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Kinetic studies of the mechanism of pig kidney aldehyde reductase

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Initial-rate measurements were made of the oxidations of pyridine-3-methanol and glycerol by $NADP^+$ and of the reduction of the corresponding aldehydes by NADPH catalysed by pig kidney aldehyde reductase. In addition, a brief survey of the specificity of the enzyme towards aldehyde substrates and its sensitivity to the inhibitors ethacrynic acid, sodium barbitone and warfarin was made. The detailed kinetic work indicates a compulsory mechanism for aldehyde reduction, with NADPH binding before aldehyde. For alcohol oxidation, however, it is necessary to postulate the formation of kinetically significant amounts of binary complexes of the type enzyme–alcohol to explain the results. Thus, for alcohol oxidation random-order addition of substrates may occur. Inhibition studies of the kinetics of aldehyde reduction in the presence of the corresponding alcohol product provide further evidence for the existence of enzyme–alcohol complexes. Finally, detailed kinetic studies were made of the inhibition of pyridine-3-aldehyde reduction by sodium barbitone. The mechanism of the inhibition is discussed.

Davidson & Flynn (1979), on the basis of product-inhibition studies over a limited concentration range, have claimed that the mechanism of pig kidney aldehyde reductase in aldehyde reduction may be explained by a strict compulsory-order mechanism, with coenzyme reacting first with the enzyme. These workers did not, however, investigate the reaction in the reverse direction, and their experiments were done with only one substrate pair, D-glyceraldehyde and glycerol. There is the possibility with an enzyme such as aldehyde reductase, which uses a wide variety of substrates, that the reaction mechanism may vary. This does happen, for example with horse liver alcohol dehydrogenase (Dalziel & Dickinson, 1966*a*), which obeys a compulsory-order mechanism with primary alcohols, but follows a partially random-order pathway with secondary alcohols.

There have been studies on aldehyde reductases from other sources, the most detailed one being by Toews (1967) on the skeletal-muscle enzyme. For this enzyme, Toews (1967) suggested that an enzyme-substitution mechanism was in operation, a conclusion based on apparently parallel plots over limited substrate concentrations for the glyceraldehyde–NADPH reaction. However, Dalziel (1975) has given an alternative conclusion more in keeping with product inhibition and other data of Toews (1967). He suggests that an ordered mechanism is in effect, with the dissociation constant for NADPH much less than its Michaelis constant. In this situation there may be only quite small changes in the slopes of primary plots so that they appear parallel. This does occur with several dehydrogenases (Dalziel, 1975).

Bronaugh & Erwin (1972) have investigated briefly the kinetics of aldehyde reductase from bovine brain and concluded that it operates with a different mechanism from both the skeletal-muscle and kidney enzymes. They found that both $NADP^+$ and *p*-nitrobenzyl alcohol were competitive inhibitors with NADPH or aldehyde. From these results, they suggested that a random order of substrate addition takes place.

In the present work we describe the results of kinetic experiments with several substrates for forward and reverse directions, the results of limited product-inhibition studies and detailed studies of inhibition by sodium barbitone. This extensive study, coupled with information about coenzyme binding described in a previous paper (Morpeth & Dickinson, 1980), has allowed us to reach conclusions about the mechanism of action of the enzyme which we believe are internally consistent and convincing.

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Experimental

NADPH and $NADP^+$ were from Boehringer Corp. (London), London W.5, U.K., and all aldehyde substrates were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Pyridine-3-methanol was a product of Aldrich Chemicals, Gillingham, Dorset, U.K. All other chemicals were of the highest grade commercially available and were from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K.

Aldehyde reductase was prepared and assayed as described previously (Morpeth & Dickinson, 1980). Biogenic aldehydes were prepared by using sheep liver mitochondrial membranes as a source of monoamine oxidase, by the method described by Smolen & Anderson (1976).

Initial-rate measurements of aldehyde reduction and alcohol oxidation

These were performed fluorimetrically by using a recording filter fluorimeter of similar design to that described by Dalziel (1962). A Perspex standard was used and the fluorimeter was calibrated as described by Dalziel (1961). All initial-rate experiments were carried out in sodium phosphate buffer, pH 7, $I = 0.1$ at 25°C, and the kinetic coefficients in eqn. (1) were estimated by the direct graphical method of Eisenthal & Cornish-Bowden (1974). For presentation purposes data are shown in double-reciprocal plots.

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \qquad (1)$$

In eqn. (1) e is the concentration of enzyme active sites, S_1 and S_2 are the coenzyme and substrate respectively and v_0 is the specific initial velocity (μM-NADPH consumed or produced/s). Primed symbols (ϕ'_0, etc.) are used for the kinetic coefficients describing aldehyde reduction by NADPH and unprimed symbols for those describing alcohol oxidation by $NADP^+$. Initial-rate experiments were performed in duplicate, and were reproducible generally to within 5% and at worst 10%, and at least two complete experiments were performed with each aldehyde and alcohol.

Results

The results of assays of our pig kidney aldehyde reductase with various substrates under standard conditions at pH 7.0 and 25°C are shown in Table 1. The dramatic decrease in K'_m on passing from D-glucose to D-glucuronate is particularly impressive, since the $V'_{max.}$ values for the two substrates are quite similar. In a second group of experiments at 25°C, pH 7.0, with a constant NADPH concentration of 70 μM and with *p*-nitrobenzaldehyde as substrate, ethacrynic acid, sodium barbitone and warfarin were found to be potent inhibitors of enzyme activity. Ethacrynic acid was an uncompetitive inhibitor, $K_i = 12\,\mu$M, whereas sodium barbitone and warfarin were mixed competitive/non-competitive inhibitors, with K_i values respectively of 17 μM and 21 μM determined from intercept effects and 38 μM and 260 μM from slope effects. These experiments show that the enzyme acts on or is inhibited by a wide range of pharmacologically active compounds. This is a general feature of aldehyde reductases. However, in view of the findings of Turner & Tipton (1972), Ris & von Wartburg (1973) and Ahmed *et al.* (1978), of isoenzymes of aldehyde dehydrogenase in brain and liver, and in view of our findings (Morpeth & Dickinson, 1980) that our preparation contains essentially only one isoenzyme, Table 1 and associated experiments will help to establish the characteristics of the enzyme studied in detail here.

Table 1. *Apparent Michaelis constants and maximum velocities for various substrates of pig kidney-cortex aldehyde reductase*

Values for K'_m and $V'_{max.}$ were determined from double-reciprocal plots. For the determination of K'_m values for aldehyde substrates, the concentration of NADPH was held constant at 0.1 mM. The maximum velocities were calculated by assuming a molecular weight for aldehyde reductase of 43 700 (Morpeth & Dickinson, 1980). All values are for 25°C and pH 7.0. The following compounds showed no activity: testosterone, 17α-epitestosterone, 5β-dihydrotestosterone, 2-carboxybenzaldehyde, D-glucose 6-phosphate, indole-3-aldehyde.

Substrate	$K'_{m,app.}$ (mM)	$V'_{max,app.}$ (s^{-1})
Pyridine-3-aldehyde	3.3	33.2
p-Nitrobenzaldehyde	0.37	16.7
D-Glyceraldehyde	3.8	9.1
L-Glyceraldehyde	4.4	4.6
Succinate semialdehyde	0.93	22.6
D-Glucose	16000	3.3
D-Glucuronate	3.92	6.5
4-Hydroxyphenylglycolaldehyde	0.35	17.5
3,4-Dihydroxyphenylglycolaldehyde	0.23	9.8
Daunomycin	0.52	0.25

Mechanistic studies

Aldehyde–NADPH reactions. The results of initial-rate studies at pH 7.0 and 25°C for the reduction of pyridine-3-aldehyde by NADPH catalysed by pig kidney aldehyde reductase are shown in Figs. 1(*a*) and 1(*b*). The reciprocal plots are linear over the concentration ranges used and the data conform to eqn. (1). The initial-rate parameters

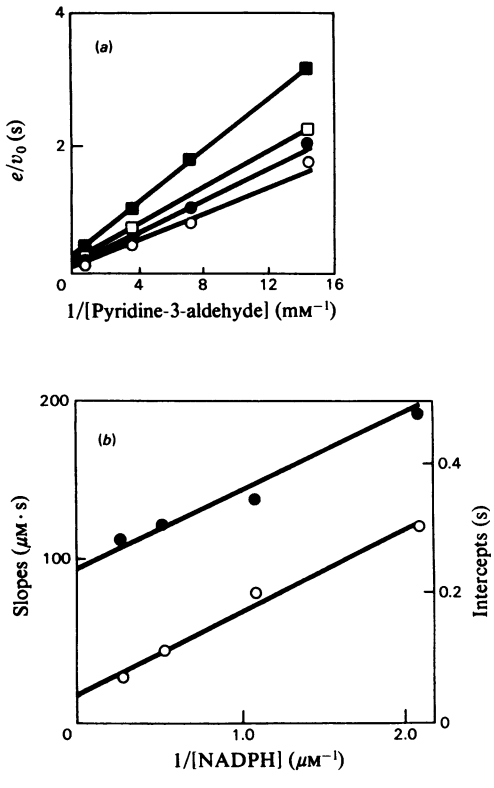


Fig. 1. *Initial-rate measurements of the reduction of pyridine-3-aldehyde by NADPH*

(*a*) Primary plots showing the variation of the reciprocal of the initial specific rate at 25°C and pH 7 with the reciprocal of pyridine-3-aldehyde concentration between 0.07mM and 1.4mM at several concentrations of NADPH. The NADPH (μM) concentrations were: ○, 3.8; ●, 1.9; □, 0.91; ■, 0.47. (*b*) Secondary plots showing the variation of the intercepts (○) and slopes (●) of the primary plots with the reciprocal of the NADPH concentration.

obtained for the pyridine-3-aldehyde–NADPH reaction are shown in Table 2, where they are included with values obtained for D- and L-glyceraldehyde reduction at pH 7.0 and 25°C. The primary and secondary plots with the latter substrates were very similar to Figs. 1(*a*) and 1(*b*).

Alcohol–NADP$^+$ reactions. Initial-rate studies of the oxidation of pyridine-3-methanol by NADP$^+$ at pH 7.0 and 25°C gave rise to the results shown in Figs. 2(*a*) and 2(*b*). The primary and secondary plots are again linear, and values for the initial-rate parameters in eqn. (1) are given in Table 3. Also included in Table 3 are values for the initial-rate parameters for glycerol oxidation by NADP$^+$ at pH 7.0 and 25°C. The primary and secondary plots for glycerol oxidation were linear over the concentration ranges used, and values for the initial-rate parameters were, within experimental error, unchanged by using chromatographically purified NADP$^+$. The changes seen in Table 3 are in any case of the opposite sign from those expected if chromatographic purification of the coenzyme resulted in the removal of inhibiting impurities. It should be noted here that there are serious difficulties in estimating the parameters ϕ_0 and ϕ_1. The difficulties arise because, as seen in Fig. 2(*a*), there are only very small changes in the intercepts of the primary plots even at very low NADP$^+$ concentrations. Estimates for the parameters were found by doing separate experiments with different concentration ranges of substrates. One experiment was over a very limited range to determine ϕ_0 and ϕ_1, and a second experiment over a wide substrate range to find ϕ_2 and ϕ_{12}. The results for ϕ_2 and ϕ_{12} proved to be reproducible, with estimates agreeing to within 10%. However, ϕ_0 and ϕ_1 varied widely, and there was as much as a 2-fold difference between values found in duplicate experiments.

Product-inhibition studies and inhibition by sodium barbitone. The results of initial-rate studies

Table 2. *Kinetic coefficients describing the reduction of various aldehydes by NADPH and aldehyde reductase at 25°C in sodium phosphate buffer, pH 7, I = 0.1*

The kinetic coefficients shown are those in the reciprocal initial-rate equation:

$$\frac{e}{v_0} = \phi'_0 + \frac{\phi'_1}{[S'_1]} + \frac{\phi'_2}{[S'_2]} + \frac{\phi'_{12}}{[S'_1][S'_2]}$$

where e is the enzyme concentration, S'_1 is NADPH and S'_2 is aldehyde. ϕ'_1/ϕ'_0 is the Michaelis constant for NADPH and ϕ'_2/ϕ'_0 is the Michaelis constant for the aldehyde; $1/\phi'_0 = V'_{max.}$

Substrate	ϕ'_0 (s)	ϕ'_1 (μM·s)	ϕ'_2 (μM·s)	ϕ'_{12} (μM^2·s)	ϕ'_1/ϕ'_0 (μM)	ϕ'_2/ϕ'_0 (mM)	ϕ'_{12}/ϕ'_2 (μM)
Pyridine-3-aldehyde	0.03	0.15	100	95	4.9	3.3	0.95
D-Glyceraldehyde	0.093	0.14	530	290	1.5	5.7	0.55
L-Glyceraldehyde	0.23	0.14	2700	1000	0.63	11.8	0.4
D-Glyceraldehyde*					9.1	4.8	1.6

* Data derived from Davidson & Flynn (1979) in 0.1M-phosphate buffer, pH 7, 25°C.

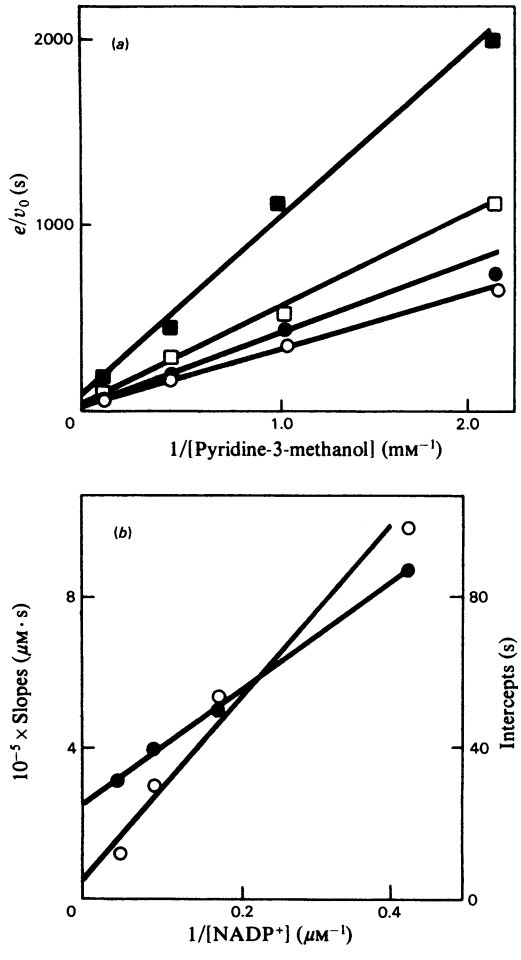


Fig. 2. *Initial-rate measurements of the oxidation of pyridine-3-methanol by NADP$^+$*

(*a*) Primary plots showing the variation of the reciprocal of the initial specific rate at 25°C and pH 7 with the reciprocal of pyridine-3-methanol concentration between 0.46mM and 9.2mM at several concentrations of NADP$^+$. The NADP$^+$ concentrations (μM) were: ○, 23.2; ●, 11.5; □, 5.7; ■, 2.3. (*b*) Secondary plots showing the variation of the intercepts (○) and slopes (●) of the primary plots with the reciprocal of the NADP$^+$ concentration.

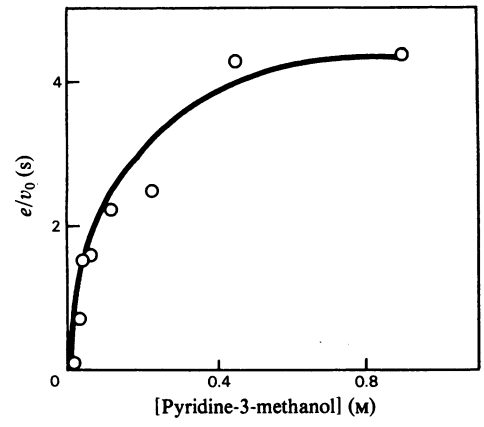


Fig. 3. *Variation of the reciprocal apparent maximum specific rate of reduction of pyridine-3-aldehyde by NADPH with aldehyde reductase on varying the pyridine-3-methanol concentration of the assays*

The reactions were carried out at 25°C and pH 7. The concentrations used were: pyridine-3-aldehyde, 30mM; NADPH, 70μM.

of the oxidation of pyridine-3-aldehyde by NADPH at pH 7.0 and 25°C in the presence of pyridine-3-methanol are presented in Fig. 3. The assays were performed with concentrations of substrates at least 10-fold greater than their respective Michaelis constants to minimize any effect from changing K'_m value at high product concentrations. The results suggest that the inhibition is hyperbolic and that the alternative pathway utilized at high pyridine-3-methanol concentrations is very much less efficient than the uninhibited route. Similar studies on the inhibition of DL-glyceraldehyde reduction by glycerol show the same kind of behaviour. In this case maximal inhibition is achieved with approx. 0.2M-glycerol, and the rate is some 10-fold lower than the uninhibited rate.

The results of initial-rate experiments of the reduction of pyridine-3-aldehyde by NADPH in the presence of various concentrations of sodium barbitone at pH 7.0 and 25°C are summarized in Figs. 4(*a*) and 4(*b*). At each concentration of sodium barbitone, primary and secondary plots were linear, like Figs. 1(*a*) and 1(*b*), and the initial-rate parameters were determined as described above. The conclusion from these experiments is that ϕ'_0 varies hyperbolically and ϕ'_2 linearly with the sodium barbitone concentration. The effect on ϕ'_2 may actually be non-linear, perhaps hyperbolic, but the deviations from linearity are probably not outside the limits of experimental error, and experiments at much higher barbitone concentrations are required to establish the point. ϕ'_{12} appears to be unchanged by the presence of barbitone, but there may be a small linear effect on ϕ'_1, indicating a K_i value of about 110μM. The effect on ϕ'_1 is, however, very uncertain. The parameters ϕ'_1 and ϕ'_{12} become much less important in eqn. (1) at high barbitone concentrations because of the very large increases in ϕ'_0 and ϕ'_2. Accordingly, the errors involved in estimating these parameters increase considerably, and it may be that the apparent increase in ϕ'_1 at high barbitone concentrations reflects the larger error associated with the measurement. At this stage we provisionally conclude that the inhibition effects of

Table 3. *Kinetic coefficients describing the oxidation of alcohols by NADP$^+$ and aldehyde reductase at 25°C in sodium phosphate buffer, pH 7, I = 0.1*

The kinetic coefficients shown are those in the reciprocal initial-rate equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}$$

where e is the enzyme concentration, S_1 is NADP$^+$ and S_2 is alcohol, ϕ_1/ϕ_0 is the Michaelis constant for NADP$^+$ and ϕ_2/ϕ_0 is the Michaelis constant for the alcohol. $1/\phi_0 = V_{max.}$.

Substrate	ϕ_0 (s)	ϕ_1 (μM·s)	ϕ_2 (μM·s)	ϕ_{12} (μM^2·s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (mM)	ϕ_{12}/ϕ_2(μM)
Glycerol	2.4	46	9.7×10^6	86×10^6	19	4040	8.7
Glycerol (purified NADP)*	4.2	72	10.5×10^6	97×10^6	17	2500	9.2
Pyridine-3-methanol	8.4	250	0.28×10^6	1.4×10^6	29	33	5.0

* Purified by the method of Dalziel & Dickinson (1966*b*).

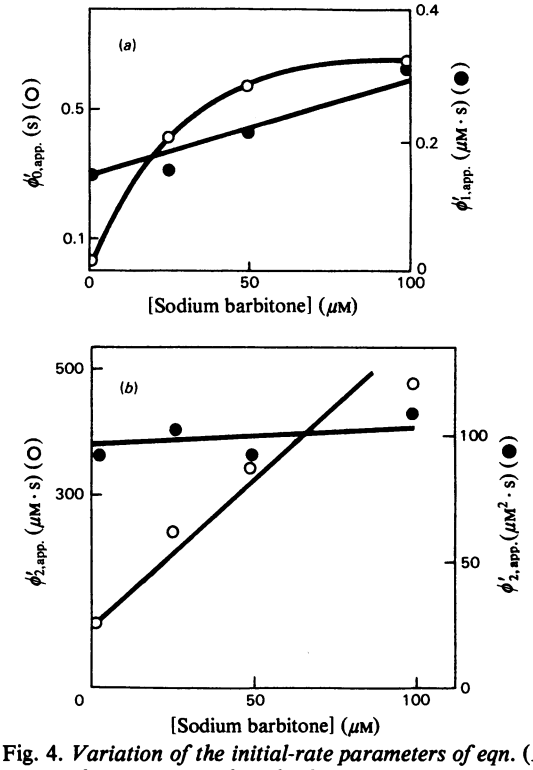


Fig. 4. *Variation of the initial-rate parameters of eqn. (1) with increasing sodium barbitone concentration*

(*a*) Variation of ϕ'_0 (○) and ϕ'_1 (●) for pyridine-3-aldehyde and NADPH with increasing sodium barbitone concentration at pH 7 and 25°C. (*b*) Variation of ϕ'_2 (○) and ϕ'_{12} (●) for pyridine-3-aldehyde and NADPH with increasing sodium barbitone concentration at pH 7 and 25°C.

sodium barbitone at pH 7.0 and 25°C are on ϕ'_0 and ϕ'_2 and that ϕ'_1 and ϕ'_{12} are unaffected by the presence of the inhibitor.

Equilibrium measurements

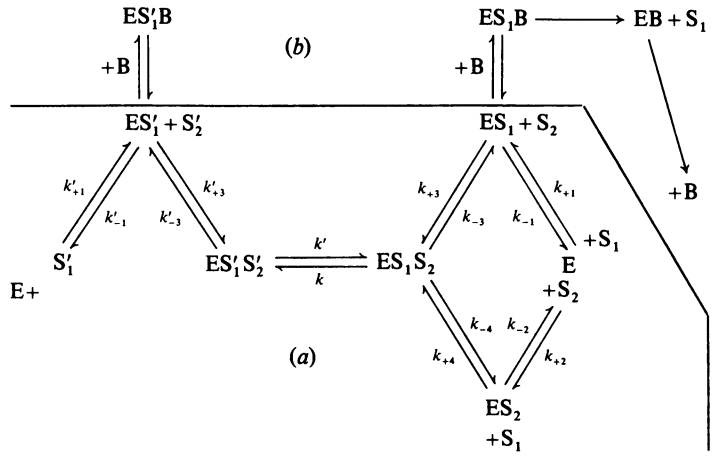
The equilibrium constant for the enzyme-catalysed oxidation of pyridine-3-methanol by NADP$^+$ at 25°C, pH 7.0, was estimated in spectrophotometric experiments with mixtures containing initially NADPH (76μM), pyridine-3-aldehyde (93μM) and pyridine-3-methanol (20–60mM). For the experiments approx. 90% of the NADPH was oxidized and values of $K_{eq.} = 0.5(\pm 0.2) \times 10^{-11}$M were found.

Discussion

The initial-rate data for the aldehyde–NADPH and alcohol–NADP$^+$ reactions in the absence of products which are given in Figs. 1 and 2 and Tables 2 and 3 may be explained by the reaction scheme shown in Scheme 1(*a*). The mechanism is very similar to that proposed for horse liver alcohol dehydrogenase (Dalziel & Dickinson, 1966*a*) and yeast alcohol dehydrogenase (Dickenson & Dickinson, 1975) on the basis of detailed initial-rate data. As the arguments used to justify the mechanism are very similar to those used in those references for the alcohol dehydrogenases, they need not be rehearsed at length here. The salient points are as follows.

(*a*) The finding of ϕ'_{12} and ϕ_{12} terms for forward and reverse reactions rules out an enzyme-substitution mechanism such as was proposed by Toews (1967) for the enzyme on the basis of very limited data. That mechanism also requires the Haldane relationship $\phi'_1\phi'_2[H^+]/\phi_1\phi_2 = K_{eq.}$ (Dalziel, 1957). As $\phi'_1\phi'_2[H^+]/\phi_1\phi_2 = 3.4 \times 10^{-15}$M at pH 7.0 and $K_{eq.} = 0.5 \times 10^{-11}$M, the Haldane relationship clearly does not hold.

(*b*) The existence of ϕ'_{12} and ϕ_{12} terms suggests some kind of ternary-complex mechanism. This is borne out by testing the Haldane relationship $\phi'_{12}[H^+]/\phi_{12} = K_{eq.}$, which is predicted for ternary-complex mechanisms (Dalziel, 1957). $K_{eq.}$ =



Scheme 1. *Proposed mechanism for pig kidney aldehyde reductase*

S'_1 = NADPH; S_1 = $NADP^+$; S'_2 = aldehyde; S_2 = alcohol; B = sodium barbitonate. (*a*) Mechanism in the absence of sodium barbitone; (*b*) complete scheme to describe mechanism in the presence of sodium barbitone.

S'_2 = aldehyde; S_2 = alcohol;

$0.5(\pm 0.2) \times 10^{-11}$ M for the pyridine-3-aldehyde–pyridine-3-methanol system and $\phi'_{12}[H^+]/\phi_{12} = 0.68 \times 10^{-11}$ M. For aldehyde reduction the data of Table 2 suggest a general compulsory mechanism, with NADPH being the first substrate to combine with the enzyme. The constancy of ϕ'_1 on changing the aldehyde substrate is required by this mechanism (Dalziel, 1957), because $\phi'_1 = 1/k'_{+1}$, and ϕ'_1 is a direct measure of the specific rate of combination of NADPH with the enzyme. This cannot change on changing the nature of the substrate. The constancy of ϕ'_1 is striking in view of the wide variation of the other parameters in the initial-rate equation. Confirmation that $\phi'_1 = 1/k'_{+1}$ has come from preliminary stopped-flow measurements at 375nm after the mixing of 20μM-aldehyde reductase and NADPH in phosphate buffer, pH 7.0 at 25°C. The results were analysed by assuming that the dissociation reaction is significant (Frost & Pearson, 1953), and a value of $k'_{+1} = 7.6 \times 10^6\ M^{-1} \cdot s^{-1}$ was obtained; $1/\phi'_1 = 7 \times 10^6\ M^{-1} \cdot s^{-1}$. There is also some evidence from protection studies against inactivation by pyridoxal 5'-phosphate, butanedione and phenylglyoxal that a compulsory order of NADH and aldehyde binding is in effect. Thus aldehyde reductase is protected from all of these reagents by NADH, but not at all by any aldehyde tested (Morpeth & Dickinson, 1980).

(*c*) A strict compulsory mechanism for aldehyde reduction requires the relationships $\phi'_{12}/\phi'_2 = K_{E\cdot NADPH}$ (the dissociation constant of the binary complex of NADPH and enzyme) and $\phi'_1\phi'_2/\phi'_{12} \leqslant \phi_0$ (Dalziel, 1957). For the pyridine-3-aldehyde–pyridine-3-methanol system $\phi'_{12}/\phi'_2 = 0.95\mu$M, $K_{E\cdot NADPH} = 1.1\mu$M (Morpeth & Dickinson, 1980) and the maximum-rate relationship is $\phi'_1\phi'_2/\phi'_{12} = 0.16\,s \ll \phi_0 = 8.4\,s$, so that the requirements are satisfied. For the D- and L-glyceraldehyde/glycerol systems, ϕ'_{12}/ϕ'_2 is not in such good agreement with $K_{E\cdot NADPH}$, though the discrepancy might not be outside the combined experimental error, but in each case $\phi'_1\phi'_2/\phi'_{12} \ll \phi_0$. The latter relationship shows that for both pyridine-3-methanol and glycerol oxidations the rate-limiting step cannot be NADPH dissociation from the terminal E·NADPH complex. The fact that ϕ_0 seems to change on changing the nature of the substrate alcohol (Table 3) argues the same conclusion. Ternary-complex interconversion or dissociation of aldehyde from the product E·NADPH·aldehyde complex seem possible candidates for the rate-limiting step.

(*d*) A compulsory mechanism in the direction of alcohol oxidation does not accord with the data, because ϕ_1 changes very substantially on changing alcohol substrates. Moreover, the requirement that $\phi_1\phi_2/\phi_{12} \leqslant \phi'_0$ is not met. For the D-glyceraldehyde–glycerol system $\phi_1\phi_2/\phi_{12} = 9.7\,s \gg \phi'_0 = 0.09\,s$, and similar very large inequalities are found for the other substrate pairs. Inequalities of this sort have been explained by compulsory mechanisms involving isomeric enzyme–coenzyme complexes (Bloomfield *et al.*, 1962) or by the presence of inhibiting impurities in coenzyme preparations (Dalziel, 1963). Neither of the explanations seems likely here. The fact that ϕ_1 varies on changing the alcohol substrate is not expected for an isomeric complex mechanism (Dalziel, 1963), and the use of specially purified coenzyme preparations does not alter the kinetic picture. A more likely explanation of the results is

that in alcohol oxidation alternative pathways of substrate and coenzyme addition are possible, as shown in Scheme 1(*a*). In general, a steady-state random-order mechanism predicts non-linear Lineweaver–Burk plots (Dalziel, 1957), although, as Pettersson (1972) has pointed out, a relationship can exist between the rate constants so that linearity in reciprocal plots is observed. On the other hand a rapid-equilibrium random mechanism predicts linear Lineweaver–Burk plots (Dalziel, 1957), as does another special case of the steady-state random mechanism, the so-called preferred-order mechanism (Dalziel & Dickinson, 1966*a*). The three cases mentioned allow the possibility that $\phi_1\phi_2/\phi_{12} > \phi'_0$, and at this stage we cannot distinguish between them. What does seem clear is that alternative pathways of substrate and coenzyme addition are possible and that complexes of the type E·alcohol are kinetically significant in the direction of alcohol oxidation.

Additional evidence for the existence of E·alcohol complexes is provided by the hyperbolic product-inhibition effects on the reciprocal apparent maximum rates of aldehyde reduction (e.g. Fig. 3). Presumably saturating product alcohol concentrations prevent effective dissociation of alcohol from the ternary E·$NADP^+$·alcohol complex, and so the alternative route is used, with dissociation of $NADP^+$ and the formation of the E·alcohol complex. The pronounced inhibition achieved by high product alcohol concentrations indicates that the alternative routes are much less effective than that followed in the uninhibited condition and that $k_{-4} \ll k_{-3}$. This condition means that, in the absence of products, product dissociation is effectively ordered. Interestingly, the same is found for the alcohol dehydrogenases (Silverstein & Boyer, 1964; Dickenson & Dickinson, 1978).

Detailed product-inhibition studies were not pursued further because the alcohol substrates of aldehyde reductase have, almost without exception, either very high K_m values or low solubility (Bosron & Prairie, 1972). Further, Plapp (1973) and Dickenson & Dickinson (1978) point out that product-inhibition data yield an estimate of the dissociation constant of substrate from ternary complexes only if coenzyme release is rate-limiting. That does not seem to be the case here, because the maximum rate of aldehyde reduction changes on changing from one aldehyde to another.

Inhibition by sodium barbitone

As an alternative to product-inhibition studies, it was decided to study the effect of sodium barbitone on the initial-rate parameters of eqn. (1). Sodium barbitone is freely soluble in water and is a powerful inhibitor of the enzyme. Unfortunately alcohol oxidation could not be studied, because in the presence of inhibitor so much enzyme was added at low coenzyme and substrate concentrations in order to get measurable rates that the steady-state requirement that [enzyme] ≪ [substrate] was violated. Also, as indicated above, the errors in determining ϕ_0 and ϕ_1 were very large and it would have been difficult to decide if apparent changes in these parameters were real.

The inhibition of pyridine-3-aldehyde reduction by sodium barbitone summarized in Fig. 4 is interpreted in the light of the following principles. Dalziel (1975) states that if an inhibitor binds only to the enzyme·product coenzyme binary complex (E·$NADP^+$) in compulsory- and preferred-order pathways, then

$$\phi'_{0,\text{app.}} = \phi'_0 + \frac{[\text{I}]}{k_{-1}K_{\text{E·NADP}^+\text{·I}}};$$

$K_{\text{E·NADP}^+\text{·I}}$ is the dissociation constant for inhibitor from the complex E·$NADP^+$·I. If the inhibitor binds only to free enzyme and competes with the coenzyme, then

$$\phi'_{1,\text{app.}} = \phi'_1\left(1 + \frac{[\text{I}]}{K_{\text{E·I}}}\right)$$

and

$$\phi'_{12,\text{app.}} = \phi'_{12}\left(1 + \frac{[\text{I}]}{K_{\text{E·I}}}\right)$$

where $K_{\text{E·I}}$ is the dissociation constant of the E·I complex. Clearly the hyperbolic inhibition seen with $\phi'_{0,\text{app.}}$ in Fig. 4(*a*) cannot be explained by an equation of the type

$$\phi'_{0,\text{app.}} = \phi'_0 + \frac{[\text{I}]}{k_{-1}K_{\text{E·NADP}^+\text{·I}}}$$

because in that case linear inhibition is expected. Hyperbolic inhibition requires an equation of the form

$$\phi_{\text{app.}} = \frac{\text{A} + \text{B[inhibitor]}}{\text{C} + \text{D[inhibitor]}} \qquad (2)$$

(Cleland, 1972; Dickenson & Dickinson, 1978), where A, B, C and D are constants.

Since ϕ'_0 and ϕ'_2 are the main parameters seen to vary, it seems that sodium barbitone inhibits by forming complexes of the type enzyme·NADP(H)·barbitone. Indeed, there is evidence for the formation of these complexes. The increase in fluorescence of a mixture of enzyme and NADPH in the presence of sodium barbitone indicates that a complex of the type enzyme·NADPH·barbitone can form (Morpeth & Dickinson, 1980). The existence of the enzyme·NADPH·barbitone and the enzyme·$NADP^+$·barbitone complexes is also inferred by studies of protection against modification by pyridoxal 5′-phosphate (F. F. Morpeth, unpublished work). However, the non-linear variation in ϕ'_0

suggests that the enzyme·$NADP^+$·barbitone complex breaks down to products by an alternative route, such as that suggested in Scheme 1(*b*).

The initial-rate equation for Scheme 1(*b*) may be obtained from the general inhibition equations of Pettersson (1974). The equation shows the required behaviour, with linear effects on ϕ'_2 and hyperbolic effects on ϕ'_0. One significance of this result is that the enzyme·$NADP^+$·barbitone ternary complex is analogous to the ternary product complex E·$NADP^+$·alcohol. Since E·$NADP^+$·barbitone apparently breaks down to yield $NADP^+$ and E·barbitone, it suggests that there may be a random order of product dissociation in aldehyde oxidation, as deduced above from the product-inhibition experiments.

The mechanism deduced from the present results differs from that arrived at by Davidson & Flynn (1979) in that we find convincing evidence for the kinetic significance of E·alcohol complexes. We agree with the previous authors in proposing a compulsory mechanism for aldehyde reduction in the absence of products, but they did not find evidence for the alternative route of product dissociation in the presence of high product alcohol (glycerol) concentrations. Davidson & Flynn (1979) observed linear inhibition by glycerol, as opposed to hyperbolic inhibition, but it is also apparent from comparison of their results with ours that they observed only small inhibition effects even at very high glycerol concentrations. The reason for the differences in our results cannot be explained at the moment, but perhaps it is important to note that they used very high glycerol concentrations (2M), whereas we worked at concentrations below 0.2M. Another difference from the results of Davidson & Flynn (1979) is that we observe hyperbolic inhibition effects with sodium barbitone, whereas they obtained linear effects when using phenobarbital. Our experiments, however, involved variation of both substrate concentrations and the inhibitor concentration, whereas Davidson & Flynn (1979) simply observed the inhibition effects of phenobarbital with fixed concentrations of NADPH and D-glyceraldehyde. In any case it may be noted that Erwin *et al.* (1971) found that a plot of percentage inhibition versus ionization phenobarbital concentration was hyperbolic with bovine brain aldehyde reductase.

Although evidence for the importance of E·alcohol-type complexes is found in our product-inhibition and sodium barbitone experiments, the most important evidence comes from our findings that consistently $\phi_1\phi_{12}/\phi_{12} \gg \phi'_0$ and that ϕ_1 varies considerably on changing the alcohol substrate. Neither of the latter findings are compatible with a strict compulsory mechanism such as that proposed by Davidson & Flynn (1979). These authors did not attempt to study the kinetics of alcohol oxidation by aldehyde reductase and therefore could not observe the inconsistencies noted above. This is another illustration of the principle given by Dalziel (1957) that to distinguish mechanisms properly by initial-rate measurements both forward and reverse reactions should be studied. It may be noted that, because of the very unfavourable equilibrium and very slow rates of reaction, studies of alcohol oxidation at neutral pH require very sensitive assay methods. The recording fluorimeter used here is designed for that purpose.

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