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# Partial Reversal of the Acetaldehyde and Butyraldehyde Oxidation Reactions Catalysed by Aldehyde Dehydrogenases from Sheep Liver

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In the presence of acetic anhydride or butyric anhydride, liver aldehyde dehydrogenases catalyse the oxidation of NADH at pH7.0 and 25°C. The maximum velocities and Michaelis constants for NADH at saturating anhydride concentrations are independent of which anhydride is used, the values being $V'_{max.} = 12 min^{-1}$ and $K_m$ for NADH = 9 $\mu$M for the mitochondrial enzyme and $V'_{max.} = 25 min^{-1}$ and $K_m$ for NADH = 20 $\mu$M for the cytoplasmic enzyme. Substitution of [4A-$^2$H]NADH for NADH resulted in 2-fold and 4-fold decreases in rate for the mitochondrial and cytoplasmic enzymes respectively.

Aldehyde dehydrogenase (EC 1.2.1.3) catalyses the $NAD^+$-linked oxidation of aldehyde to the corresponding acids, with reduction of $NAD^+$ to NADH.

The mechanism of aldehyde oxidation is thought to proceed via the formation of a thiohemiacetal between an enzyme thiol group and the aldehyde substrate (Jakoby, 1963), which is then oxidized to give a thioacyl-enzyme–NADH complex, hydrolysis of which leads to product release. The reaction is effectively irreversible. At pH7.0 and 25°C, the free-energy change for the oxidation of acetaldehyde by $NAD^+$ is −2.98 KJ/mol (Burton & Stadtman, 1953). It is the hydrolysis of the thioester that accounts for the majority of the total free-energy change, and that is thought to be the step responsible for the overall irreversibility of the reaction, typical values for thioester hydrolysis being about −1.92 kJ/mol (Faber & Reid, 1917). The aldehyde dehydrogenase reaction contrasts with that catalysed by glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), which follows the same basic mechanism (Jakoby, 1963), but which is freely reversible. In this case the product acyl phosphate, 1,3-diphosphoglycerate, is capable of bringing about the rapid acylation of the enzyme thiol group.

If the acyl-enzyme of aldehyde dehydrogenase could be formed by alternative means, the major irreversible stage of the reverse reaction would have been by-passed and it might be possible, in the presence of NADH, for partial reversal of the reaction to be demonstrated.

One potential way of doing this is to make use of the ability of aldehyde dehydrogenase to catalyse the hydrolysis of *p*-nitrophenyl esters. It is proposed that ester hydrolysis also proceeds by way of an acyl-enzyme intermediate (Feldman & Weiner, 1972), and since the rate of hydrolysis of *p*-nitrophenyl acetate is greatly increased in the presence of coenzymes (Hart & Dickinson, 1978), ester hydrolysis in the presence of NADH must proceed via an acyl-enzyme–NADH complex. However, Duncan (1977) was unable to demonstrate the oxidation of NADH by acetyl-enzyme formed through ester hydrolysis. Nor was it possible to observe oxidation of NADH by a semi-purified aldehyde dehydrogenase from pig brain in the presence of ethyl thioacetate, which might be expected to form the acetyl-enzyme by acyl transfer (Duncan, 1970).

We decided to investigate the possibility of forming the acyl-enzyme by using simple acylating reagents, acid anhydrides, and to look for oxidation of NADH by any acyl-enzyme so formed.

## Materials and Methods

$NAD^+$ (grade II) and NADH (grade I) were purchased from Boehringer Corp. (London), London W.5, U.K. Acetic anhydride and acetic acid were from Fisons Chemicals, Loughborough, Leics., U.K. Butyric anhydride was from BDH Chemicals, Poole, Dorset, U.K. [$^2H_6$]Ethanol [containing 99 atom % deuterium; 95% ethanol (w/w) in $^2H_2O$] was a product of Prochem BOC, Deer Park Road, London S.W.19, U.K. [4A-$^2$H]NADH was prepared by the yeast alcohol dehydrogenase-catalysed reduction of $NAD^+$ with [$^2H_6$]ethanol by the method of Dalziel (1962) modified in that after the enzymic reduction the mixture was placed in a boiling-water bath for 3.5 min. This step was introduced to totally inactivate the enzyme before the addition of non-labelled ethanol to the mixture. The product had the following characteristics: $A_{260}/A_{340} = 2.5$; residual $A_{340}$ after oxidation by excess acetaldehyde and yeast alcohol dehydrogenase at pH7.0 was 4%. Analysis by n.m.r. spectroscopy indicated that there had been at least 90% incorporation of deuterium into the product.

Mitochondrial aldehyde dehydrogenase from sheep liver was prepared by the method of Hart &

Dickinson (1977). The cytoplasmic enzyme was prepared basically by the method of Crow *et al.* (1974), but with two additional $(NH_4)_2SO_4$ fractionations after the Sephadex G-200 column stage. Before use enzyme solutions were dialysed against sodium phosphate buffer, pH 7.0 ($I = 0.1$), containing 100 $\mu$M-dithiothreitol. The mitochondrial enzyme had a specific activity of 0.2 units/mg and the cytoplasmic enzyme a specific activity of 0.16 units/mg when assayed at pH 8.0 and 25°C by the method of Hart & Dickinson (1977). Isoelectric focusing on 4% polyacrylamide gels with Ampholines at pH 5–8 showed that both enzymes were essentially pure and that each was uncontaminated by significant amounts of the other. The two enzymes can be clearly separated by this procedure (F. M. Dickinson & S. Berrieman, unpublished work).

Assays in the reverse direction were carried out at 25°C in 200 mM-sodium phosphate buffer, pH 7.0. After the addition of the required amounts of NADH and enzyme to a final volume of 3 ml, acetic anhydride (5 $\mu$l; 16.3 mM final concentration) or butyric anhydride (5 $\mu$l; 10.5 mM final concentration) was added, and the decrease in $A_{340}$ was monitored by using a Zeiss PMQII spectrophotometer, equipped with a Vitatron UR40 lin-log chart recorder.

## Results and Discussion

A typical progress curve for the reverse reaction catalysed by mitochondrial aldehyde dehydrogenase is shown in Fig. 1. Entirely similar behaviour is shown by the cytoplasmic enzyme. The initial rate of the reaction was proportional to enzyme concentration at least up to a final concentration of 600 nM. Enzyme assayed in the standard assay after the anhydride treatment retained 90% of its activity after corrections were made for the amount of NADH carried over into the standard assay. The pH of the solution after total hydrolysis of the added anhydride did not fall below pH 6.9.

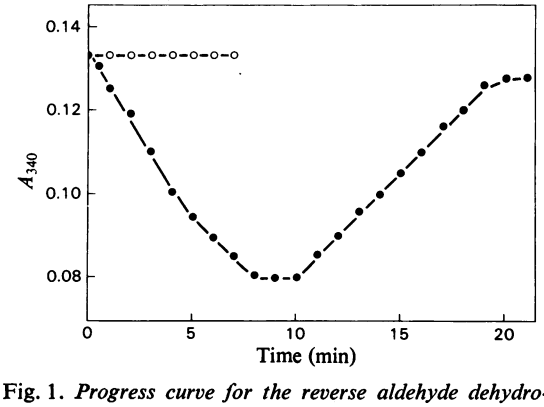


Fig. 1. *Progress curve for the reverse aldehyde dehydrogenase reaction*

Assays were carried out at pH 7.0 and 25°C in 0.2 M-phosphate buffer by the addition of acetic anhydride (●) (16.3 mM) to NADH (21.6 $\mu$M) and mitochondrial aldehyde dehydrogenase (200 nM). After 10 min, acetaldehyde (650 $\mu$M) was added. The Figure also shows an assay (○) in which acetic acid (26 mM) was added in place of acetic anhydride. Similar features were observed with the cytoplasmic enzyme.

The decrease in $A_{340}$ after the addition of anhydride to mixtures of enzyme and NADH indicates that anhydrides are able to acylate aldehyde dehydrogenase, and that acyl-enzyme formed in this way is able to oxidize NADH, leading to release of aldehyde and $NAD^+$. Addition of acetaldehyde (650 $\mu$M final concentration) at the end of the reaction causes the $A_{340}$ to return to its original value, confirming that the earlier decrease in $A_{340}$ was due to oxidation of NADH. Addition of 28 mM-acetic acid in place of acetic anhydride does not give any change in $A_{340}$. In addition to the unfavourable equilibrium and the fact that it is in a poor acylating agent, acetate might not act as a substrate in the reverse reaction because it does not bind to the enzyme. Iodoacetate does not inhibit the mitochondrial enzyme whereas the uncharged iodoacetamide is quite effective (Hart & Dickinson, 1977). Sodium acetate at concentrations as high as 20 mM does not inhibit the $F_1$ isoenzyme from horse liver (Eckfeldt & Yonetani, 1976). Enzyme that had been treated with the thiol-modifying reagent methylmethanethiosulphonate so that there was less than 5% activity remaining when the enzyme was assayed in the standard assay did not, as expected, show any detectable activity in the reverse reaction.

This indicates that the thiol groups required in the forward reaction are also essential for the reverse reaction.

Fig. 2 shows the effect on the specific initial rate of the reverse reaction of varying the NADH concentration, at a constant and saturating anhydride concentration. (Halving or doubling the amount of anhydride added does not lead to any change in rate).

Assays in which butyric anhydride (10 mM) was added in place of acetic anhydride gave rates that, within experimental error, were identical to those with acetic anhydride. Assays in which 100 $\mu$M-[4A-$^2$H]-NADH replaced 100 $\mu$M-NADH under the conditions of Fig. 2 resulted in a 1.5–2.0-fold decrease in rate for the mitochondrial enzyme and a 3.5–4.0 fold decrease for the cytoplasmic enzyme. Levy & Vennesland (1957) showed that aldehyde dehydrogenase reacts with the A-side of the nicotinamide ring of the coenzyme.

The double reciprocal plots shown in Fig. 2 provide estimates of the maximum specific rate

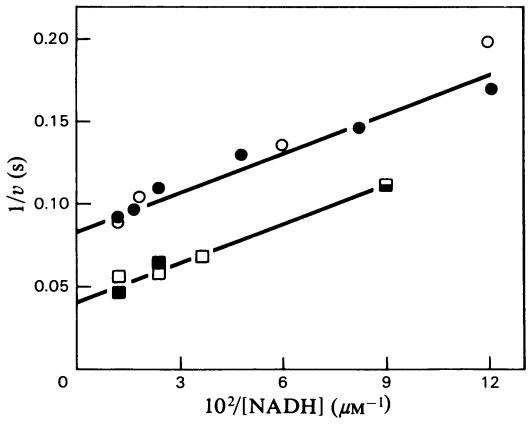


Fig. 2. *Initial-rate measurements for the reverse aldehyde dehydrogenase reaction*

Variation of the reciprocal of the specific initial rate of the oxidation of NADH at pH7.0 and 25°C in 0.2M-phosphate buffer with the reciprocal of the NADH concentration for mitochondrial aldehyde dehydrogenase (●, ○) and cytoplasmic aldehyde dehydrogenase (■, □) at constant anhydride concentrations. The anhydride concentrations were: acetic anhydride (●, ■) (16.3mM) and butyric anhydride (○, □) (10.5mM). Enzyme concentrations were calculated by using a mol.wt. of 200000.

($V'_{max.}$) and the Michaelis constants for NADH [$K_{m(NADH)}$] for the reverse reaction. For the mitochondrial enzyme $V'_{max.} = 12 min^{-1}$ and $K_{m(NADH)} = 9 \mu M$ and for the cytoplasmic enzyme $V'_{max.} = 25 min^{-1}$ and $K_{m(NADH)} = 20 \mu M$. The parameters are essentially unchanged when butyric anhydride is used instead of acetic anhydride. Kitson (1975) has shown that the mitochondrial and cytoplasmic enzymes show different inhibition behaviour when treated with tetraethylthioperoxydicarbonic diamide (disulfiram). The kinetic parameters given in the present paper provide further evidence that the two enzymes are different as do the differences in observed deuterium isotope effect.

The maximum rates of the acetic anhydride/NADH reactions are not greatly different from those observed in the acetaldehyde/$NAD^+$ reactions. For the mitochondrial enzyme at pH7.0 and 25°C, $V_{max.} = 77 min^{-1}$ (Hart & Dickinson, 1978), and preliminary investigations in our laboratory indicate that for the cytoplasmic enzyme $V_{max.} = 60 min^{-1}$. The latter value is in reasonable agreement with that of $k_{cat.} = 0.25 s^{-1}$ at pH7.6 obtained by MacGibbon *et al.* (1977*a*), if it is assumed that all four subunits of the enzyme are operative. Studies with both enzymes showed that the maximum rates were not subject to a deuterium isotope effect (MacGibbon *et al.*, 1977*b*, 1978; Hart & Dickinson, 1978). Thus the rates of hydride transfer in the direction of aldehyde oxidation must be considerably greater than the observed maximum velocities. By contrast the observed isotope effects with [4A-$^2$H]NADH show that hydride transfer is partly rate limiting in the direction of NADH oxidation and that the specific rate of hydride transfer is unlikely to exceed $50 min^{-1}$.

In our previous kinetic work with the mitochondrial enzyme (Hart & Dickinson, 1978) we found no 'burst' of NADH production in studies of aldehyde oxidation at high enzyme concentrations. The studies were made by using spectrophotometric methods and they led to the conclusion that the rate-limiting step in aldehyde oxidation probably precedes hydride transfer. In their studies with more sensitive fluorescence methods MacGibbon *et al.* (1978) did detect a small non-stoicheiometric 'burst' of NADH production at high propionaldehyde concentrations, but concluded that 'non-fluorescent enzyme-containing intermediates must be present in appreciable concentrations' in the steady state. The findings described in the present paper that the hydride-transfer step is quite reversible raised the possibility that the major rate-limiting step in aldehyde oxidation by the mitochondrial enzyme could occur after hydride transfer, if the NADH-containing species were linked via rapidly equilibrating step(s) to an enzyme–$NAD^+$–aldehyde type of complex and the equilibrium favoured the latter. However, the fact that there is a significant isotope effect affecting a maximum rate of $12 min^{-1}$ in the acetic anhydride/NADH reaction and none affecting the maximum rate of $77 min^{-1}$ in the acetaldehyde/$NAD^+$ reaction indicates that the equilibrium of the hydride-transfer step enzyme–$NAD^+$–acetaldehyde $\rightleftharpoons$ enzyme–NADH–acetyl strongly favours the NADH-containing species. It seems then that the principal rate-limiting step for acetaldehyde oxidation by the mitochondrial enzyme probably does occur before hydride transfer. We have suggested that it occurs in the formation of the enzyme–$NAD^+$–thiohemiacetal complex from the enzyme–$NAD^+$–aldehyde Michaelis complex (Hart & Dickinson, 1978). The situation for the cytoplasmic enzymes is apparently different, although again the isotope effects indicate that the equilibrium of the hydride-transfer step favours the NADH-containing species. For this enzyme there is a substantial 'burst' of NADH production in approaching the steady state at high aldehyde concentrations and the rate-limiting step occurs after hydride transfer (MacGibbon *et al.*, 1977*b*). This is a further indication of differences between the two sheep liver enzymes.

One final noteworthy point concerns the finding that maximum rates of NADH oxidation by the aldehyde dehydrogenases are independent of whether acetic or butyric anhydride is used. This suggests a common rate-limiting step, and one might suppose

that this was the dissociation of the product enzyme–$NAD^+$ complex. However, in both cases studies with [4A-$^2$H]NADH indicate that hydride transfer is partly rate-limiting (see above), and stopped-flow studies indicate that the specific rates of dissociation of the $NAD^+$ complexes are about ten times greater than the value of $V'_{max.}$ obtained in the present paper (MacGibbon *et al.*, 1977*b*, 1978). It appears that another step, possibly connected with aldehyde release, is rate-limiting and that this step is insensitive to the nature of the aldehyde involved.

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