

Kinetics and Reaction Mechanism of Potassium-Activated Aldehyde Dehydrogenase from *Saccharomyces cerevisiae*

By KEITH A. BOSTIAN* and GRAHAM F. BETTS
*Department of Plant Biology and Microbiology, Queen Mary College,
London E1 4NS, U.K.*

(Received 16 December 1977)

Data from steady-state kinetic analysis of yeast K^+ -activated aldehyde dehydrogenase are consistent with a ternary complex mechanism. Evidence from alternative substrate analysis and product-inhibition studies supports an ordered sequence of substrate binding in which NAD^+ is the leading substrate. A preincubation requirement for NAD^+ for maximum activity is also consistent with the importance of a binary enzyme- NAD^+ complex. Dissociation constant for enzyme- NAD^+ complex determined kinetically is in reasonable agreement with that determined by direct binding. The order of substrate addition proposed here differs from that proposed for a yeast aldehyde dehydrogenase previously reported. Different methods of purification produced an enzyme that showed similar kinetic characteristics to those reported here.

K^+ -activated aldehyde dehydrogenase from *Saccharomyces cerevisiae* [aldehyde- $NAD(P)^+$ oxidoreductase; EC 1.2.1.5] catalyses the oxidation of a broad range of aliphatic, aromatic and heterocyclic aldehydes to their corresponding carboxylic acids (Black, 1951; Steinman & Jakoby, 1968). Both NAD^+ and $NADP^+$ serve as electron acceptors. The reaction is dependent on the presence of a thiol and a univalent cation activator, K^+ being the most effective cation. The reaction is essentially irreversible ($\Delta G^\circ = -52.3$ kJ/mol). Enzyme stability is dependent on the presence of high concentrations of activating univalent cation (Sorger & Evans, 1966) and polyhydric alcohol (Steinman & Jakoby, 1967; Clark & Jakoby, 1970; Bradbury & Jakoby, 1972). The dependence of stability on K^+ has been interpreted in terms of a univalent-cation-induced conformation change (Springham & Betts, 1973; Springham, 1974), whereas polyhydric alcohol apparently exerts its stabilizing effect by binding in close proximity to the active site(s) of aldehyde dehydrogenase (Bradbury & Jakoby, 1972).

Previous kinetic and equilibrium-dialysis studies with an aldehyde dehydrogenase from yeast designated 'dehydrogenase A' (Bradbury & Jakoby, 1971*a,b*) have provided evidence for a ternary complex in the catalytic mechanism in which coenzyme binds only after aldehyde in a sequential order. A kinetic mechanism for a nicotinamide nucleotide-linked oxidoreductase in which coenzyme

does not form a kinetically significant binary complex with enzyme is unusual (Sund, 1970; Dalziel, 1975). In the preceding paper (Bostian & Betts, 1978), however, we reported a rapid affinity-chromatographic purification procedure for yeast aldehyde dehydrogenase which yields an enzyme that does bind NAD^+ in the absence of aldehyde. Other small differences in primary structure suggest that preparations of aldehyde dehydrogenase A might have undergone proteolytic modification during purification which could have altered its kinetic properties (Steinman & Jakoby, 1967; Clark & Jakoby, 1970; Bradbury & Jakoby, 1971*a,b*).

This work was undertaken to ascertain whether the binary complex between enzyme and NAD^+ established by direct binding studies has any significance in the kinetic mechanism. The present results indicate an ordered sequential mechanism where $NAD(P)^+$ is the first substrate bound and the carboxylic product dissociates last.

Materials and Methods

Aldehyde dehydrogenase was prepared from N.G. & S.F. yeast (British Fermentation Products, London E.C.2, U.K.) by the rapid affinity-chromatographic procedure described in the preceding paper (Bostian & Betts, 1978). Enzyme samples (1.5 or 2 ml) were equilibrated for use in appropriate buffers by passage through columns (1.25 cm \times 6.8 cm or 1.25 cm \times 9 cm) of Sephadex G-25 at 4°C. Chromatography times were approx. 4 or 8 min respectively. Except for stability studies, the enzyme sample was

* Present address: Rosenstiel Basic Medical Research Centre, Brandeis University, Waltham, MA 02154, U.S.A.

stored on ice and used immediately after chromatography. All assays in a given run were complete within 2 min.

Enzyme activity was measured at 25°C by following the rate of reduction of NAD⁺ by observing either the increase in A_{340} in a Pye– Unicam SP.1800 spectrophotometer, or for experiments involving benzaldehyde as substrate, by monitoring NADH production fluorimetrically in a double monochromator spectrofluorimeter. The standard spectrophotometric assay described in detail elsewhere (Bostian *et al.*, 1975; Bostian & Betts, 1978) varied here only in the type of aldehyde or coenzyme and concentrations of these substrates and of K⁺. In monitoring NADH fluorescence, assays were performed in a total reaction mixture volume of 0.5 ml in quartz Spectrosil fluorescence cells (5 mm × 5 mm × 40 mm). Excitation was at 340 nm with right-angle illumination and emission viewed at 465 nm. Maximum full-scale deflection (fluorescence) corresponded to an NADH concentration of 1.63 μM. In both assays, reaction was begun by the addition of aldehyde substrate immediately after the addition of not more than 100 μl (absorbance assay) or 10 μl (fluorescence assay) of enzyme.

Aldehyde dehydrogenase concentration was determined by enzyme activity calculated from a specific activity of 34 units/mg under standard assay conditions (Bostian *et al.*, 1975; Bostian & Betts, 1978); 1 enzyme unit transforms 1 μmol of substrate/min. The molarity of enzyme active sites was based on four active sites per molecule of mol.wt. 248 000 (Bostian & Betts, 1978). K⁺ concentrations were measured by flame photometry.

NAD⁺ (grade III), NADH, NADP⁺, 5'-AMP and glycolaldehyde were purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Benzaldehyde and glycerol were AnalaR grade and supplied by Hopkin and Williams, Chadwell Heath, Essex, U.K. AnalaR-grade acetaldehyde, from BDH Chemicals, Poole, Dorset, U.K., was redistilled before use and stored in portions at -18°C as a 2 M solution. All other methods and materials were as described in the preceding paper (Bostian & Betts, 1978).

Results

Initial-rate studies

Two substrate initial-rate studies of aldehyde dehydrogenase were made by using three different aldehyde substrates and two different coenzymes. In one set of experiments initial enzyme rates were measured at high and constant KCl concentration when one of the two substrates (aldehyde or coenzyme) was varied approx. 20-fold in concentration either side of K_m , at several different fixed concentrations of the non-varied substrate. Under the con-

ditions used, with all substrates except acetaldehyde, Lineweaver–Burk plots were linear with time and the data for any two substrates fitted the general rate equation for ternary complex-formation:

$$v_0 = \frac{e[A][B]}{\phi_{AB} + \phi_B[A] + \phi_A[B] + \phi_0[A][B]} \quad (1)$$

where e is the concentration of enzyme active sites, v_0 is the initial enzyme rate, A and B are coenzyme and substrate, and the ϕ terms are kinetic constants. For acetaldehyde a pronounced substrate inhibition occurred at concentration approaching K_m . Acetaldehyde inhibition became more pronounced at higher NAD⁺ concentrations and resulted in non-linear Lineweaver–Burk plots with NAD⁺ as the variable substrate (Fig. 1). Substrate inhibition with glycolaldehyde and benzaldehyde inhibition was evident only at concentrations in excess of ten times the K_m value. Further kinetic analysis was confined to these two substrates.

For the glycolaldehyde and benzaldehyde systems the crossover point in primary plots occurred below the $1/[A]$ axis in all cases. Slope and intercept secondary plots are shown in Figs. 2 and 3. The values for the four kinetic coefficients in eqn. (1) were determined by a least-squares analysis of the data in Figs. 2 and 3 as described by Dalziel (1957). Data are presented in Table 1.

Initial-rate studies were also performed by varying one of the substrates or the activating univalent cation at a high constant concentration of the non-varied substrate. Lineweaver–Burk plots of e/v_0 versus $1/[A]$ are shown in Fig. 4. In all four plots the families of lines intersect on the horizontal axis, indicating that ϕ_A/ϕ_0 for varied ligand is unaffected

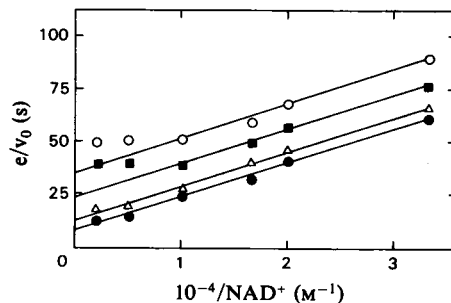


Fig. 1. Acetaldehyde oxidation by NAD⁺ with yeast aldehyde dehydrogenase

Primary plot of variation of the reciprocal specific initial rate with the reciprocal of NAD⁺ concentration for several constant acetaldehyde concentrations (mM): ○, 0.3; ■, 0.4; △, 0.8; ●, 1.0. The buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/0.1 M-KCl, at 25°C.

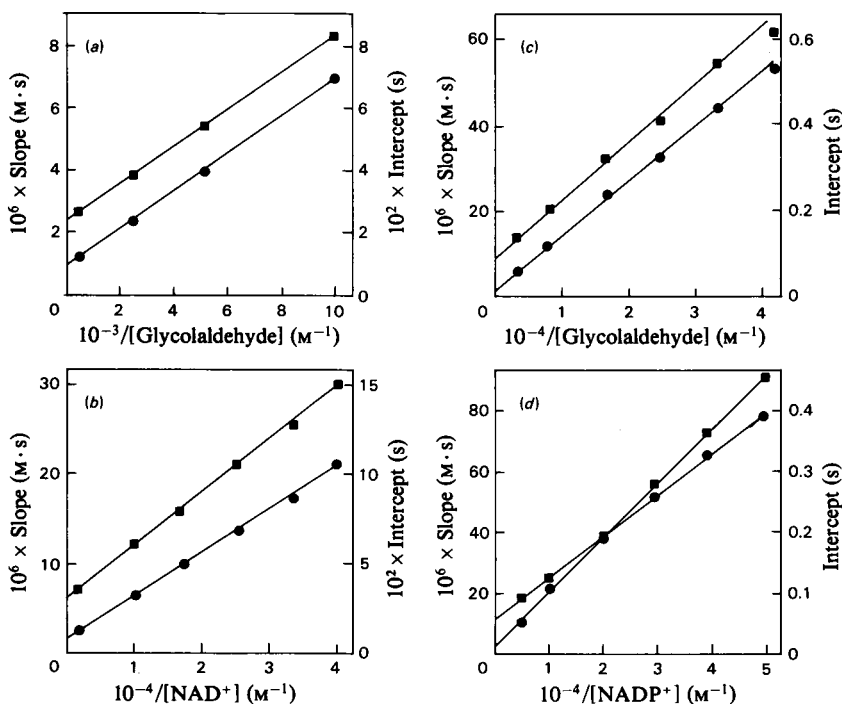


Fig. 2. Glycolaldehyde oxidation by NAD^+ or $NADP^+$ with aldehyde dehydrogenase

Secondary plots of variation of the intercepts (\bullet) and slopes (\blacksquare) for double-reciprocal plots of specific initial rate with: (a) NAD^+ concentration for several constant glycolaldehyde concentrations; (b) glycolaldehyde concentration for several constant NAD^+ concentrations; (c) $NADP^+$ concentration for several constant glycolaldehyde concentrations; (d) glycolaldehyde concentration at several constant $NADP^+$ concentrations. The buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/0.1 M-KCl, at 25°C.

by changes in non-varied substrate or activator concentration. From these data K_s for KCl can be unambiguously determined and is equal to the K_m for KCl in either plot, i.e. 7 mM.

Alternative-substrate analysis

Because evaluation of Haldane relationships by steady-state analysis (Alberty, 1953; Dalziel, 1957) and isotope exchange (Cleland, 1967) are not applicable to the essentially irreversible reaction catalysed by yeast aldehyde dehydrogenase, the approach by Wong & Hanes (1962) of alternative substrates was used to distinguish between the various homoeomorphic bisubstrate mechanisms obeying eqn. (1), i.e. ordered sequential or random rapid-equilibrium mechanisms. The dissociation constant, K_S^A , for any substrate A, with free enzyme remains invariant to a change in the nature of the B substrate. As K_S^A is given by ϕ_{AB}/ϕ_B then ϕ_{AB}/ϕ_X will be invariant to changes in substrate X if X is substrate B, but may give rise to different values if X is A. In the random rapid-equilibrium mechanism ϕ_{AB}/ϕ_X will remain

invariant when alternatives to either substrate are used.

The relationships of kinetic coefficients for aldehyde dehydrogenase shown in Table 2 show that when alternative forms of aldehyde were used, i.e. benzaldehyde or glycolaldehyde, $\phi_{AB}/\phi_{\text{benzaldehyde}}$ and $\phi_{AB}/\phi_{\text{glycolaldehyde}}$ were invariant, whereas values for $\phi_{AB}/\phi_{NAD(P)^+}$ varied, depending on the alternative form of nicotinamide nucleotide coenzyme. This is indicative of a mechanism where $NAD(P)^+$ binds to enzyme as the leading substrate in a sequential ordered mechanism. Values derived from the data of Bradbury & Jakoby (1971a) for kinetic coefficients for dehydrogenase A are shown in Table 3. By using NAD^+ as the coenzyme, with the alternative substrates benzaldehyde and glycolaldehyde, values of $\phi_{AB}/\phi_{\text{aldehyde}}$ are variant and non-hyperbolic kinetics were observed with NAD^+ as the varied substrate (Bradbury & Jakoby, 1971a). This is inconsistent with a mechanism for dehydrogenase A where NAD^+ binds to free enzyme.

In addition to the inequality of the kinetic con-

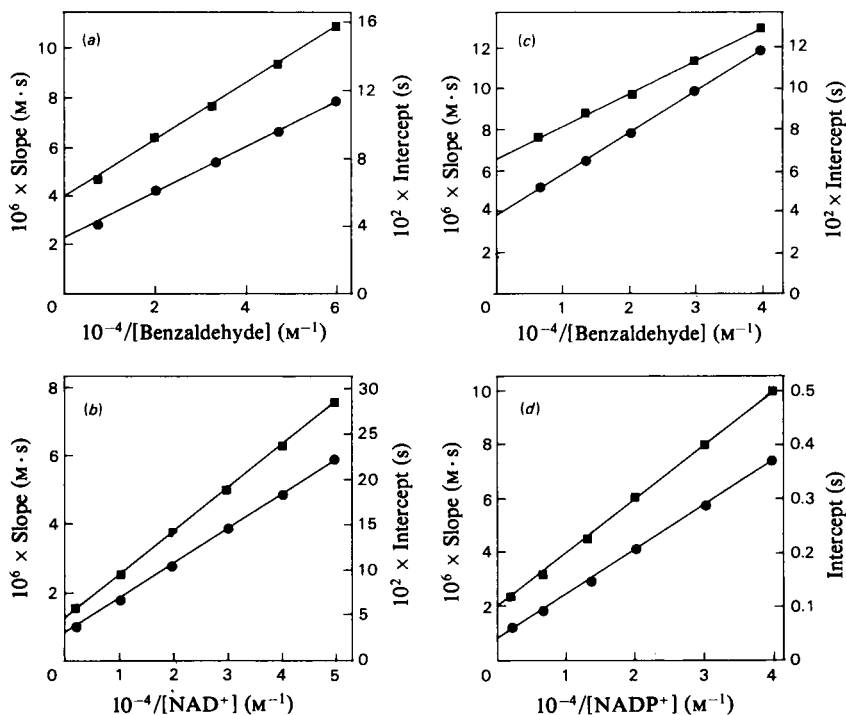


Fig. 3. Benzaldehyde oxidation by NAD^+ or $NADP^+$ with aldehyde dehydrogenase

Secondary plots of variation of the intercepts (●) and slopes (■) for double-reciprocal plots of specific initial rate with: (a) NAD^+ concentration for several constant benzaldehyde concentrations; (b) benzaldehyde concentration for several constant NAD^+ concentrations; (c) $NADP^+$ concentration for several constant benzaldehyde concentrations; (d) benzaldehyde concentration at several constant $NADP^+$ concentrations. The buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/0.1 M-KCl, at 25°C.

Table 1. Kinetic coefficients for aldehyde oxidation by NAD^+ or $NADP^+$ with aldehyde dehydrogenase
Reaction velocity was measured in 0.1 M-Tris/HCl (pH 8.0)/0.1 M-KCl/1 mM-mercaptoethanol. $NAD(P)^+$ and benzaldehyde or glycolaldehyde concentrations were varied as in Figs. 2-3, at 25°C.

Substrates	ϕ_0 (s)	ϕ_{aldehyde} ($\mu\text{M}\cdot\text{s}$)	$\phi_{NAD(P)^+}$ ($\mu\text{M}\cdot\text{s}$)	ϕ_{AB} ($\text{mm}^2\cdot\text{s}$)
NAD^+ -Glycolaldehyde	0.00897	6.19	2.42	0.000585
NAD^+ -Benzaldehyde	0.0320	1.31	3.84	0.000122
$NADP^+$ -Glycolaldehyde	0.0143	1.23	8.90	0.00133
$NADP^+$ -Benzaldehyde	0.0386	1.98	8.30	0.00020

stants and their relationships shown in Tables 2 and 3, a clear distinction between these two yeast enzyme preparations can be made on the basis of the patterns of intersecting lines in the Lineweaver-Burk plots. For dehydrogenase A the crossover point of the various lines in Lineweaver-Burk plots of e/v_0 versus $1/[A]$ or $1/[B]$ occurs on the $1/[A]$ or $1/[B]$ axis, whereas for the present preparation of aldehyde dehydrogenase the crossover occurs below the axis. Since the vertical coordinate, e/v_0 , of the crossover

point in these plots, where either substrate is varied, expresses the ratio of K_A^S to ϕ_A/ϕ_0 , by the following equation (Laidler & Bunting, 1973):

$$\frac{e}{v_0} = \frac{\phi_0}{e} \left(1 - \frac{\phi_A/\phi_0}{K_A^S} \right) \quad (2)$$

then for dehydrogenase A $\phi_A/\phi_0 = K_A^S$. However, with the enzyme as prepared by us K_A^S value is less than ϕ_A/ϕ_0 . This rules out a special case where $\phi_0 =$

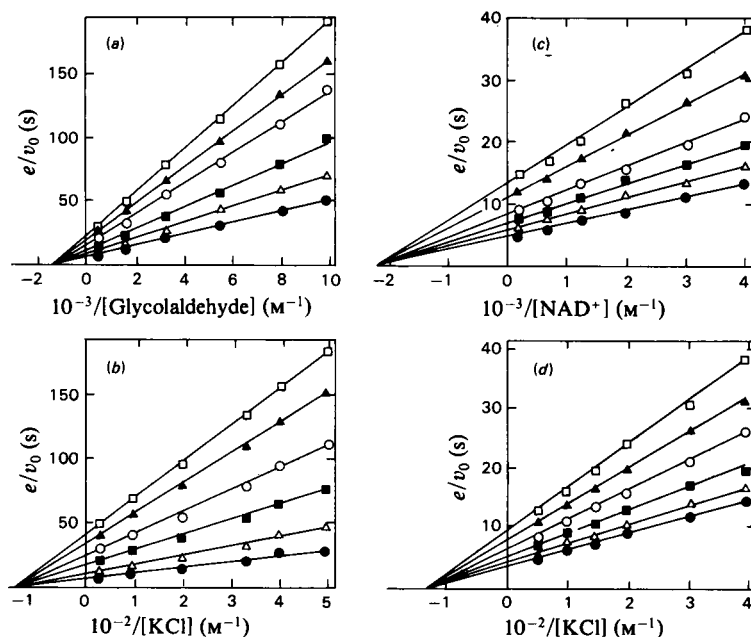


Fig. 4. Effect of K^+ on glycolaldehyde oxidation by NAD^+ with aldehyde dehydrogenase

(a) Primary plot of variation of the reciprocal specific initial rate with the reciprocal of glycolaldehyde concentration for several constant KCl concentrations (mM): \square , 2; \blacktriangle , 2.5; \circ , 3; \blacksquare , 5; \triangle , 10; \bullet , 33. (b) Primary plot of variation of the reciprocal specific initial rate with the reciprocal of KCl concentration for several constant glycolaldehyde concentrations (μM): \square , 10; \blacktriangle , 12.5; \circ , 18; \blacksquare , 30; \triangle , 60; \bullet , 200. (c) Primary plot of variation of the reciprocal specific rate with the reciprocal of NAD^+ concentration for several constant KCl concentrations (mM): \square , 2.5; \blacktriangle , 3.33; \circ , 5.0; \blacksquare , 6.66; \triangle , 10; \bullet , 20. (d) Primary plot of variation of the reciprocal specific rate with the reciprocal of KCl concentration for several constant NAD^+ concentrations (mM): \square , 0.25; \blacktriangle , 0.33; \circ , 0.5; \blacksquare , 0.8; \triangle , 1.5; \bullet , 5. The buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol at 2 mM-glycolaldehyde (a) and (b) or 5 mM- NAD^+ (c) and (d) at 25°C.

Table 2. Relationships between kinetic coefficients obtained from aldehyde oxidation by NAD^+ or $NADP^+$ with aldehyde dehydrogenase

Reaction was measured in 0.1 M-Tris/HCl (pH 8.0)/0.1 M-KCl/1 mM-mercaptoethanol. $NAD(P)^+$ and aldehyde concentrations were varied as in Figs. 2-3. e/ϕ_0 values were normalized for 1 nm-enzyme active sites.

Substrates	$\phi_{\text{aldehyde}}/\phi_0$ ($=K_m$ aldehyde) (μM)	$\phi_{\text{NAD(P)}^+}/\phi_0$ ($=K_m$ $NAD(P)^+$) (μM)	e/ϕ_0 ($=V$) (nm/s)	$\phi_{AB}/\phi_{\text{aldehyde}}$ (μM)	$\phi_{AB}/\phi_{\text{NAD(P)}^+}$ (μM)
NAD^+ -Glycolaldehyde	690	270	111	94.5	242
NAD^+ -Benzaldehyde	410	120	31.2	93.9	32
$NADP^+$ -Glycolaldehyde	860	622	62.2	108	149
$NADP^+$ -Benzaldehyde	51.3	215	25.9	101	24.1

Table 3. Relationships between kinetic coefficients obtained for aldehyde oxidation by NAD^+ or $NADP^+$ with dehydrogenase A

Kinetic constants were calculated from data of Bradbury & Jakoby (1971a). e/ϕ_0 values were normalized for 1 nm-enzyme active sites.

Substrates	$\phi_{\text{aldehyde}}/\phi_0$ (μM)	$\phi_{\text{NAD(P)}^+}/\phi_0$ (μM)	e/ϕ_0 (nm/s)	$\phi_{AB}/\phi_{\text{aldehyde}}$ (μM)	$\phi_{AB}/\phi_{\text{NAD(P)}^+}$ (μM)
NAD^+ -Glycolaldehyde	4580	122	1.85	126	4750
NAD^+ -Benzaldehyde	40.7	61	0.231	80.1	53.4

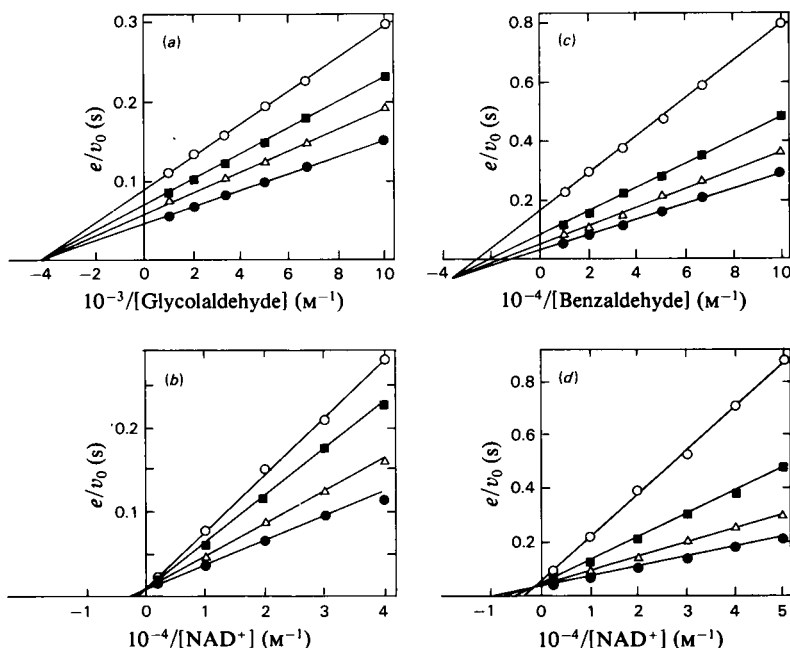


Fig. 5. Product inhibition of aldehyde oxidation by NAD^+ with aldehyde dehydrogenase

(a) Primary plot of variation of the reciprocal specific initial rate with the reciprocal of glycolaldehyde concentration for several constant NADH concentrations (μM): \circ , 150; \blacksquare , 100; \triangle , 50; \bullet , zero. Buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/0.1 M-KCl/0.5 mM- NAD^+ , at 25°C. (b) Primary plot of variation of the reciprocal specific initial rate with the reciprocal of NAD^+ concentration for several constant NADH concentrations (μM): \circ , 150; \blacksquare , 100; \triangle , 50; \bullet , zero. Buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/0.1 M-KCl/0.5 mM-glycolaldehyde, at 25°C. (c) Primary plot of variation of the reciprocal specific initial rate with the reciprocal of benzaldehyde concentration for several constant benzoate concentrations (mM): \circ , 76; \blacksquare , 38; \triangle , 15.2; \bullet , zero. Buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/0.1 M-KCl/0.1 mM- NAD^+ , at 25°C. (d) Primary plot of variation of the reciprocal specific initial rate with the reciprocal of NAD^+ concentration for several constant benzoate concentrations (mM): \circ , 76; \blacksquare , 45; \triangle , 19; \bullet , zero. Buffer used was 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/0.1 M-KCl/50 μM -benzaldehyde, at 25°C.

$\phi_A\phi_B/\phi_{AB}$ holds and the combination of enzyme with one substrate does not affect its affinity for the other.

Inhibition studies

Confirmation of the ordered sequential mechanism for the present preparation of aldehyde dehydrogenase was made by product and dead-end inhibition studies. Initial velocities at various glycolaldehyde, benzaldehyde or NAD^+ concentrations were made at different fixed concentrations of benzoate or NADH, both normal reaction products; Fig. 5 shows results as Lineweaver-Burk plots. Experiments were performed at fixed substrate concentrations of K_m values or less. These plots show that NADH is competitive with respect to NAD^+ and non-competitive with respect to either benzaldehyde or glycolaldehyde. Benzoate is non-competitive with respect to either

NAD^+ or benzaldehyde. Inhibition by either NADH or benzoate is a linear function of inhibitor concentration.

Rate predictions for the essentially irreversible reaction of yeast aldehyde dehydrogenase were derived by the rules of Cleland (1963*b,c*) for inhibitor acting as a product inhibitor only, a substrate analogue in dead-end fashion only, or as a mixed product dead-end inhibitor. The experimental data are only consistent with a sequential ordered mechanism where NAD^+ is the first substrate bound and carboxylic acid dissociates last. NADH acts as a dead-end inhibitor (with either benzaldehyde or glycolaldehyde as substrate) and benzoate acts as a product and dead-end inhibitor. Classification of the mechanism by the system of Cleland (1963*a*) is thus ordered Bi Bi.

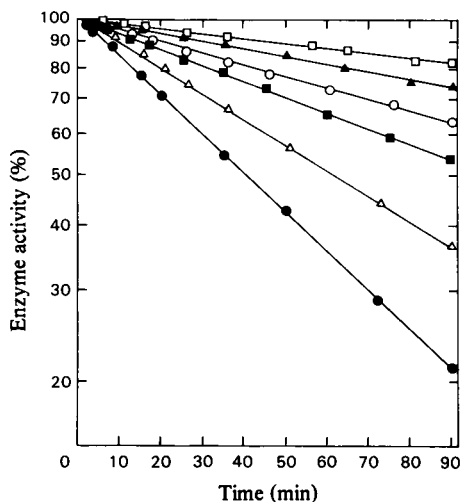


Fig. 6. Stability of aldehyde dehydrogenase at various concentrations of ethanediol

Plot of percentage enzyme activity with time at ethanediol concentrations (% v/v) of: ●, zero; △, 5; ■, 10; ○, 15; ▲, 20; □, 24. Homogeneous aldehyde dehydrogenase was equilibrated by passage through a Sephadex G-25 column (1.25 cm × 6.8 cm) into 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercapto-ethanol/0.1 M-KCl/5 mM-NAD⁺ and various ethanediol concentrations as indicated, and incubated at 25°C. Samples were removed at various times and measured for activity in standard reaction mixture after a prior 10 min incubation at 25°C in which buffer was supplemented to 24% (v/v) ethanediol.

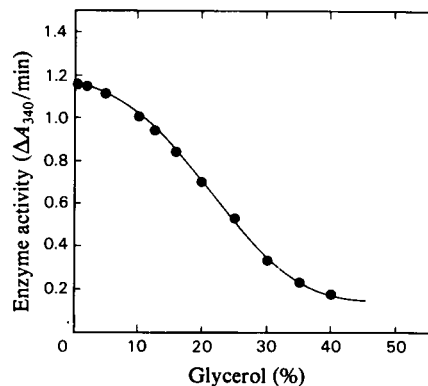


Fig. 7. Effect of glycerol on acetaldehyde oxidation by NAD⁺ with aldehyde dehydrogenase

Plot of variation of initial enzyme activity with glycerol concentration when assayed in an otherwise standard reaction mixture.

Table 4. Effect of glycerol on apparent K_m values for dehydrogenase A and homogeneous aldehyde dehydrogenase

Initial velocities for homogeneous aldehyde dehydrogenase at various substrate or cofactor concentrations were measured in 0.1 M-Tris/HCl, pH 8.0/10 mM-glutathione at 25°C with or without 25% (v/v) glycerol. K_m values were determined from double-reciprocal plots of v_0 versus substrate or cofactor concentration in a range 10-fold either side of K_m . Data for dehydrogenase A are from Bradbury & Jakoby (1972).

Substrate or cofactor	K_m (μM)			
	Dehydrogenase A		Homogeneous aldehyde dehydrogenase	
	Zero glycerol	25% (v/v) glycerol	Zero glycerol	25% (v/v) glycerol
Acetaldehyde	9	—	1000	660
Benzaldehyde	10	1	—	—
NAD ⁺	30	10	440	66
KCl	10000	—	6900	1000

With confirmation of the order of binding, the dissociation constant for enzyme binary complex with NAD⁺ or NADP⁺ can be determined by the method of Frieden (1957) from the negative reciprocal of the horizontal coordinate of the cross-over point of the various lines in Lineweaver-Burk plots. K_s for NAD⁺ and NADP⁺ are 94 and 100 μM respectively.

Inhibition studies of aldehyde dehydrogenase A by NADH at various NAD⁺ or benzaldehyde concentrations are inconsistent with the mechanism proposed above. NADH was competitive with respect to NAD⁺ but uncompetitive with respect to benzaldehyde (Bradbury & Jakoby, 1971a). These patterns are consistent with NADH behaving as a dead-end inhibitor in a sequential ordered mechanism where NAD⁺ is the second substrate bound.

Stability studies

Fig. 6 shows homogeneous enzyme stability at various ethanediol concentrations at high and constant KCl and NAD⁺ concentration. A 10 min incubation immediately before assay in which ethanediol was adjusted to 24% (v/v) resulted in no appreciable alteration in the initial velocity. The data confirm the requirement for polyhydric alcohol for stability (Steinman & Jakoby, 1967; Clark & Jakoby, 1970; Bradbury & Jakoby, 1972) even in the presence of K⁺ and NAD⁺. Decay of enzyme activity is a first-order process; without ethanediol but with 0.1 M-KCl and 5 mM-NAD⁺ the constant is 0.00029 s⁻¹. Identical experiments with glycerol as the stabilizing alcohol showed that higher concentrations of glycerol are necessary to achieve equivalent enzyme stability.

In addition to its stabilizing effect, polyhydric alcohol alters K_m values for both substrates and K^+ (Table 4), and decreases enzyme activity (Fig. 7). In a standard reaction mixture supplemented with 22.5% (v/v) glycerol or 25.5% (v/v) ethanediol, 50% inhibition of enzyme activity was observed.

Fig. 8 shows homogeneous enzyme stability at various KCl concentrations at high and constant ethanediol and NAD^+ concentration. Again, a 10 min incubation immediately before assay in which KCl is adjusted to 0.1M resulted in no appreciable alteration in initial velocity. The deviation from first-order rate decay indicates that the process is not a simple function with respect to a single enzyme species. Initial decay rate for homogeneous enzyme without K^+ and with high ethanediol and NAD^+ is $0.00057s^{-1}$.

Fig. 9 shows homogeneous enzyme stability at various NAD^+ concentrations at high and constant KCl and polyhydric alcohol concentration. Acceleration discernible in the progress curves for enzyme

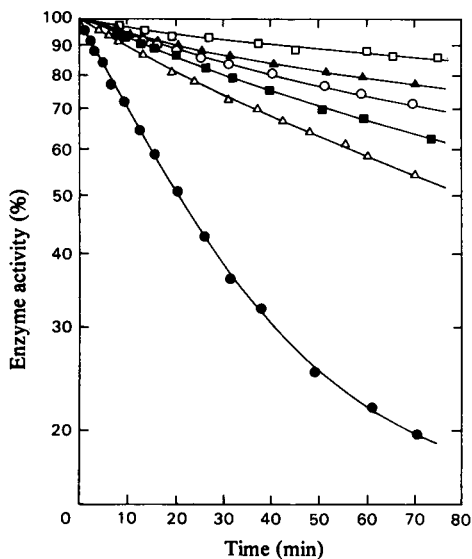


Fig. 8. Stability of aldehyde dehydrogenase at various concentrations of K^+

Plot of percentage of enzyme activity with time at KCl concentrations (mM) of: ●, zero; △, 5; ■, 7.5; ○, 10; ▲, 25; □, 50. Homogeneous aldehyde dehydrogenase was equilibrated by passage through a Sephadex G-25 column (1.25 cm × 6.8 cm) into 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/25% (v/v) ethanediol/5 mM- NAD^+ and various KCl concentrations as indicated, and incubated at 25°C. Samples were removed at various times and measured for activity in standard reaction mixture after a prior 10 min incubation at 25°C in which buffer was supplemented to 0.1 M-KCl.

incubated at lower than standard reaction mixture concentrations of NAD^+ indicate a recovery of enzyme activity during assay. When this experiment was performed with a 10 min preincubation period immediately before assay, in which the NAD^+ concentration was adjusted to 5 mM, total recoverable enzyme activities comparable with the most stable decay curve shown in Fig. 9 were observed for all concentrations of NAD^+ , including zero.

The rate of recovery of homogeneous enzyme after prolonged incubation in the absence of NAD^+ , but under otherwise saturating conditions for enzyme stability was measured after re-introduction of 5 mM- NAD^+ . The data in the insert to Fig. 9 have a rate constant of $0.005s^{-1}$.

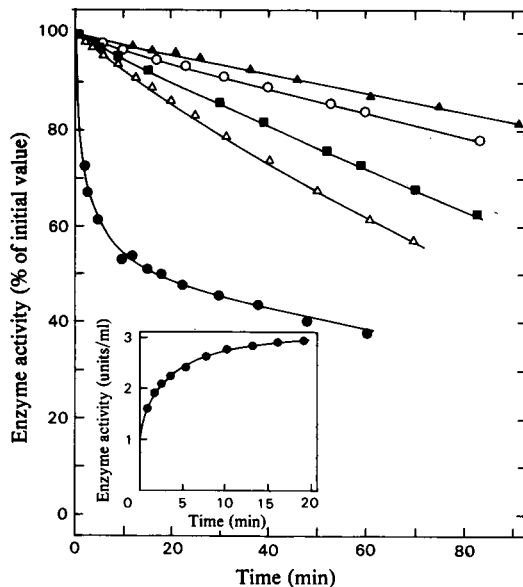


Fig. 9. Decay of initial aldehyde dehydrogenase activity at various NAD^+ concentrations

Plot of percentage of initial enzyme activity with time at ●, zero; △, 0.2 mM; ■, 0.8 mM; ○, 2.5 mM; ▲, 5 mM- NAD^+ . Homogeneous aldehyde dehydrogenase was equilibrated by passage through a Sephadex G-25 column (1.25 cm × 6.8 cm) into 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/25% (v/v) ethanediol/0.1 M-KCl and incubated at 25°C. Samples were removed at various times and assayed for initial activity in standard reaction mixture. Insert: plot of re-activation of aldehyde dehydrogenase by NAD^+ . Fraction VI aldehyde dehydrogenase was equilibrated into 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/22% (v/v) glycerol/0.1 M-KCl. After incubation at 25°C for 20 min enzyme was supplemented to 5 mM- NAD^+ and samples were removed at various times for assay of initial activity in standard reaction mixture.

In similar experiments to those in Fig. 9, 5'-AMP and NADH substituted for NAD⁺ in stabilizing initial enzyme activity. For 5'-AMP much higher concentrations than NAD⁺ were required for equivalent protection. This presumably reflects the relative affinity, paralleling the high value of K_1 for 5'-AMP (0.79 mM) compared with the K_s for NAD⁺ (0.094 mM).

A different behaviour is seen for K⁺ stabilization in the absence of polyhydric alcohol. Prolonged incubation in 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol gives rise to an enzyme that recovers activity in the reaction mixture. A 10 min incubation immediately before assay in buffer supplemented to 0.1 M-KCl eliminates the acceleration and results in higher activities. Thus in the absence of polyhydric alcohol K⁺ alone is sufficient to cause the partial recovery of activity which in the presence of polyhydric alcohol only NAD⁺ can achieve (see Fig. 9). The rate of recovery of enzyme allowed to incubate in the absence of K⁺ for 20 min, on supplementation to 0.1 M-KCl is 0.018 s⁻¹.

Initial decay rates for homogeneous enzyme in the absence of polyhydric alcohol with or without high concentrations of KCl or NAD⁺ are presented in Table 5. Also provided are values for homogeneous enzyme at high ethanediol concentration. In all cases ethanediol decreases the decay rate. K⁺ does the same either in its presence or absence. At progressively higher concentrations of both, enzyme stability increases.

The stability of dehydrogenase A, calculated from data of Bradbury & Jakoby (1972), indicates that with such preparations preincubation with NAD⁺ is not required for maximum activity. Decay of activity of dehydrogenase A in the absence of NAD⁺,

but under otherwise similar conditions, have rates comparable with homogeneous enzyme (Bostian & Betts, 1978) in the presence of high concentrations of NAD⁺.

Discussion

Data from initial-rate studies of yeast aldehyde dehydrogenase with varied substrate and coenzyme fit eqn. (1), and confirm the participation of a ternary complex in the overall catalytic mechanism. Evidence for a sequential ordered mechanism where NAD⁺ is the leading substrate was established by alternative-substrate analysis and product inhibition. This is consistent with the demonstrated binding of NAD⁺ to free enzyme (Bostian & Betts, 1978). It differs, however, from the proposed mechanism for dehydrogenase A, which has aldehyde as an obligatory leading substrate (Bradbury & Jakoby, 1971a,b). In our hands, several attempts to prepare dehydrogenase A by the method of Clark & Jakoby (1970) yielded enzyme displaying non-competitive inhibition by NADH with respect to aldehyde, and thus consistent with enzyme reported here and different from data for dehydrogenase A.

Comparison of kinetic coefficients obtained from initial-rate studies with varied coenzyme and aldehyde concentrations show, with enzyme prepared by us, that glycolaldehyde and benzaldehyde give the same values of $\phi_{AB}/\phi_{\text{benzaldehyde}}$ or $\phi_{AB}/\phi_{\text{glycolaldehyde}}$ when either NAD⁺ or NADP⁺ was used. In contrast, the $\phi_{AB}/\phi_{\text{aldehyde}}$ values for aldehyde dehydrogenase A (Bradbury & Jakoby, 1971a) differ by 60-fold for glycolaldehyde and benzaldehyde. Values of $\phi_{AB}/\phi_{\text{NAD}^+}$ and $\phi_{AB}/\phi_{\text{NADP}^+}$ for benzaldehyde were not significantly different, although for glycolaldehyde these differed by 1.6-fold. $\phi_{AB}/\phi_{\text{aldehyde}}$ or $\phi_{AB}/\phi_{\text{NAD(P)}^+}$ values in the present studies did not vary by more than a factor of 1.6 with all aldehydes investigated or with either nicotinamide nucleotide. Values of $\phi_{AB}/\phi_{\text{aldehyde}}$, the dissociation constant for E-NAD⁺ and E-NADP⁺ complexes with yeast aldehyde dehydrogenase were similar at about 100 μM . This is in reasonable agreement with values for NAD⁺ in liver alcohol dehydrogenase with either primary or secondary alcohols (Dalziel, 1975) and is 2-, 3- and 4-fold less than bovine heart lactate dehydrogenase, yeast alcohol dehydrogenase and bovine heart cytoplasmic malate dehydrogenase respectively. The dissociation constant for NAD⁺ reported here is five times greater than for horse liver aldehyde dehydrogenase (Takio *et al.*, 1974).

The original observation by Black (1951) that the enzyme is one-tenth as active with NADP⁺ as NAD⁺ is thus not verified by these findings, maximal velocity differing by a factor less than two with either glycolaldehyde or benzaldehyde. The ϕ_0 values with glycolaldehyde were similar to those with acetalde-

Table 5. Rates of decay of homogeneous aldehyde dehydrogenase in the presence or absence of ethanediol. Enzyme was incubated in 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol plus additions as listed at 25°C. Rate constants were calculated as $-2.303 \times$ initial slope from plots of log activity versus time. A 10 min incubation in 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/22% (v/v) ethanediol/0.1 M-KCl/5 mM-NAD⁺ immediately before assay was performed on all samples taken for activity assay.

Additions to incubation buffer	$10^4 \times k \text{ (s}^{-1}\text{)}$	
	Zero ethanediol	22% (v/v) ethanediol
—	36	3.5
0.1 M-KCl	3.0	0.36
5 mM-NAD ⁺	34	5.7
0.1 M-KCl and 5 mM-NAD ⁺	2.9	0.35

hyde and one-quarter of that with acetaldehyde reduction by liver alcohol dehydrogenase at optimal pH. Maximum turnover numbers ($1/\phi$) for dehydrogenases in general are greater than those reported here.

By analogy with the work of Dalziel & Dickinson (Dalziel & Dickinson, 1966; Cleland, 1967) on primary alcohol oxidation by horse liver alcohol dehydrogenase, the nearly identical maximum velocities for any of these three aldehydes, even though Michaelis constants vary by as much as 10-fold, might indicate that the rate-limiting step is the breakdown of E-product binary complex. The 60-fold difference in maximum velocity for benzaldehyde and glyceraldehyde with aldehyde dehydrogenase A indicates that there is not a single rate-limiting step in this mechanism.

All three aldehyde substrates used for initial-velocity studies show inhibition at high concentration. For benzaldehyde and glycolaldehyde (NAD^+ concentration at K_m values) deviations from hyperbolic rate behaviour is observed at aldehyde concentration at ten times the K_m values. For acetaldehyde this occurs just slightly above the K_m value. The inhibition by acetaldehyde becomes more pronounced at higher NAD^+ concentrations.

Although a detailed kinetic investigation of substrate inhibition of yeast aldehyde dehydrogenase was not attempted in this work, several generalizations can be made and two possible models proposed. Inhibition by aldehyde substrate is a common property of most aldehyde dehydrogenases thus far examined (Jakoby, 1963) and has been ascribed to hemiacetal formation with an —SH group important in catalysis (Nirenberg & Jakoby, 1960; Jakoby, 1963). If, in polyhydric alcohol, these readily reactive essential thiol groups are said to be buried (Bradbury & Jakoby, 1972), then one would expect a decrease in the observed inhibition as polyhydric alcohol concentration is increased. This is, in fact, not observed. With aldehyde dehydrogenase a 2–3-fold decrease in K_m for acetaldehyde caused by supplementing reaction mixture to 25% (v/v) glycerol is accompanied by similar decreases in acetaldehyde concentration necessary for inhibition (K. A. Bostian & G. F. Betts, unpublished work). That in one instance for aldehyde dehydrogenase (Jakoby & Scott, 1959) inhibition is specific to aldehyde substrates and not other aldehydes, suggests that the inhibition being observed might be explained by an abortive ternary complex.

Data from initial-rate studies with various concentrations of univalent cation activator show that K_m values for substrate and coenzyme are unaltered by change in activator concentration. This is consistent with the formation of a complex in which enzyme forms a bridge between cation and substrate and the binding of activator and substrate are truly

independent. Stability data also show that K^+ is able to bind in the absence of either NAD^+ or aldehyde. The ability of NAD^+ to cause some recovery of activity to enzyme incubated in the presence of polyhydric alcohol but in the absence of NAD^+ and K^+ indicates at least one binary enzyme-substrate complex forms in the absence of K^+ . The data are inconsistent with a coenzyme bridge (E- NAD^+ -M), a cation bridge (E-M- NAD^+), or a substrate bridge (E-aldehyde- K^+) in the catalytic mechanism, unless another molecule of activating cation is involved. The absence of any effect of K^+ on the K_m for aldehyde also argues against a cation bridge between enzyme and aldehyde unless aldehyde binding is absolutely dependent on cation.

In addition to previously demonstrated requirements for enzyme stability, yeast aldehyde dehydrogenase as purified by us is also dependent on preincubation with nicotinamide nucleotide for maximum enzyme rate. Yeast aldehyde dehydrogenase is not unique in its NAD^+ preincubation requirement. Several other nicotinamide nucleotide-dependent enzymes show similar behaviour (Boyer *et al.*, 1963; Sund, 1970; Boyer, 1975). In all cases enzyme has been shown to form binary complexes. With glutamate dehydrogenase for example, stabilization by nicotinamide nucleotides involves aggregational as well as conformational changes in the enzyme molecule (Eisenkraft & Veeger, 1970). Evidence for very similar preincubation requirements has also been shown for two other aldehyde dehydrogenases, aldehyde dehydrogenase from *Pseudomonas* (Jakoby, 1958), and malonic semialdehyde oxidative decarboxylase (Yamada & Jakoby, 1960). Enzyme conformation change on coenzyme binding has been implicated in lactate dehydrogenase, alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase (Boyer, 1975).

Although binary complex between enzyme and NAD^+ is implied by the aldehyde dehydrogenase stability data, it is conceivable that ubiquitous contamination by endogenous aldehydes (Bradbury & Jakoby, 1971a; Bostian & Betts, 1978) might provide a binary E-aldehyde complex with which NAD^+ can combine. However, during incubation of enzyme with saturating concentrations of NAD^+ , enzymically oxidizable aldehydes would be removed leaving only non-oxidizable aldehydes. These are present at much less than saturating concentrations (Steinman & Jakoby, 1968; Bostian & Betts, 1978). Initial stability would be expected to decrease to that approaching the situation where NAD^+ is absent. This, however, is not experimentally observed. Moreover, equilibrium dialysis shows NAD^+ binding even when such metabolizable aldehydes have long since disappeared (Bostian & Betts, 1978).

The presence of contaminating aldehydes provides

another possible mechanism for the NAD^+ activation effect. If contaminating aldehydes are substrates having high affinity but low rates of oxidation these would need to be oxidized before maximum enzyme activity is observed with added aldehydes. However, the time required to oxidize enzymically the low amount of enzymically reactive contaminating aldehydes present (i.e. 1 h) is greater than the time required for recovery of activity by preincubation with NAD^+ (i.e. under 10 min) after incubation in polyhydric alcohol and KCl. Moreover, with 5'-AMP and NADH, both of which prevent the reversible loss of initial enzyme activity, contaminant aldehydes are clearly not being removed by enzymic oxidation.

A mechanism of NAD^+ activation suggested by Jakoby (1963) for malonic semialdehyde oxidative decarboxylase and aldehyde dehydrogenase from *Pseudomonas* proposed that NAD^+ preincubation is required to prevent and/or reverse indiscriminate hemiacetal formation between aldehyde substrates and an —SH group involved in the binding of NAD^+ at the active site. This model would be consistent with any order of substrate addition dependent only on whether aldehyde is also bound at its correct binding site.

Kinetic estimates of dissociation constants for coenzyme agree well with calculations from equilibrium or rate of dialysis binding experiments (Bostian & Betts, 1978). Values for dissociation constants from the two types of dialysis binding studies were 120 and $68 \mu\text{M}$ respectively for each of four binding sites per enzyme molecule. Bradbury & Jakoby (1971*b*) equated the two binding sites calculated for reduced coenzyme on aldehyde dehydrogenase A with binding sites for NAD^+ in catalysis. Specific activity and molecular weight of aldehyde dehydrogenase A and the preparation reported here are very similar. Thus these binding studies can only reflect the number of active sites if the two sites of dehydrogenase A have a turnover number twice as great as the four sites of the present preparation. With the finding that 4 mol of NAD^+ are bound/mol of tetrameric enzyme it is reasonable to assume that there is one active site per identical subunit. However, there is no reason as such why this need be. For horse liver alcohol dehydrogenase Weiner (1969) used a paramagnetic NAD^+ analogue to show that 8 mol of NAD^+ bind per dimer, although there are only two active sites. Weiner *et al.* (1974) suggested that there may be fewer than four active sites in tetrameric horse liver aldehyde dehydrogenase. This suggestion was prompted in part by analogy with the data of Bradbury & Jakoby (1971*b*) for reduced coenzyme binding to yeast aldehyde dehydrogenase A in the presence of aldehyde. However, for the horse liver enzyme there are two sets of binding sites with dissociation constants 6 and $50 \mu\text{M}$, each binding

2 mol of NADH, and yet there is no evidence from kinetic studies of two different binding affinities.

Unequal binding stoichiometry and 'functional active sites' have been observed for a number of enzymes: glyceraldehyde 3-phosphate dehydrogenase, alkaline phosphatase, acetoacetate decarboxylase, pyridoxamine pyruvate transaminase and alcohol dehydrogenase (Bernhard *et al.*, 1970). In contrast with the proposed mechanism of half-sites reactivity for alcohol dehydrogenase (Bernhard *et al.*, 1970), it is possible that a similar negative co-operativity with aldehyde dehydrogenase A is not operative with the enzyme preparation reported here. Alternatively only two of the four NAD^+ -binding sites in enzyme prepared by us may be active in the catalytic oxidation of aldehydes. A possible role for the remaining bound NAD^+ could be to influence activity by inducing an enzyme-conformation change. This would be consistent with the failure of Jakoby and co-workers (Clark & Jakoby, 1970; Bradbury & Jakoby, 1971*b*) to observe a requirement for nicotinamide nucleotide preincubation in their enzyme preparations. Identical activity of four subunits which seem to be structurally identical would suggest a symmetrical arrangement for their quaternary structure. A model for yeast aldehyde dehydrogenase consisting of two active sites per molecule, however, is easier to equate with the unique appearance of enzyme prepared by us in negatively stained electron micrographs (K. A. Bostian, N. Wriggley & G. F. Betts, unpublished results). These reveal a high degree of asymmetry and a highly asymmetric oligomeric structure atypical of most cyclic and dihedrally arranged tetrameric protein molecules. Heterologous bonding of subunits is also supported by the appearance of dimer after sodium dodecyl sulphate dissociation (Bostian & Betts, 1978).

Non-availability of the NAD^+ -binding sites of aldehyde dehydrogenase A until substrate is bound would represent an important and unique distinction from other dehydrogenases. Changing physiological or nutritional status may have considerable effect on cellular aldehyde concentration. If nicotinamide nucleotide only binds in the presence of substrate, and substrate can vary between zero concentration and ten times K_m values, this will have the effect of changing free nicotinamide nucleotide by the concentration of enzyme active sites times the saturation by nicotinamide nucleotide. Since aldehyde dehydrogenase constitutes as much as 0.1% of dry cell weight, this change in nicotinamide nucleotide pool by aldehyde dehydrogenase could be as large as 5%. This may be a physiological reason why dehydrogenase binds coenzymes in the absence of substrate, i.e. there is no rapid fluctuation in nicotinamide nucleotide concentrations affecting a whole range of metabolic processes on a change in the substrate status of one dehydrogenase. If the present enzyme

and aldehyde dehydrogenase A are both forms of the enzyme newly synthesized *in vivo*, or modified by proteolysis in response to changes in physiological status, then this may be active in metabolic regulation.

References

- Alberty, R. A. (1953) *J. Am. Chem. Soc.* **75**, 1928–1932
- Bernhard, S. A., Dunn, M. F., Luisi, P. L. & Schack, P. (1970) *Biochemistry* **9**, 188–192
- Black, S. (1951) *Arch. Biochem. Biophys.* **34**, 86–97
- Bostian, K. A. & Betts, G. F. (1978) *Biochem. J.* **173**, 773–786
- Bostian, K. A., Betts, G. F., Man, W. K. & Hughes, M. N. (1975) *FEBS Lett.* **59**, 88–91
- Boyer, P. D. (ed.) (1975) *Enzymes 3rd Ed.* **11**
- Boyer, P. D., Lardy, H. & Myrbäch, K. (eds.) (1963) *Enzymes 2nd Ed.* **7**
- Bradbury, S. L. & Jakoby, W. B. (1971a) *J. Biol. Chem.* **246**, 1834–1840
- Bradbury, S. L. & Jakoby, W. B. (1971b) *J. Biol. Chem.* **246**, 6920–6932
- Bradbury, S. L. & Jakoby, W. B. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2373–2376
- Clark, J. F. & Jakoby, W. B. (1970) *J. Biol. Chem.* **245**, 6065–6074
- Cleland, W. W. (1963a) *Biochim. Biophys. Acta* **67**, 104–137
- Cleland, W. W. (1963b) *Biochim. Biophys. Acta* **67**, 173–187
- Cleland, W. W. (1963c) *Biochim. Biophys. Acta* **67**, 188–196
- Cleland, W. W. (1967) *Annu. Rev. Biochem.* **36**, 77–112
- Dalziel, K. (1957) *Acta Chem. Scand.* **11**, 1706–1723
- Dalziel, K. (1975) *Enzymes 3rd Ed.* **11**, 2–52
- Dalziel, K. & Dickinson, F. M. (1966) *Biochem. J.* **100**, 34–46
- Eisenkraft, B. & Veeger, C. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), pp. 271–276, Springer-Verlag, New York
- Frieden, C. (1957) *J. Am. Chem. Soc.* **79**, 1894–1896
- Jakoby, W. B. (1958) *J. Biol. Chem.* **232**, 75–87
- Jakoby, W. B. (1963) *Enzymes 2nd Ed.* **7**, 203–221
- Jakoby, W. B. & Scott, E. M. (1959) *J. Biol. Chem.* **234**, 937–940
- Laidler, K. J. & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, pp. 116–119, Clarendon Press, Oxford
- Nirenberg, M. & Jakoby, W. B. (1960) *Proc. Natl. Acad. Sci. U.S.A.* **46**, 206–212
- Sorger, G. J. & Evans, H. J. (1966) *Biochim. Biophys. Acta* **118**, 1–8
- Springham, M. G. (1974) Ph.D. Thesis, University of London
- Springham, M. G. & Betts, G. F. (1973) *Biochim. Biophys. Acta* **309**, 233–236
- Steinman, C. R. & Jakoby, W. B. (1967) *J. Biol. Chem.* **242**, 5019–5023
- Steinman, C. R. & Jakoby, W. B. (1968) *J. Biol. Chem.* **243**, 730–734
- Sund, H. (ed.) (1970) *Pyridine Nucleotide Dependent Dehydrogenases*, Springer-Verlag, New York
- Takio, K., Saho, Y. & Yonetani, T. (1974) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Yonetani, T., Williamson, J. R. & Chance, B., eds.), pp. 115–123, Academic Press, New York
- Weiner, H. (1969) *Biochemistry* **8**, 526–533
- Weiner, H., King, P., Hu, J. H. J. & Bensch, W. R. (1974) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Yonetani, T., Williamson, J. R. & Chance, B., eds.), pp. 101–113, Academic Press, New York
- Wong, J. T. & Hanes, C. S. (1962) *Can. J. Biochem. Physiol.* **40**, 763–804
- Yamada, E. W. & Jakoby, W. B. (1960) *J. Biol. Chem.* **235**, 589–594