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The Characterization of Two Reduced Nicotinamide–Adenine Dinucleotide Phosphate-Linked Aldehyde Reductases from Pig Brain

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1. NADPH-linked aldehyde reductase from pig, ox and rat brain exhibits non-linear reciprocal plots when partially purified enzyme preparations are studied. 2. In pig brain this non-linearity is due to the presence of two distinct aldehyde reductases, which can be separated by DEAE-cellulose chromatography. 3. These two enzymes can be distinguished by several criteria, including pH optima, Michaelis constants for substrates and their inhibitor sensitivity. 4. The probable role of these enzymes in the metabolism of the aldehydes derived from the biogenic amines is discussed.

The product of the oxidative deamination of catecholamines and phenethylamines in brain is an aldehyde (Blaschko, 1952), which may then be oxidized by NAD^+-linked aldehyde dehydrogenase or reduced by NADPH-linked aldehyde reductase (Rutledge & Jonason, 1967; Breese *et al.*, 1969*a*). Phenylglycolaldehydes are preferentially reduced, whereas phenylacetaldehydes are mainly oxidized both *in vivo* and *in vitro* (Breese *et al.*, 1969*a*,*b*). Thus the major metabolite of exogenous noradrenaline in brain is probably 4-hydroxy-3-methoxyphenethylene glycol (Mannarino *et al.*, 1963) or its sulphate conjugate (Schanberg *et al.*, 1968). In brain slices 5-hydroxytryptamine (serotonin) is converted both into 5-hydroxyindoleacetic acid and into 5-hydroxytryptophol (Eccleston *et al.*, 1969) suggesting that similar metabolic routes are available to the indolethylamines.

Increased metabolism of the amines to the alcohols, at the expense of acid formation, can be induced by aldehyde dehydrogenase inhibitors such as disulfiram (Smith & Wortis, 1960) or 2-chloroacetophenone (Rutledge & Deitrich, 1971). Reserpine treatment (Sandler & Youdim, 1968) and ethanol ingestion (Tacker *et al.*, 1970) also increases the production of the alcohols.

The partial purification of the NADPH-linked aldehyde reductase from ox brain has been reported by Tabakoff & Erwin (1970). More recently, Turner & Tipton (1972*b*) have characterized an NADPH-linked aldehyde reductase from pig brain. Although the ox and pig brain enzymes appeared similar with respect to pH optima and coenzyme specificity, the quoted Michaelis constants for aldehyde substrates differed considerably between the two species. To examine whether this difference was due to a species variation or to the presence of two distinct enzymes in brain, the aldehyde reductase content of brain has been re-examined. Pig brain was shown to contain two separable NADPH-linked aldehyde reductases, which could be distinguished by their substrate specificity and inhibitor sensitivity. A similar situation probably exists in ox and rat brain. Some of the work described here has been briefly reported (Turner & Tipton, 1972*a*).

Experimental

Materials

Coenzymes and proteins. All coenzymes were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany) and were stored desiccated at 4°C. Horse liver alcohol dehydrogenase (EC 1.1.1.1), rabbit muscle lactate dehydrogenase (EC 1.1.1.27), pig heart malate dehydrogenase (EC 1.1.1.37), ox liver glutamate dehydrogenase (EC 1.4.1.3), ox liver catalase (EC 1.11.1.6) and horse heart cytochrome *c* were also obtained from Boehringer. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Ox chymotrypsinogen A was purchased from Seravac Laboratories Ltd., Maidenhead, Berks., U.K., and aldehyde dehydrogenase (EC 1.2.1.3) was prepared from ox liver by the method of Deitrich *et al.* (1962).

Amines and aldehydes. Tyramine [*p*-(2-aminoethyl)-phenol] hydrochloride, DL-octopamine [DL-α-(aminomethyl)-*p*-hydroxybenzyl alcohol] hydrochloride and 5-hydroxytryptamine (serotonin) creatinine sulphate were obtained from Ralph N. Emanuel Ltd., Wembley, Middx., U.K. Indol-3-ylacetaldehyde

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was obtained as the sodium bisulphite compound from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. *p*-Nitrobenzaldehyde was purchased from British Drug Houses Ltd., Poole, Dorset, U.K., and was recrystallized twice from water before use.

p-Hydroxyphenylacetaldehyde and *p*-hydroxyphenylglycolaldehyde were prepared from tyramine–HCl and octopamine–HCl respectively by the method of Tabakoff & Erwin (1970). 5-Hydroxyindol-3-ylacetaldehyde was prepared from 5-hydroxytryptamine by a modification (R. J. S. Duncan, personal communication) of the method of Renson *et al.* (1964) as follows. Frozen rat liver mitochondria prepared by the method of Schneider & Hogeboom (1950) were thawed and washed three times and then suspended in 0.01M-sodium phosphate buffer, pH7.2. Suspended mitochondria equivalent to 40mg of protein were incubated for 1h at 30°C with 2mM-5-hydroxytryptamine, 20μl of a 20mg/ml solution of catalase and 0.1M-sodium phosphate buffer, pH7.2, in a final volume of 5ml. After incubation, the mixture was centrifuged at 23000*g* for 15min at 4°C and the supernatant was extracted three times with 7.5ml of diethyl ether. To the combined ether extracts was added 2ml of 0.1M-sodium phosphate buffer, pH7.2, and the ether was removed under a stream of N_2. The final concentration of 5-hydroxyindol-3-ylacetaldehyde was approx. 0.2mM.

Other chemicals. Pyrazole was purchased from Eastman–Kodak Co. Ltd., Kirkby, Liverpool, U.K. Chlorpromazine was from May and Baker Ltd., Dagenham, Essex, U.K. Disulfiram [bis(diethylthiocarbamoyl) disulphide] was from Albion Laboratories Ltd., London N.W.5, U.K. Calcium phosphate gel was obtained from Sigma (London) Chemical Co. Ltd. Whatman microgranular ion-exchange cellulose DE-52 was supplied by H. Reeve Angel (London) Ltd., London E.C.4, U.K., and Sephadex G-25 and G-100 were obtained from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden. All other chemicals were obtained from British Drug Houses Ltd. The chemicals used were of the highest grade commercially available.

Methods

Enzyme assay. Routine assays of aldehyde reductase activity were carried out at 30°C in 0.1M-sodium phosphate buffer, pH7.2, containing 75μM-NADPH and 0.6mM-*p*-nitrobenzaldehyde in a total volume of 3ml. The reaction was monitored continuously by following the decrease in E_{340} in a Gilford model 240 spectrophotometer. The reaction rate was linear for at least 5min and the initial rate was proportional to enzyme concentration.

A unit of enzyme activity is defined as the amount that catalyses the oxidation of 1μmol of NADPH/min at 30°C.

Substrate assays. Solutions of NADPH and of aldehydes were prepared immediately before use. A 0.01M solution of the bisulphite complex of indol-3-ylacetaldehyde was treated by the method of Erwin *et al.* (1971) to obtain a bisulphite-free aldehyde solution. Acetaldehyde was distilled under N_2 before use. Stock NADPH solutions were assayed by using glutamate dehydrogenase (Klingenberg, 1965). Aldehydes were assayed with liver aldehyde dehydrogenase or alcohol dehydrogenase (Racker, 1957). Wherever checked these methods gave identical results.

Determination of protein. The eluates of chromatographic columns were monitored by measuring the E_{280}. In all other cases the micro biuret method was used (Goa, 1953). Bovine serum albumin was used as the protein standard.

Molecular-weight determination. The molecular weight of pig brain aldehyde reductases was determined by gel-filtration experiments (Andrews, 1970). A column of Sephadex G-100 was packed and run in 0.1M-sodium phosphate buffer, pH7.2. The following reference proteins with the molecular weights shown in parentheses were used for calibration of the column: cytochrome *c* (12400), ox chymotrypsinogen A (25000), malate dehydrogenase (65000), bovine serum albumin (67000) and lactate dehydrogenase (130000). The molecular weights assumed were taken from Andrews (1970).

Polyacrylamide-gel electrophoresis. Electrophoresis in 7.5% polyacrylamide gels was performed by the method of Zweig & Whitaker (1967). The buffer used was 0.01M-tris–glycine, pH8.5, and 0.01% Bromthymol Blue was used as tracking dye. Samples were applied, by layering on to the gel surface in 20% (v/v) glycerol, and subjected to electrophoresis with a constant current of 3mA per tube until the tracking dye had moved the length of the gel. Before being stained for enzyme activity, the gels were washed with 0.1M-glycine–NaOH buffer, pH9.5. Gels were then incubated at room temperature in the dark with fresh buffer containing the following mixture: $NADP^+$, 0.4mg/ml; propane-1,2-diol, 1.0M; phenazine methosulphate, 0.04mg/ml; Nitro Blue Tetrazolium, 0.4mg/ml. Control gels were incubated without propane-1,2-diol.

Preparation of aldehyde reductases. The first two steps were carried out at 2–6°C.

Step 1: initial extraction. Pig brains were collected within 2h of death and transported to the laboratory on ice. The brains were de-fatted and surface blood vessels were removed. The brains were then homogenized in a Kenwood Mix-master with an equal volume of 0.1M-sodium phosphate buffer, pH7.4. The homogenate was centrifuged at 23000*g* for 40min and the precipitate discarded.

Step 2: $(NH_4)_2SO_4$ fractionation. To each 100ml of the supernatant 20g of $(NH_4)_2SO_4$ was slowly added.

After addition of the salt, the solution was stirred for a further 30min and then centrifuged at 23000*g* for 30min, and the precipitate was discarded. Then 16g of $(NH_4)_2SO_4$/100ml of supernatant solution was added slowly with stirring. After centrifugation the precipitate was dissolved in the minimum volume of 0.01M-triethanolamine–HCl buffer, pH7.7, and dialysed overnight against 10 litres of this buffer.

Step 3: DEAE-cellulose chromatography. The microgranular ion-exchanger DE-52 was equilibrated in 0.01M-triethanolamine–HCl buffer, pH7.7, and packed into a column (1.5cm×8cm). The dialysed $(NH_4)_2SO_4$ fraction was centrifuged at 100000*g* at 4°C for 30min to remove insoluble protein and then 150–200mg of this fraction was applied to the column. Non-adsorbed protein was eluted with 0.01M-triethanolamine–HCl buffer, pH7.7, and then enzyme activity was eluted by applying a linear concentration gradient of buffer (100ml each of 0.01M- and 0.2M-triethanolamine–HCl buffer, pH7.7). Fractions (3ml) were collected and enzyme activity appeared as two peaks (Fig. 2). Each peak was pooled separately and precipitated by addition of $(NH_4)_2SO_4$ to 75% saturation. The protein was then dissolved in a minimal volume of 0.1M-sodium phosphate buffer, pH7.2, and dialysed against this buffer to remove residual $(NH_4)_2SO_4$. The two enzymes prepared in this manner were stable for 3–4 days at 4°C and for a longer period if stored frozen.

Results

The dialysed $(NH_4)_2SO_4$ fraction (from step 2 of the purification) did not oxidize NADPH in the absence of added aldehyde. This fraction was examined for its ability to reduce *p*-nitrobenzaldehyde over a 200-fold concentration range. The Lineweaver–Burk plot obtained has a distinct biphasic shape (Fig. 1). Since the enzyme purified by Turner & Tipton (1972*b*) exhibited a linear reciprocal plot over this concentration range, this effect was presumed to be due to the presence of two or more enzymes with different affinities for *p*-nitrobenzaldehyde. The high-speed supernatant (3×10^6*g*-min) of a pig brain homogenate showed similar biphasic kinetics when *p*-nitrobenzaldehyde reduction was examined. By extrapolation of the linear portions of the curve to infinite aldehyde concentration, it was estimated that the ratio of 'high-K_m' to 'low-K_m' aldehyde reductase activity at pH7.2 was approx. 9:1. Apparent K_m values of 2.5×10^{-4}M and 4×10^{-6}M for *p*-nitrobenzaldehyde were obtained by extrapolation. The 'high-K_m' value is consistent with

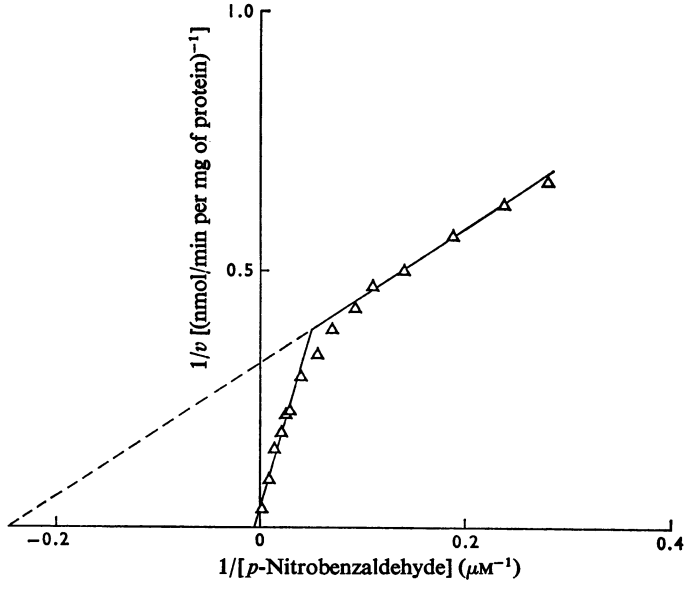

Fig. 1. *Reduction of p-nitrobenzaldehyde by the '$(NH_4)_2SO_4$ fraction' from pig brain*

Aldehyde reductase from step 2 of the purification (specific activity 0.012 unit/mg of protein) was assayed in 0.1M-sodium phosphate buffer, pH7.2, in the presence of 75μM-NADPH and various concentrations of *p*-nitrobenzaldehyde. Results are presented as a double-reciprocal plot.

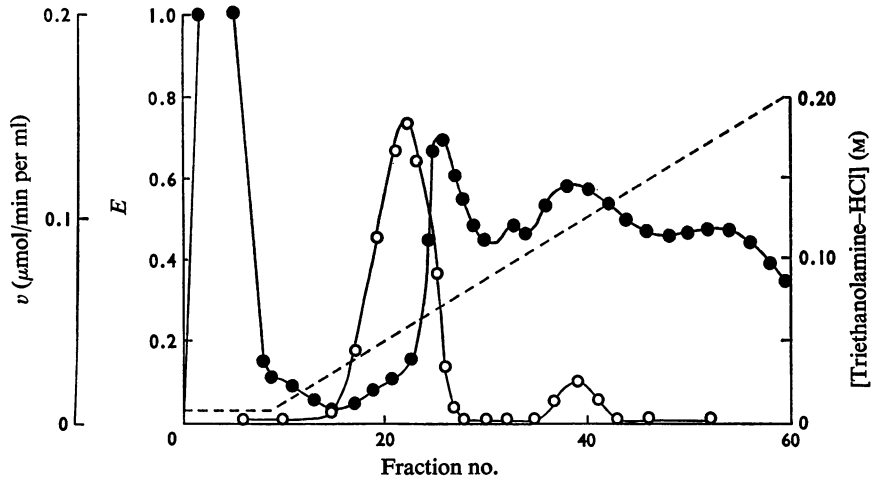

Fig. 2. *DEAE-cellulose chromatography of the '$(NH_4)_2SO_4$ fraction' from pig brain*

Aldehyde reductase (150–200mg) from step 2 of the purification (specific activity 0.012 unit/mg of protein) was chromatographed on a DEAE-cellulose column as described in the text. Fractions (3ml) were collected and E_{280} (●) and enzyme activity (○) of each fraction were measured. The concentration gradient is indicated by the broken line.

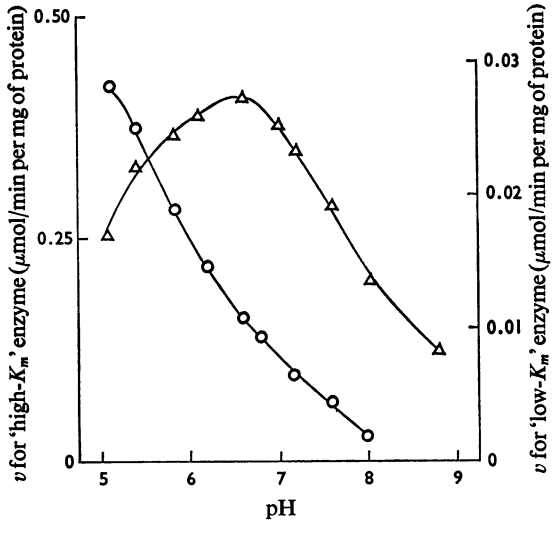

Fig. 3. *pH–activity curves for pig brain aldehyde reductases*

The activities of the 'low-K_m' (○) and 'high-K_m' (△) aldehyde reductases were measured at various pH values in 0.1M-sodium phosphate buffer in the presence of 75μM-NADPH and 0.6mM-*p*-nitrobenzaldehyde. The pH of the assay mixture was checked both before and after the assay.

the value obtained previously (Turner & Tipton, 1972*b*).

DEAE-cellulose chromatography of the $(NH_4)_2SO_4$ fraction separates two peaks of enzyme activity (Fig. 2). The major peak corresponds to the 'high-K_m' enzyme, and if this peak is re-chromatographed under identical conditions only a single peak of activity is obtained. The two separated enzymes exhibit linear reciprocal plots with respect to *p*-nitrobenzaldehyde.

pH–activity curves

The two enzymes show very different pH–activity profiles when assayed in 0.1M-sodium phosphate buffer (Fig. 3). However, the 'high-K_m' enzyme was not assayed at saturating concentrations of *p*-nitrobenzaldehyde because of solubility problems, and this may account for the observed differences. For the purposes of comparison all kinetic experiments were carried out at pH7.2.

Reversibility of the reaction

It had been observed previously that purified pig brain aldehyde reductase would catalyse the $NADP^+$-dependent oxidation of propane-1,2-diol to lactaldehyde in 0.1M-glycine–NaOH buffer, pH9.8, but this reaction proceeds at less than 5% of the maximal rate of reduction of lactaldehyde at pH7.2

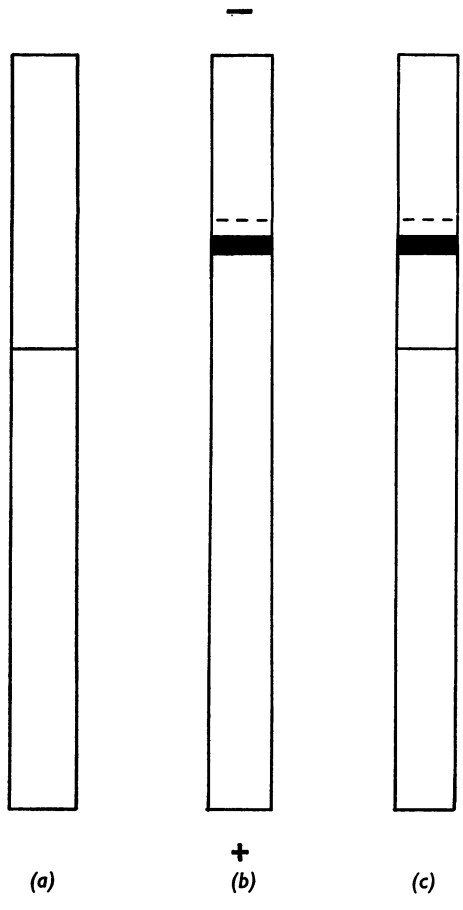

Fig. 4. *Separation of brain aldehyde reductases by polyacrylamide-gel electrophoresis*

Pig brain aldehyde reductases were separated by electrophoresis on polyacrylamide gels (0.5cm × 7.0cm) and stained for activity as described in the text. Samples applied were: (*a*) 500μg of 'low-K_m' enzyme (specific activity 0.008 unit/mg of protein); (*b*) 200μg of 'high-K_m' enzyme (specific activity 0.34 unit/mg of protein); (*c*) 500μg of 'low-K_m' enzyme plus 200μg of 'high-K_m' enzyme.

(Turner & Tipton, 1972*b*). Both the enzymes separated here will catalyse the NADPH-dependent reduction of lactaldehyde and the $NADP^+$-dependent oxidation of propane-1,2-diol. However, no oxidation of 0.6mM-*p*-nitrobenzyl alcohol or of 1.0M-ethanol was observed in the presence of NAD^+ or $NADP^+$ at pH9.8. Thus these enzymes would seem to function physiologically as aldehyde reductases rather than as alcohol dehydrogenases.

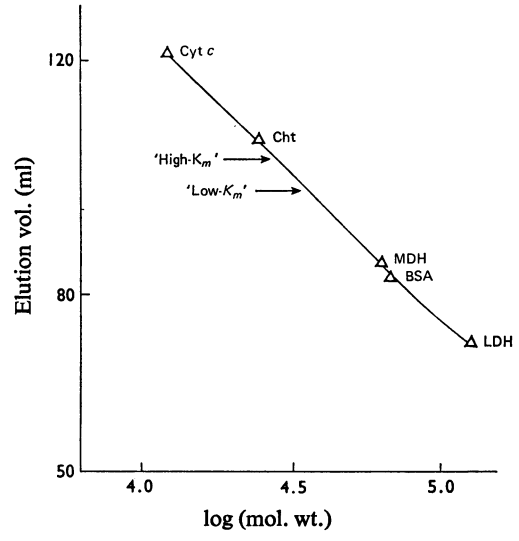

Fig. 5. *Molecular-weight determination of pig brain aldehyde reductases by gel filtration on a Sephadex G-100 column*

Plot of elution volume against log (mol.wt.) of the proteins used. The marker proteins are cytochrome *c* (Cyt *c*), bovine chymotrypsinogen A (Cht), malate dehydrogenase (MDH), bovine serum albumin (BSA) and lactate dehydrogenase (LDH). The arrows indicate the elution positions of the 'high-K_m' and 'low-K_m' aldehyde reductases.

Polyacrylamide-gel electrophoresis

The ability to catalyse the propane-1,2-diol-dependent reduction of $NADP^+$ was used to locate enzyme activity on polyacrylamide gels (Fig. 4). The two enzymes showed very different mobilities. A faint second band of activity was also visible in the freshly prepared 'high-K_m' fraction, which may represent a third, more labile, form of aldehyde reductase present in pig brain. No activity bands were observed in the absence of propane-1,2-diol.

Molecular-weight determination

From the calibration curve obtained, the molecular weights of the 'low-K_m 'and 'high-K_m' enzymes were determined as 36000 and 29000 respectively (Fig. 5). An NADPH-dependent aldehyde reductase purified to homogeneity from pig liver was found to have a molecular weight of 30200 from ultracentrifugation studies (Bosron & Prairie, 1971).

Table 1. *Substrate specificity of pig brain NADPH-linked aldehyde reductases*

K_m values for the 'low-K_m' and 'high-K_m' aldehyde reductases (specific activities 0.008 unit/mg and 0.34 unit/mg respectively) were determined from double-reciprocal plots. NADPH was kept constant at 75 μM while aldehyde concentrations were varied, and *p*-nitrobenzaldehyde concentration was kept constant at 0.6 mM while NADPH concentrations were varied. Assays were carried out in 0.1 M-sodium phosphate buffer, pH 7.2, as described in the text.

	'Low-K_m' reductase		'High-K_m' reductase	
Substrate	Apparent K_m (M)	$V_{max.}$ (% of rate with *p*-nitrobenzaldehyde)	Apparent K_m (M)	$V_{max.}$ (% of rate with *p*-nitrobenzaldehyde)
p-Nitrobenzaldehyde	1.3×10^{-6}	100	2.9×10^{-4}	100
p-Hydroxyphenylacetaldehyde	2.3×10^{-6}	67	8.5×10^{-5}	40
p-Hydroxyphenylglycolaldehyde	0.7×10^{-6}	120	6.5×10^{-5}	260
Indol-3-ylacetaldehyde	5.9×10^{-6}	74	2.0×10^{-3}	53
Hydroxyindol-3-ylacetaldehyde	3.8×10^{-6}	40	—	—
NADPH	1.2×10^{-6}	100	2.5×10^{-6}	70

Table 2. *Effect of certain drugs on the activitity of pig brain aldehyde reductases*

The effects of various drugs were tested on the activities of the 'low-K_m' and 'high-K_m' aldehyde reductases (specific activities 0.008 unit/mg and 0.34 unit/mg respectively). The 'low-K_m' reductase was assayed in the presence of 75 μM-NADPH and 10 μM-*p*-nitrobenzaldehyde and the 'high-K_m' reductase in the presence of 75 μM-NADPH and 0.6 mM-*p*-nitrobenzaldehyde. The reaction was started by addition of enzyme.

	Rate (% of control)	
Drug	'Low-K_m' reductase	'High-K_m' reductase
Sodium barbitone (0.1 mM)	84	23
Chlorpromazine (0.5 mM)	68	100
Reserpine (0.02 mM)	97	99
Disulfiram (5 μM)	98	100
Ethanol (0.05 M)	100	99

Substrate specificity

The aldehyde and coenzyme specificity of the two enzymes was examined (Table 1). Although NADPH was the preferred coenzyme, the 'low-K_m' aldehyde reductase could reduce *p*-nitrobenzaldehyde in the presence of 75 μM-NADH but only at about 20% of the rate of reduction with 75 μM-NADPH. The NADH-linked reduction was not inhibited by 1 mM-pyrazole, a potent inhibitor of liver and brain NAD^+-linked alcohol dehydrogenase (Li & Theorell, 1969; Raskin & Sokoloff, 1970), and thus this activity may be distinguished from that of the NAD^+-dependent enzyme. No reduction of 0.6 mM-*p*-nitrobenzaldehyde was observed in the presence of 75 μM-NADH and the 'high-K_m' aldehyde reductase. Both enzymes could reduce several of the aldehydes derived from the biogenic amines, although no reduction of 5-hydroxyindol-3-ylacetaldehyde was observed, at concentrations of up to 30 μM, by the 'high-K_m' aldehyde reductase.

Putative enzyme inhibitors

Ox brain aldehyde reductase was reported to be inhibited by barbiturates (Erwin *et al.*, 1971) and by chlorpromazine (Bronaugh & Erwin, 1972) but not by pyrazole (Tabakoff & Erwin, 1970). The effect of these agents on pig brain aldehyde reductases is shown in Table 2. The effect of various agents that are known to modify biogenic amine metabolism *in vivo* was also examined. None of these significantly affected aldehyde reductase activity. Disulfiram, ethanol and reserpine in the concentrations given in Table 2 also did not affect aldehyde reductase activities if preincubated with enzyme at 30°C for 15 min before the addition of substrates.

Acetaldehyde (75 μM) could be reduced by either aldehyde reductase, but only at less than 1% of the rate of reduction of *p*-nitrobenzaldehyde. This concentration of acetaldehyde did not significantly inhibit the reduction of 0.6 mM-*p*-nitrobenzaldehyde by either enzyme. A maximal concentration of approx. 75 μM-acetaldehyde has been reported in rat brain after ethanol administration (Ridge, 1963).

Aldehyde reductase in rat and ox brain

Rat brain aldehyde reductase was prepared by centrifugation ($3 \times 10^6 g$-min) of a homogenate of 10 g of rat brain in an equal volume of 0.1 M-sodium

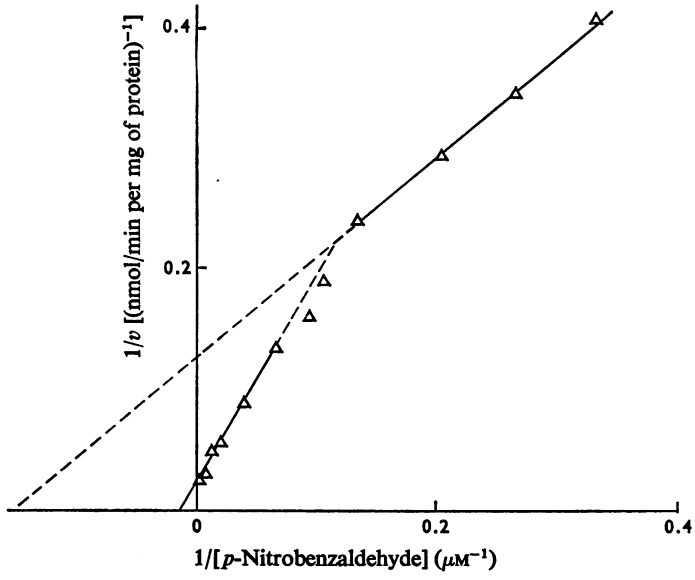

Fig. 6. *Reduction of p-nitrobenzaldehyde by ox brain aldehyde reductase*

Aldehyde reductase purified from ox brain to a specific activity of 0.05 unit/mg of protein by the method of Tabakoff & Erwin (1970) was assayed in 0.1M-sodium phosphate buffer, pH7.2, in the presence of 75μM-NADPH and various concentrations of *p*-nitrobenzaldehyde. Results are presented as a double-reciprocal plot.

phosphate buffer, pH7.4. The brains were homogenized within 10min of death. The high-speed supernatant was gel-filtered through Sephadex G-25 into 0.1M-sodium phosphate buffer, pH7.2, and the kinetics of *p*-nitrobenzaldehyde reduction were examined. As with pig brain, a biphasic reciprocal plot was obtained and by extrapolation the ratio of 'high-K_m' to 'low-K_m' aldehyde reductase activity at pH7.2 was estimated as approx. 4:1.

Ox brain aldehyde reductase was purified to a specific activity of 0.05 unit/mg of protein by $(NH_4)_2SO_4$ fractionation and calcium phosphate gel–cellulose chromatography as described by Tabakoff & Erwin (1970). This preparation also exhibited a non-linear reciprocal plot (Fig. 6), suggesting the presence of more than one aldehyde reductase in ox brain. Thus kinetic studies carried out on such partially purified preparations might be misleading. It is important to check the linearity of the reciprocal plots before undertaking kinetic studies on these enzymes.

Discussion

The evidence presented here suggests that NADPH-linked aromatic aldehyde reductase activity exists in more than one form in the brain of several species. These enzymes can be distinguished by several criteria including pH optima, substrate specificity and sensitivity to inhibition by chlorpromazine and barbiturates. The 'high-K_m' enzyme appears identical with the highly purified aldehyde reductase studied previously by us (Turner & Tipton, 1972*b*). The 'low-K_m' enzyme is apparently not carried through this previous purification procedure. The K_m values that have been reported for the ox brain aldehyde reductase (Tabakoff & Erwin, 1970) appear to lie between the values reported here for the 'low-K_m' and 'high-K_m' pig brain aldehyde reductases.

Several drugs that promote the conversion of the biogenic amines into their alcohol metabolites do not appear to affect aldehyde reductase activity *in vitro*. An alternative mechanism may therefore be proposed to explain the effect of these drugs on amine metabolism.

Under normal conditions brain aldehydes are presumably metabolized by aldehyde dehydrogenase and the less active 'low-K_m' aldehyde reductase. Under conditions of increasing aldehyde concentrations (for example after inhibition of aldehyde dehydrogenase) the more active 'high-K_m' aldehyde reductase could come into play, resulting in an increased proportion of reduced product. The preferential reduction of *p*-hydroxyphenylglycolaldehyde in brain (Breese *et al.*, 1969*a*,*b*) appears to be reflected in

the marked difference in kinetic constants between this aldehyde and *p*-hydroxyphenylacetaldehyde for both aldehyde reductases.

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