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Inhibition of a Reduced Nicotinamide Adenine Dinucleotide Phosphate-Linked Aldehyde Reductase from Bovine Brain by Barbiturates

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SUMMARY

The partially purified NADPH-linked aldehyde reductase (alcohol:NADP oxidoreductase, EC 1.1.1.2) from bovine brain has been further characterized, and inhibition of the enzyme by barbiturates has been investigated. In addition to substituted benzaldehydes and phenylethanols, free indoleacetaldehyde was observed to be a substrate for the enzyme. In the present studies, it was found that brain aldehyde reductase is markedly inhibited by barbital, phenobarbital, pentobarbital, and amobarbital, with K_i values ranging from 50 to 400 μM. Inhibition by these agents was reversible and of a noncompetitive type with respect to *p*-nitrobenzaldehyde, indoleacetaldehyde, *p*-hydroxyphenylglycolaldehyde, or NADPH. The enzyme was not markedly inhibited by amobarbital alcohol, a normal metabolite of amobarbital, or by barbituric acid; K_i values for these compounds were found to be 0.6 and 4 mM, respectively. Evidence is presented suggesting that inhibition of aldehyde reductase by the barbiturates is due to the ionized form of the inhibitors. Pyrazole, a potent inhibitor of alcohol dehydrogenase, did not inhibit aldehyde reductase, and the barbiturates had no effect on alcohol dehydrogenase. The differential sensitivity of aldehyde reductase to the inhibitory effects of barbiturates and pyrazole is used to distinguish this enzyme from liver alcohol dehydrogenase.

INTRODUCTION

It is well known that the biogenic monoamines serotonin, dopamine, and norepinephrine are involved in central nervous system function. In the brain these amines may be converted by the action of monoamine oxidase to their corresponding aldehyde intermediates. These aldehyde intermediates may be either oxidized or reduced to their corresponding acid or alcohol metabolite, respectively (1–3). Evidence to date indicates that the biogenic aldehydes as well as the biogenic amines may be involved in CNS function. Sabelli *et al.* (4) have reported that 5-hydroxyindoleacetaldehyde and indoleacetaldehyde produced sedation in 4-day-old chicks, and 5-hydroxyindoleacetaldehyde has been implicated as the chemical mediator of paradoxical sleep (5). In addition, Erwin[2] has shown that the

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[2] In unpublished experiments and in *Dissertation Abstracts* [26, 6096 (1965–1966)] it has been noted that 50–100 mg of 3,4-dihydroxyphenylacetaldehyde administered intraperitoneally to rats together with 75 mg of calcium carbamide (an inhibitor of aldehyde dehydrogenase) per kilogram of body weight produces marked sedation with a loss of righting reflex. Animals regain their righting reflex in 30–40 min.

administration of aldehyde derivatives of dopamine and norepinephrine concomitantly with calcium carbamide causes CNS depression in rats. A series of aliphatic aldehydes which were postulated to undergo aldol condensation to form β-hydroxycarbonyl compounds were found to alter the excitability of an isolated neuron and to slow the various phases of the action potential (6). *p*-Hydroxyphenylglycolaldehyde and 3,4-dihydroxyphenylglycolaldehyde, which are formed from octopamine and norepinephrine in brain, are β-hydroxycarbonyl compounds and, as such, could alter membrane properties of neurons.

Tabakoff and Erwin (7) have recently shown that bovine and rat brain contain an NADPH-dependent aldehyde reductase (alcohol:NADP oxidoreductase, EC 1.1.1.2) which catalyzes the following reaction.

$$RCHO + NADPH + H^+ \rightleftharpoons RCH_2OH + NADP^+$$

They postulated that the enzyme may be responsible for the conversion of the aldehyde intermediates of brain biogenic amines to their corresponding alcohol metabolites. In addition, it was shown that this enzyme was inhibited by amobarbital and pentobarbital. Inasmuch as the barbiturates, which have long been identified as CNS depressants, were inhibitors of the NADPH-dependent aldehyde reductase (7, 8), the present study was undertaken to characterize the effects of barbiturates on this enzyme. In addition, since alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) and NADPH-dependent aldehyde reductase catalyze similar reactions, it was of interest to distinguish between these enzymes further by means of inhibitors.

EXPERIMENTAL PROCEDURE

Materials. All chemicals used were of the highest quality commercially available. Barbituric acid obtained from Eastman Organic Chemicals was recrystallized three times from hot water and dried in a vacuum desiccator over sulfuric acid. Amobarbital alcohol, 5-ethyl-5-(3′-hydroxy-3′-methylbutyl)barbituric acid, was kindly supplied by Dr. Everett W. Maynert, University of Illinois School of Medicine, Chicago. The nucleotide cofactors NAD, NADP, NADH, NADPH, and octopamine, and horse liver alcohol dehydrogenase, were obtained from Sigma Chemical Company. *p*-Nitrobenzaldehyde and indoleacetaldehyde sodium bisulfite complex were purchased from Aldrich Chemical Company. A solution of indoleacetaldehyde was prepared free of sodium bisulfite, just prior to use, by extracting a 0.01 M solution of the sodium bisulfite complex (the solution contained 0.03 M sodium pyrophosphate, pH 9.6) three times with equal volumes of ether. The combined ether extracts were washed three times with water, and the volume was reduced under a stream of nitrogen to one-half the volume of the original solution. The ether extract was then shaken with an equal volume of 0.1 M sodium phosphate, pH 7.0, and the ether phase was completely removed. *p*-Hydroxyphenylglycolaldehyde was prepared from octopamine as described previously (7). The concentration of free indoleacetaldehyde or *p*-hydroxyphenylglycolaldehyde in the 0.1 M sodium phosphate (pH 7.0) solution was assayed by the use of rat liver aldehyde dehydrogenase as previously described by Deitrich, Hellerman, and Wein (9).

Procedure. NADPH-dependent aldehyde reductase was obtained from bovine brain by procedures previously described (7). Bovine brain homogenates were centrifuged at 27,000 × *g* for 30 min, and the resulting supernatant fluid was subjected to fractionation with ammonium sulfate. The protein which precipitated between 40 and 55% ammonium sulfate saturation was chromatographed on a calcium phosphate gel column as previously described, and the enzyme activity eluted from the column was used for kinetic studies. The bovine brain aldehyde reductase used in the present studies was shown not to possess any aldehyde dehydrogenase activity, since no reduction of NAD was observed when the enzyme was incubated with *p*-nitrobenzaldehyde in pyrophosphate buffer at pH 9.6.

Aldehyde reductase activity was assayed spectrophotometrically using a Gilford model 2400 spectrophotometer. The standard reaction mixture consisted of enzyme protein (0.1–0.2 mg), 0.16 mM NADPH, and 0.65

mM aldehyde in sufficient 0.1 M sodium phosphate, pH 7.0, to make a final volume of 3.0 ml. The reaction was initiated by the addition of substrate, and initial rates of NADPH oxidation were followed at 340 mμ and 25°. For studies of inhibition of bovine brain aldehyde reductase or horse liver alcohol dehydrogenase activity by various agents, enzyme, NADPH or NADH, and inhibitor were incubated in 0.1 M sodium phosphate buffer, pH 7.0, for 2 min at room temperature before the reaction was initiated by the addition of aldehyde substrate. Alcohol oxidation by the aldehyde reductase or alcohol dehydrogenase was measured by monitoring NADP or NAD reduction at 340 mμ in a system containing 1.7 mM NADP or NAD, enzyme protein, and 8.6 mM *p*-hydroxyphenylethanol in 0.1 M sodium pyrophosphate, pH 9.6.

RESULTS

Inhibition of bovine brain aldehyde reductase by barbiturates. As shown in Table 1, under the assay conditions employed, 0.1 mM sodium phenobarbital or sodium pentobarbital inhibited the rate of oxidation of NADPH by aldehyde reductase approximately 50% with *p*-nitrobenzaldehyde as substrate. Under similar conditions, 33 μM barbiturate produced 30–40% inhibition of enzyme activity. When indoleacetaldehyde was the substrate, inhibition by the barbiturates was somewhat less pronounced; similar results were obtained with *p*-hydroxyphenylglycolaldehyde as the substrate. Incubation, at 25°, of the enzyme protein with either sodium phenobarbital or sodium pentobarbital for 20 min before addition of substrate did not increase the extent of inhibition. The inhibition produced by either of these barbiturates was completely reversed by dialysis against 200 volumes of sodium phosphate, pH 7.0 (Table 2), or by dilution of the inhibitor.

When the enzyme kinetic data were plotted according to the Lineweaver-Burk method, the inhibition of aldehyde reductase by the barbiturates was of a noncompetitive nature with respect to *p*-nitrobenzaldehyde, indoleacetaldehyde, *p*-hydroxyphenylglycolaldehyde, or NADPH (Figs. 1 and 2). Inhibitor constants (K_i values) for the various barbiturates were calculated from the graphic treatment of the kinetic data (10). At least two concentrations of inhibitor

TABLE 1

Inhibition of aldehyde reductase by barbiturates

Enzyme activity was determined as described in the text. Sufficient substrate to give a final concentration of 0.65 mM was added to initiate the reaction. The assay system contained 0.11 mg of enzyme protein (specific activity, 90 nmoles of NADPH oxidized per minute per milligram of protein), 0.16 mM NADPH, various concentrations of inhibitors as indicated, and 0.1 M sodium phosphate, pH 7.0, in a final volume of 3.0 ml.

Substrate	Inhibitor	Inhibitor concentration	Inhibition
		$M \times 10^4$	%
p-Nitrobenzaldehyde	Sodium phenobarbital	10.0	72.3
p-Nitrobenzaldehyde	Sodium phenobarbital	1.0	49.8
p-Nitrobenzaldehyde	Sodium phenobarbital	0.33	30.0
p-Nitrobenzaldehyde	Sodium pentobarbital	10.0	74.8
p-Nitrobenzaldehyde	Sodium pentobarbital	1.0	52.8
p-Nitrobenzaldehyde	Sodium pentobarbital	0.33	39.4
Indoleacetaldehyde	Sodium phenobarbital	3.3	37.5
Indoleacetaldehyde	Sodium phenobarbital	1.0	20.7
Indoleacetaldehyde	Sodium pentobarbital	3.3	44.4
Indoleacetaldehyde	Sodium pentobarbital	1.0	31.0

were used to obtain the values listed in Table 3. The K_i values ranged from 4.0 mM for barbituric acid with *p*-nitrobenzaldehyde as substrate to 60 μM for sodium pentobarbital. The relatively high K_i value for barbituric acid shows that this unsubstituted malonylurea derivative is a poor inhibitor of aldehyde reductase when compared to the other barbiturates. The alcohol derivative of amobarbital, 5-ethyl-5-(3′-hydroxy-3′-methylbutyl)barbituric acid, which is considered to be the normal metabolite of amobarbital *in vivo* (12), was found to have a K_i value approximately 2 times higher than sodium amobarbital.

TABLE 2

Reversal of inhibition of bovine brain aldehyde reductase by dialysis against buffer

Enzyme protein, 0.5 mg (specific activity, 90 nmoles of NADPH oxidized per minute per milligram of protein), was incubated with a final concentration of barbiturate of 0.167 mM for 5 min. A sample was then withdrawn, and the activity was determined with *p*-nitrobenzaldehyde as substrate. The remainder of the protein was then dialyzed against 200 volumes of 0.05 M sodium phosphate, pH 7.0, at 4° for 24 hr. The dialysis medium was changed three times.

Inhibitor	Aldehyde reductase activity	
	Before dialysis	After dialysis
	%	%
None	100	100
Sodium pentobarbital	51	99
Sodium phenobarbital	63	97

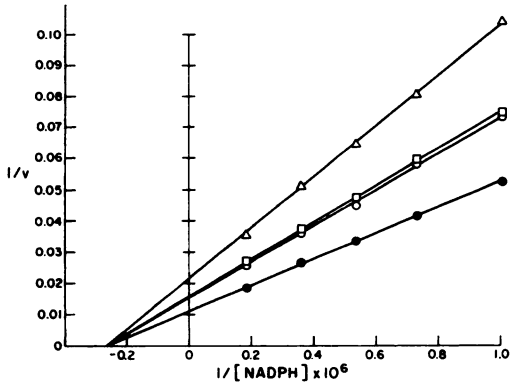

FIG. 1. *Kinetics of bovine brain aldehyde reductase inhibition by barbiturates*

The reaction mixtures were as described in Table 1, and the assay conditions were as described in the text. The ordinate gives the reciprocal of the velocity (nanomoles of NADPH oxidized per minute per milligram of protein), and the abscissa gives the reciprocal of the molarity of NADPH. ●——●, control without inhibitor; □——□, 33 μM pentobarbital; ○——○, 33 μM phenobarbital; △——△, 100 μM phenobarbital.

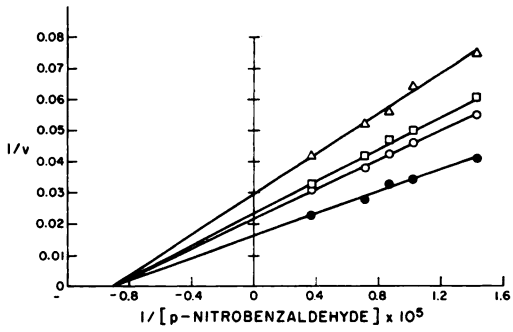

FIG. 2. *Kinetics of aldehyde reductase inhibition by barbiturates*

Experimental conditions were as described in Fig. 1, except that the specific activity of the enzyme was 62.5 nmoles of NADPH oxidized per minute per milligram of protein. The abscissa gives the reciprocal of the molarity of *p*-nitrobenzaldehyde. ●——●, control without inhibitor; □——□, 33 μM pentobarbital; ○——○, 33 μM phenobarbital; △——△, 100 μM phenobarbital. Similar results were obtained when indoleacetaldehyde or *p*-hydroxyphenylglycolaldehyde was the substrate in place of *p*-nitrobenzaldehyde.

Indoleacetaldehyde as a substrate for aldehyde reductase. When indoleacetaldehyde sodium bisulfite was incubated with the enzyme system, no oxidation of NADPH could be observed (7). In the present study sodium bisulfite was shown to be a potent inhibitor of aldehyde reductase (Table 4) when the initial rates of aldehyde oxidation or alcohol reduction (Table 5) were measured (see DISCUSSION). Free indoleacetaldehyde was found to be a substrate when incubated with aldehyde reductase and NADPH. A Michaelis-Menten constant of 40 μM was determined for indoleacetaldehyde.

Effect of pH on inhibition of aldehyde reductase by barbiturates. The extent of inhibition of aldehyde reductase by sodium pheno-

TABLE 3

Inhibitor constants for various barbiturates

K_i values were determined from Lineweaver-Burk plots similar to those shown in Figs. 1 and 2, and as described in the text. The values reported are averages of values obtained with at least two inhibitor concentrations.

Inhibitor	pK_a[a]	Substrate[b]	k_i[c]	k_i'[d]
			$M \times 10^4$	$M \times 10^5$
Phenobarbital	7.41	NADPH	1.2	3.4
	7.41	*p*-Nitrobenzaldehyde	1.5	4.2
	7.41	Indoleacetaldehyde[e]	3.0	8.4
	7.41	*p*-Hydroxyphenylglycolaldehyde	1.9	5.3
Pentobarbital	8.11	NADPH	0.6	0.4
	8.11	*p*-Nitrobenzaldehyde	1.5	1.0
	8.11	Indoleacetaldehyde	4.0	2.8
	8.11	*p*-Hydroxyphenylglycolaldehyde	2.9	2.0
Amobarbital	7.94	NADPH	2.9	3.0
	7.94	*p*-Nitrobenzaldehyde	2.8	2.9
Barbital	7.91	NADPH	1.0	1.1
	7.91	*p*-Nitrobenzaldehyde	1.2	1.3
Amobarbital alcohol		*p*-Nitrobenzaldehyde	6.0	
Barbituric acid	4.98	*p*-Nitrobenzaldehyde	40.0	396.4

[a] Values from ref. 11.

[b] Substrate refers to the reactant which was varied while the other factors were kept constant.

[c] K_i values are based on total inhibitor concentrations.

[d] The K_i values were calculated using the concentrations of ionized form of barbiturate present in the reaction mixture as described in the text.

[e] The K_m value for indoleacetaldehyde was 40 μM.

barbital was shown to be dependent on the pH of the assay system (Table 6). The concentration of the ionized form of phenobarbital in each system was determined by the use of the Henderson-Hasselbalch relationship, utilizing a pK_a value of 7.4 (11). The data presented in Fig. 3 show that the amount of inhibition of aldehyde reductase activity was dependent upon the concentration of the ionized form of sodium phenobarbital. Various concentrations of the ionized barbiturate were obtained by varying the pH of the assay system or by varying the total inhibitor concentration at pH 7.0. As shown in Table 3, the K_i values, based on the concentration of the ionized form of various barbiturates, ranged from approximately 4 mM for barbituric acid to approximately 4 μM for pentobarbital.

Comparison of bovine brain aldehyde reductase with horse liver alcohol dehydrogenase. As shown in Table 4, sodium bisulfite inhibits the reduction of *p*-nitrobenzaldehyde by liver alcohol dehydrogenase or aldehyde reductase. In addition, the initial rate of *p*-hydroxyphenylethanol oxidation by aldehyde reductase or alcohol dehydrogenase was also inhibited by sodium bisulfite (Table 5). These results indicate that inhibition of the aldehyde reductase activity by sodium bisulfite is not totally due to a reduction of available substrate by the formation of a bisulfite adduct with the aldehyde. Pyrazole, 0.01 M, was shown to inhibit purified liver alcohol dehydrogenase activity approximately 96 and 65% with NADH and NADPH as cofactors, respectively (Table 4). However, 0.01 M pyrazole

TABLE 4

Comparison of inhibition of aldehyde reductase and alcohol dehydrogenase by various agents

The reaction mixtures for the determination of horse liver alcohol dehydrogenase activity consisted of enzyme protein (0.025 mg), 0.16 mM NADH or NADPH, and 0.5 mM *p*-nitrobenzaldehyde in 0.05 M sodium phosphate, pH 7.0. The reaction mixture for determination of bovine brain aldehyde reductase activity consisted of enzyme protein (0.115 mg), 0.16 mM NADPH, and 0.5 mM *p*-nitrobenzaldehyde in 0.05 M sodium phosphate, pH 7.0. The reaction mixtures were incubated with inhibitor for 2 min at room temperature before the addition of substrate to start the reaction. The rates of oxidation of reduced nucleotide cofactors were monitored spectrophotometrically at 340 mμ and 25°.

Inhibitor	Concentration	Inhibition	
		Alcohol dehydrogenase	Aldehyde reductase
	M	%	%
Pyrazole	10^{-2}	96.0	
	10^{-2}	65.0[a]	0
Sodium phenobarbital	10^{-3}	0	
	10^{-3}	0[a]	76.0
Sodium bisulfite	10^{-3}	55.1	
	5×10^{-4}	37.0	76.0
	2.5×10^{-4}	30.6	64.7
	1.7×10^{-4}		48.2

[a] NADPH was the cofactor for alcohol dehydrogenase instead of NADH.

had no effect on bovine brain aldehyde reductase activity, indicating that this enzyme preparation was free of any classic alcohol dehydrogenase activity. Sodium phenobarbital, 1 mM, on the other hand, inhibited aldehyde reductase 76%, while no significant inhibition of alcohol dehydrogenase was observed with either NADH or NADPH as the cofactor.

When aldehyde reductase obtained by ammonium sulfate fractionation was incubated in the presence of 0.2 mM *p*-chloromercuribenzoate, inhibition of 53% was ob-

TABLE 5

Comparison of inhibition of alcohol-oxidizing capacities of bovine brain aldehyde reductase and horse liver alcohol dehydrogenase by sodium bisulfite

Aldehyde reductase activity was determined with 0.35 mg of enzyme protein (specific activity, 90 nmoles of NADPH oxidized per minute per milligram of protein), with 1.7 mM $NADP^+$ and 8.6 mM *p*-hydroxyphenylethanol as substrates, in 0.03 M sodium pyrophosphate, pH 9.6. Alcohol dehydrogenase activity was determined with 0.17 mM NAD^+ and 8.6 mM *p*-hydroxyphenylethanol as substrates in 0.1 M sodium pyrophosphate, pH 9.0. The reaction mixtures were incubated with inhibitor for 2 min at room temperature before substrate was added to initiate the reaction.

Sodium bisulfite (1 mM)	Aldehyde reductase activity[a]	Alcohol dehydrogenase activity[b]
−	2.5	135
+	0	102

[a] Expressed as nanomoles of $NADP^+$ reduced per minute per milligram of protein.

[b] Expressed as nanomoles of NAD^+ reduced per minute per milligram of protein.

TABLE 6

Effect of pH on inhibition of aldehyde reductase activity by phenobarbital

The reaction mixtures contained 0.115 mg of enzyme protein (specific activity, 90 nmoles of NADPH oxidized per minute per milligram of protein), 0.16 mM NADPH, and 0.65 mM *p*-nitrobenzaldehyde in 0.1 M sodium phosphate, at the pH values indicated, in a final volume of 3 ml. The phenobarbital concentration was 1 mM.

pH	Inhibition[a]
	%
5.8	38.0
6.0	58.0
6.45	69.7
7.0	77.0
7.5	82.0

[a] Values are expressed as percentages of the rate of enzyme activity at various pH values in the absence of inhibitor.

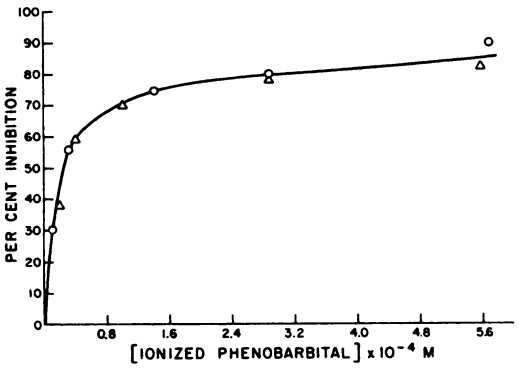

FIG. 3. *Inhibition of aldehyde reductase activity by various concentrations of ionized phenobarbital*

The experimental conditions were similar to those described in Table 6. △, values obtained by determining the enzyme activity of reaction mixtures containing 1 mM phenobarbital at various pH values; ○, values obtained by varying the total concentration of sodium phenobarbital in the assay mixture at pH 7.0. The concentration of ionized phenobarbital in the reaction mixture was calculated from the Henderson-Hasselbach equation as described in the text and plotted on the abscissa.

tained after 10 min. Although inhibited by this sulfhydryl reagent, the relative crudeness of the enzyme preparation may not reveal its full sensitivity to this compound.

DISCUSSION

It has been shown that pyrazole inhibits alcohol dehydrogenase by the formation of a stable complex among NAD, enzyme, and pyrazole (13). With NADPH as a cofactor the enzyme was not markedly inhibited by low concentrations of pyrazole, but at higher concentrations inhibition of alcohol dehydrogenase was observed even with NADPH as a cofactor. No inhibition of aldehyde reductase could be demonstrated with pyrazole concentrations as high as 0.01 M. Although pyrazole was ineffective as an inhibitor of aldehyde reductase, a series of barbiturates were found to be potent inhibitors of this enzyme. Alcohol dehydrogenase, on the other hand, was not inhibited by the barbiturates with either NADH or NADPH as the cofactor. The barbiturates have recently been shown to interact specifically with adenine derivatives by the formation of hydrogen bonds with these compounds (14). However, the reduction of available cosubstrate cannot explain the differential inhibition of aldehyde reductase and alcohol dehydrogenase. A biuret-like reaction between metal ions and high concentrations of barbital has been reported (15), but again it is unlikely that such a reaction could account for the inhibition of aldehyde reductase, particularly since the barbiturates were ineffective as inhibitors of alcohol dehydrogenase. Raskin and Sokoloff (16) suggested that brain tissue might contain an alcohol dehydrogenase similar to liver alcohol dehydrogenase. The use of barbiturates and pyrazole as selective inhibitors of NADPH-dependent aldehyde reductase and alcohol dehydrogenase may provide a method for distinguishing between these enzymes.

The K_i values obtained for the various physiologically active barbiturates are low, i.e., 10–100 μM. These levels are consistent with levels determined in brains of rats after the administration of sodium pentobarbital (17). Barbiturates are classified as to their therapeutic effectiveness according to the time of onset and the duration of the hypnotic effects they produce. Such properties are related to distribution of barbiturates between lipid-water systems and the rate of metabolism of these compounds. These parameters, which are many times related to the potency of a barbiturate, cannot be determined in a system *in vitro* such as the one employed in these experiments, but the duration of action of a barbiturate and the intensity of its effect on a neuron are not necessarily equivalent. Thus, the barbiturates used in this study were compared with their metabolites and with the physiologically inactive barbituric acid as to their efficiency in inhibiting aldehyde reductase. The alcohol derivative of amobarbital has been reported to be only one-half as potent a hypnotic as the parent compound (18), and other alcohol derivatives of the barbiturates have been shown to have no hypnotic action (19). In the present study the alcohol derivative of amobarbital was less effective than sodium amobarbital as an inhibitor of aldehyde reductase. Barbituric

acid, which is devoid of hypnotic activity, was a poor inhibitor of aldehyde reductase and was shown to have a K_i value of approximately 4 mM. The undissociated forms of barbituric acid derivatives have been shown to be transported across cellular membranes, and these same forms have been postulated to be active intracellularly in preventing cellular division (20). Hardman, Moore, and Lum (21) have shown the unionized form of the barbiturates to depress cardiac tissue. The present study, however, indicates that the ionized form of phenobarbital is the inhibitor of bovine brain aldehyde reductase. The possible physiological significance of these findings is currently under investigation, with the working hypothesis that the CNS-depressant effects of the barbiturates may be causally related to an increase in brain biogenic aldehydes as a result of inhibition of the enzyme aldehyde reductase.

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