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# INHIBITION OF ALDEHYDE REDUCTASE ISOENZYMES IN HUMAN AND RAT BRAIN*

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**Abstract**—Anticonvulsant drugs, such as barbiturates, open-chained analogues of barbiturates, glutethimide, succinimides and hydantoins have been tested *in vitro* as inhibitors of the isoenzymes of aldehyde reductase from human and rat brain. One major isoenzyme of both species is highly sensitive to the ionizable forms of these drugs containing the —CO—NH—CO— grouping and a minimal lipophilic substitution. Differences between the isoenzymes were observed with respect to the absolute configuration of various succinimides as inhibitors. One isoenzyme of both species exerts activity with NADH as well as NADPH. The NADH-dependent activity of the human enzyme is inhibited in a noncompetitive way by NADP with a $K_i$-intercept of $2{\cdot}2 \times 10^{-7}$M. A mixed inhibition is obtained with the biogenic acid, 4-hydroxyphenylacetic acid, as inhibitor of the main human isoenzyme. The inhibition is uncompetitive up to $[I] = 1 \times 10^{-3}$ M and yields a $K_i$ value of $4{\cdot}2 \times 10^{-4}$ M.

Recently, the heterogeneity of NADPH-dependent aldehyde reductase from human and rat brain has been investigated [1]. Four multiple molecular forms have been isolated from human and two from rat brain. They differ with respect to their substrate and coenzyme specificity. The main enzyme of each species is inhibited by barbiturates and thus is similar to the enzyme isolated from bovine [2] and pig brain [3]. A minor enzyme form in each species is characterized by its ability to catalyze the reduction of aliphatic as well as aromatic aldehydes with either NADPH or NADH.

Lately, Erwin and Deitrich [4] have shown that not only barbiturates but also other drugs exhibiting anticonvulsant activity are potent inhibitors of bovine brain aldehyde reductase. They suggest that the anticonvulsant action of these drugs *in vivo* could be related to their inhibitory action on this enzyme. Hence, it seemed to be of interest to extend these studies to the multiple molecular forms of human and rat brain aldehyde reductase and to further elucidate the structure-activity relationship for inhibition of the enzymes by anticonvulsant compounds. A preliminary report on some of the results has been given [5].

Biogenic aldehydes (i.e. those aldehydes arising from biogenic amines) are very reactive. In brain, they may bind to neuronal components [6, 7]. Furthermore, it has been theorized, that they play a role in sleep mechanisms [8–10] and the control of body temperature [11, 12]. Biogenic aldehydes have been reported to inhibit both, $Na^+$–$K^+$ and $Mg^{2+}$-activated ATPases in brain synaptosomes [13] and mitochondrial cytochrome *c* oxidase [14]. Aldehyde reductase as well as aldehyde dehydrogenase are involved in the degradation of these aldehydes. Biogenic acids, the products of the aldehyde dehydrogenase reaction, were found in a preliminary study to inhibit some forms of human and rat brain aldehyde reductase [1]. Based on this observation, further possibilities for regulation of aldehyde reductase were studied and NADP was found to exert an inhibition on the NADH-linked aldehyde reductase activity.

## EXPERIMENTAL

*Materials.* Human brains were obtained from legal medical autopsies and rats from the RAC strain from the Tierfarm AG, Sisseln, Switzerland, were used. All chemicals were at least reagent grade. All coenzymes were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Resolved *d*- and *l*-mephobarbitals were kindly supplied by Dr. E. Gordis, Rockefeller University, New York, N.Y. and the R- and S-succinimides by Dr. J. Knabe, Institut für Pharmazeutische Chemie der Universität des Saarlandes, Saarbrücken, Germany. All the other chemicals were purchased from Fluka, Buchs, Switzerland, or from Merck, Darmstadt, Germany. Deionized water was used for all experiments.

*Enzyme preparations.* NADPH-dependent aldehyde reductase was obtained from human and rat brain by procedures previously described [1]. Brain homogenates were centrifuged for 2 hr at 45,000 *g*, dialyzed and recentrifuged under the same conditions. The resulting supernatant was subjected to fractionation on a DEAE–cellulose column. The pooled active fractions of each enzyme form were further purified by chromatography on CM–cellulose and by gel chromatography on Sephadex G-100.

*Enzyme assay.* Aldehyde reductase (alcohol : NADP oxidoreductase; EC 1.1.1.2) was assayed by measuring the oxidation of NADPH or NADH at 334 or 340 nm in an Eppendorf or Unicam SP 1800 spectrophotometer at 25°. The standard reaction mixture consisted of enzyme protein, 0·16 mM coenzyme, 0·5

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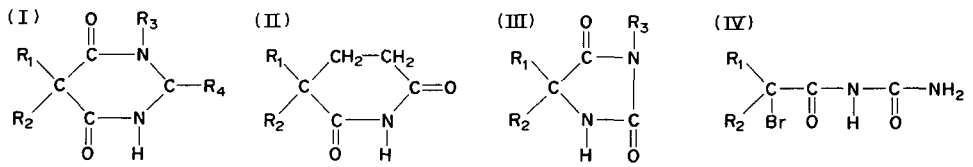
Table 1. Inhibition of NADPH-linked aldehyde reductases from human and rat brain by various derivatives of barbiturates, glutarimides, hydantoins and open-chained analogues of barbiturates




| Inhibitor | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Concn (mM) | % Inhibition H 4·1 | H 4·2 | H 4·3 | H 4·4 | R 4·1 | R 4·2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | | | | | | | | | | | |
| Phenobarbital | $C_6H_5$ | $C_2H_5$ | H | O | 1·0 | 6 | 55 | 88 | 33 | 95 | 34 |
| Mephobarbital | $C_6H_5$ | $C_2H_5$ | $CH_3$ | O | 1·0 | 17 | 0 | 74 | 28 | 73 | 13 |
| Primidone | $C_6H_5$ | $C_2H_5$ | H | $H_2$ | 1·0 | 0 | 0 | 10 | 6 | 19 | 3 |
| II | | | | | | | | | | | |
| Glutethimide | $C_6H_5$ | $C_2H_5$ | | | 0·5 | 0 | 8 | 52 | 8 | 62 | 39 |
| Phenglutarimide | $C_6H_5$ | $C_2H_4$ $N(C_2H_5)_2$ | | | 0·5 | 26 | 5 | 0 | 19 | 0 | 6 |
| III | | | | | | | | | | | |
| Diphenylhydantoin | $C_6H_5$ | $C_6H_5$ | H | | 0·5 | 8 | 49 | 89 | 16 | 98 | 74 |
| Mesantoin | $C_6H_5$ | $C_2H_5$ | $CH_3$ | | 0·5 | 4 | 13 | 9 | 8 | 23 | 13 |
| Nirvanol® | $C_6H_5$ | $C_2H_5$ | H | | 0·5 | 0 | 29 | 88 | 23 | 84 | 33 |
| IV | | | | | | | | | | | |
| Carbromal | $C_2H_5$ | $C_2H_5$ | | | 0·5 | 3 | 24 | 94 | 33 | 98 | 46 |
| Bromisoval | H | $CH(CH_3)_2$ | | | 0·5 | 37 | 32 | 90 | 38 | 94 | 54 |

Enzyme activity was determined as described under Experimental. 0·5 mM *p*-nitrobenzaldehyde was used as substrate. H stands for human enzymes, R for rat enzymes.

mM *p*-nitrobenzaldehyde and 0·1 M sodium phosphate buffer, pH 7·0 in a volume of 1 ml.

## RESULTS

*Effect of various inhibitors on aldehyde reductase from human and rat brain.* The four multiple molecular forms of aldehyde reductase from human brain and the two from rat brain were isolated and the inhibitory capacity of four groups of anticonvulsant compounds was tested with these isoenzymes. As detailed in Table 1, most compounds tested which contain the partial structure —CO—NH—CO— are inhibitory to human isoenzyme H 4·3 and rat isoenzyme R 4·1. Primidone, which does not have the structure of anticonvulsant drugs but is known to have anticonvulsant activity, is not an inhibitor of aldehyde reductase. However, the anticonvulsant activity has been attributed to a biotransformation to phenobarbital [15]. The open-chained analogues of the barbiturates, such as carbromal and bromisoval, are potent inhibitors. From the group of the glutarimides, glutethimide also inhibits mainly the enzymes H 4·3 and R 4·1. Phenglutarimide, an anticholinergic drug differs by its substitution with a longer chain and is not inhibitory, indicating steric hindrance. From the hydantoins tested, diphenylhydantoin is a potent inhibitor. Mesantoin and likewise other *N*-alkylated compounds, such as ethotoin, methsuccimide and phensuccimide, are not inhibitory. Nirvanol® (5-ethyl, 5-phenylhydantoin) known to be the demethylated primary metabolite of mesantoin *in vivo* [15], shows the expected inhibitory effect on enzymes H 4·3 and R 4·1. These results indicate that only the ionizable, *N*-dealkylated forms of these drugs are able to significantly inhibit aldehyde reductase *in vitro*. The restriction of the inhibitory properties of phenobarbital to one isoenzyme in each species is maintained throughout all anticonvulsant compounds tested.

Since both, *d*- and *l*-mephobarbital possess anticonvulsant activity but only the *l*-form shows hypnotic activity as well [16], the *d*- and *l*-isomers were tested with respect to inhibition of human and rat brain aldehyde reductases. No difference between the isomers was found with the enzymes H 4·3 and R 4·1 confirming the results obtained with the main bovine brain enzyme [4]. Since no other isoenzyme was inhibited, it seems unlikely that the hypnotic activity is related to inhibition of any of these enzymes.

As is shown in Table 2, both enantiomers of differently substituted succinimides inhibit the human isoenzyme H 4·3 to approximately the same extent. The least lipophilic compound 2-ethyl, 2-methylsuccinimide exerts less inhibition with some stereospecificity. Sensitivity to absolute configuration is observed with the human enzyme H 4·2, where inhibitions comparable to phenobarbital and diphenylhydantoin are obtained (see Table 1) with the S(−)-forms. In contrast to these, it is the R(−)-form of 2-ethyl, 2-methylsuccinimide, which is inhibitory. It should be noted that the absolute configuration of the R-form of this compound corresponds to the S-form of the other compounds [17]. No significant inhibition with any succinimide is observed with the human isoenzymes H 4·1 and H 4·4. The experiments with rat enzymes were carried out at smaller inhibitor concentrations, revealing a higher sensitivity of both rat isoenzymes when compared to the human. Furthermore, both isoenzymes show some stereospecificity.

Table 3 shows a list of compounds from different classes, which do not inhibit any form of human and

Table 2. Inhibition of human and rat brain aldehyde reductases by different succinimides

| Compound | Isomer | % Inhibition H 4·1 | H 4·2 | H 4·3 | H 4·4 | R 4·1 | R 4·2 |
|---|---|---|---|---|---|---|---|
| 2-Phenyl, 2-methylsuccinimide | R(+) | 4 | 0 | 89 | 13 | 33 | 16 |
| | S(−) | 6 | 55 | 95 | 13 | 77 | 31 |
| 2-Phenyl, 2-ethylsuccinimide | R(+) | 3 | 5 | 97 | 18 | 77 | 22 |
| | S(−) | 1 | 52 | 90 | 11 | 66 | 24 |
| 2-Cyclohexen(1)yl, 2-methylsuccinimide | R(+) | 0 | 0 | 84 | 11 | 12 | 7 |
| | S(−) | 7 | 54 | 94 | 5 | 74 | 24 |
| 2-Ethyl, 2-methylsuccinimide | S(+) | 0 | 0 | 8 | 0 | 3 | 3 |
| | R(−) | 1 | 20 | 44 | 11 | 27 | 4 |

Enzyme activity was determined as described under Methods with 0·5 mM *p*-nitrobenzaldehyde as substrate. Results represent mean values of three experiments. For experiments with human isoenzymes the inhibitor concentration was 250 μM, for rat isoenzymes 25 μM; 100 μM 2-ethyl,2-methylsuccinimide was used with the rat isoenzymes.

rat brain aldehyde reductase. The only exceptions are found in the group of neuroleptic drugs, chlorpromazine, thioridazine and chlorprothixene each tested at a concentration of 0·5 mM inhibit all human and rat brain aldehyde reductases between 40 and 60% under standard assay conditions.

*Inhibition of aldehyde reductases from human brain by NADP.* In accord with previous results [1], the replacement of 160 μM NADPH as coenzyme by the same concentration of NADH yields approximately 80% of the activity with enzyme H 4·2 but less than 10% with the other isoenzymes. The addition of increasing amounts of NADP to the NADPH-linked enzymes H 4·1, H 4·3 and H 4·4 leads to increasing product inhibition. Isoenzyme H 4·3 is the least sensitive, whereas up to 50% inhibition is observed for enzyme H 4·1 with a NADP concentration aequimolar with the coenzyme (Table 4). A similar inhibition is observed with enzyme H 4·2 when NADPH is used as coenzyme. However, with NADH as coenzyme, 92% inhibition is observed already at the lowest NADP concentration (16 μM).

Starting with this observation, kinetic studies with enzyme H 4·2 were carried out, using NADH as variable substrate, *p*-nitrobenzaldehyde as constant substrate and NADP as inhibitor (Fig. 1). A noncompetitive inhibition is observed and a $K_i$ of $2{\cdot}2 \times 10^{-7}$ M can be calculated from a secondary plot of the intercepts. With this enzyme apparent $K_m$ values for NADPH and NADH of $7{\cdot}3 \times 10^{-6}$ M and $5{\cdot}3 \times 10^{-4}$ M are obtained.

Table 3. Substances not inhibiting human and rat brain aldehyde reductase

| Group of substances | Compounds tested |
|---|---|
| Antidepressive drugs | Imipramine<br>Dimethacrine<br>Amphetamine<br>Methamphetamine<br>Ephedrine |
| Tranquillizers | Diazepam<br>Oxazepam<br>Medazepam<br>Meprobamate<br>Phenprobamate |
| Neuroleptic drugs | Promazine<br>Prothiadene<br>Clozapine<br>Haloperidol |
| Monoamineoxidase inhibitors | Iproniazide |
| β-Receptor blocking agents | Propranolol |
| Acid amides and acids | Phenylacetamide<br>Nicotinamide<br>Nicotinic acid<br>γ-aminobutyric acid |
| —CONHCO—<br>Containing substances | Hydantoin<br>Uracil<br>Thymidine monophosphate<br>Uridine monophosphate |

Assay conditions were as described under Experimental, using 0·5 mM *p*-nitrobenzaldehyde as substrate, 0·16 mM NADPH, and 0·5 mM of each compound tested as inhibitor.

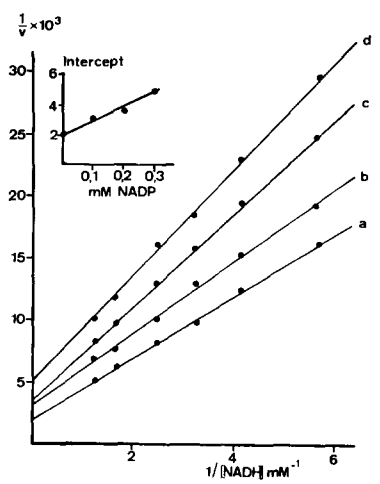



Fig. 1. Kinetics of human brain aldehyde reductase H 4·2 inhibition by NADP. The reaction mixtures and assay conditions were as described under Methods. *p*-Nitrobenzaldehyde, final concentration, was 0·5 mM with varying NADH concentration from 96 to 800 μM in 0·1 M sodium phosphate, pH 7·0 and enzyme protein in a final volume of 1 ml. Results are presented in and calculated from Lineweaver–Burk plots, the ordinate giving the reciprocal of the velocity (ΔE 340/min per ml × $10^3$). (a) without inhibitor, (b) 0·1 μM NADP, (c) 0·2 μM NADP, (d) 0·3 μM NADP.

Table 4. Effect of NADP on aldehyde reductase activity from human brain

| NADPH (μM) | NADH (μM) | NADP (μM) | % Inhibition H 4·1 | H 4·2 | H 4·3 | H 4·4 |
|---|---|---|---|---|---|---|
| 160 | — | 16 | 7 | 9 | 9 | 12 |
| 160 | — | 50 | 25 | 15 | 9 | 23 |
| 160 | — | 100 | 38 | 25 | 12 | 25 |
| 160 | — | 160 | 50 | 55 | 16 | 37 |
| — | 160 | 16 | — | 92 | — | — |

Enzyme activity was measured as described under Methods using 0·5 mM *p*-nitrobenzaldehyde as substrate.

*Inhibition of aldehyde reductase from human brain by 4-hydroxyphenylacetic acid.* Biogenic acids are one of the physiological end products of biogenic amine metabolism. Compounds of this type have previously been found to inhibit aldehyde reductase [1]. Hence, a kinetic study with enzyme H 4·3 and 4-hydroxyphenylacetic acid as a model inhibitor was carried out, NADPH being the constant and *p*-nitrobenzaldehyde the variable substrate (Fig. 2). A mixed inhibition pattern is observed. Up to an inhibitor concentration of $1 \times 10^{-3}$ M it is uncompetitive becoming noncompetitive at higher concentrations. The apparent $K_m$ value for *p*-nitrobenzaldehyde is $1{\cdot}3 \times 10^{-4}$ M and the $K_i$-intercept value for 4-hydroxyphenylacetic acid is $4{\cdot}2 \times 10^{-4}$ M in the uncompetitive range. Analogous results were obtained with the corresponding human liver isoenzyme, the $K_m$ for *p*-nitrobenzaldehyde being $9{\cdot}7 \times 10^{-5}$ M and the $K_i$-intercept for 4-hydroxyphenylacetic acid $4{\cdot}0 \times 10^{-4}$ M.

## DISCUSSION

Multiple molecular forms of brain aldehyde reductase have been observed in several species, such as bovine [2, 18], pig [3], rat and human [1]. One isoenzyme of each species has the following common characteristics: (1) it contributes a major part to the total aldehyde reductase activity in brain; (2) it occurs in the cytosol; (3) it is NADPH-dependent; (4) it prefers aromatic to aliphatic aldehydes; and (5) it has a high sensitivity to barbiturate inhibition. A minor isoenzyme in human and rat brain utilizes both NADPH and NADH as coenzyme, and aliphatic aldehydes serve as substrates as well as aromatic aldehydes. These multiple forms are less sensitive to barbiturate inhibition and one major isoenzyme of human brain (H 4·1) is not inhibited by barbiturates.

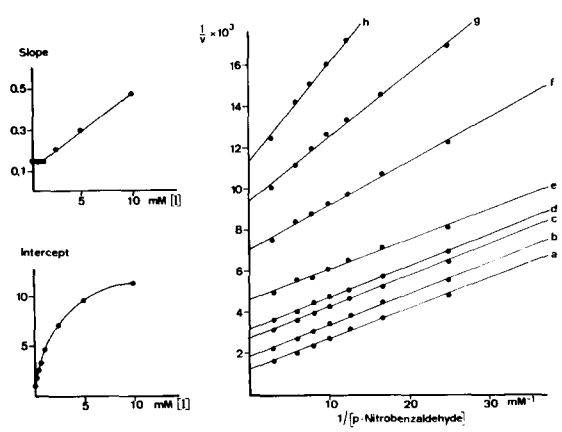



Fig. 2. Kinetics of human brain aldehyde reductase H 4·3 inhibition by 4-hydroxyphenylacetic acid. The assay conditions were as described under Methods. The reaction mixtures consisted of 0·16 mM NADPH, enzyme protein and varying concentrations of *p*-nitrobenzaldehyde from 40 to 325 μM in 0·1 M sodium phosphate pH 7·0 in a final volume of 1 ml. Results are plotted according to Lineweaver and Burk, the ordinate representing the reciprocal of the velocity (ΔE 340/min per ml × $10^3$). (a) without inhibitor, (b) $10^{-4}$ M, (c) $3{\cdot}6 \times 10^{-4}$ M, (d) $6 \times 10^{-4}$ M, (e) $10^{-3}$ M, (f) $2{\cdot}5 \times 10^{-3}$ M, (g) $5 \times 10^{-3}$ M and (h) $10^{-2}$ M 4-hydroxyphenylacetic acid.

Recently, Erwin and Deitrich [4] reported on the inhibition of the NADPH-dependent bovine brain aldehyde reductase by anticonvulsant drugs. It was of interest to determine whether or not the isoenzyme specificity of barbiturate inhibition extends to anticonvulsants in general. Our results show that *in vitro* all anticonvulsants tested with the minimal structure —CO—NH—CO— and a lipophilic group are potent inhibitors mainly of the NADPH-specific barbiturate sensitive isoenzyme from human and rat brain. The finding that *N*-alkylated compounds are poor inhibitors is consistent with the hypothesis of Erwin and Deitrich [4] that only the ionized form of these compounds is inhibitory. However, it is suggested that *in vivo* only the *N*-dealkylated primary metabolites exhibit anticonvulsant activity [15]. It was shown that mesantoin [19] and tridione are demethylated *in vivo* [20]. Primidone known as an anticonvulsant drug does not inhibit aldehyde reductase *in vitro*. In the case of primidone it has been shown that it is oxidized *in vivo* to phenobarbital [21, 22]. Diazepam is no inhibitor of the enzymes *in vitro* although it has some anticonvulsant properties *in vivo*. Since it is known to be demethylated *in vivo* [23], this metabolite may account for this pharmacological property. Chlorpromazine and related compounds inhibit aldehyde reductase without specificity with respect to the isoenzymes, suggesting a different mechanism of inhibition.

The human NAD(P)H-dependent enzyme (H 4·2) is less sensitive to inhibition by anticonvulsant compounds, but exhibits a remarkable sensitivity to the absolute configuration of substituted succinimides. Nothing is known about the pharmacology of these isomers. Therefore it is impossible to predict whether an inhibition of this enzyme in addition to the inhibition of isoenzyme H 4·3 is related to therapeutic usefulness. The fact that the major human isoenzyme H 4·1 is insensitive to inhibition by anticonvulsants obviates a high degree of specificity in the action of these drugs *in vitro*. This increases the likelihood for a relationship of this *in vitro* inhibition and the mechanism of action *in vivo*.

The human brain aldehyde reductase H 4·2 which can use both NADPH and NADH as coenzyme shows $K_m$ values of $7{\cdot}3 \times 10^{-6}$ M for NADPH and $5{\cdot}3 \times 10^{-4}$ M for NADH. This indicates that NADPH is the preferred coenzyme. This study reveals that NADP concentrations in the range of $10^{-7}$ M are able to strongly inhibit the NADH activity of this enzyme. From this the question arises whether NADH can serve as a coenzyme for this enzyme *in vivo*. Lowry *et al.* [24] found the following coenzyme concentrations in brain (μmoles/kg): 322 for NAD, 95 for NADH, 7·2 for NADP and 22·3 for NADPH. In view of these coenzyme levels it is likely that only NADPH functions as coenzyme *in vivo*.

Among others, 4-hydroxyphenylacetic acid, the derivative of tyramine, acts as an inhibitor of human brain aldehyde reductases [1]. The present kinetic study of the potency of such inhibitors was carried out in order to elucidate a possible physiological significance. A $K_i$ value for 4-hydroxyphenylacetic acid with enzyme H 4·3 of $4 \times 10^{-4}$ M was found. At lower concentrations of the inhibitor the type is uncompetitive. These observations lead to the question of a regulation mechanism between the enzymes involved in biogenic aldehyde metabolism. Tabakoff *et al.* [25] have shown that acid derivatives of the biogenic amines (i.e. 5-hydroxyindoleacetic acid) inhibit aldehyde-stimulated metabolism of glucose by the pentose phosphate shunt in brain. Probenecid is well known to increase biogenic acids in cerebrospinal fluid. Furthermore, a single dose or prolonged administration of ethanol has been shown to produce an elevation in brain 5-hydroxyindoleacetic acid levels in mice [26]. An increase in biogenic acid levels after ethanol administration has also been noted in the cerebrospinal fluid of cats [27] and in human alcoholics during ethanol intoxication [28]. In view of the relatively high inhibition constant found for 4-hydroxyphenylacetic acid it seems questionable whether the increase in biogenic acid levels provoked by probenecid or alcohol intake is sufficient to divert the metabolism of biogenic aldehydes from the reductive to the oxidative pathway *in vivo*.

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