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ANTI-CONVULSANTS AND BRAIN ALDEHYDE METABOLISM

INHIBITORY CHARACTERISTICS OF OX BRAIN ALDEHYDE REDUCTASE

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Abstract—The major isoenzyme of aldehyde reductase has been purified from ox brain by affinity chromatography. Carbamazepine ($K_i = 7.3 \times 10^{-4}$ M) and phenacemide ($K_i = 2.5 \times 10^{-4}$ M), in common with all other established anti-convulsant drugs tested, have been shown to inhibit the activity of this enzyme. A selection of structural analogues of the anti-convulsant sodium valproate were found to be potent inhibitors of the reductase (K_i values in the range 10^{-3} M – 5×10^{-5} M) and these analogues also showed anti-convulsant activity in the mouse maximal electroshock test. A third group of compounds, the flavonoids, constitute the most potent group of aldehyde reductase inhibitors yet reported. Quercetin and morin exhibited K_i values less than 1 μM. The possible relationship between aldehyde metabolism and anti-convulsant action is discussed and structural characteristics pre-disposing to potent inhibition of aldehyde reductase are described.

The inhibitory characteristics of NADPH-dependent aldehyde reductase (alcohol : NADP oxidoreductase, EC 1.1.1.2) have attracted considerable interest since the initial observations that this enzyme is inhibited by anti-convulsant barbiturates [1, 2]. Erwin and Deitrich subsequently extended these studies [3] and demonstrated that aldehyde reductase from ox brain is sensitive to the major groups of anti-convulsant drugs including hydantoins, succinimides and oxazolidinediones. More recently, other anti-convulsant drugs, including benzodiazepines and sodium valproate have also been shown to inhibit this enzyme [4, 5]. The sensitivity of aldehyde reductase to anti-convulsant drugs of widely differing structures has led to the suggestion that modifications in brain aldehyde metabolism may be relevant to the physiological action of anti-convulsants [4, 6] and that inhibition of aldehyde reductase *in vitro* may provide a simple screening test for potential anti-epileptic drugs [3]. These studies have been complicated, however, by the multiplicity of aldehyde reductases in brain, and their different sensitivities to anti-convulsants [2, 7]. The major isoenzyme of aldehyde reductase in brain (referred to as AR1 or 'High-K_m' form) is located in the cytosol and is the isoenzyme most sensitive to inhibition by such drugs [7, 8]. The physiological function of this enzyme, though, is unclear. Under normal circumstances it does not appear to be involved in the reduction of catecholamine-derived aldehydes [8, 9] nor in *O*-alkyl lipid metabolism*. It may play a role in the formation of γ-hydroxybutyrate from GABA or act to detoxify aldehydes in general [8, 10].

In order to investigate further the potential correlation between anti-convulsant drugs and inhibition of aldehyde reductase, the present study examines the effects of a range of compounds on the activity of aldehyde reductase purified from ox brain. The compounds chosen include proven anti-convulsant drugs not previously tested as inhibitors of aldehyde reductase as well as a range of structural analogues of sodium valproate. The third group of compounds examined have previously been shown to inhibit aldose reductase (alditol : NADP oxidoreductase, EC 1.1.1.21) [11, 12]. This enzyme catalyses the NADPH-dependent reduction of aldoses to alditols and therefore overlaps in substrate specificity with the major isoenzyme of aldehyde reductase.

MATERIALS AND METHODS

All chemicals were of the highest grade commercially available and, unless stated otherwise, were obtained from British Drug Houses, Ltd., Poole, Dorset, U.K. Succinic semialdehyde, NADPH, diphenylacetic acid, quercetin and rutin were purchased from Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K. 2-Phenylbutyric acid, 2-phenylpropionic acid and phenylacetylurea (phenacemide) were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Quercitrin was obtained from ICN Pharmaceuticals Inc., Plainview, N. Jersey, U.S.A. and 2-ethylbutyric acid and 2-methylhexanoic acid from Fluorochem Ltd., Glossop, Derbyshire, U.K. DEAE-Cellulose DE 52 was purchased from Whatman Ltd., Maidstone, Kent, U.K., and Sepharose 4B and 2′5′ ADP-Sepharose from Pharmacia (Gt. Britain) Ltd., London, U.K. Sodium valproate and carbamazepine were gifts from Dr. D.

* P.S. Sastry (personal communication)

S. Walter, Reckitt & Colman Pharmaceutical Division, Hull, U.K., and Procion Red HE-3B was a gift from ICI Organics Division, Manchester, U.K. 7-Hydroxyethyl quercetin, 7-hydroxyethyl rutin, 7,3′,4′,-trihydroxyethyl rutin and 5,7,3′,4′, tetrahydroxyethyl rutin were kindly donated by Zyma (United Kingdom) Ltd., Macclesfield, Cheshire, U.K. Less soluble flavonoids were dissolved in alkali and the pH of the solution readjusted to pH 7.2. Procion Red HE-3B Sepharose 4B was synthesised as described elsewhere [13].

Assay of aldehyde reductase activity. Aldehyde reductase assays were performed at 30° in 100 mM sodium phosphate buffer (pH 7.2) containing 100 μM NADPH and variable amounts of succinic semialdehyde in a total volume of 3 ml. The decrease in absorbance at 340 nm was monitored continuously in a Gilford Model 240 spectrophotometer connected to a Varian Servoscribe potentiometric recorder. The reaction rate was shown to be linear with respect to protein concentration in the range used, and with respect to time for at least 5 min.

Purification of ox brain aldehyde reductase. Ox brains were obtained from M. H. Rigg (Butcher) Rawdon, W. Yorkshire, and were transported to the laboratory on ice where they were cleaned rapidly of extraneous membrane and blood vessels. The brains were then either used immediately or stored at −70° until required.

Aldehyde reductase was purified as described by Whittle and Turner [5]. Briefly, a 35–65% ammonium sulphate fraction was subjected to chromatography on hydroxylapatite and DEAE-cellulose, following by two affinity chromatographic steps. The first involved application to a column of Procion Red HE-3B Sepharose, and elution with a linear gradient of KCl. The final step in the purification procedure involved chromatography on 2′5′ADP Sepharose, to remove any NADH-utilising isoenzymes such as those described in other species [2]. The enzyme was purified over 900-fold by this procedure and showed no evidence of contamination by minor isoenzyme forms as judged from polyacrylamide gels stained for enzyme activity [2].

Anti-convulsant screening. These tests were kindly performed by Dr. D. S. Walter in the laboratories of Reckitt and Colman Pharmaceutical Division, Hull. Mice, dosed with the compound under test, were subjected to an electric current passed across the brain via eye electrodes, using a constant voltage set at 80 V and a 0.3 sec train of pulses, 0.4 msec pulse width at 50 Hz. The number of mice to show tonic extension of the hind limbs in the drug treated group was compared with the number in a control group dosed with the drug vehicle only.

RESULTS

Inhibition of aldehyde reductase by proven anticonvulsant drugs

Although the inhibitory effects of many of the well-established anti-convulsant drugs are known, the effects of carbamazepine and phenacemide had not previously been reported. Both these drugs were shown to inhibit aldehyde reductase although they are an order of magnitude less effective than phenobarbitone, diphenylhydantoin or sodium valproate. Both drugs were shown to be noncompetitive inhibitors of aldehyde reductase.

Inhibition constants (K_i values) were calculated by plotting slope (K_I) and intercept (K_{II}) values, obtained from the Lineweaver–Burk double reciprocal plot, against inhibitor concentration, and are shown in Table 1. K_i values obtained for phenobarbitone and diphenylhydantoin using the same enzyme preparation are included for comparison.

Inhibition by sodium valproate analogues

The effects of analogues of sodium valproate were examined in order to determine structure–activity relationships for branched chain fatty acids. All the compounds tested were shown to be inhibitors, although only three of these, 2-ethylhexanoic acid, 2-phenylbutyric acid and diphenylacetic acid showed K_i values in the same range as sodium valproate. The inhibition pattern shown by 2-ethylhexanoic acid was similar to that observed with sodium valproate. Diphenylacetic acid showed non-competitive inhibition with respect to the aldehyde substrate whereas all the other analogues tested showed mixed-type inhibition. The K_i values for these compounds are shown in Table 2; sodium valproate is included for comparison. Typical examples of double-reciprocal plots obtained in these studies are shown in Fig. 1. The three most potent inhibitors in this group were screened for anticonvulsant activity. All showed similar potency to sodium valproate in the mouse maximal electroshock test.

Table 1. Inhibition of aldehyde reductase by proven anti-convulsant drugs

Drug	Type of inhibition	K_i Values (M) K_I	K_{II}
Phenobarbitone	Non-competitive	4.0×10^{-5}	
Diphenylhydantoin	Linear mixed	3.75×10^{-5}	4.8×10^{-6}
Carbamazepine	Non-competitive	7.3×10^{-4}	
Phenacemide	Non-competitive	2.5×10^{-4}	

Ox brain aldehyde reductase was assayed as described in Materials and Methods, using succinic semialdehyde as variable substrate. Values for the inhibition constants K_I and K_{II} ($K_I = K_{II}$ for non-competitive inhibition) were obtained from re-plots of slope and intercept against inhibitor concentration as described in the text. The inhibition of all the compounds tested was shown to be reversible by dilution.

Table 2. Inhibition of aldehyde reductase by analogues of sodium valproate

Inhibitor	Type of inhibition	K_i Values (M) K_I	K_{II}
Sodium valproate	Un-competitive		8.5×10^{-5}
Diphenylacetic acid	Non-competitive	9.3×10^{-5}	
2-Phenylpropionic acid	Mixed	1.4×10^{-3}	3.2×10^{-4}
2-Phenylbutyric acid	Mixed	7.4×10^{-4}	4.6×10^{-5}
2-Methylbutyric acid	Mixed	1.1×10^{-3}	5.5×10^{-4}
2-Ethylbutyric acid	Mixed	4.6×10^{-4}	2.3×10^{-4}
2-Methylhexanoic acid	Mixed	1.0×10^{-3}	2.4×10^{-4}
2-Ethylhexanoic acid	Un-competitive		5.5×10^{-5}

Assays and determination of inhibition constants were carried out as described in the legend to Table 1.

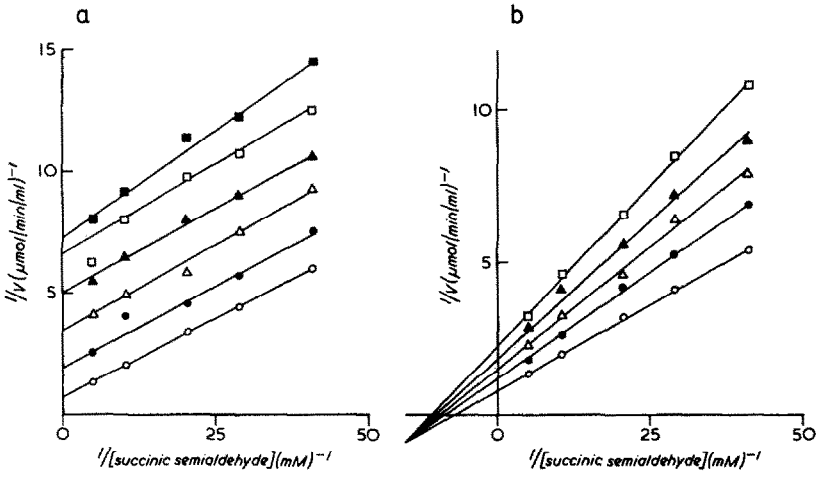

Fig. 1. Inhibition of aldehyde reductase by representative branched-chain fatty acids. (a) Double-reciprocal plot of inhibition of aldehyde reductase by 2-ethylhexanoic acid. Succinic semialdehyde was used as variable substrate. Concentrations of 2-ethylhexanoic acid were: ○, 0; ●, 0.1 mM; △, 0.2 mM; ▲, 0.3 mM; □, 0.4 mM; ■, 0.5 mM. (b) Inhibition of aldehyde reductase by 2-ethylbutyric acid. Concentrations of inhibitor were: ○, 0; ●, 0.1 mM; △, 0.2 mM; ▲, 0.3 mM; □, 0.4 mM.

Table 3. Inhibition of aldehyde reductase by inhibitors of aldose reductase

Inhibitor	Type of inhibition	K_i Values (M) K_I	K_{II}
AY 22,284	Non-competitive	8.9×10^{-3}	
Quercetin	Un-competitive		4.3×10^{-7}
Morin	Un-competitive		4.0×10^{-7}
Quercitrin	Un-competitive		2.0×10^{-5}
Rutin	Mixed	$\approx 2 \times 10^{-5}$	
7-Hydroxyethyl quercetin	Mixed	1.4×10^{-5}	9.5×10^{-6}
7-Hydroxyethyl rutin	Mixed	3.3×10^{-5}	4.0×10^{-5}
7,3',4'-trihydroxyethyl rutin	Mixed	4.6×10^{-5}	5.2×10^{-5}
5,7,3',4'-Tetrahydroxyethyl rutin	Mixed	5.5×10^{-5}	7.9×10^{-5}

Structures of these compounds are shown in Fig. 3. Assays and determination of inhibition constants were carried out as described in the legend to Table 1. Secondary plots of slope against inhibitor concentration were non-linear for rutin. An estimate of the K_i for rutin was obtained by extrapolation of the linear portion of the curve at low concentrations ($< 10\ \mu M$) of rutin.

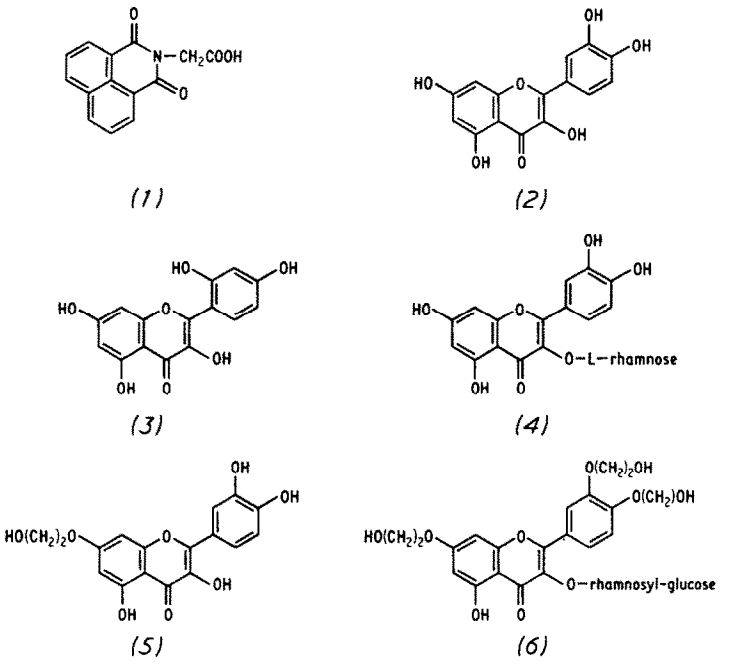

Fig. 2. Structures of aldose reductase inhibitors. (1) AY 22,284. (2) Quercetin. (3) Morin. (4) Quercitrin. (5) 7-Hydroxyethyl quercetin. (6) 7,3′4′-Trihydroxyethyl rutin.

Inhibition by aldose reductase inhibitors

It has been reported [11, 12] that dioxo-1H-benz-[de]-isoquinoline-2(3H) acetic acid (AY 22,284) and many flavonoid compounds are potent inhibitors of aldose reductase. In view of the reported similarities between this enzyme and aldehyde reductase the effects of a selection of these compounds on aldehyde reductase activity were investigated. In addition to the flavonoids previously shown to be aldose reductase inhibitors, four water-soluble flavonoids were also tested. These are hydroxyethyl-substituted analogues of quercetin and rutin. The structures of some of these compounds are shown in Fig. 2, and the results of this study are shown in Table 3. All the aldose reductase inhibitors tested were shown to be potent inhibitors of aldehyde reductase. AY 22,284, quercitrin, rutin, and three of the soluble flavonoids were shown to have K_i values in the same range as sodium valproate. 7-Hydroxyethyl quercitin was about 10-fold more potent and quercetin and morin about 100-fold more potent than valproate. AY 22,284 showed noncompetitive inhibition. Morin, quercetin and quercitrin showed uncompetitive inhibition, whereas all the water-soluble flavonoids showed mixed-type inhibition (Fig. 3). None of these compounds showed any anticonvulsant activity in the mouse maximal electroshock tests at dosages up to 400 mg/kg.

DISCUSSION

It is remarkable that all anti-convulsant drugs tested, including carbamazepine and phenylacetylurea (phenacemide) which had not previously been

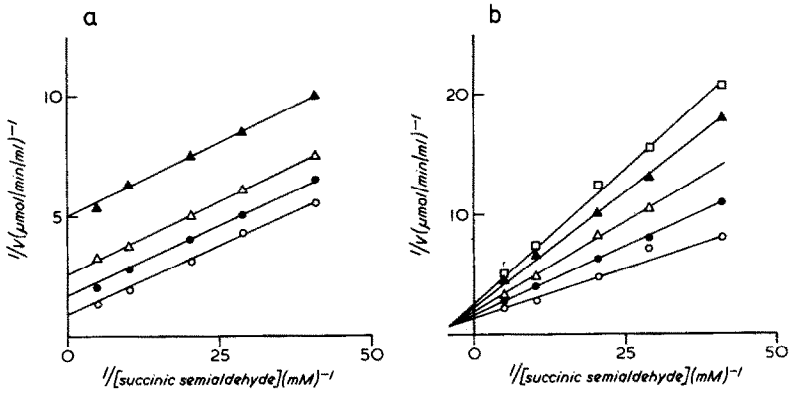

Fig. 3. Inhibition of aldehyde reductase by representative flavonoids. (a) Double-reciprocal plot of inhibition of aldehyde reductase by quercetin. Assays were performed as described in Materials and Methods. Concentrations of quercetin were: ○, 0; ●, 0.5 μM; △, 1.0 μM; ▲, 2.0 μM. (b) Inhibition of aldehyde reductase by 7-hydroxyethyl quercetin. Concentrations of inhibitor were: ○, 0; ●, 2.5 μM; △, 5.0 μM; ▲, 7.5 μM; □, 10.0 μM.

examined, are inhibitors of the major isoenzyme of aldehyde reductase. The comparative potency of these compounds is documented in Tables 1 and 2. The inhibition constants obtained here for phenobarbitone and diphenylhydantoin are considerably lower than those reported by other workers [3]. This discrepancy may be due to differences in the methods of enzyme preparation, contamination of earlier preparations with other isoenzyme forms, or to the use of different substrates. In this study, succinic semialdehyde was chosen as substrate rather than the more common but non-physiological *p*-nitrobenzaldehyde since reduction of succinic semialdehyde may be an important physiological role of aldehyde reductase in brain [10].

All the branched chain fatty acids tested were found to be inhibitors of aldehyde reductase, although few were as effective as valproate itself. The most potent was 2-ethylhexanoic acid which showed a similar inhibition pattern to that reported for sodium valproate [5], being apparently uncompetitive at low inhibitor concentrations, but becoming of mixed type at higher concentrations. Diphenylacetic acid and 2-phenylbutyric acid also showed K_i values similar to valproate, although they exhibited different modes of inhibition. The presence of a bulky hydrophobic region on the molecule appears to favour potent inhibition since fatty acids of short chain length (e.g. 2-methylbutyric acid) were less effective inhibitors than those with longer side chains (e.g. 2-ethylhexanoic acid). The substitution of one or both of the aliphatic side chains by a phenyl group does not appear to affect significantly its inhibitory potency (cf. 2-phenylpropionic acid with 2-methylhexanoic acid). This similarity is also seen between 2-phenylbutyric acid and 2-ethylhexanoic acid. Thus, the overall size of the hydrophobic region may be more important than its conformation. Another factor which appears to favour potent inhibition is equality in the size of the two hydrophobic side chains (see Table 2). Although the precise conformation of the hydrophobic regions may not be important, symmetry of the molecule does appear to be so. It has further been suggested that the inhibition of aldehyde reductase by biogenic acids as well as some branched chain fatty acids reflects the presence of a positively charged group in or near the substrate binding site of the enzyme [15]. All the branched chain fatty acids tested have been shown to possess anti-convulsant properties. This observation raises the possibility that sodium valproate is only one of a range of fatty acids which might be clinically effective in the prevention of seizures.

Flavonoids, which forms a large class of natural products synthesized by ferns and flowering plants, have been shown to inhibit a variety of adenine-nucleotide requiring enzymes, for example ATPases, cyclic nucleotide phosphodiesterases, malate dehydrogenase and hexokinase was well as aldose reductase [15, 16]. A number of flavonoids have been shown to have anti-asthmatic activity and to produce coronary vasodilation. They have been used clinically in the treatment of venous insufficiency. All the flavonoid compounds tested were shown to be effective inhibitors of ox brain aldehyde reductase, as was AY 22,284. Quercetin and morin are the most potent inhibitors yet reported with K_i values below 1 μM. It is interesting to note that the potency of inhibition is opposite to that reported for lens aldose reductase, for which quercitrin is the more potent inhibitor [12]. There appears to be no simple correlation between flavonoid structure and potency of inhibition, although some generalisations can be made. The addition of sugar residues to the flavonoid appears to cause a major decrease in potency, e.g. quercetin is approx. 100-fold more potent than the sugar-substituted derivatives quercitrin and rutin. The addition of a 7-hydroxyethyl residue also considerably decreases the inhibitory effect of quercetin, but a similar substitution on the rutin molecule has little or no effect (Table 3). Further substitutions on other hydroxyl groups also have relatively little effect. The flavonoids exhibited no anti-convulsant effects in the mouse maximal electro-shock test, which may partly be due to problems of absorption of these compounds. Water-insoluble flavonoids (e.g. quercetin, morin) are known to be poorly absorbed from the alimentary tract [18]. In cases where quercitrin and AY22,284 have been reported to reduce polyol accumulation in galactosaemic and diabetic rats [11, 18], the oral dosage was greatly in excess of those used in anti-convulsant screening tests, being over 700 mg/kg for quercitrin and 1.1 g/kg for AY 22,284. In the case of the water-soluble flavonoids which are known to be absorbed from the gut (e.g. 7-hydroxyethylquercetin) it is probable that they are unable to cross the blood–brain barrier [19]. It seems unlikely in view of the present data that the flavonoids will provide further insight into the possible role of aldehyde reductase in the action of anti-convulsant drugs. However, they remain of interest as extremely potent inhibitors of this enzyme *in vitro*, and may be useful in examining the physiological functions of the reductase.

Although the major groups of anti-convulsants all inhibit aldehyde reductase activity substantially, there is little evidence to date to support a direct association between modifications in brain aldehyde levels and prevention of seizures in animals. Javors and Erwin [4] have reported a correlation between inhibition of brain aldehyde reductase activity and the anti-convulsant activity of a range of benzodiazepines. They attribute the anti-convulsant effect to alterations in brain levels of β-hydroxylated biogenic aldehydes [4]. It is likely, however, that the major isoenzyme of aldehyde reductase examined in these studies has little functional role in this metabolic pathway [20, 21]. Sodium valproate, for example, does not appear to modify the metabolism of normetanephrine to its glycol product in brain homogenates [21]. Aldehyde reductase may be of importance in the reduction of succinic semi-aldehyde to γ-hydroxybutyrate. The latter compound has been shown to be an endogenous metabolite of γ-aminobutyric acid and was reported to cause an epileptic-like stupor, similar to petit mal epilepsy when administered to animals [6, 22]. Inhibition of γ-hydroxybutyrate formation may therefore provide a link between aldehyde reductase and anti-convulsant action. However, several enzymes in brain are capable of reducing succinic semialdehyde and their relative contributions *in vivo* to this metabolic con-

version have not been assessed [10, 21, 23, 24]. Until a definitive physiological role has been assigned to the major isoenzyme of brain aldehyde reductase, it will remain difficult to correlate its inhibition with anti-convulsant action. Our current studies are directed towards this goal.

The present work demonstrates a variety of structural features that predispose towards potent inhibition of aldehyde reductase. These and other data [14] suggest that many of these inhibitors may be binding to a hydrophobic region of the enzyme in close juxtaposition to a positively charged amino acid residue in or near the substrate-binding site. This study also highlights some of the difficulties encountered in trying to correlate enzyme inhibition *in vitro* with the physiological consequences of this inhibition. For any physiological effect to be observed, the enzyme must become rate-limiting in its metabolic pathway. In addition, the inhibitors must be able to gain access to their specific site of action in an unmetabolized form. Thus, it will be necessary to demonstrate a correlation between brain levels of the anti-convulsants during the period of seizure protection and reduced activity of aldehyde reductase. Only then would a strong case exist for the involvement of brain aldehyde metabolism in seizure mechanisms. The demonstration here of the inhibitory characteristics of brain aldehyde reductase is a first step in this direction.

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