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Kinetics and Mechanism of Action of Aldehyde Reductase from Pig Kidney

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An improved procedure for purifying aldehyde reductase is described. Utilization of Blue Dextran–Sepharose 4B and elimination of hydroxyapatite chromatography greatly improves the yield and ease of purification. Starting with 340g of kidney tissue (two pig kidneys) approx. 50mg of purified reductase may be routinely and reproducibly obtained. The purified reductase was used to establish the kinetic reaction mechanism of the enzyme. Initial-velocity analysis and product-inhibition data revealed that pig kidney aldehyde reductase follows an Ordered Bi Bi reaction mechanism in which NADPH binds first before D-glyceraldehyde. The limiting Michaelis constants for D-glyceraldehyde and NADPH were 4.8 ± 0.7mM and 9.1 ± 2.1μM respectively. The mechanism is similar to that of another monomeric oxidoreductase, octopine dehydrogenase, towards which aldehyde reductase exhibits several similarities, but differs from that of other aldehyde reductases. Phenobarbital is a potent inhibitor of aldehyde reductase, inhibiting both substrate and cofactor non-competitively ($K_i = 80.4 \pm 10.5$μM and 66.9 ± 1.6μM respectively). Barbiturate inhibition seems to be a common property of NADPH-dependent aldehyde reductases.

Aldehyde reductase from pig kidney is an NADPH-dependent monomeric oxidoreductase of broad specificity (Bosron & Prairie, 1972; Flynn *et al.*, 1975). Several physical and chemical properties of the enzyme have been examined and the enzyme has been shown to bear striking similarities to octopine dehydrogenase, a monomeric NAD^+-dependent oxidoreductase from mollusc muscle (Flynn *et al.*, 1975). A similar finding has been reported for human liver aldehyde reductase (Wermuth *et al.*, 1977).

In an attempt to gain some insight into the mechanism of action of the pig kidney reductase we have shown that the stereospecificity of substrate reduction was similar to that of liver alcohol dehydrogenase (Flynn *et al.*, 1975). In addition, more recently, we have obtained evidence to suggest that the mammalian aldehyde reductases are compositionally more similar to liver alcohol dehydrogenase than they are to other oligomeric oxidoreductases (Davidson *et al.*, 1978). In order to make further comparisons we have determined the kinetic mechanism of aldehyde reductase and have compared it with that of alcohol dehydrogenase (Wratten & Cleland, 1963; Plapp, 1970) and octopine dehydrogenase (Doublet & Olomucki, 1975).

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Experimental

Materials

$NADP^+$, NADPH, D-glyceraldehyde, glycerol, Blue Dextran and *NNN'N'*-tetramethylethylenediamine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phenobarbital (Na^+ salt) was a gift from the Department of Pharmacology, Queen's University. Sepharose 4B and Sephadex G-100 were purchased from Pharmacia (Canada) Ltd., Montreal, Que., Canada. Acrylamide and bisacrylamide were products of BDH (Canada) Ltd., Toronto, Ont., Canada. All other reagents were of the highest grade commercially available. Pig kidneys were obtained from a local slaughterhouse.

Purification of enzyme

Pig kidney aldehyde reductase was purified by a modification of the procedure previously described (Flynn *et al.*, 1975). Kidneys were cut into small pieces, any fat or capsule was removed, and the tissue homogenized in 3 vol. of 0.15M-KCl containing 2-mercaptoethanol (2ml/litre) for 1–2min. The homogenate was centrifuged at 30000*g* for 60min and the resultant supernatant, after filtration through glass wool to remove surface lipids, was retained. Solid $(NH_4)_2SO_4$ was added to the supernatant (0.23g/ml) and the solution stirred for 90min. The mixture was

then centrifuged at 30000*g* for 40min and the supernatant retained. $(NH_4)_2SO_4$ was added to this supernatant (0.142g/ml) and after stirring for 90min the mixture was centrifuged at 30000*g* for 40min. The supernatant from this centrifugation was discarded, and the pellets were suspended in 0.1M-sodium phosphate buffer, pH7.0, containing 2.5mM-2-mercaptoethanol. The suspended protein was then applied to a Sepnadex G-100 column (10cm × 85cm) equilibrated with 0.01M-phosphate buffer, pH7.0. Fractions (12ml) were assayed for enzyme activity with D-glyceraldehyde (5mM) as substrate and NADPH (133μM) as cofactor. The protein elution profile was monitored by recording the A_{280}, and protein concentrations were determined from the A_{280} and A_{260} by using the conversion formula of Layne (1957). Enzymically active fractions were pooled, concentrated by ultrafiltration and applied to a column (2.5cm × 40cm) containing Blue Dextran–Sepharose 4B, which was prepared by the method of Ryan & Vestling (1974). After application of the protein solution the column was eluted with 0.1M-phosphate buffer, pH7.0 (1 litre), followed by elution with a linear 0–0.5M-NaCl gradient (1 litre) in 0.1M-phosphate buffer. Active fractions were pooled, concentrated by ultrafiltration to approx. 20ml and then applied to a Sephadex G-100 column (5cm × 85cm). The column was again eluted with 0.01M-phosphate buffer, pH7.0. Enzymically active fractions were again pooled, concentrated by ultrafiltration to about 10ml and then applied to a column (2.5cm × 30cm) of DEAE-cellulose (DE-52) equilibrated with 0.005M-phosphate buffer, pH7.0. The column was eluted with 0.01M-phosphate buffer, pH7.0. Aldehyde reductase activity was associated with the major protein peak. Active fractions were pooled, dialysed exhaustively (48h with 4 × 2 litres of water) and then freeze-dried.

Kinetic measurements

The activity of the enzyme was measured by determining the change in A_{340} in the presence of D-glyceraldehyde and NADPH. The incubation mixture in a total volume of 3.0ml contained 100mM-phosphate buffer, pH7.0. The concentrations of NADPH, D-glyceraldehyde and inhibitors are given in the legends describing the specific experiments. Controls contained all reagents except D-glyceraldehyde. All measurements of enzyme activity were made on a Beckman 25 recording spectrophotometer with maximum full-scale expansion of 0–0.1 *A* unit. The reaction was initiated by addition of enzyme, and initial rates were measured for 1–2min. The reaction was carried out at 25°C, and all reagents and enzyme solutions were maintained at this temperature before the actual measurement of activity.

Kinetic analysis

The data were fitted in the non-reciprocal form without weighting to appropriate rate equations (see the Results section) by non-linear regression on a Burroughs B6700 computer at the Queen's University Computing Centre. The program supplied values of the kinetic constants with their standard deviations and the sum of the squares of the residual errors. The data obtained are shown in the form of double-reciprocal plots. The straight lines on these plots were drawn from the predicted values of the experimental points supplied by the computer program.

Results

Purification of enzyme

Table 1 summarizes the purification procedure, which yields approx. 50mg of purified enzyme starting with 300–400g of kidney tissue. The procedure differs significantly from that previously used (Flynn *et al.*, 1975), principally in the introduction of the Blue Dextran–Sepharose 4B column and the elimination of the Bio-Gel HTP and Serva hydroxyapatite (3:2, w/w) column. In addition, passage through Sephadex G-100 after $(NH_4)_2SO_4$ fractionation effected separation of the reductase from higher-molecular-weight oligomeric dehydrogenases and acted as a desalting column by removing $(NH_4)_2SO_4$ from the eluted enzyme.

Criteria of purity

When the enzyme was subjected to polyacrylamide-disc-gel electrophoresis on 7% gels as previously

Table 1. *Purification of pig kidney aldehyde reductase*
For details see the text.

Fraction	Volume (ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Recovery (%)	Purification (fold)
Supernatant from crude extract	1010	28600	977	0.034	100	1.0
Sephadex G-100	1150	6330	779	0.123	80	3.6
Blue Dextran–Sepharose 4B	430	258	583	2.230	60	66.0
Sephadex G-100	225	124	588	4.900	60	138.0
DEAE-cellulose (DE-52)	128	51	278	5.450	28	267.0

described (Flynn *et al.*, 1975) and stained with Amido Black a single band was evident. A single band was also evident when the gel was stained for enzyme activity by the procedure of Kormann *et al.* (1972). Thus the minor protein band present after the previous procedure was completely eliminated. Gel electrophoresis in the presence of 1% sodium dodecyl sulphate as described previously (Flynn *et al.*, 1975) revealed a single protein band, and by comparison with several proteins of different molecular weight, gel electrophoresis in sodium dodecyl sulphate allowed an estimation of the molecular weight of aldehyde reductase, which was 36700. The behaviour of aldehyde reductase in these gel systems and the estimation of molecular weight agree very closely with the previously (Flynn *et al.*, 1975) obtained values.

This purified aldehyde reductase was used in the kinetic studies described below.

Initial-velocity studies

When NADPH was the variable substrate, D-glyceraldehyde being held constant at concentrations ranging from 0.25 to 5.0mM, double-reciprocal plots of initial velocity versus NADPH concentration gave a family of straight lines intersecting in the lower left quadrant (Fig. 1). With D-glyceraldehyde as the variable substrate and NADPH kept constant at values ranging from 5 to 50μM double-reciprocal plots of initial velocity versus D-glyceraldehyde concentration again yielded a series of straight lines intersecting in the lower left quadrant. These results indicate that the apparent Michaelis constant for one substrate is dependent on the concentration of the other substrate, and the data obtained from the double-reciprocal plots fitted eqn. (1):

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

where A is the concentration of NADPH, B is the concentration of D-glyceraldehyde, v and V are the initial and maximum velocities respectively and K_m^A and K_m^B are the limiting Michaelis constants for NADPH and D-glyceraldehyde respectively. K_s^A is the dissociation constant for NADPH. The values of K_m^A, K_m^B and K_s^A were determined from a computer program for non-linear regression analysis by using a Burroughs B6700 computer and are shown in Table 2. Secondary plots of slopes and intercepts obtained from the double-reciprocal plots versus the reciprocal of the concentration of the fixed substrate were linear in each case (Fig. 2).

Table 2. *Kinetic constants for pig kidney aldehyde reductase*

K_s^A, dissociation constant for NADPH	1.6 ± 0.9μM
K_m^A, limiting Michaelis constant for NADPH	9.1 ± 2.1μM
K_m^B, limiting Michaelis constant for D-glyceraldehyde	4.8 ± 0.7μM
K_q, inhibition constant for $NADP^+$	39.3 ± 6.8μM
K_p, inhibition constant for glycerol	4.1 ± 0.5M
K_{Phe}, inhibition constant for phenobarbital with respect to NADPH	66.9 ± 1.6μM
K_{Phe}, inhibition constant for phenobarbital with respect to D-glyceraldehyde	80.4 ± 1.5μM

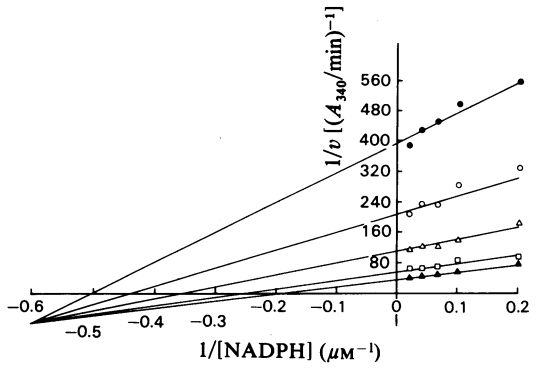


Fig. 1. *Initial-velocity analysis of pig kidney aldehyde reductase with NADPH as the variable substrate*
The D-glyceraldehyde concentration was held constant at 0.25mM (▲), 0.5mM (□), 1.0mM (△), 2.5mM (○) and 5.0mM (●).

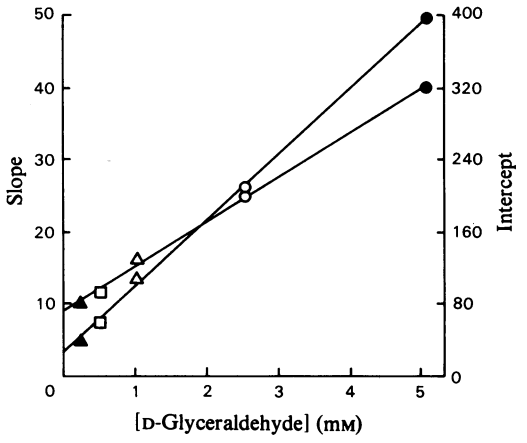


Fig. 2. *Replots of slopes and intercepts from Fig. 1 versus D-glyceraldehyde concentration*

Product-inhibition studies

With NADPH as the variable substrate and D-glyceraldehyde held constant at 5.0mM, $NADP^+$ gave a linear competitive-inhibition pattern (Fig. 3). A replot of the slopes versus $NADP^+$ concentration was also linear. The data obtained from these plots fitted eqn. (2):

$$v = \frac{V \cdot A}{K_A\left(1 + \frac{Q}{K_q}\right) + A} \quad (2)$$

where K_A is the apparent Michaelis constant for NADPH and Q is the concentration of NADPH. K_q is the inhibition constant for $NADP^+$, the value for which is shown in Table 2. When the concentration of NADPH was kept constant at either 25μM or 50μM and D-glyceraldehyde was the variable substrate, $NADP^+$ (30–120μM) gave no detectable inhibition.

Glycerol was found to be a non-competitive inhibitor when D-glyceraldehyde was the variable substrate and NADPH was maintained at a concentration of 100μM (Fig. 4). Replots of intercepts and slopes of these data versus glycerol concentration were linear. The data obtained from these observations fitted eqn. (3):

$$v = \frac{V \cdot B}{\left(K_B + B\right)\left(1 + \frac{P}{K_p}\right)} \quad (3)$$

where K_B is the apparent Michaelis constant for D-glyceraldehyde, P is the concentration of glycerol and K_p is the inhibition constant for glycerol. The value of K_p is given in Table 2.

Inhibition of barbiturate

It has been previously shown that phenobarbital inhibits aldehyde reductase (Davidson *et al.*, 1978), but the nature of the inhibition of pig kidney aldehyde reductase has not been established. With the NADPH concentration maintained constant at 100μM and the concentration of D-glyceraldehyde varied from 0.5 to 7.5mM at different concentrations of phenobarbital, a double-reciprocal plot of initial velocity against D-glyceraldehyde concentration showed that phenobarbital is a non-competitive inhibitor of D-glyceraldehyde (Fig. 5). Replots of the intercepts and slopes of these graphs versus phenobarbital concentrations were linear. The data obtained from this experiment fitted eqn. (4):

$$v = \frac{V \cdot B}{\left(K_B + B\right)\left(1 + \frac{\text{Phe}}{K_{\text{Phe}}}\right)} \quad (4)$$

where Phe is the concentration of phenobarbital and K_{Phe} is the inhibition constant for phenobarbital. The value of K_{Phe} is given in Table 2.

Phenobarbital is also a non-competitive inhibitor of NADPH. Fig. 6 shows the effects of varying the NADPH concentration at fixed concentrations of phenobarbital when the D-glyceraldehyde concentration was kept constant at 5mM. Replots of the slopes and intercepts of the lines in Fig. 6 were linear. The data obtained from these experiments fitted eqn. (5):

$$v = \frac{V \cdot A}{\left(K_A + A\right)\left(1 + \frac{\text{Phe}}{K_{\text{Phe}}}\right)} \quad (5)$$

The value for K_{Phe} is given in Table 2.

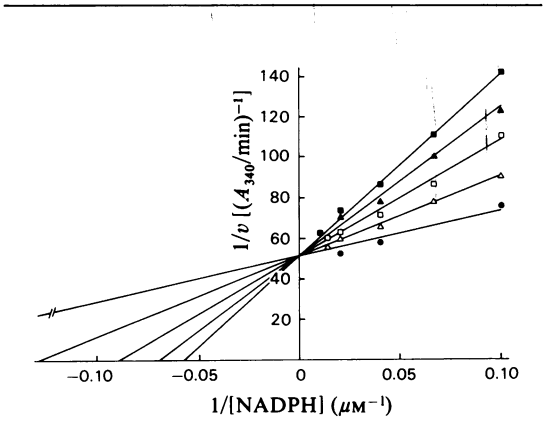


Fig. 3. *Product inhibition of pig kidney aldehyde reductase by $NADP^+$ with NADPH as the variable substrate*
D-Glyceraldehyde was held constant at 5mM. The concentration of $NADP^+$ was 0 (●), 30μM (△), 60μM (□), 90μM (▲) and 120μM (■).

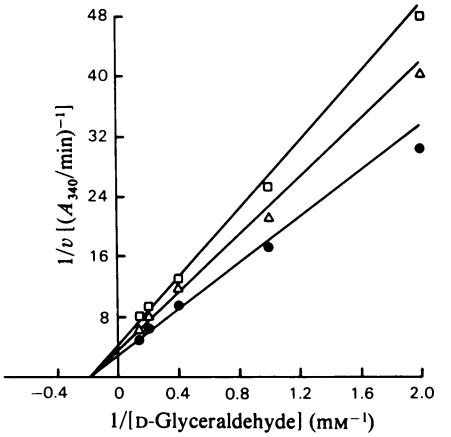


Fig. 4. *Product inhibition of pig kidney aldehyde reductase by glycerol with D-glyceraldehyde as the variable substrate*
NADPH was held constant at 100μM. The concentration of glycerol was 0 (●), 1M (△) and 2M (□).

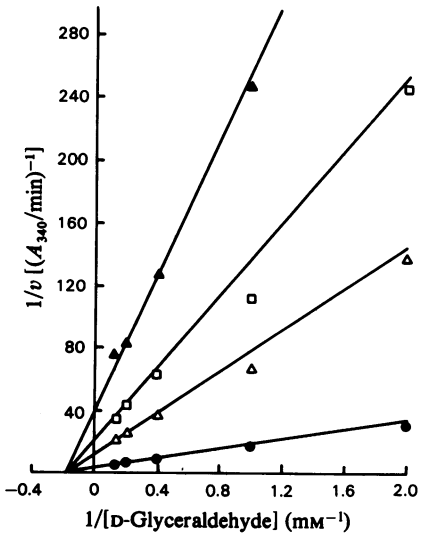


Fig. 5. *Inhibition of pig kidney aldehyde reductase by phenobarbital with* D-*glyceraldehyde as the variable substrate*

NADPH was held constant at 100μM. The concentration of phenobarbital was 0 (●), 0.25mM (△), 0.5mM (□) and 1.0mM (▲).

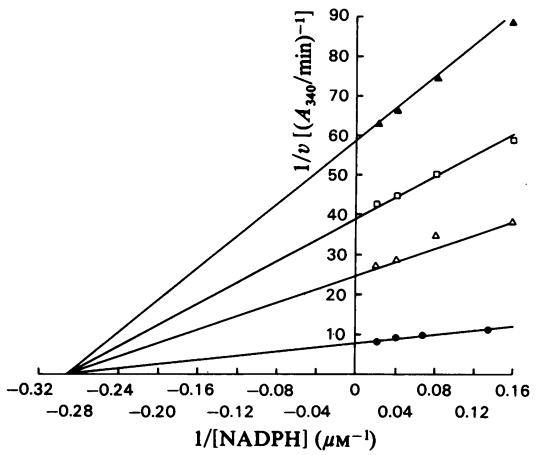


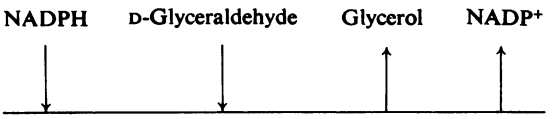
Fig. 6. *Inhibition of pig kidney aldehyde reductase by phenobarbital with NADPH as the variable substrate*

D-Glyceraldehyde was held constant at 7.5mM. The concentration of phenobarbital was 0 (●), 0.15mM (△), 0.3mM (□) and 0.45mM (▲).

Discussion

The procedure described in this paper for purifying aldehyde reductase from pig kidney is an improvement on the one previously used (Flynn *et al.*, 1975). The introduction of a Blue Dextran–Sepharose 4B column and the elimination of the hydroxyapatite step greatly improved the yield and ease of purification. Thompson *et al.* (1975) have shown that Blue Dextran–Sepharose columns function as affinity columns for proteins with nucleoside phosphate-binding sites that are formed by the $NAD(P)^+$-binding domain. This property has been used effectively here for the purification of pig kidney aldehyde reductase and also previously for the purification of aldehyde reductase from other species (Davidson *et al.*, 1978). Starting with 340g of kidney tissue (two pig kidneys) approx. 50mg of purified reductase may be obtained. The final enzyme preparation is free of the small amount of additional enzyme material previously described (Flynn *et al.*, 1975).

With the purified enzyme, initial-velocity studies were consistent with several kinetic mechanisms, but a Ping Pong mechanism would be excluded on the grounds that such a mechanism would produce parallel lines for reciprocal plots when either D-glyceraldehyde or NADPH was the variable substrate and the other was kept constant (Cleland, 1963). Similarly, a Random Bi Bi mechanism could be ruled out, as the replots of the intercepts obtained in the initial-velocity plots (Fig. 1) would in that case have been non-linear (Cleland, 1963). The limiting Michaelis constants for the substrate and cofactor are dependent on one another, and these results, taken in conjunction with the linear secondary plots, are consistent with an Ordered Bi Bi, a Random Rapid Equilibrium Bi Bi or a Theorell–Chance mechanism. Product-inhibition studies ruled out the Random Rapid Equilibrium Bi Bi and the Theorell–Chance mechanisms, as in these cases glycerol would be a competitive inhibitor of D-glyceraldehyde and clearly it was found to be non-competitive. Using fluorescence techniques, Flynn *et al.* (1975) showed that pig kidney aldehyde reductase does bind NADPH, which substantiates the proposal that the enzyme follows an Ordered Bi Bi reaction mechanism. In this scheme NADPH is bound by the enzyme first, and only then can the substrate be added. The reduced substrate is first to leave, followed by the oxidized cofactor, as depicted below.



Many oligomeric dehydrogenases (Wratten & Cleland, 1963; Holbrook & Gutfreund, 1973) follow Ordered Bi Bi reaction mechanisms. The monomeric oxidoreductases, octopine dehydrogenase (Doublet & Olomucki, 1975) and saccharopine dehydrogenase (Fujioki & Nakatoni, 1970), also follow sequential mechanisms in which the cofactor is bound to the enzyme before the substrates. However, the mono-

meric aldehyde reductases, which had been investigated previously, do not appear to follow this type of mechanism. Bronaugh & Erwin (1972) determined that NADPH-dependent aldehyde reductase from bovine brain has a Random Bi Bi mechanism, and Toews (1967) has suggested that the reaction mechanism for rat skeletal-muscle glycerol dehydrogenase (aldehyde reductase) is either Ping Pong Bi Bi or Iso Ping Pong Bi Bi. Neither of these mechanisms can account for the direct transfer of hydrogen from the cofactor to the substrate, and this throws doubt on this mechanism, particularly since every dehydrogenase examined to date has been shown to have a mechanism that proceeds by direct transfer of a hydride ion. Toews (1967) has suggested that the enzymes from skeletal muscle and cardiac muscle are similar, and that they differ from the enzymes from kidney and liver, which follow an Iso Theorell–Chance reaction mechanism. However, if his initial mechanism is wrong, then this interpretation is open to question. The kinetic studies on pig kidney aldehyde reductase suggest that this enzyme and the NADPH-dependent aldehyde reductase from bovine brain can be differentiated by their kinetic mechanism, even though they are similar in many respects and would seem to belong to the same class of enzymes. On the other hand, pig kidney aldehyde reductase and octopine dehydrogenase bear striking similarities to one another, and, as shown in the present work, from the point of view of classical steady-state kinetics have similar sequential mechanisms. Recently, however, a more complex mechanism has been suggested for octopine dehydrogenase (Monneuse-Doublet *et al.*, 1978). Liver alcohol dehydrogenase and pig kidney aldehyde reductase both catalyse the transfer of the A hydrogen from coenzyme to the *re* face of a carbonyl group on the substrate (Flynn *et al.*, 1975). The fact that alcohol dehydrogenase (Wratten & Cleland, 1963) and pig kidney reductase follow the same reaction mechanism suggest that there may be further similarities among these enzymes.

One of the characteristic properties of mammalian NADPH-dependent aldehyde reductases is their inhibition by barbiturates (Erwin *et al.*, 1971; Ris *et al.*, 1975; Erwin & Dietrich, 1973) and the enzymes from chicken kidney, frog kidney and fruit fly are also barbiturate-sensitive (Davidson *et al.*, 1978). The kinetic studies on pig kidney aldehyde reductase revealed that phenobarbital is a non-competitive inhibitor of both substrate and cofactor, with inhibition constants in the range 60–90 μM. Similar results were obtained for bovine brain aldehyde reductase (Erwin *et al.*, 1971; Erwin & Dietrich, 1973) and for daunorubicin reductase from rat liver (Turner & Hick, 1975). Aldehyde reductases have been implicated in the metabolism of 'biogenic aldehydes', derived from the biogenic amines, such as adrenaline, which have a β-hydroxy group. 'Biogenic aldehydes' lacking this hydroxy group are oxidized to the corresponding acid, whereas 'biogenic aldehydes' with a β-hydroxy group are reduced by an aldehyde reductase (Breese *et al.*, 1969). The importance of the 'biogenic aldehydes' is not understood, but it has been theorized that they play a role in sleep mechanisms, may control body temperature and are known to inhibit both the $(Na^+ + K^+)$- and Mg^{2+}-activated adenosine triphosphatases (Ris & von Wartburg, 1973). The discovery that barbiturates inhibit aldehyde reductases led to the hypothesis that the mechanism of action of these drugs may be causally related to an increase in the concentration of 'biogenic aldehydes' (Erwin *et al.*, 1971). The reduction of 'biogenic aldehydes' by aldehyde reductase, however, may be only one of the roles performed by this enzyme. As has been discussed previously (Davidson *et al.*, 1978), the broad substrate specificity of aldehyde reductase (Bosron & Prairie, 1972; Davidson *et al.*, 1978) allows the enzyme a variety of functions in relation to aldehyde metabolism.

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