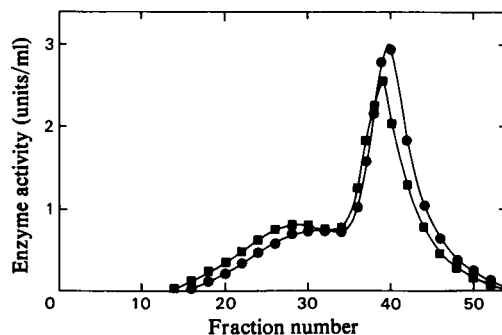


Fig. 5. Affinity chromatography of aldehyde dehydrogenase on 5'-AMP-Sepharose 4B

Chromatography was performed in  $0.1 \text{ M-K}_2\text{HPO}_4$  (pH 8.0)/22% (v/v) glycerol/1 mM-mercaptoethanol/1.2 mM-phenylmethanesulphonyl fluoride buffer, with ( $\bullet$ ) or without ( $\blacksquare$ ) 2 mM-acetaldehyde; 17 units of fraction V aldehyde dehydrogenase were used in each case, and buffer was supplemented with 5 mM-5'-AMP at fraction 20. All other conditions were as in Fig. 3.

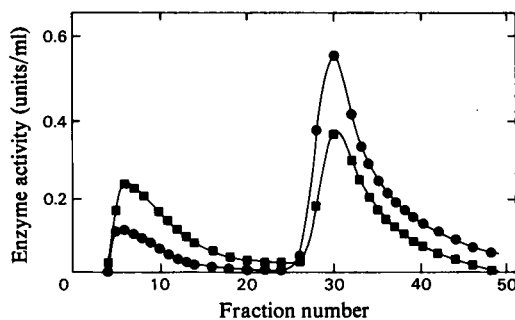


Fig. 6. Affinity chromatography of aldehyde dehydrogenase on Blue Dextran-Sepharose 4B

Chromatography was performed in  $10 \text{ mM-Tris/HCl}$  (pH 8.0)/0.4 mM-mercaptoethanol buffer, with ( $\bullet$ ) or without ( $\blacksquare$ ) 2 mM-acetaldehyde; 12 units of fraction V aldehyde dehydrogenase in a volume of 1.5 ml were used in each case. Elution buffer [ $0.1 \text{ M-K}_2\text{HPO}_4$  (pH 8.0)/22% (v/v) glycerol/0.4 mM-mercaptoethanol/5 mM- $\text{NAD}^+$ ] was added at fraction 25 in each case. Column dimensions were  $1.2 \text{ cm} \times 7 \text{ cm}$ , and the volume of each fraction was 1.2 ml. Flow rate was  $0.8 \text{ ml/min}$ . Activity was determined in the standard assay mixture.

removed from the reaction mixture at various time intervals provide an estimated initial aldehyde concentration of  $100 \mu\text{M}$ , with no reduction during the reaction period, in spite of the fact that  $40 \mu\text{M}$ -aldehyde is removed by enzymic oxidation. The data suggest that the 3-methyl-2-benzothiazolone hydrazone test (as used here) is not a reliable test for contaminating aldehyde.

Contamination of glycerol by enzymically reactive aldehyde present in the affinity-chromatography buffer is  $26\ \mu\text{M}$ . When 10% (v/v) ethanediol was substituted for glycerol in the affinity-chromatography buffer, a 10-fold reduction in enzymically reactive contaminating aldehyde (to  $3\ \mu\text{M}$ ) was achieved without altering column capacity and elution profiles of enzyme from  $\text{NAD}^+$  and 5'-AMP columns. 3-Methyl-2-benzothiazolone hydrazone-estimated aldehyde contamination by 10% (v/v) ethanediol is  $10\ \mu\text{M}$  based on the standard curve for acetaldehyde.

In these affinity-chromatographic investigations, if aldehyde is to be an obligatory leading ligand before the binding of immobilized  $\text{NAD}^+$ , 5'-AMP or Blue Dextran, then for biospecific separation to be unchanged by the addition of saturating concentrations of aldehyde, contaminating aldehyde must be close to saturation for enzyme binding sites. Moreover, since a 10–50-fold decrease in contaminating aldehyde does not result in an alteration of biospecific elution, within experimental error, the aldehyde sites would have to be 99% saturated. Thus at a total contaminating aldehyde concentration measured enzymically or colorimetrically of  $10\ \mu\text{M}$  or less the  $K_s$  for contaminating aldehyde would have to be less than  $0.1\ \mu\text{M}$ . On this basis the actual  $K_m$  values for good aldehyde substrates would have to be less than that determined with these omnipresent contaminating aldehydes by a factor of  $1 + 1/K_i$ , or 100-fold. This requires that true  $K_m$  values for at least two aldehyde substrates, isobutyraldehyde and *p*-nitrobenzaldehyde (Steinman & Jakoby, 1968), are less than  $10\ \text{nm}$ , an unreasonably high affinity for such enzyme substrate complexes.

The binding of fraction VI enzyme to  $\text{NAD}^+$  in the absence of added aldehyde was determined directly by equilibrium dialysis and by the rate of dialysis method (Colowick & Womack, 1969). Protein was determined by the method of Lowry *et al.* (1951) and molarity calculated with a mol.wt. of 248000. For both experiments enzyme was approx.  $5\ \mu\text{M}$ , and  $\text{NAD}^+$  varied from 1 to  $0.01\ \text{mM}$ . Scatchard-type plots of the binding data for enzymes from N.G. & S.F.-supplied yeast are shown in Fig. 7. A least-squares fit to the equilibrium-dialysis data in Fig. 7(a) provides a dissociation constant (1/slope) of  $120\ \mu\text{M}$  for each of 4 coenzyme-binding sites per molecule. Data for the rate of dialysis experiment in Fig. 7(b), performed under similar experimental conditions, provide a dissociation constant of  $68\ \mu\text{M}$  for each of 4 coenzyme-binding sites per molecule.  $\text{NAD}^+$  binding to fraction-VI enzyme purified from Anheuser-Busch yeast determined by an identical equilibrium-dialysis procedure gave a dissociation constant of  $200\ \mu\text{M}$  for each of 4 coenzyme-binding sites per molecule.

### Discussion

Evidence is presented above that the enzyme purified from N.G. & S.F.-supplied yeast as described here is different in primary structure from that previously reported (Steinman & Jakoby, 1967; Clark & Jakoby, 1970a). The most unequivocal indication of a difference is the *N*-terminal amino acid valine for this enzyme preparation, compared with serine for the enzyme designated dehydrogenase A (Clark & Jakoby, 1970a). The total amino acid analysis indicates minor differences between the

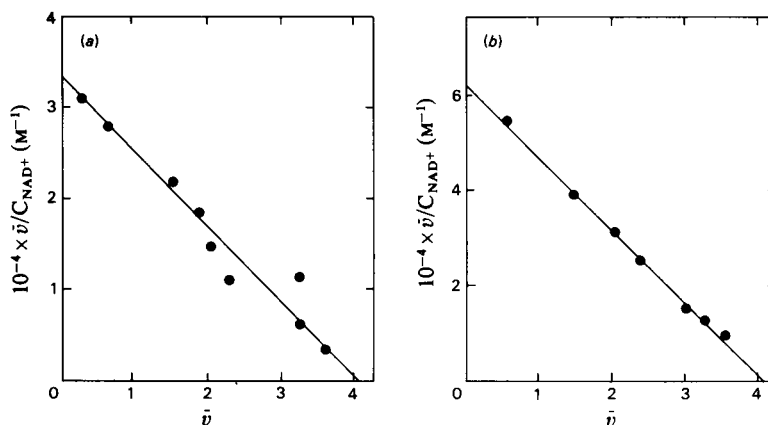


Fig. 7. Binding of  $\text{NAD}^+$  to aldehyde dehydrogenase

(a) Scatchard type plot of variation of  $\bar{v}$  with  $\bar{v}/C_{\text{NAD}^+}$  from equilibrium dialysis data; (b) Scatchard type plot of variation of  $\bar{v}$  with  $\bar{v}/C_{\text{NAD}^+}$  from rate of dialysis data. Dialysis was performed in  $0.1\ \text{M-Tris/HCl}$  (pH 8.0)/20% (v/v) ethanediol/1 mM-mercaptoethanol, at  $4^\circ\text{C}$ . Aldehyde dehydrogenase was obtained from fraction VI prepared in the presence of  $0.024\%$  (w/v) phenylmethanesulphonyl fluoride. Dialysis was performed as in the Materials and Methods section.

preparations with only arginine and tyrosine content being significantly different.

SDS/polyacrylamide-gel electrophoresis gives a mol.wt. of 62 500 for the monomer, compared with 56 000 reported for dehydrogenase A (Clark & Jakoby, 1970*b*), determined by the same method. Molecular-sieve chromatography gives a mol.wt. of 240 000 for the intact enzyme, compared with 214 000 reported for dehydrogenase A (Clark & Jakoby, 1970*a*). However, in our hands enzyme preparations from yeast supplied by either N.G. & S.F. or Anheuser-Busch and purified by the method reported here or as previously (Clark & Jakoby, 1970*a*) all co-migrate on SDS/polyacrylamide gels.

These data might reflect a minor difference in the form of the proteins *in vivo* isolated from two different yeast strains. An alternative hypothesis in view of considerations below is that both proteins have the same primary structure *in vivo* and that differences between the isolated proteins are the result of differential proteolytic digestion during purification which results in the removal of a segment too small to modify the migration rate on SDS/polyacrylamide gels.

Highly specific proteolytic modification of yeast aldehyde dehydrogenase *in vitro* resulting in the production of distinct but active enzyme forms has been demonstrated both in the original work of Clark & Jakoby (1970*a*) and by the findings presented here (Fig. 1). By avoiding autolytic procedures and/or by the addition of high concentrations of proteinase inhibitors di-isopropyl phosphorofluoridate or phenylmethanesulphonyl fluoride, a single electrophoretically homogeneous enzyme activity is observed. It has been clearly documented, however, in an exhaustive review by Pringle (1976) that no single precaution, such as the inclusion of di-isopropyl phosphorofluoridate or phenylmethanesulphonyl fluoride, is completely adequate for safe-guarding against modification. In many cases, proteinases show residual activity or are unaffected by the presence of these agents, and therefore, any procedure that speeds the separations of proteinases from the required enzyme is likely to result in a lesser amount of the degraded forms.

The slowest steps in published purification procedures of yeast aldehyde dehydrogenase are ion-exchange chromatography on DEAE-Sephadex A-50 and hydroxyapatite (Steinman & Jakoby, 1967; Clark & Jakoby, 1970*a*). These were used at a time when it was recognized that proteolytic activity persisted in the preparation even in the presence of phenylmethanesulphonyl fluoride or di-isopropyl phosphorofluoridate. Moreover, to allow rapid equilibration and sample binding to the ion-exchange material it is necessary to lower glycerol and K<sup>+</sup> concentrations, both of which are required for enzyme stability.

As a possible alternative to these lengthy ion-exchange chromatographic steps, we attempted to introduce an early rapid affinity-chromatography step. After the report of a compulsory sequential mechanism in which aldehyde is the leading substrate (Bradbury & Jakoby, 1971*a,b*) and the report that direct binding studies reveal no nucleotide binding in the absence of aldehyde (Bradbury & Jakoby, 1971*a*), we made several unsuccessful attempts to immobilize an aldehyde on a polysaccharide for affinity chromatography. However, enzyme does bind to columns containing immobilized coenzyme, partial coenzyme (AMP) and probe for dinucleotide fold (Blue Dextran). Unfortunately, dispensing with the ion-exchange steps completely and proceeding from (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation directly to affinity chromatography results in preparations demonstrating multiple protein bands on disc polyacrylamide gel electrophoresis of which only one band has aldehyde dehydrogenase activity. Presumably an ion-exchange step removes other NAD<sup>+</sup>-linked enzymes. With a bulk procedure for DEAE-Sephadex A-50 ion exchange rather than column chromatography, the purification step is marginally less efficient, but only takes one-tenth of the time. The enzyme can then be purified to homogeneity (as judged by polyacrylamide-gel electrophoresis and *N*-terminal amino acid analysis) by a relatively rapid NAD<sup>+</sup> affinity chromatography.

A slow step (4h) in the present purification is the initial resuspension of acetone-dried powder. Although it is true that proteinases are present in higher concentration at this stage than any other, so too are the yeast's own proteinase inhibitors. A pH was purposely chosen at which proteinase-inhibitor complexes are most stable (Pringle, 1976). The high concentration of other proteins present at this stage offers some protection. Moreover, it has been shown that an alternative glass-bead breakage procedure for extraction yields an enzyme with apparently identical characteristics. This more direct and rapid method of extraction is the method of choice at the moment as it was recently made applicable to large amounts of cells.

An attempt was made to ascertain whether differences in protein characteristics between the present preparation and that previously reported (Steinman & Jakoby, 1968; Clark & Jakoby, 1970*a*; Bradbury & Jakoby, 1971*a*) are due to differences in isolation methods rather than to source. Thus the present more rapid isolation methods were used on Anheuser-Busch yeast (that used by Clark & Jakoby, 1970*a*) to see if an enzyme could be obtained having characteristics of the present preparation. *N*-Terminal amino acid analysis was ambiguous. In none of the Anheuser-Busch yeast enzyme preparations was a single  $\alpha$ -amino derivative of amino acids observed. Aspartic acid and glutamic acid comprised

about 50% of the  $\alpha$ -amine derivatives, with the next most prevalent spots corresponding to Dns-valine and Dns-serine. These results were observed regardless of the method of purification. In di-isopropyl phosphorofluoridate preparations of dehydrogenase A only 80% of the  $\alpha$ -amine derivatives of amino acids were serine, and in purification with phenylmethanesulphonyl fluoride as little as 60% (W. Jakoby, personal communication). The generally higher proteinase activity in Anheuser-Busch yeast enzyme preparations indicated by *N*-terminal amino acid analysis is also reflected in the lower enzyme activity recoverable during purification, even in phenylmethanesulphonyl fluoride (Clark & Jakoby, 1970a; the present work).

To explain these results one must assume that even when careful attempts have been made to follow the dehydrogenase A-type purification outline, some alteration in procedure (e.g. breakage with glass beads rather than Manton-Gaulin homogenization, or a change in the Anheuser-Busch strain of yeast, either physiologically or genetically, avoids a proteolysis or is otherwise responsible for modifying the enzyme.

A significant feature of the above work is that a purification technique involving binding to  $\text{NAD}^+$ , AMP or Blue Dextran affinity columns has been successfully applied to yeast aldehyde dehydrogenase in spite of the report of Bradbury & Jakoby (1971a) of a compulsory order of substrate addition in which aldehyde is the leading substrate. Moreover, our enzyme preparations bind to these affinity columns in buffers similar to those that failed to demonstrate  $\text{NAD}^+$  binding to dehydrogenase A (Bradbury & Jakoby, 1971a). Supplementation with aldehyde at concentrations optimal for activity does little to enhance enzyme affinity toward 5'-AMP columns and only slightly increases affinity for Blue Dextran columns. Conceivably, this might indicate prior binding by contaminating aldehydes rather than binary enzyme- $\text{NAD}^+$  complex. Aldehyde determinations reveal the presence of contaminating aldehyde in the chromatography buffer. These are introduced predominantly by polyhydric alcohol. However, best estimates indicating a 10-50-fold decrease in contaminating aldehyde concentration by removal of glycerol or its substitution by ethanediol does not alter the biospecific affinity of aldehyde dehydrogenase for these columns.

For several reasons (Porath & Kristiansen, 1975) it is not possible to determine accurately the effective ligand concentration of the affinity gels used here. However, the total amount of immobilized ligand present is  $50\ \mu\text{mol/g}$  and  $150\ \mu\text{mol/g}$  of dry gel for immobilized  $\text{NAD}^+$  and 5'-AMP respectively. Assuming that total immobilized ligand approximates to effective ligand concentration, and by using bovine serum albumin as a protein for which bio-

affinity is zero, behaviour can be compared with predicted binding and elution for simulated binary systems of enzyme and immobilized ligand (Graves & Wu, 1974). The behaviour of aldehyde dehydrogenase is consistent with a dissociation constant for immobilized  $\text{NAD}^+$  and 5'-AMP in the range 1-0.10 mM. The  $K_i$  for 5'-AMP is 0.79 mM (Fig. 4), and under the chromatographic conditions described the  $K_s$  for  $\text{NAD}^+$  has been determined by us as 0.12 mM by equilibrium dialysis. These data demonstrate that a specific interaction between aldehyde dehydrogenase and immobilized  $\text{NAD}^+$  or 5'-AMP is taking place. The introduction of non-specific interactions at low ionic strength is advantageously used to increase this affinity, and therefore the amount of enzyme bound to either column, with no other obvious alteration in chromatographic behaviour.

The qualitative data on binding to Blue Dextran columns as well as kinetic inhibition data indicate that at least some portion of the dinucleotide fold common to all dehydrogenases thus far investigated (Thompson *et al.*, 1975) is present in aldehyde dehydrogenase.

Binding to columns of immobilized AMP, Blue Dextran or  $\text{NAD}^+$  is not a critical test of binding to free  $\text{NAD}^+$ . However, the data (Fig. 7) clearly show that  $\text{NAD}^+$  interacts with aldehyde dehydrogenase in the absence of added aldehyde. Respective values for coenzyme dissociation constants from the two dialysis studies were 120 and  $68\ \mu\text{M}$  for each of four coenzyme-binding sites per tetrameric molecule. This implies one active site per monomer, a general case for most enzymes with fewer than six subunits.

Known reactive aldehydes are present to a certain extent in the rate of dialysis experiments, but have been completely oxidized during equilibrium dialysis. In either case an  $\text{NAD}^+$ -regenerating system was included so the system was not measuring the binding of product NADH. The concentration of enzymically unreactive aldehyde is negligible with the buffer system used for equilibrium dialysis and is unlikely to exceed 10% saturation of aldehyde sites. The presence of additional enzymically reactive aldehydes in the rate of dialysis experiments does not alter the number of available binding sites per enzyme molecule. Thus unless unreasonable assumptions are made about contaminating aldehyde concentrations,  $\text{NAD}^+$  binds to the free enzyme.

Once again studies have been made in an attempt to resolve the discrepancy in  $\text{NAD}^+$ -binding characteristics between this enzyme preparation and that previously reported (Bradbury & Jakoby, 1971a). This has been done both by using our purification procedures on the yeast used previously and by varying the preparation procedure of enzyme from N.G. & S.F.-supplied yeast in an attempt to obtain enzyme with identical coenzyme-binding charac-

teristics to those previously reported. Enzyme prepared rapidly from Anheuser-Busch yeast was found to bind to the affinity columns in a fashion identical with enzyme rapidly purified from N.G. & S.F.-supplied yeast. NAD<sup>+</sup> binding was subsequently confirmed by equilibrium dialysis. Variations in the source of yeast or the method of purification have always resulted in enzyme that binds to coenzyme analogue affinity columns and where tested binds to NAD<sup>+</sup> during equilibrium dialysis.

The implication from this work is that enzyme-NAD<sup>+</sup> binary complex does form, and that this might therefore reflect an important binary complex in the kinetic mechanism. This is consistent with the known mechanisms of all other dehydrogenases sufficiently investigated. Alcohol dehydrogenases, lactate dehydrogenase, malate dehydrogenase, formate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase all have ordered mechanism with coenzyme as the leading substrate (Dalziel, 1975). The kinetic mechanisms of glutamate dehydrogenase and isocitrate dehydrogenase are consistent with a random-order mechanism where free enzyme binds coenzyme (Dalziel, 1975). Coenzyme has also been shown to bind with free rat and horse liver aldehyde dehydrogenase (Takio *et al.*, 1974; Tottmar *et al.*, 1974; Weiner *et al.*, 1974). Such a distinct difference in the proposed inability of free yeast aldehyde dehydrogenase to bind NAD<sup>+</sup> in a kinetically significant pathway has led us to a complete kinetic investigation of our enzyme preparations. The data are presented in the following paper (Bostian & Betts, 1978).

We thank Mr. P. Poole for his ready technical assistance and Dr. C. Bruton for his generous help in the determination of *N*-terminal amino acid. We also thank Mr. Kenneth Davy and Professor C. J. O. R. Morris for performing the amino acid analysis, and Ms. Diana Gorringer for making available a calibrated Sepharose 6B CL column. This work was supported, in part, by U.S. Public Health Service grant GM20755 awarded to D. J. Tipper.

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