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KINETIC STUDIES ON THE MAJOR FORM OF ALDEHYDE REDUCTASE IN OX KIDNEY: A GENERAL KINETIC MECHANISM TO EXPLAIN SUBSTRATE-DEPENDENT MECHANISMS AND THE INHIBITION BY ANTICONVULSANTS

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The inhibition of the major form of ox kidney aldehyde reductase (AR 1) by sodium barbitone revealed linear mixed kinetics. This behaviour is distinct from the non-linear intercept effect we reported for valproate [Daly and Mantle (1982) *Biochem. J.* **205**, 381]. 4-Carboxybenzaldehyde exhibits partial uncompetitive substrate inhibition. These results are discussed in terms of a model that involves nucleotide-induced isomerization and an additional flux (with some substrates and inhibitors) through an enzyme. nucleotide.substrate/inhibitor ternary complex.

KEY WORDS: Aldehyde reductase, valproic acid, barbitone.

INTRODUCTION

Multiple forms of aldehyde reductase (E.C. 1.1.1.2) exist in many tissues (see reference [1] for a recent review). They generally exhibit wide and overlapping substrate specificities and are monomers with M_r values in the range 30,000–40,000. The major form, which occurs in large amounts in liver and kidney[1], is particularly sensitive to inhibition by barbiturates[2,3] and sodium valproate[4,5]. The sensitivity of the major form of aldehyde reductase, including the brain enzyme, to inhibition by anticonvulsant drugs has led to the suggestion that this enzyme may be a pharmacologically important site of action of these drugs[5,6]. However, attempts to delineate the reaction mechanism of the major form of aldehyde reductase and to determine the mechanism of inhibition by various anticonvulsants have not been totally consistent[7–12]. There is general agreement that in the direction of aldehyde reduction the addition of substrates is ordered[7–11], however, the mechanism of product release has been described as ordered[7,9,11] and random[8,10,12]. This may result from substrate-dependent differences in kinetic mechanism[8,12,13] as the studies reported in references [7–12] utilised a wide range of substrates. In addition, different sources of enzyme have been used, including pig kidney[7,8], ox kidney[11], pig liver[12], human liver[10] and rat brain[9] so that species-dependence with regard to kinetic mechanism remains a possible explanation for some of the differences observed.

Barbiturate inhibition of the major form of aldehyde reductase from rat liver[14] and pig kidney[7], measuring enzyme activity with daunorubicin and glyceraldehyde

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respectively, exhibits linear mixed (non-competitive) kinetics. However, hyperbolic kinetics have been reported for sodium barbitone inhibition of the pig kidney enzyme using pyridine-3-aldehyde as substrate[8]. This results from an additional flux through the ternary complex enzyme.NADP.barbiturate[8]. A similar mechanism has recently been proposed to explain the hyperbolic intercept effect seen with sodium valproate inhibition of the ox kidney enzyme[11].

We have, therefore, examined the kinetics of barbitone inhibition of the ox kidney enzyme and in addition report details of the kinetic mechanism with 4-carboxybenzaldehyde as substrate. A comparison of our results with D-glucuronic acid and 4-carboxybenzaldehyde as substrates and barbitone and valproate as inhibitors allows us to propose a general mechanism for the enzyme and its inhibition by anticonvulsants.

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MATERIALS AND METHODS

NADPH and NADP were purchased from Boehringer, D-glucuronic acid was supplied by Sigma and 4-carboxybenzaldehyde by Ralph Emanuel Ltd., Wembley, London, U.K. Sodium barbitone was obtained from May and Baker, Dagenham, U.K. The major form of ox kidney aldehyde reductase (AR 1) was purified to homogeneity as previously described[15]. Details of the spectrophotometric[15] and fluorimetric[11] assays for aldehyde reductase activity have been previously reported.

All initial rate data were plotted in double reciprocal form. The slopes (K_m/V) and intercepts ($1/V$) of each line were estimated by fitting the data to eqn. (1):

$$v = VS/K_m + S \tag{1}$$

by non-linear regression using the method of Wilkinson[16]. Slope and intercept replots were fitted to straight lines by linear regression and the respective K_i values were obtained by extrapolating to the inhibitor concentration axis. Initial rate data were also fitted to eqn. (2):

$$v = VAB/K_{ia}K_b + K_bA + K_aB + AB \tag{2}$$

by non-linear regression[17].

RESULTS

The effect of sodium barbitone on AR 1 in the forward direction was examined at pH 7. When either NADPH or glucuronic acid was the variable substrate, linear double reciprocal plots were obtained which intersected in the upper left quadrant showing that sodium barbitone was a mixed inhibitor in each case. Slope and intercept replots were linear. The resultant K_i values from the slope and intercept replots with glucuronic acid as the variable substrate were $40.5 \pm 6.4\,\mu$M and $34.2 \pm 5.8\,\mu$M respectively. With NADPH as the variable substrate the corresponding values were $90.9 \pm 25.3\,\mu$M and $54.5 \pm 11.7\,\mu$M.

A double reciprocal plot with 4-carboxybenzaldehyde as the variable substrate shows lines which exhibit upward curvature at high substrate concentrations (Figure 1). Extrapolation of the linear portion of these lines results in an intersection in the upper left quadrant. When NADPH was the variable substrate linear double

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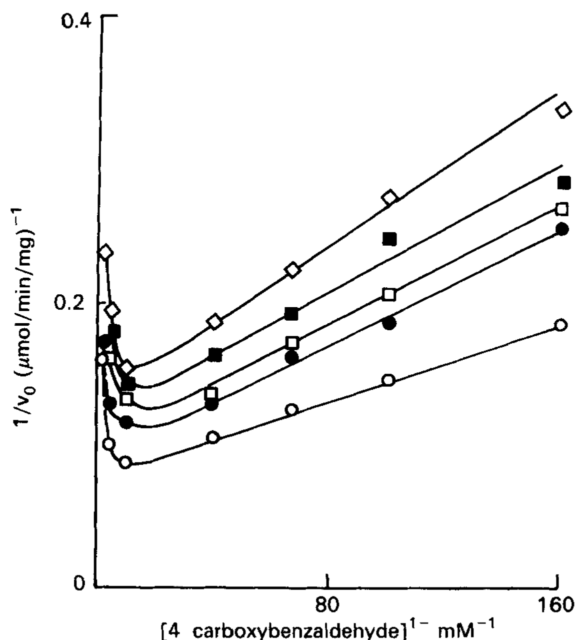

FIGURE 1 Double reciprocal plots of initial velocities with varying 4-carboxybenzaldehyde concentrations. The NADPH concentrations used were 6.72 μM (○), 2.24 μM (●), 1.68 μM (□), 1.12 μM (■) and 0.84 μM (◇). For each assay, 10 μl of purified AR 1 (1.02 mg/ml), diluted 1 in 20 was used.

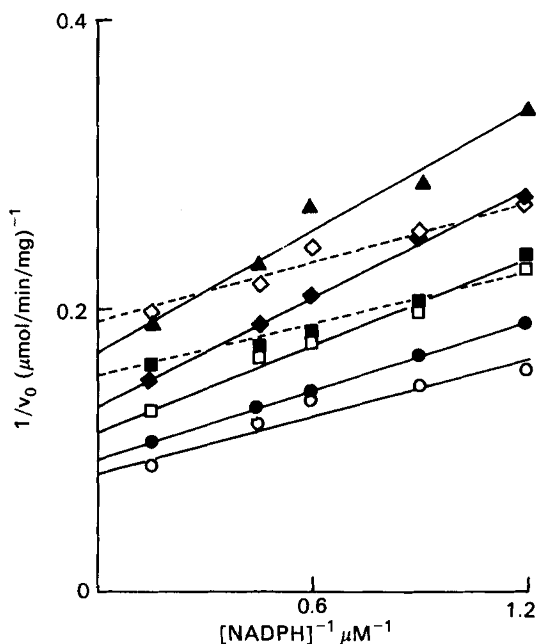

FIGURE 2 Double reciprocal plots of initial velocities with varying NADPH concentrations. The concentrations of 4-carboxybenzaldehyde used were 6.25 μM (▲), 10 μM (◆), 15 μM (□), 25 μM (●), 100 μM (○), 250 μM (■) and 750 μM (◇). For each assay, 10 μl of purified AR 1 (1.02 mg/ml), diluted 1 in 20 was used.

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TABLE I
The kinetic constants for AR 1[a]

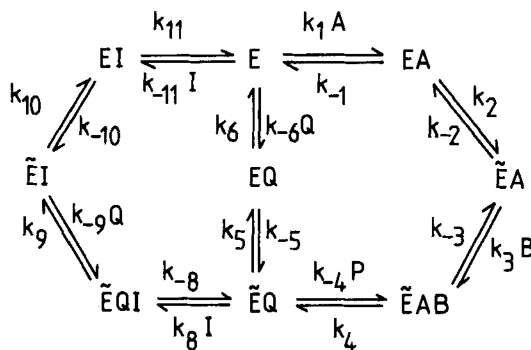
Substrate	K_a (μM)	K_{ia} (μM)	K_b (mM)	V (μmol/min/mg)
4-Carboxybenzaldehyde	1.01 ± 0.16	0.70 ± 0.35	0.009 ± 0.002	13.99 ± 0.77
D-Glucuronic acid[b]	2.11 ± 0.18	0.92 ± 0.15	2.37 ± 0.2	16.2 ± 0.66
L-Gulonic acid[b]	0.67 ± 0.19	9.61 ± 1.02	7.11 ± 0.69	0.13 ± 0.004
	V_m/K_a	D-Gluc 4.17 $\mu M^{-1} s^{-1}$		
		4CB 7.69 $\mu M^{-1} s^{-1}$		

[a]All kinetic constants were determined by fitting to eqn. (2).
[b]Taken from reference [11].

reciprocal plots were obtained which intersected in the upper left quadrant at low concentrations of 4-carboxybenzaldehyde (Figure 2). The data obtained at sub-inhibitory levels of 4-carboxybenzaldehyde were fitted to eqn. (2) and the kinetic parameters obtained are shown in Table I. At high concentrations of 4-carboxybenz-aldehyde the intercept replot for the NADPH kinetics became non-linear (Figure 3). Dixon plots[18] for 4-carboxybenzaldehyde showed upward curvature at substrate-inhibitory levels of 4-carboxybenzaldehyde and the linear portions of these lines, obtained with various NADPH concentrations, were parallel showing that the sub-strate inhibition was uncompetitive (results not shown). Although the Dixon plot was approximately linear in the region between 0.2–1 mM 4-carboxybenzaldehyde, at higher concentrations there was a pronounced deviation from linearity (Figure 4) indicating partial substrate inhibition.

DISCUSSION

The linear mixed inhibition of ox kidney AR 1 by sodium barbitone is consistent with a mechanism where barbitone binds both to the free enzyme and the enzyme.NADP complex. A more realistic and general scheme, which takes account of our previous observations with sodium valproate[11] as inhibitor is shown in Scheme 1.



SCHEME 1

In this scheme it is normally the nucleotide-induced conformer ($\tilde{E}$) of the enzyme that binds the aldehyde substrate (B) in complexes such as $\tilde{E}AB$ and $\tilde{E}QB$, although we would argue that barbitone, valproate and certain substrates, e.g., 4-carboxybenz-aldehyde, can bind to the free enzyme (i.e., $k_{-11} \neq 0$) although they are not capable

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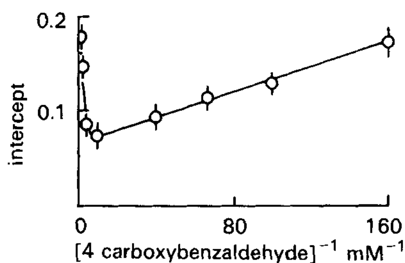

FIGURE 3 Intercept replot from Figure 2.

of inducing the conformational change to $\tilde{E}$ (i.e., $k_{-10} = 0$). In the case of the ox kidney enzyme it appears that NADP cannot dissociate from the enzyme.NADP. barbitone complex (i.e., k_9 must be negligible) as we see no evidence for an alternative flux (we obtain linear intercept replots for sodium barbitone inhibition against either substrate). The inclusion of the enzyme isomerisation step in Scheme 1 provides an explanation for omitting nucleotide binding to an enzyme.barbiturate complex in a simpler scheme and is consistent with our previous work[11]. In the model shown in Scheme 1, nucleotides can bind to the $\tilde{E}I$ complex (i.e., $k_9 \neq 0$) but not to the EI complex as we have assumed barbiturates are not capable of inducing the conformational step characterised by k_{-10}. In the case of the pig kidney enzyme with pyridine 3-aldehyde as substrate dissociation of NADP from the enzyme.NADP. barbitone complex apparently occurs (i.e., $k_9 \neq 0$) as there is an alternative flux

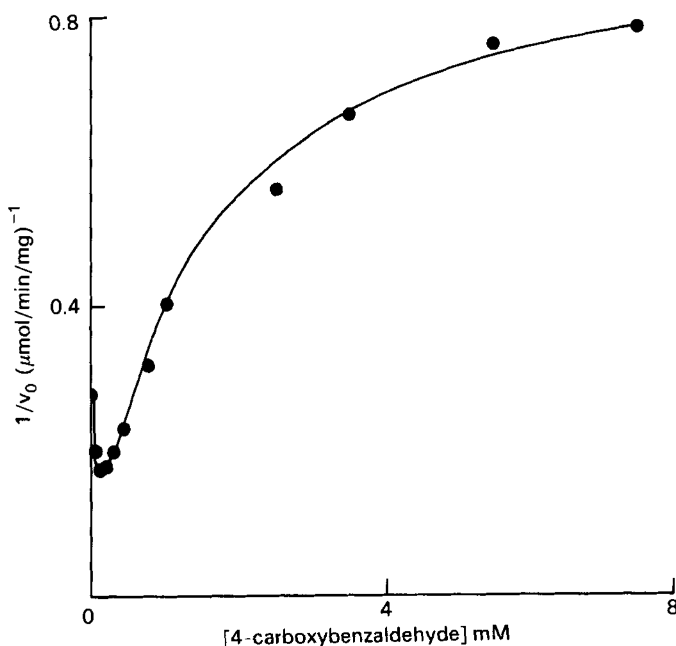

FIGURE 4 Dixon plot for substrate inhibition of AR 1 with 4-carboxybenzaldehyde as the variable substrate. NADPH was held constant at 150 µM. For each assay, 50 µl of purified AR 1 (1.02 mg/ml), diluted 1 in 20 was used.

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through this ternary complex operative at high concentrations of sodium barbitone[8]. It should be noted that this phenomenon is apparently not observed with glyceraldehyde as substrate[7].

The partial uncompetitive substrate inhibition we observe with 4-carboxybenzaldehyde as substrate suggests that an enzyme.NADP.4-carboxybenzaldehyde complex ($\tilde{E}BQ$; analogous to $\tilde{E}QI$) is formed and breaks down via $\tilde{E}B$ and EB to free enzyme and 4-carboxybenzaldehyde. Similar results have been reported for the pig liver enzyme[12] and are analogous to those previously reported for sodium valproate inhibition[11]. Formation of $\tilde{E}BQ$ complexes has been observed with succinic semialdehyde and the rat brain enzyme[9], and with 4-carboxybenzaldehyde and the enzyme from pig liver[12] and ox kidney (present work). In the case of the ternary complexes involving 4-carboxybenzaldehyde, nucleotide dissociation occurs (reference [12] and the present study) although this apparently does not occur with succinic semialdehyde as a substrate for the rat brain enzyme[9].

In conclusion our results are consistent with Scheme 1 as a general model for the mechanism of ox kidney aldehyde reductase and its inhibition by anticonvulsants. A similar conclusion has also been reached in the case of the pig kidney enzyme[12].

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