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INHIBITION OF BOVINE BRAIN ALDEHYDE REDUCTASE BY ANTICONVULSANT COMPOUNDS *IN VITRO**

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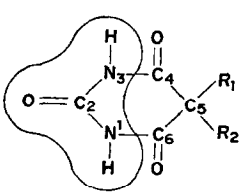
Abstract—The catalytic activity of partially purified NADPH-linked aldehyde reductase (alcohol: NADP oxidoreductase, EC 1.1.1.2) from bovine brain was markedly inhibited *in vitro* by anticonvulsant compounds. In general, the ability of these drugs to inhibit aldehyde reductase *in vitro* paralleled their anticonvulsant activity. Inhibition by various barbiturates, hydantoins or succinimides was non-competitive with either NADPH or aldehyde as the variable substrate, whereas the 2,4-oxazolidinediones produced a mixed type of inhibition. Inhibition by all ionizable compounds was found to vary with the pH of the reaction mixture, while the non-ionizable substances, paradione, trimethadione, methsuximide, 3-methyl-5-ethyl-5-phenylhydantoin, were not inhibitory. At pH 7·0 the inhibitor constants (K_i values) for phenobarbital, 5,5-diphenylhydantoin, 5,5-dimethyloxazolidinedione and ethosuximide were $1{\cdot}2 \times 10^{-4}$ M, $1{\cdot}7 \times 10^{-4}$ M, $4{\cdot}7 \times 10^{-4}$ M and $5{\cdot}5 \times 10^{-3}$ M respectively. The possibility that inhibition of brain NADPH-linked aldehyde reductase by these agents is concerned with their anticonvulsant actions is discussed.

IT HAS BEEN shown that the catalytic activity of NADPH-linked aldehyde reductase from brain tissue is inhibited by various barbituric acid derivatives.[1,2] In these studies, we[2] showed that the K_i values for the hypnotic-anticonvulsant barbiturates were $1–3 \times 10^{-4}$ M, while the K_i value for barbituric acid was 4×10^{-3} M.

The barbituric acid derivatives possess the "ureido"-pharmacophoric grouping:



This is noted not only as a chemical grouping related to hypnotic activity but also as an entity concerned with anticonvulsant activity.[3] Anticonvulsant substances other

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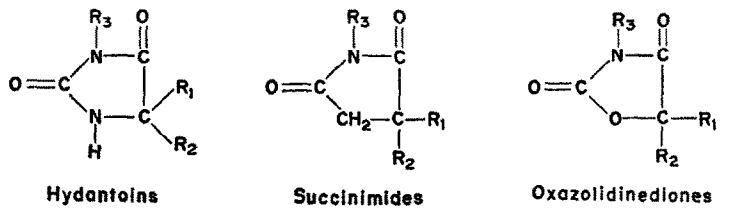
than the barbiturates which possess similar groupings are the hydantoin derivatives, the oxazolidinedione compounds and the succinimide congeners:



The effects of some of these compounds on biochemical processes in the brain, including inhibition of succinic semialdehyde dehydrogenase,[4] inhibition of protein synthesis,[5] and uncoupling of phosphorylation from oxidation[6] have been reported. However, relatively high concentrations were required to produce significant inhibitions of these processes. Certainly, the biochemical mechanism by which these agents elicit their anticonvulsant actions is unknown.

It was the purpose of the present study to characterize the effects of various structurally similar anticonvulsant agents on brain NADPH-linked aldehyde reductase activity.

EXPERIMENTAL

Materials. All chemicals were of the highest quality commercially available. Hydantoin was obtained from Eastman Organic Chemicals. All other hydantoin derivatives were obtained from Parke-Davis & Company, and the oxazolidinedione derivatives were furnished by Abbot Laboratories. The *d*- and *l*-mephobarbital was kindly supplied by Dr. Enoch Gordis.* The optical rotation was determined by Dr. J. Cann.† The nucleotide co-factor NADPH and calcium phosphate-gel were obtained from Sigma Chemical Company and *p*-nitrobenzaldehyde was purchased from Aldrich Chemical Company. In all studies, the concentration of aldehyde solutions was assayed by the use of rat liver aldehyde dehydrogenase, as previously described by Deitrich *et al.*[7] The concentration of NADPH solutions was determined spectrophotometrically, assuming a molar absorptivity value of $6{\cdot}22 \times 10^3$ at 340 nm.

Procedure. NADPH-linked aldehyde reductase was obtained from bovine brain by procedures previously described.[1] 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 per cent ammonium sulfate saturation was chromatographed on a calcium phosphate-gel cellulose column as previously described, and enzyme activity eluted from the column was used for kinetic studies. This preparation was shown to be devoid of NAD-linked aldehyde dehydrogenase[8] and the NADH-linked aldehyde reductases.[9]

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 with a specific activity of approximately 40 nmoles NADPH

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oxidized per min per mg of protein), 0·16 mM NADPH, 0·25 mM *p*-nitrobenzaldehyde and 0·1 mM sodium phosphate, pH 7·0, in a total volume of 3·0 ml. The reaction was initiated by the addition of substrate, and the initial rates of NADPH oxidation were followed at 340 nm and 25°. In some instances the rate of NADPH oxidation was determined fluorometrically with a Farrand spectrophotofluorometer equipped with a Sargent SRL recorder.

RESULTS

As shown in Figs. 1 and 2, the *d*- and *l*-isomers of mephobarbital were inhibitors of the bovine brain NADPH-linked aldehyde reductase. The K_i values for these isomers (Table 1) are comparable to those obtained with other hypnotic-anticonvulsant

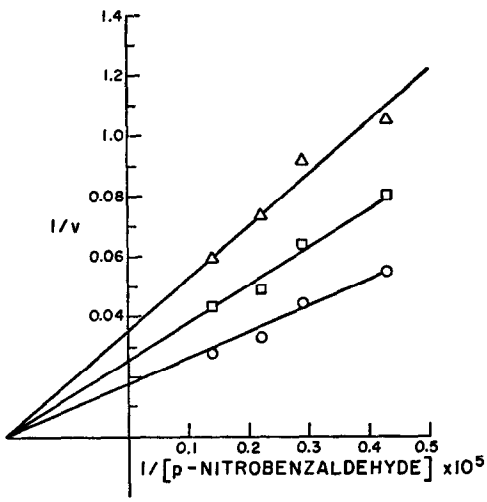


FIG. 1. Kinetics of bovine brain aldehyde reductase inhibition by *d*-mephobarbital. The reaction mixtures and assay conditions were as described in the text. NADPH, final concentration, was 0·16 mM with varying concentrations of *p*-nitrobenzaldehyde in 0·1 M sodium phosphate, pH 7·0, and with 0·2 mg enzyme protein (specific activity approximately 40 nmoles NADPH oxidized/min/mg protein) in a final volume of 3·0 ml. The ordinate gives the reciprocal of the velocity (nmoles NADPH oxidized/min/mg protein). (○) Control without inhibitor; (□) $3{\cdot}3 \times 10^{-4}$ M *d*-mephobarbital; (△) 10^{-3} M *d*-mephobarbital.

barbiturates. Inasmuch as Buch *et al.*,[10] and Gordis[11] reported that *d*-mephobarbital (*d*-3-*N*-methyl-5-ethyl-5′-phenylbarbituric acid) possessed little hypnotic activity, these results would indicate that inhibition of the enzyme *in vitro* may not be correlated with the central nervous depressant activity of these compounds. However, whether both optical isomers inhibit the enzyme activity *in vivo* must be known before such a conclusion is proven. On the other hand, the structural activity relationships for inhibition of the enzyme by various barbiturates may be correlated with the anticonvulsant actions of these compounds; particularly since it has been shown that both

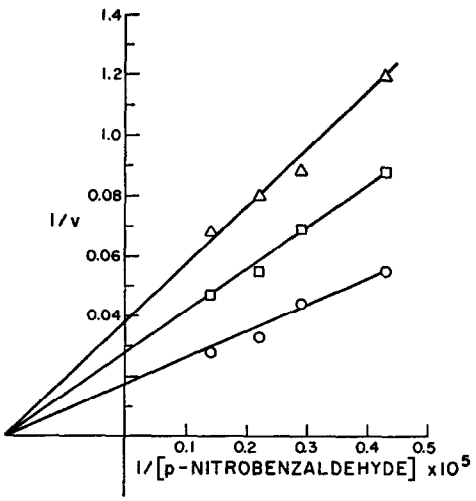


FIG. 2. Kinetics of bovine brain aldehyde reductase inhibition by *l*-mephobarbital. Experimental conditions were as described in Fig. 1, except that the inhibitor was *l*-mephobarbital. (○) Control without inhibitor; (□) $3{\cdot}3 \times 10^{-4}$ M *l*-mephobarbital; (△) 10^{-3} M *l*-mephobarbital.

the *d*- and *l*-isomers of mephobarbital possess anticonvulsant activity.* These observations led to a study of the effects of other anti-convulsants on brain NADPH-linked aldehyde reductase activity.

TABLE 1. COMPARISON OF INHIBITOR CONSTANTS FOR VARIOUS BARBITURATES WITH NADPH-LINKED ALDEHYDE REDUCTASE*

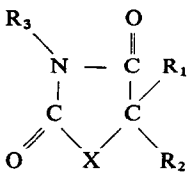
Inhibitor	K_i ($M \times 10^{-4}$)	Inhibitor	K_i ($M \times 10^{-4}$)
Pentobarbital	1·5	Barbituric acid	40·0
Phenobarbital	1·2	*d*-Mephobarbital	8·1
Barbital	1·2	*l*-Mephobarbital	6·6
Amobarbital	2·8		

* K_i values were determined from Lineweaver–Burk plots, as shown in Figs. 1 and 2, with two different concentrations of inhibitors. *p*-Nitrobenzaldehyde was the variable substrate and the NADPH final concentration was 0·16 mM. The reaction conditions were as described in the text and the values reported are averages of at least three separate experiments.

The data presented in Table 2 shows that various compounds with known anticonvulsant activity produced a marked inhibition of NADPH oxidation with *p*-nitrobenzaldehyde as substrate. These data indicate that the non-ionizable compounds, ethotoin, trimethadione and paramethadione, and methsuximide were extremely poor

* In a personal communication, Dr. F. Hoffmeister, Bayer Company, Wuppertal, West Germany, noted that the hypnotic dose for *d*-mephobarbital (300 mg/kg body wt) was approximately 3-fold greater than that obtained for *l*-mephobarbital (89 mg/kg). However, the ED_{50} values for protection against maximal electroshock seizures, when administered subcutaneously 30 min before electroshock, were quite similar for the *d*- and *l*-isomers. The ED_{50} values for *d*-mephobarbital and *l*-mephobarbital were 20·8 and 14·9 mg/kg body weight respectively.

TABLE 2. INHIBITION OF NADPH-LINKED ALDEHYDE REDUCTASE BY VARIOUS HYDANTOIN, OXAZOLIDINEDIONE AND SUCCINIMIDE ANTICONVULSANTS*



X = N for hydantoins
X = O for oxazolidinedione
X = CH_2 for succinimides

Inhibitor	R_3	Inhibitor concn ($M \times 10^4$)	Inhibition (%)
Diphenylhydantoin	H	1·0	40·6
Ethotoin	C_2H_5	10·0	5·0
Dimethadione	H	3·3	53·6
Trimethadione	CH_3	10·0	12·0
Paramethadione	CH_3	10·0	9·8
Ethosuximide	H	3·3	40·3
Methsuximide	CH_3	10·0	15·2

* Enzyme activity was determined as described in the text and Table 1, with a *p*-nitrobenzaldehyde concentration of 0·25 mM. The assay system contained the concentrations of various inhibitors as indicated.

inhibitors compared with their dealkylated, ionizable derivatives. It was previously reported that inhibition of brain NADPH-linked aldehyde reductase activity by barbiturates was due to the ionized form of these compounds.[2] Likewise, it was of interest to determine whether inhibition of the enzyme activity by various hydantoins, oxazolidinediones and succinimides was due to the ionized form of these substances. As shown in Table 3, when the pH of the reaction mixture was increased from 6·0 to 8·0, the per cent inhibition by the ionizable anticonvulsant compounds, dimethadione, hydantoin, diphenylhydantoin, ethosuximide, also increased. However, the per cent inhibition by compounds with substitutions at position 3, which prevent ionization, such as trimethadione, paramethadione, ethotoin and methsuximide, was not altered by varying the pH from 6·0 to 8·0. The concentration of the ionized form of the various compounds in each assay system was determined by the use of the Henderson–Hasselbach relationship utilizing the p*Ka'* values listed in Table 4 for the various ionizable compounds. 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 the various anticonvulsant compounds. Various concentrations of the ionized anticonvulsant compounds were obtained by varying the pH of the assay system or by varying the total inhibitor concentration at pH 7·0.

Kinetics of inhibition of aldehyde reductase by diphenylhydantoin, ethosuximide or dimethadione are shown in Fig. 4. Inhibition by these compounds was of a non-competitive type with either NADPH or *p*-nitrobenzaldehyde as the variable substrate. A similar type of inhibition was observed with all hydantoin and succinimide, as well as barbiturate derivatives (Figs. 1 and 2). Dimethadione produced an inhibition

TABLE 3. EFFECT OF pH ON INHIBITION OF ALDEHYDE REDUCATSE ACTIVITY BY VARIOUS ANTICONVULSANTS*

pH	Inhibition (%)					
	DMO†	TMO‡	DPH§	ETH‖	ETS¶	Pb**
6·0	33	5	3	7	32	58
6·5	55	8	30	9	44	69
7·0	57	14	40	9	53	77
7·5	61	5	47	8	69	82
8·0	63	0	63	8	81	

* The reaction mixtures contained 0·15 mg enzyme protein (specific activity, 40 nmoles NADPH oxidized/min/mg of protein), 0·16 mM NADPH and 0·25 mM *p*-nitrobenzaldehyde in 0·1 M sodium phosphate, at the pH values indicated, in a final volume of 3 ml. The concentrations of various inhibitors were as indicated in the footnotes. Values for inhibition are expressed as percentages of the rate of enzyme activity at various pH values in the absence of inhibitor.

† 5,5-Dimethyloxazolidinedione (dimethadione), $3·3 \times 10^{-4}$ M.

‡ 3-Methyl-5,5-dimethyloxazolidinedione (trimethadine), 10^{-3} M.

§ 5,5-Diphenylhydantoin, $6·6 \times 10^{-5}$ M.

‖ 3-Ethyl-5′-phenyl-5-ethylhydantoin (ethotoin), 10^{-3} M).

¶ 5-Phenyl-5-ethyl-succinimide (ethosuximide), 10^{-3} M.

** Phenobarbital, 10^{-3} M.

TABLE 4. INHIBITOR CONSTANTS FOR VARIOUS ANTICONVULSANTS*

Anticonvulsant	p*K*a†	Type of inhibition	K_i‡ (M × 10^4)	K'_i§ (M × 10^4)
Phenobarbital	7·41	Noncompetitive	1·2	0·24
Diphenylhydantoin	8·05	Noncompetitive	1·7	0·14
Dimethadione	6·2	Mixed	4·7	3·8
5-Ethyl-methyl-oxazolidinedione	6·2	Mixed	5·8	4·7
Trimethadione		Noncompetitive	23·0	
Paramethadione		Noncompetitive	49·0	
Ethosuximide	9·1	Noncompetitive	5·4	0·036
Methsuximide		Noncompetitive	50·5	
Phensuximide		Noncompetitive	70·0	
Ethotoin		Noncompetitive	400·0	
Hydantoin	9·12	Noncompetitive	120·0	0·78

* K_i values were determined from Lineweaver–Burk plots similar to those shown in Fig. 4, and as described in the text. The values reported are averages of values obtained with at least two inhibitor concentrations.

† Values from references 12, 13, 14. Value for the p*K*a for hydantoin was determined from the change in absorption of aqueous solutions at 250 nm as a function of pH values.

‡ K_i values were calculated using total inhibitor concentrations at pH 7·0, and the values were calculated from the intercepts of the Lineweaver–Burk plots with NADPH or aldehyde as the variable substrate.

§ K'_i values were calculated using the concentrations of ionized form of inhibitor.

of the type often referred to as a "mixed inhibition"[15] but also considered to be of a noncompetitive nature.[16]

As shown in Table 4, the K_i value for diphenylhydantoin was comparable to that obtained for phenobarbital and was 70- to 235-fold lower than the values obtained

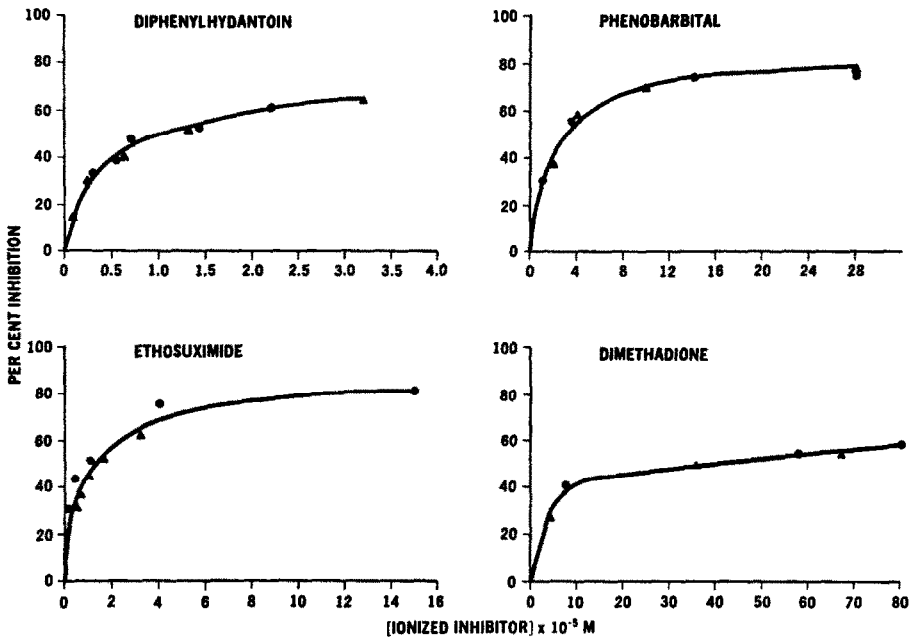


FIG. 3. Inhibition of aldehyde reductase activity by various concentrations of ionized anticonvulsant compounds. The experimental conditions were similar to those described in Table 3. (▲) Values obtained by determining the enzyme activity of reaction mixtures containing 1 mM ethosuximide or phenobarbital, $6{\cdot}6 \times 10^{-5}$ M diphenylhydantoin, or $3{\cdot}3 \times 10^{-4}$ M dimethadione at various pH values; (●) values obtained by varying the total concentration of inhibitor in the assay mixture at pH 7·0. The concentrations of ionized inhibitor in the reaction mixture were calculated from the Henderson–Hasselbach equation as described in the text and plotted on the abscissa.

for hydantoin and ethotoin respectively. Inhibition produced by dimethadione and 5-ethyl, 5-methyl-2,4-oxazolidinedione was more marked than inhibition by either trimethadione or paramethadione. As shown in Table 4, the succinimide anticonvulsant, ethosuximide, produced marked inhibition of aldehyde reductase activity (K_i value of 0·54 mM), whereas the N_3-methyl derivatives, methsuximide and phensuximide, were weak inhibitors with K_i values of 5·0 and 7·0 mM respectively. The K_i values based on the concentrations of the ionized form of the various compounds (K'_i values) were $1{\cdot}4 \times 10^{-5}$ M, $3{\cdot}6 \times 10^{-6}$ M, $2{\cdot}4 \times 10^{-5}$ M and $3{\cdot}8 \times 10^{-4}$ M for diphenylhydantoin, ethosuximide, phenobarbital and dimethadione respectively.

DISCUSSION

In earlier work[1,2,17] we postulated that the barbiturate-sensitive NADPH-linked aldehyde reductase from brain tissue may be responsible for the conversion of biogenic aldehydes to their corresponding alcohol metabolites. Recently this suggestion was confirmed by the reports of Davis *et al.*[18] and Tabakoff *et al.*[19] Davis *et al.*[18] demonstrated that the primarily NADPH-dependent conversion of ^{14}C-norepinephrine to the 3,4-dihydroxyphenylglycol metabolite in brain stem homogenates was inhibited by barbiturates. A working hypothesis for the present studies is that the highly reactive aldehyde intermediates of biogenic amine metabolism may be concerned with some

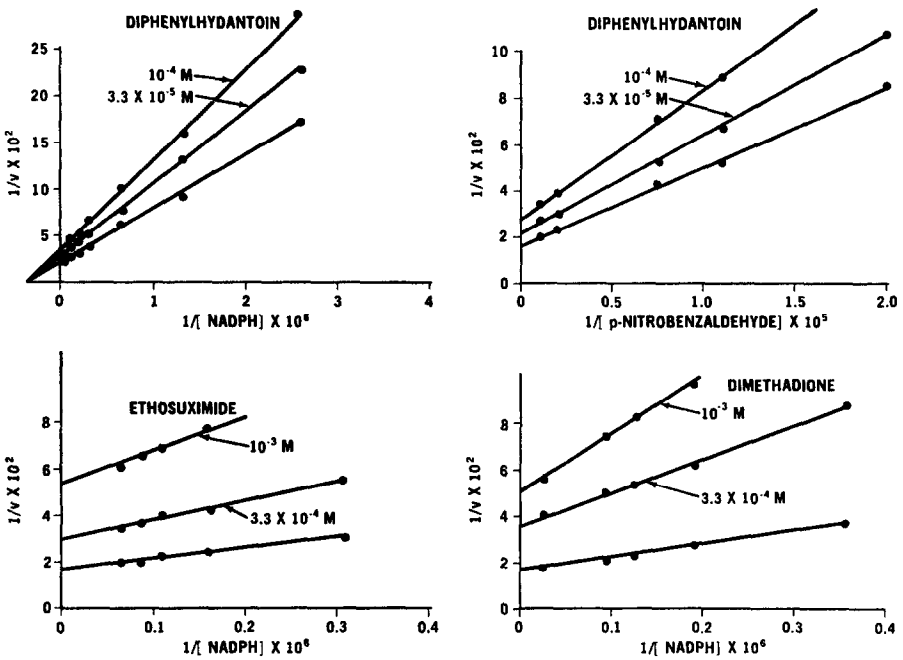


FIG. 4. Kinetics of bovine brain aldehyde reductase inhibition by various anticonvulsants. Experimental conditions were as described in Figs. 1 and 2. The concentrations of the various inhibitors are as indicated; control values, obtained without inhibitor, are indicated by the absence of an inhibitor concentration. Either *p*-nitrobenzaldehyde or NADPH was the variable substrate. When present as the nonvariable substrate, *p*-nitrobenzaldehyde or NADPH was 0·25 or 0·16 mM respectively. The ordinate gives the reciprocal of the velocity (nmoles NADPH oxidized per min per mg protein).

of the actions of various agents which are inhibitors of the metabolism of these aldehydes. In the past several years it has been shown that biogenic aldehydes formed *in vitro*[20] or *in vivo*[21] may become what appears to be irreversibly bound to various cellular components. In addition, inhibitors of biogenic aldehyde metabolism have been shown to enhance the formation of various isoquinoline derivatives in brain tissue *in vitro*.[18,22] Although the significance of these observations remains to be determined, they nonetheless must be considered in accounting for the biochemical basis of action of the barbiturates[2] and other agents which inhibit biogenic aldehyde metabolism.

In the present study we have shown that *d*-mephobarbital, which possesses little hypnotic activity,[10,11] has practically the same K_i value (Table 1) for inhibition of bovine brain aldehyde reductase as the hypnotically active *l*-isomer. However, both isomers are potent anticonvulsants. Also, as shown in Table 1 (see reference 2), a correlation exists between the extent of inhibition of aldehyde reductase and the anti-convulsant activity of various barbiturates. The data presented in Tables 2 and 4 clearly show that other anticonvulsants of the hydantoin, succinimide and oxazolidinedione types are inhibitors of bovine brain NADPH-linked aldehyde reductase. The data indicate that those compounds possessing an alkyl substitution at N_3, which are not capable of ionizing, are extremely weak inhibitors of the enzyme

activity. Substitution at the 5-position not only alters the p*K*a value, but in the case of hydantoin and 5,5-diphenylhydantoin, substitution causes a decrease in the K'_i value (Table 4) of 5·5-fold. It has been suggested by various authors[23] that trimethadione, paramethadione, ethotoin and methsuximide are converted *in vivo* to their corresponding N_3-dealkyl metabolites and that these metabolites may be responsible for some of the anticonvulsant actions of the parent molecules. Certainly the data presented in the present investigation are consistent with such a hypothesis. Also, from these studies it may be concluded that the extent of inhibition of the NADPH-linked aldehyde reductase at physiological pH is not only governed by the total inhibitor concentration but more importantly is directly related to the p*K*a of these compounds, that is, the concentration of the ionized form at physiological pH.

It is well known that the various anticonvulsants do not all prevent the same type of seizure: diphenylhydantoin blocking tonic hind-limb extension produced by maximal electroshock (MES), trimethadione blocking metrazol-induced seizures, and phenobarbital blocking convulsions due to MES or metrazol. Any biochemical mechanism will ultimately have to account for such differences in anticonvulsant action. It is of interest that phenobarbital and diphenylhydantoin were considerably more potent inhibitors of aldehyde reductase than were dimethadione or ethosuximide and that the latter are required in much higher doses to block convulsions.

Whether inhibition of bovine aldehyde reductase *in vitro* might be utilized as a tool to screen for compounds possessing anticonvulsant properties or whether inhibition of the enzyme *in vivo* is concerned with the ability to block convulsions remains to be determined. Studies related to this hypothesis, including studies of the inhibition of aldehyde reductase *in vivo*, are currently being conducted in our laboratory.

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REFERENCES

1. B. Tabakoff and V. G. Erwin, *J. biol. Chem.* **245,** 3263 (1970).
2. V. G. Erwin ,B. Tabakoff and R. L. Bronaugh, *Molec. Pharmac.* **7,** 169 (1971).
3. E. W. Maynert, in *Drill's Pharmacology in Medicine* (Ed. J. R. DiPalma), p. 250. McGraw-Hill, New York (1971).
4. K. Nakamura and F. Bernheim, *Jap. J. Pharmac.* **11,** 37 (1961).
5. T. Yanagihara and A. Hamberger, *Expl. Neurol.* **32,** 152 (1971).
6. T. M. Brody and J. A. Bain, *Proc. Soc. expl. Biol. Med.* **77,** 50 (1951).
7. R. A. Deitrich, L. Hellerman and J. Wein, *J. biol. Chem.* **237,** 560 (1962).
8. V. G. Erwin and R. A. Deitrich, *J. biol. Chem.* **241,** 3533 (1966).
9. V. G. Erwin, W. D. W. Heston and B. Tabakoff, *J. Neurochem.* **19,** 2269 (1972).
10. H. Buch, N. Buzello, O. Neurohr and W. Rummel, *Biochem. Pharmac.* **17,** 2391 (1968).
11. E. Gordis, *Biochem. Pharmac.* **20,** 246 (1971).
12. W. J. Doran, in *Medicinal Chemistry* (Ed. G. Maxwell), Vol. 4, p. 40. Wiley, New York (1959).
13. M. Zief, and J. T. Edsall, *J. Am. chem. Soc.* **59,** 2245 (1937).
14. H. Erlenmeyer and A. Kliber, *Helv. chim. Acta* **22,** 851 (1939).
15. M. Dixon and E. C. Webb, *Enzymes*, p. 324. Academic Press, New York (1959).
16. H. R. Mahler and E. H. Cordes, in *Biological Chemistry*, p. 253. Harper & Row, New York (1966).
17. R. L. Bronaugh and V. G. Erwin, *Biochem. Pharmac.* **21,** 1457 (1972).
18. V. E. Davis, A. C. Collins, J. C. Cashaw and R. R. McLaughlin, *Trans. Am. Soc. Neurochem.* **3,** 68 (1972).
19. B. Tabakoff, R. Anderson, F. Ungar and S. G. A. Alivisatos, *Trans. Am. Soc. Neurochem.* **3,** 126 (1972).

20. S. G. A. ALIVISATOS and F. UNGAR, *Biochemistry, N.Y.* **7**, 185 (1968).
21. S. G. A. ALIVISATOS, F. UNGAR, P. K. SETH and L. P. LEVITT, *Biochem. Pharmac.* **19**, 401 (1970).
22. M. J. WALSH, V. E. DAVIS and Y. YAMANAKA, *J. Pharmac. expl. Ther.* **174**, 388 (1970).
23. J. E. P. TOMAN, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. GOODMAN and A. GILMAN), p. 205. MacMillan, New York (1970).