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Kinetic studies with the low-K_m aldehyde reductase from ox brain

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Initial-rate studies of the low-K_m aldehyde reductase-catalysed reduction of pyridine-3-aldehyde by NADPH gave families of parallel double-reciprocal plots, consistent with a double-displacement mechanism being obeyed. Studies on the variation of the initial velocity with the concentration of a mixture of the two substrates were also consistent with a double-displacement mechanism. In contrast, the initial-rate data indicated that a sequential mechanism was followed when NADH was used as the coenzyme. Product-inhibition studies, however, indicated that a compulsory-order mechanism was followed in which NADPH bound before pyridine-3-aldehyde with a ternary complex being formed and the release of pyrid-3-ylcarbinol before $NADP^+$. The apparently parallel double-reciprocal plots obtained in the initial-rate studies with NADPH and pyridine-3-aldehyde were thus attributed to the apparent dissociation constant for the binary complex between the enzyme and coenzyme being finite but very low.

Brain and other tissues from a number of sources have been shown to contain more than one form of $NADP^+$-dependent aldehyde reductase (see, e.g., Turner & Tipton, 1972; Wermuth *et al.*, 1982; Flynn, 1982). The function of the major form, which has been termed the high-K_m aldehyde reductase (Turner & Tipton, 1972), is still uncertain, and it appears that it is the less abundant low-K_m form that will play the dominant role in the reduction of the aldehydes derived from the oxidation of neurotransmitter amines (Turner *et al.*, 1974).

The low-K_m aldehyde reductase also catalyses the conversion of a number of aldoses into their corresponding alcohol derivatives, and it has been suggested that it may be identical with aldose reductase (EC 1.1.1.21) (see Flynn, 1982). The activity of this latter enzyme in lens has been implicated in the development of diabetic cataract (see Gabay, 1975). The low-K_m aldehyde reductase, which has also been termed aldehyde reductase 1 (Tulsiani & Touster, 1977), aldehyde reductase 2 (Branlant & Biellmann, 1980; Hara *et al.*, 1983) and aldehyde reductase 3 (Daly & Mantle, 1982), may also be identical with the glycerol dehydrogenase that has been described by other workers (Cromlish & Flynn, 1983). The uncertainty about the relationship among these different enzymes, the possible existence of multiple forms of aldose reductase (see Branlant & Biellmann, 1980; Flynn, 1982; Wermuth *et al.*, 1982; Hara *et al.*, 1983) and the possibility of considerable inter-species variations (Davidson & Flynn, 1979) has complicated comparisons of data obtained with different preparations.

There have been few detailed studies on the steady-state kinetics of members of this group of enzymes, and these have yielded conflicting results, with a double-displacement mechanism (Toews, 1967), a compulsory-order mechanism (Boghosian & McGuinness, 1981) and a complex random-order mechanism (Doughty & Conrad, 1982) having variously been reported to be obeyed. Our previous studies on the roles of the different aldehyde-metabolizing enzymes in the oxidation and reduction of the neurotransmitter amines in brain (Turner *et al.*, 1974) were carried out in the absence of any detailed understanding of the kinetic behaviour of the enzymes involved. The behaviour of this pathway may have valuable implications in connection with the diagnosis of affective disorders and with possible physiological actions of the aldehydes involved (for review see Tipton *et al.*, 1977). In order to refine our understanding of the factors that may influence its operation, we have carried out a detailed steady-state kinetic study of the behaviour of the low-K_m aldehyde reductase from ox brain.

Materials and methods

The low-K_m aldehyde reductase was purified to apparent homogeneity from ox brain by the proce-

dure reported elsewhere (Ryle *et al.*, 1984). Pyridine-3-aldehyde was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Its concentration in solutions was determined by the method of Racker (1957) with the use of aldehyde dehydrogenase that had been partially purified from ox liver by the procedure described by Houslay & Tipton (1973). Pyrid-3-ylcarbinol was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and coenzymes were from Boehringer–Mannheim, Mannheim, Germany.

Enzyme activity was determined spectrophotometrically at 30°C by monitoring the decrease in absorbance at 340nm in reaction mixtures containing, in a total volume of 2ml, 100mM-sodium phosphate buffer, pH7.0, and the appropriate concentrations of NAD(P)H, pyridine-3-aldehyde, products and enzyme. Protein concentration was determined by the Lowry method, with bovine serum albumin as the standard.

Initial-rate data were fitted by the method of Wilkinson (1961) as adapted by Cleland (1967) and results are presented as double-reciprocal plots for illustrative purposes. Secondary plots of these data (Florini & Vestling, 1957) and of the results obtained from product-inhibition data (see Dixon & Webb, 1979, pp. 96–103) were fitted to straight lines by linear regression. In no case was a significant improvement of the fit obtained if these results were fitted to hyperbolic or parabolic curves.

Results and discussion

Under the assay conditions used the initial velocity was found to be proportioned to enzyme concentration. Fig. 1(*a*) shows the effects of variation of the NADPH concentration at a series of fixed concentrations of pyridine-3-aldehyde, and Fig. 1(*b*) shows these data with pyridine-3-aldehyde as the substrate whose concentration was varied. The linearity of the plots probably excludes a mechanism in which the two substrates bind to the enzyme in a random order under steady-state conditions, although departure from linearity under such conditions may not always be apparent (see Pettersson, 1970).

Behaviour of this type in which families of apparently parallel lines are obtained is often inter-

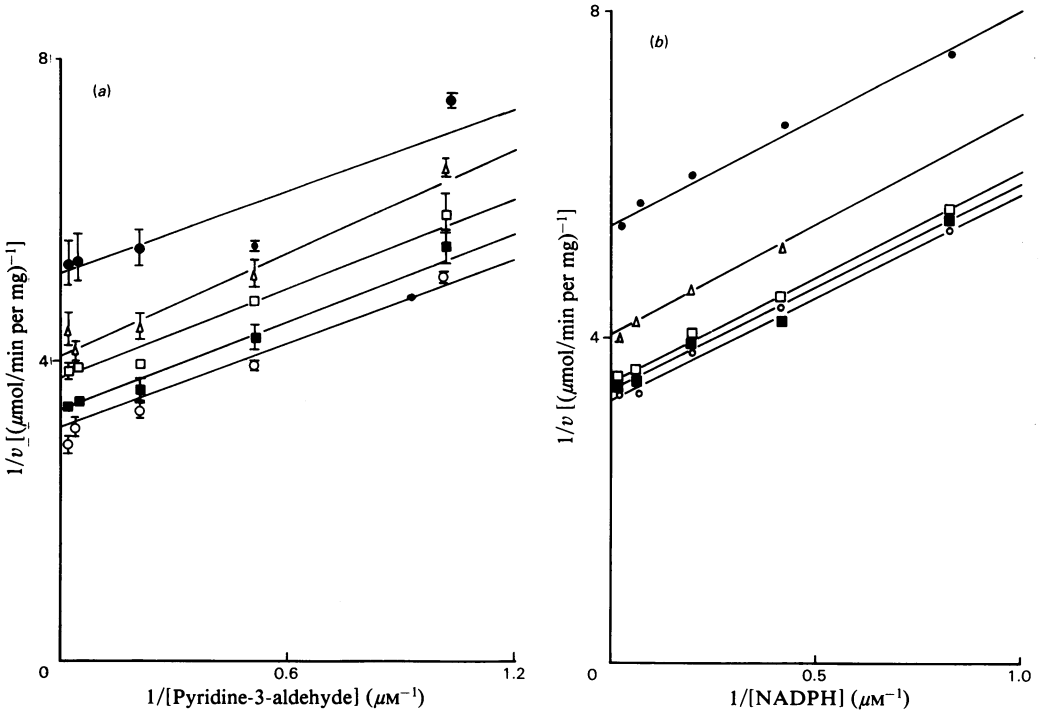

Fig. 1. *Double-reciprocal plots from initial-rate measurements of the reduction of pyridine-3-aldehyde by NADPH catalysed by low-K_m aldehyde reductase*

Details of the assay procedure were as described in the text. (*a*) The NADPH concentrations were 30μM (○), 18μM (■), 5μM (□), 2.4μM (△) and 1.2μM (●). Values are means±range for two separate determinations. (*b*) The pyridine-3-aldehyde concentrations were 48.3μM (○), 24.15μM (■), 4.83 (□), 1.93μM (△) and 0.996μM (●). Values are means for two separate determinations.

preted in terms of a double-displacement ('ping-pong') mechanism and indeed Toews (1967) has suggested that such a mechanism may be followed by the related enzyme glycerol dehydrogenase from rabbit skeletal muscle. Although Dalziel (1975) has suggested that it is unlikely that an NAD(P)$^+$-dependent dehydrogenase will be found to obey such a mechanism, there would appear to be no reason *a priori* to exclude such a possibility.

For an enzyme-catalysed reaction involving two substrates that obeys Michaelis–Menten kinetics, the kinetic equation will take the form (see Dixon & Webb, 1979, pp. 82–93):

$$v = \frac{V}{1 + \frac{K_m^A}{a} + \frac{K_m^B}{b} + \frac{K_s^A K_m^B}{ab}} \quad (1)$$

where a and b represent the substrate concentrations, K_m^A and K_m^B are the Michaelis constants for these substrates and V is the maximum velocity. In a double-displacement mechanism the value of K_s^A, the apparent dissociation constant for the first substrate to bind, is zero, but apparently parallel lines may be obtained if this constant were finite but very low (see, e.g., Dalziel, 1975). A method that has been used in attempts to distinguish between these possibilities is to vary the concentrations of the two substrates together in such a way that the ratio of their concentrations is kept constant (see Dixon & Webb, 1979, pp. 93–96). In this case the double-displacement mechanism would be expected to give a linear plot of reciprocal velocity against the reciprocal of the concentration of the substrate mixture, whereas such a plot for a sequential mechanism (i.e. one in which K_s^A is finite) would be expected to be parabolic. Fig. 2 shows such a plot in which different amounts of a mixture of the two substrates, each at a concentration of $100K_m$, were assayed. Within the limits of experimental error there was no evidence for deviation from linearity.

Fig. 3 shows the primary kinetic plots obtained when NADH was used as the coenzyme. The families of intersecting lines obtained are clearly indicative of a sequential mechanism. Thus, if it is considered unlikely that a change of the coenzyme would result in such a radical change in mechanism, it might be reasonable to conclude that a sequential mechanism is followed with both coenzymes but that the value of K_s^A is extremely small when NADPH is used. In view of the failure of the experiments shown in Fig. 2 to provide any support for this contention, however, further evidence in its justification would be required, and this was provided by the results of studies on the inhibition afforded by the products of the reaction.

The patterns of inhibition given by the products

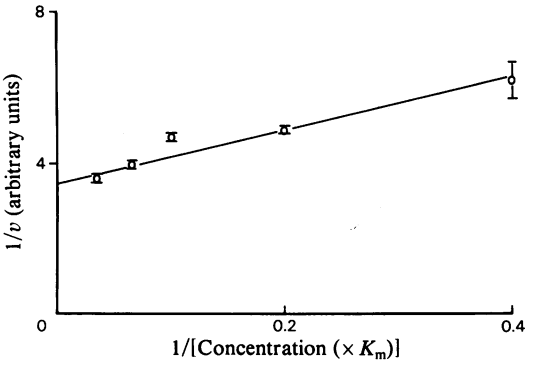

Fig. 2. *Double-reciprocal plot from initial-rate measurements of the reaction catalysed by low-K_m aldehyde reductase in the presence of various amounts of a mixture of NADPH and pyridine-3-aldehyde*

A stock solution of the two substrates, each 100 times its K_m concentration, was prepared and initial rates were determined in the presence of various quantities of this mixture. Substrate concentration is shown as a function of the K_m values and each value is the mean ± range for duplicate determinations.

NADP$^+$ and pyrid-3-ylcarbinol are summarized in Table 1, and the kinetic constants calculated from the experimental data are shown in Table 2. The competitive inhibition given by NADP$^+$ with respect to NADPH and the mixed inhibition given by this product towards pyridine-3-aldehyde (results not shown) would be consistent with the coenzymes binding to the free enzyme, although a Theorell–Chance mechanism in which the coenzyme was the second substrate to bind cannot be excluded by these data. The K_i values determined from the competitive inhibition, with respect to NADPH, by this product would represent the dissociation constants for its binding to the enzyme. If the substrates were to bind to the enzyme in a random order, this product would be expected to bind to the free enzyme and to the enzyme–pyridine-3-aldehyde binary complex, and thus the K_i value would depend on the concentration of that substrate. Such a variation would be expected to be large in view of the great dependence of the apparent K_m value for NADP$^+$ on the concentration of this substrate (Fig. 1), whereas the values shown in Table 2 suggest that large changes in the pyridine-3-aldehyde concentration have little or no significant effect on the K_i value for NADP$^+$.

Pyrid-3-ylcarbinol was found to be a mixed inhibitor with respect to both NADPH (Fig. 4) and pyridine-3-aldehyde (Fig. 5*a*) when the other substrate was held at a fixed non-saturating concentration. When the inhibition of this product was studied at saturating concentrations of the fixed substrate, the type of inhibition shown towards

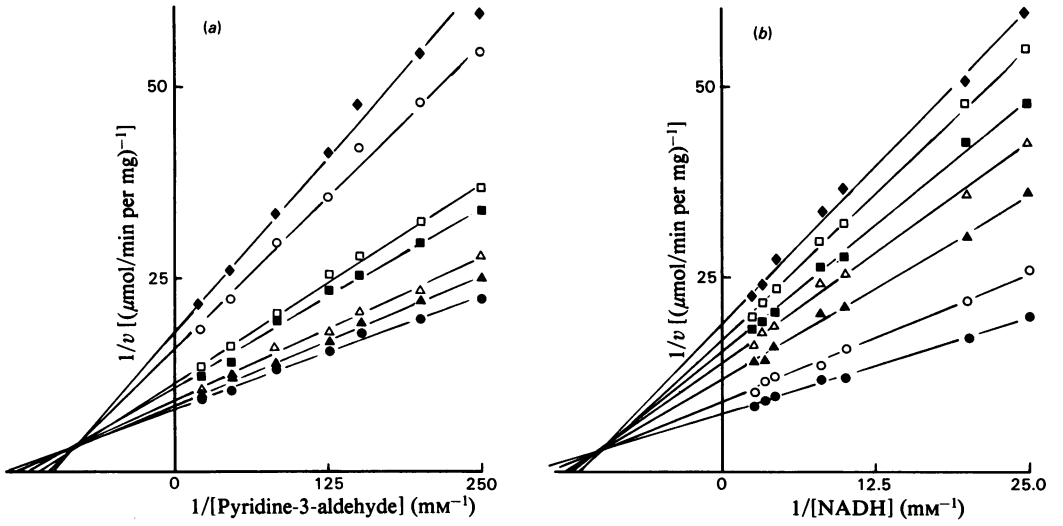

Fig. 3. *Double-reciprocal plots from initial-rate measurements of the reduction of pyridine-3-aldehyde by NADH catalysed by low-K_m aldehyde reductase*

Details of the assay procedure were as described in the text. (*a*) The NADH concentrations were 400 μM (●), 300 μM (▲), 250 μM (△), 125 μM (■), 100 μM (□), 50 μM (○) and 40 μM (◆). (*b*) The pyridine-3-aldehyde concentrations were 50 μM (●), 25 μM (○), 12.5 μM (▲), 8 μM (△), 6.5 μM (■), 5 μM (□) and 4 μM (◆).

Table 1. *Product inhibition of ox brain low-K_m aldehyde reductase*

Saturating and non-saturating concentrations of the fixed substrates were as given in Table 2.

Inhibitor	Varied substrate	Fixed substrate	Concentration	Inhibition
$NADP^+$	NADPH	Pyridine-3-aldehyde	Non-saturating	Competitive
$NADP^+$	NADPH	Pyridine-3-aldehyde	Saturating	Competitive
$NADP^+$	Pyridine-3-aldehyde	NADPH	Non-saturating	Mixed
$NADP^+$	Pyridine-3-aldehyde	NADPH	Saturating	None
Pyrid-3-ylcarbinol	NADPH	Pyridine-3-aldehyde	Non-saturating	Mixed
Pyrid-3-ylcarbinol	NADPH	Pyridine-3-aldehyde	Saturating	Uncompetitive
Pyrid-3-ylcarbinol	Pyridine-3-aldehyde	NADPH	Non-saturating	Mixed
Pyrid-3-ylcarbinol	Pyridine-3-aldehyde	NADPH	Saturating	Mixed

Table 2. *Inhibitor constants for product inhibition of ox brain low-K_m aldehyde reductase*

K_{is} and K_{ii} values were determined from the variations of the slopes and intercepts respectively of double-reciprocal plots with the inhibitor concentration as described in the text.

Inhibitor	Varied substrate	Fixed substrate	K_{is} (μM)	K_{ii} (μM)
$NADP^+$	NADPH	Pyridine-3-aldehyde (0.966 μM)	0.8 ± 0.16	—
$NADP^+$	NADPH	Pyridine-3-aldehyde (96.6 μM)	1.3 ± 0.08	—
$NADP^+$	Pyridine-3-aldehyde	NADPH (2.24 μM)	5.0 ± 2.0	2.9 ± 0.17
$NADP^+$	Pyridine-3-aldehyde	NADPH (100 μM)	—	—
Pyrid-3-ylcarbinol	Pyridine-3-aldehyde	NADPH (2.5 μM)	136 ± 58	150 ± 39
Pyrid-3-ylcarbinol	Pyridine-3-aldehyde	NADPH (90 μM)	3404 ± 296	1850 ± 97
Pyrid-3-ylcarbinol	NADPH	Pyridine-3-aldehyde (2.4 μM)	1262 ± 106	635 ± 7
Pyrid-3-ylcarbinol	NADPH	Pyridine-3-aldehyde (242.5 μM)	—	950 ± 243

pyridine-3-aldehyde was unchanged (Fig. 5*b*), whereas that towards pyridine-3-aldehyde was uncompetitive (Fig. 6).

Such behaviour is characteristic of an ordered mechanism of the type:

$$E \rightleftharpoons EA \rightleftharpoons EAB \rightleftharpoons EPQ \rightleftharpoons EP \rightleftharpoons E \quad (2)$$

where A and P represent NADPH and $NADP^+$ re-

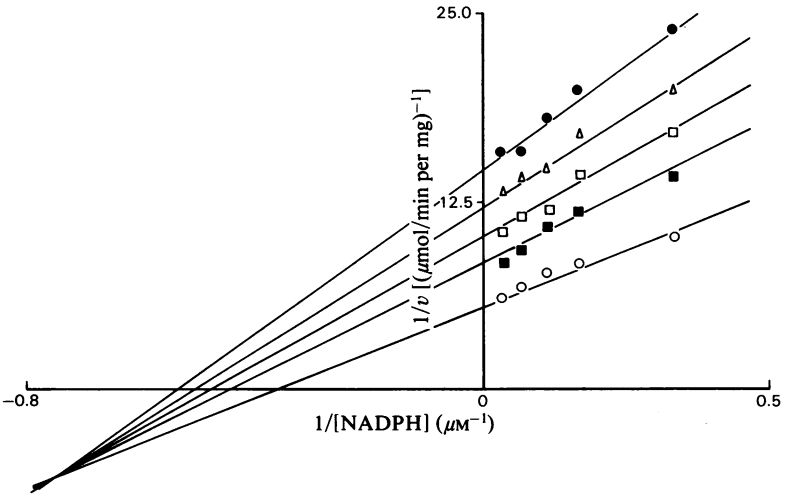

Fig. 4. *Inhibition of low-K_m aldehyde reductase by pyrid-3-ylcarbinol with respect to NADPH at a non-saturating concentration of pyridine-3-aldehyde*

The pyridine-3-aldehyde concentration was 2.4 μM and the pyrid-3-ylcarbinol concentrations were 0 (○), 0.25 mM (■), 0.5 mM (□), 0.75 mM (△) and 1.0 mM (●).

Table 3. *Kinetic constants for ox brain low-K_m aldehyde reductase*

The constants were determined from the data in Figs. 1 and 3 as described in the text and are expressed in terms of the mechanism shown in eqn. (2) and the initial-rate eqn. (1).

Substrate	K_s (μM)	K_m (μM)	V (μmol/min per mg)
NADPH	Very low	0.76±0.05	—
NADH	328±17	804±29	—
Pyridine-3-aldehyde	—	0.65±0.03*	0.32±0.01*
Pyridine-3-aldehyde	—	8.6±2.8†	0.69±0.06†

* Values determined with NADPH as the coenzyme.
† Values determined with NADH as the coenzyme.

spectively, and B and Q represent pyridine-3-aldehyde and pyrid-3-ylcarbinol respectively. The kinetic constants determined from secondary plots of the initial-rate data are given in terms of eqn. (1) in Table 3.

For this mechanism the K_i value determined from the intercept replot (K_{ii}) for $NADP^+$ acting as a mixed inhibitor with respect to pyridine-3-aldehyde will be related to the dissociation constant for this product binding to the free enzyme (K_i) by the relationship:

$$K_{ii} = K_i(1 + a/K_m^A) \quad (3)$$

The value obtained from substituting K_{ii} from Table 2 into this equation is 0.74 μM, in good agreement with the values of 0.8 and 1.3 μM determined directly.

Thus the kinetic data are consistent with the low-K_m aldehyde reductase from ox brain obeying a compulsory-order mechanism involving the formation of a ternary complex, although the complementary, but more elaborate, iso-Theorell–Chance mechanism cannot be excluded by initial-rate data of this type (see Boghosian & McGuinness, 1981). It is difficult to compare the results from these studies with those of previous work because of the possible existence of different forms of this enzyme (see Branlant & Biellmann, 1980; Daly & Mantle, 1982; Wermuth *et al.*, 1982; Hara *et al.*, 1983) the possibility of considerable inter-species variations (Davidson & Flynn, 1979) and uncertainties about the identities of enzymes that have been given different names by different workers (see Flynn, 1982). However, the data available are confusing. The results obtained by Boghosian & McGuinness (1981) with pig brain aldose reductase are consistent with those reported in the present paper, although they investigated only five of the possible eight conditions of product inhibition reported in the present study. In contrast, Toews (1967) pro-

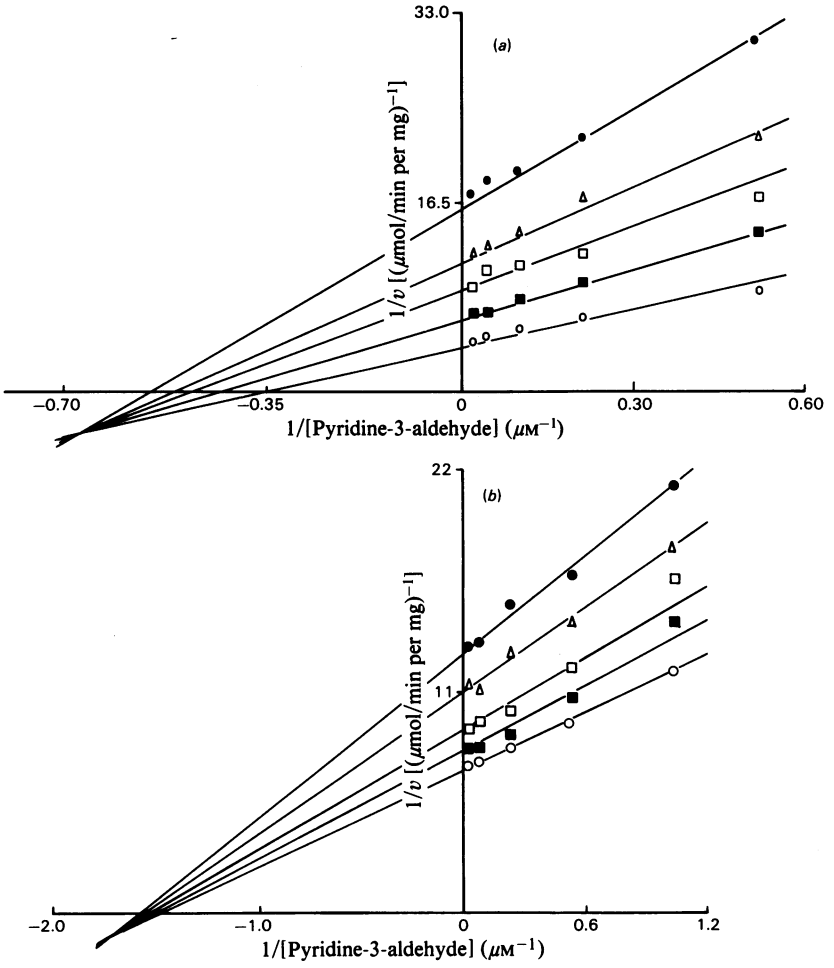

Fig. 5. *Inhibition of low-K_m aldehyde reductase by pyrid-3-ylcarbinol*

(*a*) The NADPH concentration was 2.5 μM (non-saturating) and the pyrid-3-ylcarbinol concentrations were 0 (○), 187.5 μM (■), 250 μM (□), 375 μM (△) and 500 μM (●). (*b*) The NADPH concentration was 90 μM (saturating) and the pyrid-3-ylcarbinol concentrations were 0 (○), 375 μM (■), 750 μM (□), 1.25 mM (△) and 1.75 mM (●).

posed a double-displacement mechanism for the glycerol dehydrogenase from rabbit skeletal muscle. This conclusion rested mainly on the observation of apparently parallel double-reciprocal plots for the glyceraldehyde–NADPH reaction, and the data shown in Fig. 1 of the present work indicate that it would be easy to reach such a conclusion unless further studies were carried out.

Doughty & Conrad (1982) proposed a more elaborate mechanism to account for the behaviour of ox and rat lens aldose reductase with glyceraldehyde as the substrate. This involved the random ordered binding of substrates and release of products with the additional formation of an abortive complex. In that work non-linear double-reciprocal plots were observed with glyceraldehyde, and, although it has been suggested that this may have been due to inhomogeneity of the enzyme preparation used (Cromlish & Flynn, 1983), similar behaviour has been observed with the, apparently homogeneous, preparations of ox liver low-K_m aldehyde reductase (Ryle *et al.*, 1984) and ox lens aldose reductase (Halder & Crabbe, 1984). In the latter case it was proposed that the addition of substrates was essentially random but that the pathway involving the binding of NADPH before the aldehyde was dominant when glucose was used as the substrate (Halder & Crabbe, 1984). In contrast, O'Brien & Schofield (1980) reported glycerol to be a competitive inhibitor of human brain aldose reductase with respect to glyceraldehyde, whereas Wermuth *et al.* (1982) found it to be a mixed inhibitor with a more highly purified enzyme from the same source. Further work will be

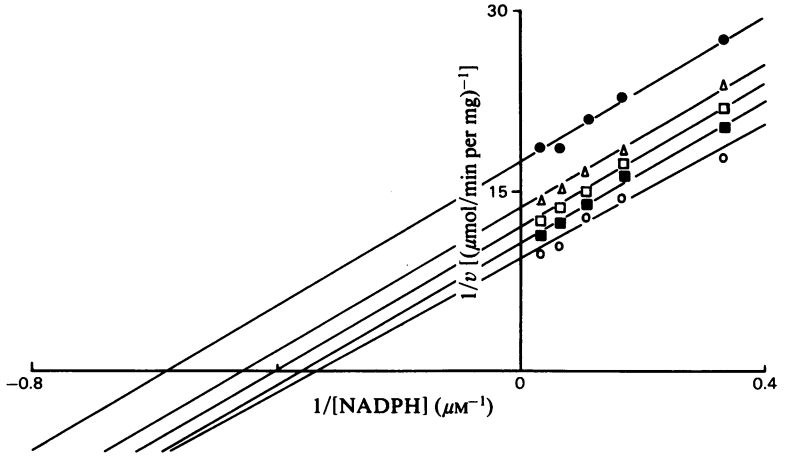

Fig. 6. *Inhibition of low-K_m aldehyde reductase by pyrid-3-ylcarbinol with respect to NADPH at a saturating concentration of pyridine-3-aldehyde*

The pyridine-3-aldehyde concentration was 242.5 μM and the pyrid-3-ylcarbinol concentrations used were 0 (○), 0.25 mM (■), 0.44 mM (□), 0.625 mM (△) and 0.875 mM (●).

necessary in order to understand the apparently complex behaviour of the ox brain enzyme with this substrate.

There have been conflicting reports on the efficiency with which the low-K_m enzyme can use NADH instead of NADPH (see Turner & Tipton, 1972; Wermuth *et al.*, 1982; Daly & Mantle, 1982; Cromlish & Flynn, 1983; Hara *et al.*, 1983). The differences in the K_m values and their remarkably different dependence on the concentration of the aldehyde substrate (Figs. 1 and 2), together with the greater sensitivity of the activity of the former coenzyme to ionic-strength effects (Ryle *et al.*, 1984), will result in the relative activities with these two coenzymes being critically dependent on the assay conditions used. The much lower K_m value for NADPH suggests, however, that this coenzyme will be dominant under conditions prevailing *in vivo*.

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References

Boghosian, R. A. & McGuinness, E. T. (1981) *Int. J. Biochem.* **13**, 909–914
Branlant, G. & Biellmann, J.-F. (1980) *Eur. J. Biochem.* **129**, 99–104
Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 1–32
Cromlish, J. A. & Flynn, T. G. (1983) *J. Biol. Chem.* **258**, 3416–3424
Daly, A. K. & Mantle, T. J. (1982) *Biochem. J.* **205**, 373–380
Dalziel, K. (1975) *Enzymes 3rd Ed.* **11**, 1–61
Davidson, W. S. & Flynn, T. G. (1979) *J. Mol. Evol.* **14**, 251–258
Dixon, M. & Webb, E. C. (1979) *Enzymes*, Longman, London
Doughty, C. C. & Conrad, S. M. (1982) *Biochim. Biophys. Acta* **708**, 358–364
Florini, J. R. & Vestling, C. S. (1957) *Biochim. Biophys. Acta* **25**, 575–578
Flynn, T. G. (1982) *Biochem. Pharmacol.* **31**, 2705–2712
Gabay, K. H. (1975) *Annu. Rev. Med.* **26**, 521–536
Halder, A. B. & Crabbe, M. J. C. (1984) *Biochem. J.* **219**, 33–39
Hara, A., Deyashiki, Y., Nakayama, T. & Sawada, H. (1983) *Eur. J. Biochem.* **133**, 207–214
Houslay, M. D. & Tipton, K. F. (1973) *Biochem. J.* **135**, 173–186
O'Brien, M. M. & Schofield, P. J. (1980) *Biochem. J.* **187**, 21–30
Pettersson. G. (1970) *Acta Chem. Scand.* **24**, 1271–1279
Racker, E. (1957) *Methods Enzymol.* **3**, 293–296
Ryle, C. M., Dowling, T. G. & Tipton, K. F. (1984) *Biochim. Biophys. Acta* **791**, 155–163
Tipton, K. F., Houslay, M. D. & Turner, A. J. (1977) *Essays Neurochem. Neurobiol.* **1**, 103–139
Toews, C. J. (1967) *Biochem. J.* **105**, 1067–1073
Tulsiani, D. R. P. & Touster, O. (1977) *J. Biol. Chem.* **252**, 2545–2550
Turner, A. J. & Tipton, K. F. (1972) *Biochem. J.* **130**, 765–772
Turner, A. J., Illingworth, J. A. & Tipton, K. F. (1974) *Biochem. J.* **144**, 353–360
Wermuth, B., Burgisser, H., Bohren, K. & von Wartburg, J.-P. (1982) *Eur. J. Biochem.* **127**, 279–284
Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332