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FURTHER CHARACTERIZATION OF A REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE-DEPENDENT ALDEHYDE REDUCTASE FROM BOVINE BRAIN

INHIBITION BY PHENOTHIAZINE DERIVATIVES*

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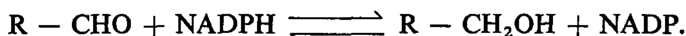
Abstract—The catalytic activity of partially purified NADPH-linked aldehyde reductase (alcohol:NADP oxidoreductase, EC 1.1.1.2) from bovine brain was inhibited by certain phenothiazines. The inhibition was competitive with respect to either NADPH or aldehyde as substrate and was found to vary with the pH of the reaction mixture. At pH 7·4 the inhibitor constants (K_i values) for chlorpromazine, trifluoperazine, and thioridazine were $7{\cdot}0 \times 10^{-4}$ M, $3{\cdot}3 \times 10^{-4}$ M, and $8{\cdot}0 \times 10^{-4}$ M respectively. Promethazine (K_i value of $45{\cdot}8 \times 10^{-4}$ M) was approximately 10 times less effective than chlorpromazine in producing inhibition of enzyme activity. In addition, chlorpromazine sulfoxide did not inhibit the rate of aldehyde reduction at concentrations as high as 10^{-3} M. When the K_i values for the various phenothiazines were compared with the reported ability of these compounds to abolish the unconditioned avoidance response in rats, it was observed that compounds with the lowest K_i values had the lowest ED_{50} for abolition of this response.

Studies of product inhibition of bovine brain aldehyde reductase showed that either NADP or *p*-nitrobenzylalcohol were competitive inhibitors with NADPH or aldehyde as the variable substrate. These observations, together with the nature of inhibition produced by the phenothiazine compounds, strongly indicate a random order of addition of substrates to the enzyme.

RECENTLY, Tabakoff and Erwin[1] reported the isolation and partial characterization of an NADPH-dependent aldehyde reductase‡ (EC 1.1.1.2) from bovine brain capable of catalyzing the conversion of various aldehydes to their corresponding alcohols as follows:



$$R - CHO + NADPH \rightleftharpoons R - CH_2OH + NADP.$$

These investigators proposed that this enzyme may be responsible for the production of the alcohol intermediates derived from the biogenic amines as reported by Kopin *et al.*[2] and Rutledge, Breese *et al.*[3,4] The enzyme was shown to differ from alcohol dehydrogenase in substrate and cofactor specificities, and pyrazole was not an

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† This work was performed in partial fulfillment for the Ph.D. requirement.

‡ Since this enzyme is an alcohol dehydrogenase (although ethanol is not a substrate) and inasmuch as pyrazole is not an inhibitor, it may be referred to as a pyrazole-insensitive NADP-linked alcohol dehydrogenase.

inhibitor of the enzyme. Also, bovine brain aldehyde reductase was reported to be inhibited by pentobarbital and chlorpromazine. Further studies by Erwin *et al.*[5] have shown the inhibition by barbiturate derivatives to be non-competitive with respect to both aldehyde substrate and NADPH with K_i values as low as 10^{-5} M. In these studies barbiturates were ineffective as inhibitors of horse liver alcohol dehydrogenase (EC 1.1.1.1). However, Khouw *et al.*[6] have shown that horse liver and rabbit liver alcohol dehydrogenase are inhibited by chlorpromazine. In light of these studies and inasmuch as it has been observed that chlorpromazine alters brain biogenic amine metabolism[7,8] and, consequently possibly alters biogenic aldehyde metabolism, the present studies were performed to characterize the inhibition of bovine brain aldehyde reductase by various phenothiazine derivatives.

MATERIALS AND METHODS

Chemicals. NADPH, NADH and octopamine were obtained from Sigma Chemical Company. The phenothiazines were kindly supplied by the drug manufacturers. *p*-Nitrobenzaldehyde was purchased from Matheson, Coleman, & Bell, and the *p*-nitrobenzylalcohol was obtained from Eastman Chemicals. *p*-Hydroxyphenylglycolaldehyde was prepared from octopamine as described previously.[1] The concentration of free aldehyde in 0·1 M sodium phosphate, pH 7·0, was assayed with excess NAD and rat liver aldehyde dehydrogenase by the method of Deitrich *et al.*[9] All aldehyde and inhibitor solutions were prepared, in triple distilled water, immediately prior to their use.

Enzyme preparation. NADPH-dependent aldehyde reductase was obtained from bovine brain by procedures previously described.[1] The homogenate was centrifuged for 30 min at 27,000 *g*, and the resultant supernatant fluid was fractionated with ammonium sulfate. The protein which precipitated between 40 and 55 per cent saturation with the ammonium sulfate was resuspended, dialyzed and placed on a calcium phosphate-gel cellulose column. The eluate containing the partially purified enzyme was collected and was concentrated by means of an Amicon diaflo apparatus. The resulting enzyme preparation (specific activity of approximately 100 nmoles NADPH oxidized/min/mg protein) was used for various studies as indicated.

Enzyme assay. The catalytic activity of aldehyde reductase was determined by observing the decrease in NADPH absorbance at 340 nm spectrophotometrically. A Gilford Model 2400 spectrophotometer was used to measure initial (within the first minute) changes in absorbancy of the reaction mixtures at 25°. The reaction mixtures consisted of enzyme protein (0·02–0·1 mg) and 0·05 M sodium phosphate, pH 7·0, in a final volume of 3·0 ml. Cofactor, aldehyde and inhibitor concentrations in the final assay mixtures are given in the tables and figures.

Protein concentrations were determined by the method of Murphy and Keis.[10]

RESULTS

Inhibition of aldehyde reductase by phenothiazines. As shown in Figs. 1 and 2, various phenothiazines, including chlorpromazine, trifluoperazine and thioridazine, inhibited bovine brain aldehyde reductase. Data presented in Figs. 1 and 2 show that inhibition by these compounds was competitive with NADPH or aldehyde as the

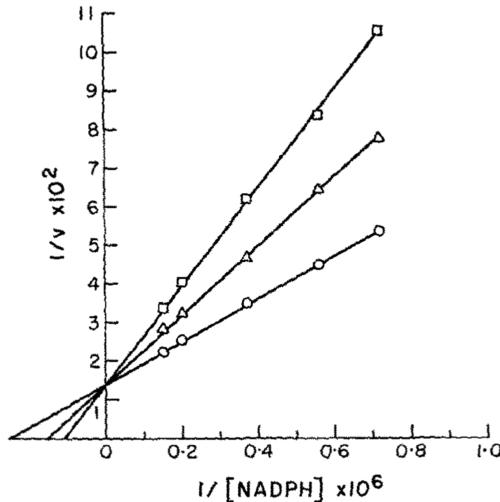


Fig. 1. Kinetics of bovine brain aldehyde reductase inhibition by chlorpromazine. The assay conditions were as described in the text. The reaction mixtures consisted of 0·028 mg protein (specific activity of 86 nmoles of NADPH oxidized/min/mg protein), $2{\cdot}5 \times 10^{-4}$ M *p*-nitrobenzaldehyde and 0·05 M sodium phosphate, pH 7·0, in a final volume of 3·0 ml. NADPH and inhibitor concentrations were varied as indicated. The abscissa gives the reciprocal of the molarity of NADPH and the ordinate gives the reciprocal of the velocity (nanomoles NADPH oxidized per min per milligram protein). As illustrated, ○——○ represents the control without inhibitor, △——△ represents chlorpromazine, $1{\cdot}0 \times 10^{-4}$ M, and □——□ represents chlorpromazine, $5{\cdot}0 \times 10^{-4}$ M. Similar results were obtained using trifluoperazine, thioridazine, and promethazine as inhibitiors.

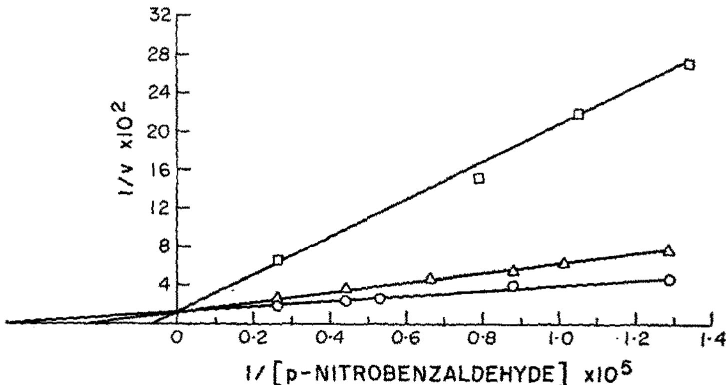


Fig. 2. Kinetics of aldehyde reductase inhibitions by chlorpromazine. The abscissa gives the reciprocal of the molarity of *p*-nitrobenzaldehyde. The assay systems contained 0·093 mg enzyme protein (specific activity of 71·6 nmoles NADPH oxidized/min/mg protein), $1{\cdot}8 \times 10^{-4}$ M NADPH and 0·05 M sodium phosphate, pH 7·0, in a final volume of 3·0 ml. As illustrated, ○——○ represents the control without inhibitor, △——△ represents chlorpromazine, $5{\cdot}0 \times 10^{-4}$ M, and □——□ represents chlorpromazine $1{\cdot}0 \times 10^{-3}$ M. Similar results were obtained when *p*-hydroxyphenylglycolaldehyde was the substrate in place of *p*-nitrobenzaldehyde.

variable substrate. Inhibition of enzyme activity by the phenothiazine compounds was completely reversible by dilution of the reaction mixture.

Inhibitor constants (K_i values) for the various phenothiazines were calculated from the Lineweaver–Burk plots of the kinetic data.[11] The values listed in Table 1 were obtained with at least two concentrations of inhibitor. At pH 7·0, trifluoperazine and chlorpromazine have the lowest K_i values ($3{\cdot}2 \times 10^{-4}$ M) while promethazine has a

TABLE 1. INHIBITOR CONSTANTS FOR VARIOUS PHENOTHIAZINES*

Inhibitor	Substrate†	$K_i \times 10^4$ M	
		pH 7·0	pH 7·4
Chlorpromazine HCl	NADPH	3·2	7·0
Chlorpromazine NCl	*p*-Nitrobenzaldehyde	3·7	—
Chlorpromazine HCl	*p*-Hydroxyphenylglycolaldehyde	3·6	—
Trifluoperazine 2 HCl	NADPH	3·2	3·3
Thioridazine HCl	NADPH	5·1	8·0
Promethazine HCl	NADPH	45·8	—

* The K_i values were obtained from Lineweaver–Burk plots and are an average of values obtained with at least two inhibitor concentrations. Experimental conditions were as described in Figs. 1 and 2.
† Substrate refers to the reactant which was varied while the other factors were kept constant.

value more than 10-fold higher. Chlorporomazine sulfoxide was not an inhibitor of aldehyde reductase at concentrations up to 1×10^{-3} M. At pH 7·4, the K_i value for trifluoperazine ($3{\cdot}3 \times 10^{-4}$ M) is about twice as low as the value for chlorpromazine ($7{\cdot}0 \times 10^{-4}$ M).

Characterization of the aldehyde reductase catalyzed reaction. Since the nature of inhibition of aldehyde reductase and alcohol dehydrogenase[6] by chlorpromazine differed, and inasmuch as the liver alcohol dehydrogenase catalyzed reaction proceeds by an ordered addition of substrate, studies were performed to determine the reaction

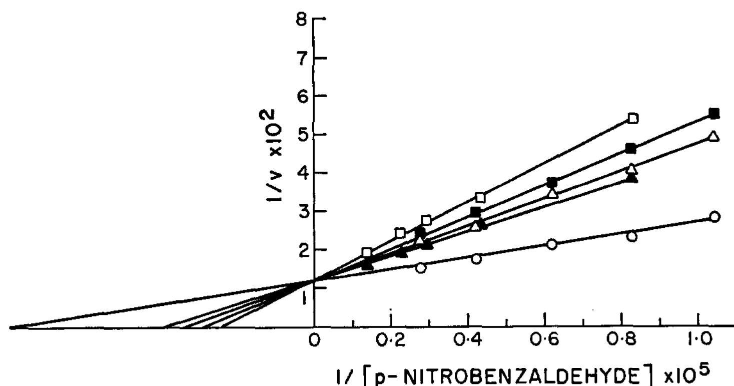


FIG. 3. Product inhibition of bovine brain aldehyde reductase. Assay conditions and systems are as reported in the text with 0·034 mg protein (specific activity of 97·2 nmoles/min/mg) and with $4{\cdot}0 \times 10^{-6}$ M NADPH. The pH of the reaction mixture is 7·0. The abscissa gives the reciprocal of the molarity of *p*-nitrobenzaldehyde. ○——○ Represents the control without inhibitor, ▲——▲ represents *p*-nitrobenzylalcohol, $5{\cdot}0 \times 10^{-4}$ M, △—△ represents *p*-nitrobenzylalcohol, $1{\cdot}0 \times 10^{-3}$ M, ■——■ represents NADP, $1{\cdot}7 \times 10^{-5}$ M, and □——□ represents NADP, $6{\cdot}0 \times 10^{-5}$ M.

mechanism for aldehyde reductase. As shown in Fig. 3 either NADP or *p*-nitrobenzylalcohol, when added to the reaction mixture, produced a competitive inhibition with *p*-nitrobenzaldehyde as the variable substrate. Similarly, when NADPH was the variable substrate, NADP or *p*-nitrobenzylalcohol produced a competitive type inhibition (Fig. 4). As described by Cleland,[12] these product inhibition data suggest a random order of addition of substrate to the enzyme. Further data in support of this proposal are presented in Figs. 5 and 6. When *p*-nitrobenzaldehyde was

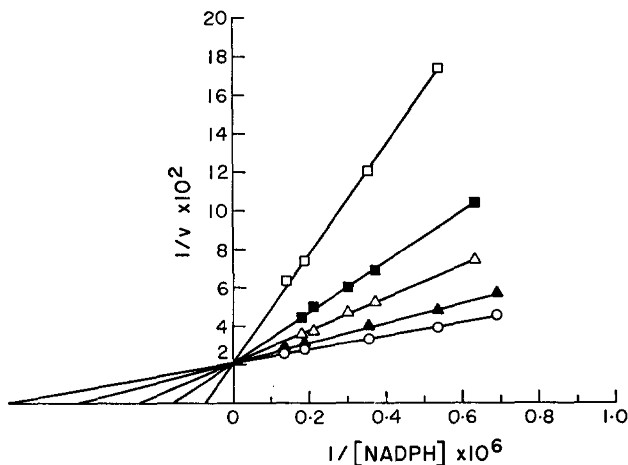


FIG. 4. Product inhibition of bovine brain aldehyde reductase. Assay conditions and reaction mixtures are as described in Fig. 3, except that the assays contained 0·067 mg protein with a specific activity of 69·8 nmoles/min/mg, and 1·9 $\times$ 10^{-5} M *p*-nitrobenzaldehyde. The abscissa gives the reciprocal of the molarity of NADPH. As illustrated, ○——○ represents the control without inhibitor, ▲——▲ represents *p*-nitrobenzylalcohol, 1·0 $\times$ 10^{-4} M, △——△ represents *p*-nitrobenzylalcohol, 5·0 $\times$ 10^{-4} M, ■——■ represents NADP, 5·0 $\times$ 10^{-5} M, and □——□ represents NADP, 1·6 $\times$ 10^{-4} M.

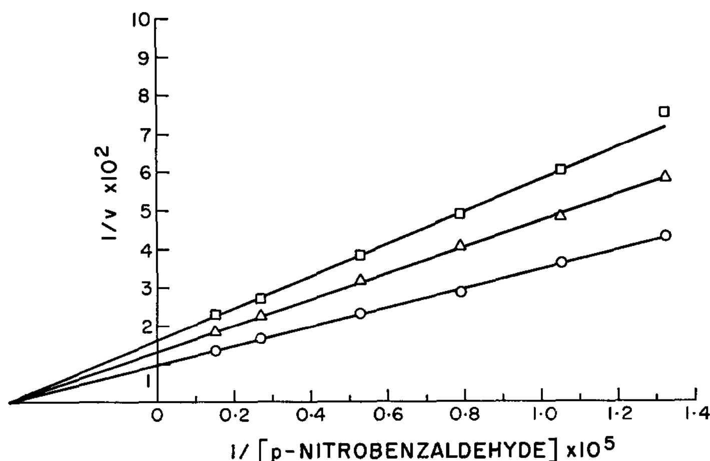


FIG. 5. Kinetics of aldehyde reductase with both substrates varied. The abscissa gives the reciprocal of the molarity of *p*-nitrobenzaldehyde. The reaction mixtures were as described in the text with 0·093 mg protein (specific activity of 106 nmoles NADPH oxidized/min/mg protein), and with the following concentrations of NADPH: ○——○, 1·44 $\times$ 10^{-4} M; △——△, 2·88 $\times$ 10^{-6} M; and □——□, 1·44 $\times$ 10^{-6} M.

the variable substrate, the lines of the Lineweaver–Burk plots at various NADPH concentrations intersected on the abscissa (Fig. 5). Similar results were obtained when NADPH was the variable substrate (Fig. 6). These results demonstrate that the

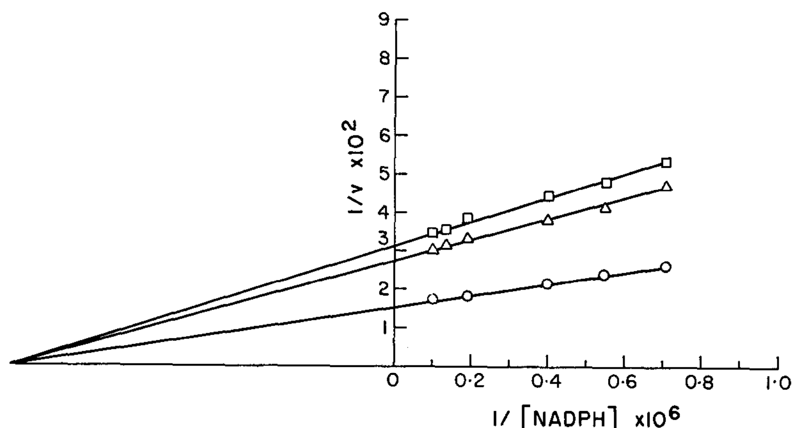


FIG. 6. Kinetics of aldehyde reductase with both substrates varied. The abscissa gives the reciprocal of the molarity of NADPH. The assay systems were the same as in Fig. 5 with 0·062 mg protein (specific activity of 45 nmoles NADPH oxidized/min/mg protein), and with the following concentrations of *p*-nitrobenzaldehyde: ○——○, $1{\cdot}69 \times 10^{-4}$ M; △——△, $3{\cdot}38 \times 10^{-5}$ M; and □——□, $1{\cdot}69 \times 10^{-5}$ M.

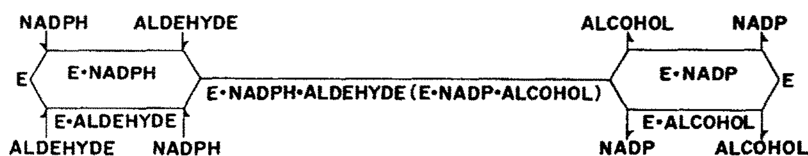
Michaelis–Menten (K_m) value for one substrate is not dependent on the concentration of the other substrate.

DISCUSSION

It has been observed that chlorpromazine and other phenothiazines inhibit many enzyme systems, including glutamate dehydrogenase,[13] $Na^{+}K^{+}$-activated adenosine triphosphatase,[14] aldehyde oxidase[15] and liver alcohol dehydrogenase.[6] In the present study it was found that various pharmacologically active phenothiazine derivatives inhibit bovine brain aldehyde reductase, a pyrazole-insensitive alcohol dehydrogenase. Similar to studies with other enzyme systems,[14] a causal relationship between inhibition of aldehyde reductase and the sedative properties of the phenothiazines has not been established. However, it is of interest that at pH 7·4 trifluoperazine was a more potent inhibitor of aldehyde reductase than chlorpromazine. The extent of inhibition of enzyme activity produced by chlorpromazine was more pronounced than that caused by promethazine. Also, it was observed that chlorpromazine sulfoxide which is known to exert little activity on the central nervous system[16] did not inhibit enzyme activity. The ED_{50} for trifluoperazine in suppression of unconditioned avoidance response in rats was approximately four to five times lower than the ED_{50} for chlorpromazine.[17] On the other hand, promethazine has been reported to be much less effective than chlorpromazine in blocking this response.[18] Apparently, the variation of potency of these agents is not related to differences in the brain permeability or in the rate of metabolism and excretion of these compounds.[19] Whether inhibition of aldehyde reductase activity might account for the sedative effects of the

phenothiazine derivatives remains to be elucidated. It is worthy of note that other sedatives, i.e. barbiturates, also inhibit bovine brain aldehyde reductase.[5]

Studies of the inhibition of aldehyde reductase by the various phenothiazines led to further characterization of the nature of the enzyme-catalyzed reaction. As shown in Figs. 1 and 2, inhibition of aldehyde reductase by chlorpromazine was competitive with either aldehyde or NADPH as the variable substrate. Theorell *et al.*[20] have shown that the reaction catalyzed by liver alcohol dehydrogenase proceeds by an ordered sequence; NAD or NADH binds with the enzyme protein before alcohol or aldehyde respectively. The kinetics of phenothiazine inhibition presented in the present study are inconsistent with an ordered reaction mechanism as described by Cleland.[12] However, the phenothiazine inhibition data are consistent with a random order of addition of substrate to the enzyme as follows:



Therefore, further studies (Figs. 3–6) were performed in order to determine whether the aldehyde reductase-catalyzed reaction proceeded by a random order of addition of substrates.

The above model indicates that either substrate or either product may combine with free enzyme (E). The data presented in Figs. 3 and 4 show that either product (alcohol or NADP) of the reaction caused a competitive inhibition when either aldehyde or NADPH was the variable substrate. Certainly these data are consistent with the random order of addition model. Further evidence for this model is presented in Figs. 5 and 6 where it is indicated that the presence of one substrate does not influence the K_m value of the other substrate. Obviously, binding studies[20] must be performed in order to completely establish a random order of addition of substrate to the enzyme; however, these studies must await a further purification of the enzyme.

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