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ISOLATION AND CHARACTERIZATION OF RAT LIVER ALDEHYDE REDUCTASE

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Summary

A systematic investigation of potential ligands for the affinity purification of aldehyde reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2) has been carried out. The most suitable nucleotide ligands tested were $NADP^+$ and 2′,5′-ADP. Adsorbed enzyme could be eluted with NADPH but not NADH. The chlorotriazinyl dyes Cibacron Blue F3GA and Procion Red HE3B also proved effective as 'affinity' ligands when immobilized to Sepharose 4B. The free dyes and also Blue Dextran (Cibacron Blue F3GA coupled to dextran) were all potent inhibitors of aldehyde reductase. The inhibition by Blue Dextran was shown to be competitive with respect to NADPH ($K_i = 1.8 \cdot 10^{-7}$ M). The enzyme was sensitive to inhibition by glutaric acid derivatives, flavonoids and a range of anti-convulsants.

Introduction

The oxidative deamination of biogenic amines by monoamine oxidase (amine:oxygen oxidoreductase (deaminating) (flavin-containing, EC 1.4.3.4) in mammalian tissues generates an aldehyde moiety that may be reduced in vivo to the corresponding alcohol metabolite [1]. This reaction is effected by an NADPH-dependent aldehyde reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2) that can be distinguished by its substrate specificity and inhibitor sensitivity from the classical alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) [2,3]. The ability of aldehyde reductase from rat liver to reduce certain cancer chemotherapeutic antibiotics has also suggested a role for this enzyme in drug metabolism [4,5]. The involvement of aldehyde reductase in these and other processes has been reviewed elsewhere [6,7].

Mammalian aldehyde reductases purified from a variety of sources are

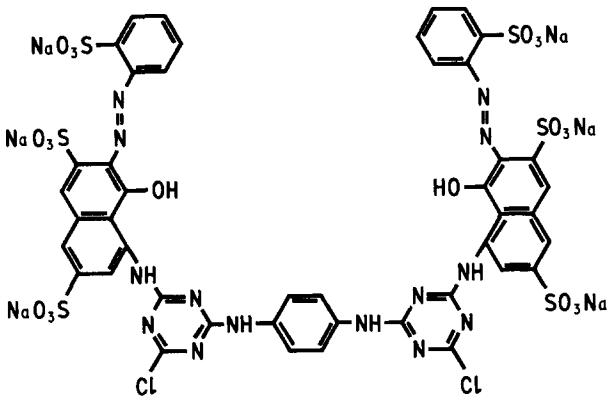

Fig. 1. Structure of the chlorotriazinyl dye, Procion Red HE3B.

monomeric proteins. They may therefore provide a useful model for evolutionary studies of dehydrogenases, and in particular for structural comparisons with alcohol dehydrogenases [8—10]. The development of suitable affinity chromatographic procedures for the rapid purification of large quantities of enzyme would be of value in such studies. A number of reports have demonstrated the utility of group-specific matrices for the isolation of NAD^+-dependent dehydrogenases [11]. Far fewer studies have been reported using $NADP^+$-dependent enzymes. This paper therefore compares the value of a number of nucleotide affinity matrices for the purification of aldehyde reductase from rat liver. In addition, the chlorotriazinyl dyes Cibacron Blue F3GA and Procion Red HE3B (Fig. 1) have been assessed as potential immobilized ligands for isolation of the enzyme. Dyes of this type have previously been shown to interact with the nucleotide-binding domain of certain dehydrogenases [12] and have been used in the purification of a range of nucleotide-requiring enzymes [13].

A number of properties of aldehyde reductase from rat liver are reported and compared with related enzymes from other mammalian tissues. A preliminary account of aspects of this work has been reported elsewhere [14,15].

Materials

All nucleotides used in this work were obtained from Sigma Chemical Co. (Kingston-Upon-Thames, U.K.) and were stored as recommended by the manufacturers. Diphenylhydantoin, 2,4- and 3,3-dimethylglutaric acids and 1,1-cyclopentanediacetic acid (3,3-tetramethylene glutaric acid) were obtained from the Aldrich Chemical Co. (Gillingham, U.K.). Chlorpromazine and trifluperazine hydrochlorides were donated by May and Baker Ltd. (Dagenham, U.K.) and morin and quercitrin were purchased from Eastman-Kodak Co. (Liverpool, U.K.). Pyridine-3-aldehyde, Blue Dextran, succinic semialdehyde, 5β-dihydrotestosterone, metyrapone, 2-decalone, 4-hydroxy 3-methoxyphenylacetic acid (homovanillic acid), quercetin, phenylmethylsulphonyl fluoride and bovine serum albumin were all from Sigma Chemical Co. Daunorubicin hydro-

chloride was a gift from Dr. S. Algeri (Mario Negri Institute for Pharmacological Research, Milan, Italy). The dyes Procion Red HE3B and Cibacron Blue F3GA were supplied by ICI Organics Division (Manchester, U.K.) and Ciba-Geigy (Basel, Switzerland), respectively. AY22.284 (1,3-dioxo-1H-benz(de)-isoquinoline-2(3H)acetic acid) was donated by Dr. D. Dvornik, Ayerst Research Laboratories (Montreal, Canada). Sodium valproate (Epilim^R) was supplied by Dr. D. Walters, Reckitt and Colman Ltd. (Hull, U.K.). Sepharose 4B, 5′-AMP-Sepharose 4B and 2′,5′-ADP-Sepharose 4B were purchased from Pharmacia Fine Chemicals Ltd. (Uppsala, Sweden). All other chemicals were obtained from Sigma Chemical Corporation or British Drug Houses Ltd. (Poole, U.K.). 4-Hydroxyphenylacetaldehyde and 4-hydroxyphenylglycolaldehyde were prepared from tyramine-HCl and octopamine-HCl as described previously [16].

Methods

Affinity adsorbents. NAD^+ and $NADP^+$ were coupled to Sepharose 4B using the method of Lamed et al. [17] (ribose-linked) or that of Larsson and Mosbach [18] (carbodiimide-linked). 2′AMP Sepharose 4B was prepared as described by Watson and Wootton [19]. The ligand concentrations of the synthesized affinity resins were in the range 2—4 μmol ligand per g moist weight Sepharose 4B; Blue Dextran-Sepharose 4B and Procion Red HE3B-Sepharose 4B were prepared by the methods of Ryan and Vestling [20] and Baird et al. [21], respectively.

Subcellular localisation studies. The livers from male (Wistar albino) rats were rinsed in 0.25 M sucrose and then a portion (5 g) of liver was homogenized in 10 ml 0.25 M sucrose-5 mM Tris/HCl-0.5 mM EDTA (pH 7.2), and fractionated as described by Tottmar et al. [22]. The fractions obtained were stored at −70°C until assayed for enzyme activity and protein content. The activities of the marker enzymes glutamate dehydrogenase, NADPH-cytochrome *c* reductase and acid phosphatase were estimated as described previously [22] and lactate dehydrogenase was measured spectrophotometrically by following the pyruvate-dependent oxidation of NADH [23]. Concentrations of protein were measured by the method of Lowry et al. [24] using bovine serum albumin as standard.

Purification of aldehyde reductase. Rat liver aldehyde reductase was purified to apparent homogeneity by a modification [4] of the method of Felsted et al. [25]. This modification involved the substitution of gel filtration on Sephadex G-100 for that on Bio-Gel P150 and subsequent application of the enzyme on a column (0.5 × 5 cm) of $NADP^+$-Sepharose equilibrated with 10 mM sodium phosphate buffer (pH 7.5). The column was washed with 5 vols. equilibration buffer followed by buffer containing 0.1 mM NADPH. Aldehyde reductase activity was eluted from the column when NADPH was present in the irrigation buffer. The purified product was concentrated by ultrafiltration, dialysed extensively to remove NADPH and stored at −18°C in 0.1 M sodium phosphate buffer (pH 7.2), containing 30% (v/v) glycerol to stabilise the enzyme. The purified reductase has previously been shown to be apparently homogeneous when subjected to polyacrylamide gel electrophoresis [4] and each preparation was routinely checked in this way. A single N-terminal residue (glycine) was

obtained using the dansylation procedure described by Gray [26]. The purified enzyme preparation was used in the subsequent studies on substrate specificity and inhibitor sensitivity.

Aldehyde reductase assay. Routine assays for aldehyde reductase were performed at 30°C in 0.1 M sodium phosphate buffer (pH 7.2), containing 0.1 mM NADPH and 1.2 mM pyridine-3-aldehyde unless stated otherwise. For kinetic studies, addition of aldehyde was normally used to start the reaction, which was monitored continuously by following the decrease in absorbance at 340 nm in a Gilford Model 240 spectrophotometer coupled to a Servoscribe 8 inch (20 cm) chart recorder. The reaction rate was linear for at least 5 min and the initial rate was proportional to enzyme concentration within the range used in these experiments. One unit of enzyme activity catalyses the oxidation of 1 μmol NADPH/min.

Results and Discussion

Subcellular localization

The distributions of NADPH-dependent aldehyde reductase and the relevant marker enzymes among the post-nuclear subcellular fractions are shown in Table I. The reductase activity with either pyridine-3-aldehyde or 4-hydroxyphenylglycolaldehyde as substrate was predominantly localized in the cytosol. A similar subcellular location has been shown for aldehyde reductase from pig kidney [8], although in brain a significant proportion of the enzyme activity is found in the nerve-ending (synaptosomal) fraction [3]. A distinct isozyme of aldehyde reductase has been reported to be present in brain mitochondria [27], although we found barely detectable activities in liver mitochondria (Table I) using either NADPH or NADH as cofactor. On the basis of the observed subcellular distribution all subsequent enzyme preparations used liver cytosol obtained by centrifugation of liver homogenates for 1 h at 100 000 × *g*.

TABLE I

SUBCELLULAR LOCALISATION OF ALDEHYDE REDUCTASE IN RAT LIVER

Rat liver (5 g) was homogenized and the post-nuclear supernatant was separated into mitochondrial, lysosomal, microsomal and cytosolic fractions according to the procedure of Tottmar et al. [22]. The activities of various marker enzymes and of NADPH-dependent aldehyde reductase were determined in each subcellular fraction. Aldehyde reductase activity was measured in the presence of 1 mM pyrazole either with pyridine-3-aldehyde (PA) or 0.5 mM 4-hydroxyphenylglycolaldehyde (HPGA) as substrate. The results are the means of three separate experiments and are expressed as percent total recovered activity. Overall recovery expresses percent recovery of activity present in post-nuclear supernatant.

Enzyme	Activity recovered in each fraction (%)				Overall recovery (%)
	Mitochondrial	Lysosomal	Microsomal	Cytosol	
Glutamate dehydrogenase	84	12	2	2	104
Lactate dehydrogenase	2	2.6	5.4	90	110
NADPH-cytochrome *c* reductase	14.5	23	56	6.5	94
Acid phosphatase	22	32	11	35	95
Aldehyde reductase (PA)	2	3	4	91	93
Aldehyde reductase (HPGA)	1.5	3.5	2	93	96

Affinity chromatographic procedures

For these investigations a partially purified extract of aldehyde reductase was used. This extract was prepared by homogenization of rat liver in 3 vols. 0.1 M sodium phosphate buffer (pH 7.5), followed by centrifugation of the homogenate at 100 000 × *g* for 1 h. The supernatant was then subjected to fractionation with solid $(NH_4)_2SO_4$ at 4°C. The fraction that precipitated between 30% and 65% saturation was dissolved in 10 mM sodium phosphate buffer (pH 7.5), and dialysed overnight against that buffer. Samples (10 mg) of extract were applied to columns (0.5 × 5 cm) of affinity adsorbent at a flow rate of 10 ml/h. No adsorption of enzyme was observed to 5′-AMP-Sepharose, NAD^+-

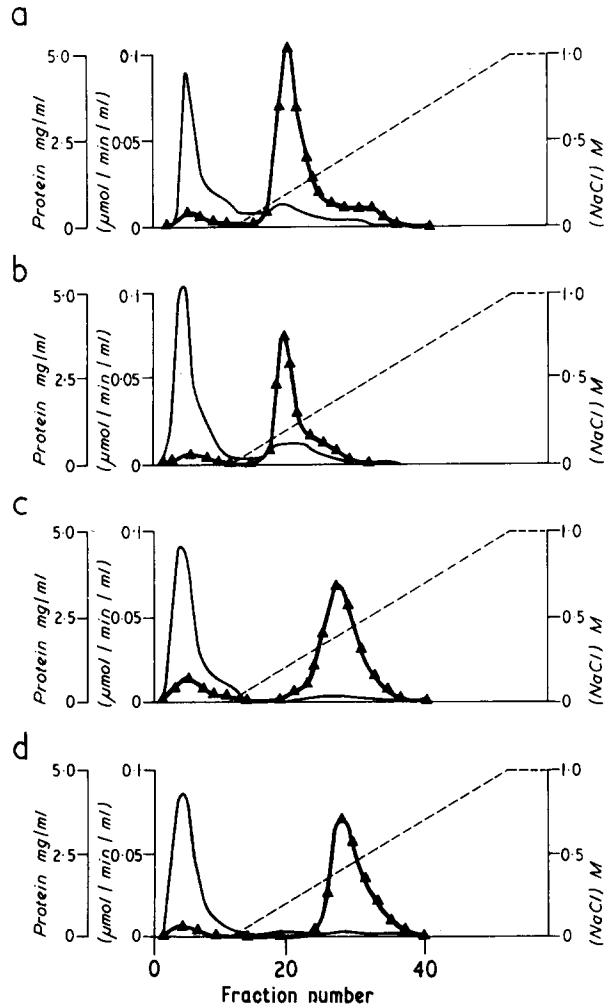

Fig. 2. Chromatography of liver aldehyde reductase on various affinity resins. Samples of extract (10 mg) were applied to columns (0.5 × 5 cm) of affinity adsorbent as described in the text. Enzyme activity was eluted with a linear gradient of KCl (0—1 M) at pH 7.5. Fraction (1 ml) were assayed for activity (▲———▲) and protein content. The chromatographic behaviour of aldehyde reductase is shown on the following affinity resins: a, NADP-Sepharose, (carbodimide-linked); b, 2′,5′-ADP-Sepharose; c, Procion Red HE3B-Sepharose, d, Blue Dextran-Sepharose.

TABLE II

INHIBITION OF RAT LIVER ALDEHYDE REDUCTASE BY NUCLEOTIDES AND RELATED COMPOUNDS

Assays were conducted in 0.1 M sodium phosphate buffer (pH 7.2), containing 1.2 mM pyridine-3-aldehyde and 5 μM NADPH. Additives were at a concentration of 0.1 mM unless otherwise stated. The results are the mean of three separate experiments which did not differ by more than 5%.

Additive	Activity (% of control)
None	100
$NADP^+$	39
NAD^+	99
2′-AMP	63
5′-AMP	97
5′-ADP	75
2′5′-ADP	12
Adenosine	105
Blue Dextran (0.01 mM)	8
Procion Red HE3B (0.01 mM)	<5
Cibacron Blue F3GA (0.01 mM)	<5

Sepharose (ribose-linked or carbodiimide linked), or to hexanoyl-Sepharose. Aldehyde reductase was retarded behind the main (void volume) peak of protein but not completely retained on a column of 2′-AMP-Sepharose at either pH 6.5 or 7.5. However, enzyme activity was found to adsorb to $NADP^+$-Sepharose (ribose-linked or carbodiimide-linked) and 2′,5′-ADP-Sepharose under the above conditions. Elution of enzyme could be effected either with a pulse of NADPH (0.1 mM) or a linear gradient of KCl (0—1 M) (see Fig. 2). 0.1 mM NADH was unable to elute any reductase activity from the affinity resins suggesting a bio-specific interaction between enzyme and affinity adsorbent. The purification achieved (15—20-fold) was similar for $NADP^+$-Sepharose and 2′,5′-ADP-Sepharose and the yield was greater than 80%. The chromatographic behaviour of aldehyde reductase is similar to that of the $NADP^+$-dependent enzyme 6-phosphogluconate dehydrogenase when applied to a number of affinity matrices [28].

Blue Dextran and Procion Red HE3B immobilized to Sepharose 4B were also effective 'pseudo-affinity' matrices for the purification of aldehyde reductase. Activity could be eluted from these resins using a linear gradient of KCl (0—1 M) (Fig. 1) with a recovery of greater than 80%. Millimolar concentrations of NADPH would also elute activity. The ability of immobilized nucleotides and dyes to act as effective affinity adsorbents appears to be reflected in the ability of these compounds to inhibit aldehyde reductase activity in vitro (Table II). Blue Dextran, Cibacron Blue F3GA (the chromophoric dye present in Blue Dextran) and Procion Red HE3B were all potent inhibitors of aldehyde reductase. Because of the heterogeneity and reactivity of the free dyes, kinetic studies of the inhibition were carried out using Blue Dextran. The mode of inhibition by Blue Dextran was found to be reversible and competitive with respect to the coenzyme Fig. 3) with an inhibition constant, K_i = 0.18 μM. The dyes may therefore interact at the nucleotide-binding site, as has been shown

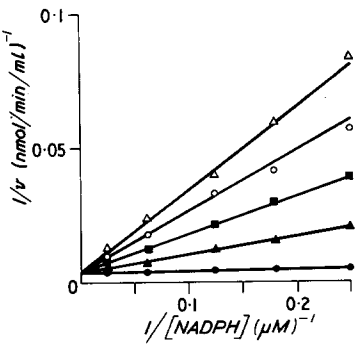

Fig. 3. Double reciprocal plot of the inhibition of aldehyde reductase by Blue Dextran. NADPH was the variable substrate and pyridine-3-aldehyde was held constant at 1.2 mM. The concentrations of Blue Dextran used were: (●———●), 0; (▲———▲), 2.5 μM; (■———■), 5 μM; (○———○), 7.5 μM; (△———△), 10 μM. The concentrations of Blue Dextran were estimated spectrophotometrically [43]. The experimental data were initially fitted to reciprocal plots by eye to determine linearity and kinetic constants were obtained using a modification of the computer program of Cleland [44], as described previously [45]. Data were fitted to the appropriate rate equation to obtain the best fit for the data and the most valid values of kinetic parameters.

previously for Cibacron Blue F3GA with a number of dehydrogenases [12]. $NADP^+$ and 2′,5′-ADP were both competitive inhibitors of aldehyde reductase when NADPH was the variable substrate and K_i values of 28 μM and 7 μM respectively were obtained for these nucleotides.

When liver cytosol was applied to a column (0.5 × 5 cm) of Procion Red-Sepharose 4B at pH 7.5 and enzyme activity was eluted with a linear gradient of KCl (0—1 M), a purification of 95-fold was obtained in a single step (mean of three experiments). Peak enzyme activity eluted at a concentration of

TABLE III

SUBSTRATE SPECIFICITY OF RAT LIVER ALDEHYDE REDUCTASE

Assays were carried out as described in the text. D-glucose (10 mM), phenylpyruvic acid (1 mM), 2-decalone (1 mM), 4-nitroacetophenone (1 mM) and 5β-dihydrotestosterone (0.1 mM) were not reduced. The carbonyl-containing drugs Warfarin (1 mM), metyrapone (1 mM), oxisuran (1 mM) and benzylidene acetophenone (0.1 mM) were also not reduced. The apparent K_m for NADPH was 1.5 μM when pyridine-3-aldehyde was held constant at 1.2 mM.

Substrate	Apparent K_m (mM)	Maximal velocity (V_m) (% rate with *p*-nitro-benzaldehyde)
4-Carboxybenzaldehyde	0.031	87
4-Hydroxyphenylglycolaldehyde	0.059	114
4-Nitrobenzaldehyde	0.23	100
Pyridine-3-aldehyde	0.26	105
4-Hydroxyphenylacetaldehyde	0.91	37
DL-Glyceraldehyde	1.30	45
D-Glucuronate	2.20	70
Benzaldehyde	4.50	11
Chloral hydrate	29	72
D-Xylose	670	45

TABLE IV

INHIBITION OF ALDEHYDE REDUCTASE BY VARIOUS ADDITIVES

Assays were conducted in 0.1 M sodium phosphate buffer (pH 7.2), containing 1.2 mM pyridine-3-aldehyde and 0.1 M NADPH. Additives were at a concentration of 1 mM unless otherwise stated. The results represent the mean of three separate experiments, which did not differ by more than 5%.

Additive	Activity (% of control)
Glutaric acid derivatives	
Glutaric acid	58
2,4-Dimethylglutaric acid	16
3,3-Dimethylglutaric acid	17
Tetramethylene glutaric acid	16
AY22, 284A (0.1 mM)	37
Anti-convulsants	
Barbitone	<5
Phenobarbitone	8
Diphenylhydantoin	<5
Sodium valproate	<5
Flavonoids	
Quercetin (0.01 mM)	<5
Quercetin (0.01 mM)	15
Morin (0.01 mM)	<5
Other	
Homovanillic acid	26
Chlorpromazine (0.1 mM)	79
Trifluperazine (0.1 mM)	102
Propranolol	97
Pyrazole	96

approx. 0.4 M KCl. A rather lower purification (45-fold) was achieved with Blue Dextran-Sepharose 4B.

Substrate specificity and inhibitor sensitivity

The wide specificity of the reductase for aldehydes is illustrated by the selection of substrates in Table III. The substrate specificity of the rat liver enzyme appears to be similar to that reported for aldehyde reductases purified from other sources [2,3,8–10,29–31]. The inhibitor sensitivity of rat liver aldehyde reductase is shown in Table IV. Similarities have previously been noted [3] between aldehyde reductases and aldose reductase and therefore a number of aldose reductase inhibitors [32] were tested on the activity of rat liver aldehyde reductase. Glutaric acid derivatives, the drug AY22 284 and certain flavonoids all produced substantial inhibition (Table IV). The two flavonoids quercetin and morin are among the most potent reductase inhibitors known with inhibition constants $K_i < 1$ μM.

Conclusions

The studies presented here demonstrate the usefulness of two distinct nucleotide-affinity matrices ($NADP^+$-Sepharose and 2′,5′-ADP-Sepharose) for the purification of aldehyde reductase. However, equally effective as 'affinity'

matrices were the immibolized triazinyl dyes. In particular, Procion Red HE3B-Sepharose was less liable to ligand leakage from the matrix than Blue-Sepharose and gave excellent purification and enzyme recovery in a single step. Indeed, data from Dean et al. [33] have suggested that immobilized Procion Red HE3B may be the 'affinity' resin of choice for the purification of $NADP^+$-dependent enzymes. Immobilized triazinyl dyes show additional advantages over conventional nucleotide-affinity resins. They are cheap and simple to prepare, less liable to bacterial degradation and have much greater capacities for enzyme adsorption [13,33]. The blue dye appears to interact at the nucleotide-binding domain of aldehyde reductase, which suggests that this monomeric enzyme may possesses a dinucleotide fold similar to that found in oligomeric dehydrogenases [12].

Aldehyde reductases have now been obtained in a highly purified form from a number of mammalian sources [2,3,8—10,25,29—31]. In some tissues more than one form of the enzyme has been shown to exist [16,29,31,34—38], although the major isoenzyme in all tissues examined appears similar in properties to the rat liver enzyme reported here. A number of enzymes, previously designated as hexonate dehydrogenase, daunorubicin reductase and mevaldate reductase have now been shown to be identical with aldehyde reductase [4,37, 39] and should all be classified as such. Aldose reductase overlaps to some degree in substrate specificity and inhibitor sensitivity with aldehyde reductase. However, it can be distinguished by a number of features, principally a much lower Michaelis constant for D-xylose (9—30 mM) [40—42]. Although immunologically distinct from aldehyde reductase [40,41], aldose reductase should perhaps be considered as an isoenzyme of the former enzyme. The characteristic features of aldehyde reductases are a cytosolic location, low specificity for aldehyde substrates, preference for NADPH as cofactor, sensitivity to inhibition by anti-convulsants and molecular weights in the range 29 000—44 000. Purification of rat liver or brain aldehyde reductase in the presence of 1 mM phenylmethylsulphonyl fluoride and EDTA to inhibit protease activity does not affect the specific activity or the apparent molecular weight of the enzyme as determined by gel filtration on Sephadex G-100 (Turner, A.J., unpublished data), suggesting that significant proteolysis does not occur during purification.

The wide substrate specificity of aldehyde reductase makes it difficult to define a single physiological function for this enzyme. Indeed it may subserve distinct functions in different tissues. Among the roles suggested for this enzyme are in the metabolism of biogenic amines, γ-aminobutyric acid, aldoses, long chain fatty aldehydes, certain drugs and dietary aldehydes [4,6,7,30,31, 38]. The demonstration of a range of inhibitors of the enzyme, of which flavonoids were the most potent (Table III), may allow more detailed examination of the metabolic roles of aldehyde reductases in various tissues.

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