Sodium-Potassium-Activated Adenosine Triphosphatase of Brain Microsomes: Modification of Sodium Inhibition by Diphenylhydantoins

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ABSTRACT Effects of diphenylhydantoins on ($Na^+ + K^+$)-ATPase activity in rat and cat brain microsomes were studied. 5,5-diphenylhydantoin (DPH) in concentrations of 5–20 $\mu g \cdot ml^{-1}$ produces an apparent stimulation of the rat brain ($Na^+ + K^+$)-activated ATPase of 55–65% in media containing 50 mM Na^+, 0.15 mM K^+, 3 mM Mg^{++}, and 3 mM ATP. No effects are found on the Mg-ATPase. At constant K^+ levels of 0.05 mmole/liter and 0.15 mmole/liter, increasing the Na^+ concentration activates the enzyme similarly with and without DPH. However, Na^+ concentrations greater than 5 mmoles/liter and 10 mmoles/liter, respectively, which are inhibitory in these low K^+ media, produce less inhibition in the presence of DPH. In media containing 10 mM Na^+, the K^+ activation, on the other hand, is potentiated by DPH. In preparations from cat brain qualitatively similar results are obtained. No effect of DPH is seen on the inhibition produced by high K^+ in low Na^+ media. DPH produces an approximately constant apparent stimulation of 45% in the ($Na^+ + K^+$) increments when these ions are varied simultaneously at a fixed ratio of 150 Na^+: 1 K^+ with cat brain extracts. 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH) has the same potency as DPH in reducing the Na^+ inhibition at high Na : K ratios. The hydantoins appear to act by decreasing the Na^+ inhibition that occurs at high Na : K ratios.

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INTRODUCTION

Diphenylhydantoin (DPH)[1] has been observed to reduce the intracellular sodium ion concentration and to increase Na^+ concentrations in brains of rats subjected to electroshock seizures. It was therefore proposed that the anti-seizure action of DPH results from stimulation of a metabolically linked process of active Na^+ extrusion (1). There is now considerable evidence that active Na^+ transport is linked to ($Na^+ + K^+$)-activated adenosine triphosphatase (2, 3). Studies on this enzyme have shown that DPH inhibits the enzyme activity in brain microsome extracts (4) and in synaptosomal preparations (5, 6) under conditions of low Na : K ratios although it appears to stimulate enzyme activity in synaptosomes under high Na : K ratios (5). In addition, DPH stimulates K^+ transport under certain conditions in synaptosomes (7). The present study is a further attempt to characterize the effects of hydantoins on brain microsomal ($Na^+ + K^+$)-ATPase. It is found that DPH and its hydroxylated derivative, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH), both act to decrease the inhibition of ($Na^+ + K^+$)-ATPase produced by high Na : K ratios.

METHODS

Materials. Tris-ATP and the sodium salt of 5,5-diphenylhydantoin (DPH) were obtained from Sigma Chemical Co., St. Louis, Mo.; [γ-^{32}P]-ATP was obtained from International Chemical and Nuclear Corp., Irvine, Calif.; and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH) was a generous gift from Dr. Henn Kutt (8). Diazepam was

[1] *Abbreviations used in this paper:* ATPase, adenosine triphosphatase; DPH, diphenylhantoin; EDTA, ethylenediaminetetraacetate; HPPH, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin.

generously supplied by Roche Laboratories, Nutley, N. J. Sodium Phenobarbital Injection, (130 mg/ml in 67.8% propyleneglycol, 10% ethanol, and 1.5% benzyl alcohol) was obtained from USV Pharmaceutical Corp., New York. This was diluted 4000-fold in the enzyme assay media. DPH and HPPH suspensions in water were made at the time of use. In some experiments where noted, sodium DPH was dissolved in Parke, Davis Solvent for Dilantin (40% propylene glycol, 10% ethanol, adjusted with NaOH to pH 12; Parke, Davis & Co., Detroit, Mich.) this solvent was diluted 1750-fold in the enzyme assay media. Diazepam was dissolved in the Parke, Davis Solvent and similarly diluted in the enzyme assay.

Microsomal $(Na^+ + K^+)$-ATPase was prepared from rat and cat brains in a procedure using NaI (9) and stored in liquid nitrogen. Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (10).

Assay of $(Na^+ + K^+)$-ATPase. Brain microsomes were diluted at the time of assay in 0.05 M Tris-HCl (pH 7.4) containing 0.1 mM Tris-EDTA. Microsomes, 5–30 μg protein, were incubated at 37°C for 30 min in 0.05 M Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 3 mM Tris-[γ-^{32}P]-ATP (specific activity 10^5 cpm/μmole) plus NaCl and KCl as indicated, in final volumes of 40 μl. The enzyme was preincubated for 15 min at 0°C in the test agent plus the indicated salts before initiation of reaction with ATP. Reactions were terminated by the addition of 10 μl of 50% cold trichloroacetic acid. Inorganic phosphate was extracted in isobutanol and the radioactivity measured as described (11). $(Na^+ + K^+)$-ATPase activity was obtained by subtracting the hydrolytic rate in the presence of Mg^{++} from the total rate in Mg^{++}, Na^+, and K^+. It was ascertained that pretreatment of microsomes with 0.1 mM ouabain in 3 mM $MgCl_2$ for 30 min at O°C abolished 95% of the $(Na^+ + K^+)$ increments.

RESULTS

During preliminary experiments, it was found that DPH stimulates $(Na^+ + K^+)$-ATPase activity of rat or cat brain microsomes in the presence of 150 mM Na^+ and 1 mM K^+.[2] Apparent stimulation of the $(Na^+ + K^+)$ increments in ATPase activity is produced by DPH or HPPH in concentrations as low as 3–5 $\mu g \cdot ml^{-1}$ (Table I). The largest effects are seen with concentrations of 20–25 $\mu g \cdot ml^{-1}$. No significant effects on the Mg^{2+}-ATPase activity are seen.

TABLE I

Stimulation of Rat Brain $(Na^+ + K^+)$-ATPase by Hydantoins

DPH	ΔMg^{2+}	$\Delta(Na^+ + K^+)$*	HPPH	$\Delta(Na^+ + K^+)$‡	ΔMg^{2+}
$\mu g \cdot ml^{-1}$	% control		$\mu g \cdot ml^{-1}$	% control	
2.5	101	105	1.5	98	
5.0	104	153	3.1	141	
10.0	108	155	12.5	171	
20.0	95	167	25.0	171	105

Other conditions as described in Methods. Control values ($nmoles \cdot mg^{-1} \cdot min^{-1}$): DPH experiment, $\Delta Mg^{2+} = 58$, $\Delta(Na^+ + K^+) = 43$; HPPH experiment, $\Delta Mg2^+ = 122$, $\Delta(Na^+ + K^+) = 119$.

* 50 mM NaCl plus 0.15 mM KCl.

‡ 150 mM NaCl plus 1 mM KCl.

[2] Under the same cation conditions, DPH, 20 $\mu g \cdot ml^{-1}$, had no significant effect on $(Na^+ + K^+)$-ATPase in electroplax microsomes from *Electrophorus electricus.*

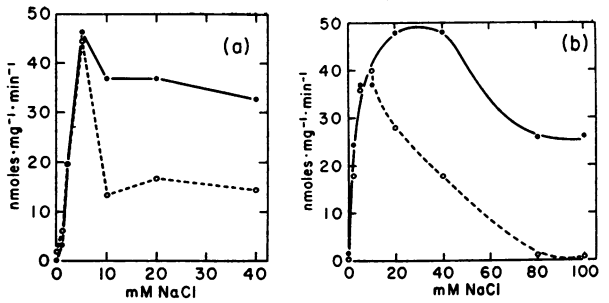

FIGURE 1 Effects of DPH on rat brain $(Na^+ + K^+)$-ATPase under varying Na^+ concentration (*a*) 0.05 mM KCl. (*b*) 0.15 mM KCl. Other conditions as described in Methods. O - - O, control; ●—●, DPH 20 $\mu g \cdot ml^{-1}$.

The activation of ATPase activity by sodium ion is shown in Fig. 1*a* and *b*. In separate experiments performed in constant K^+ concentrations of 0.05 mmole/liter and 0.15 mmole/liter, sodium ion is inhibitory in the range of 10 and 20 mmoles/liter, respectively. However, in both cases, the sodium inhibition is reduced in the presence of DPH 20 $\mu g \cdot ml^{-1}$. On the other hand, the sodium activation is not significantly altered by the DPH.

Fig. 2 shows the effect of DPH on potassium ion activation in 10 mM NaCl. DPH appears to potentiate activation by the low K^+ concentrations where the Na:K ratio is high relative to the ratio which produces optimal activation. This effect is negligible when

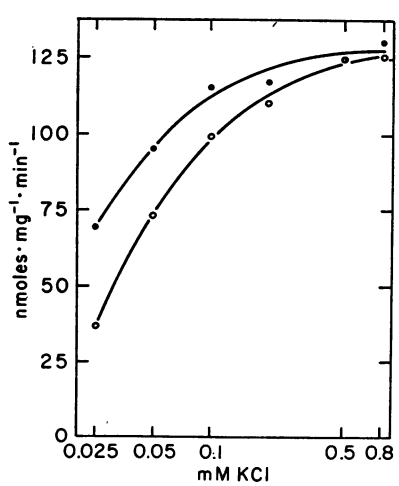

FIGURE 2 Effect of DPH on rat brain $(Na^+ + K^+)$-ATPase under varying K^+ concentration in 10 mM NaCl. Other conditions as described in Methods. O—O, control; ●—●, DPH 20 $\mu g \cdot ml^{-1}$.

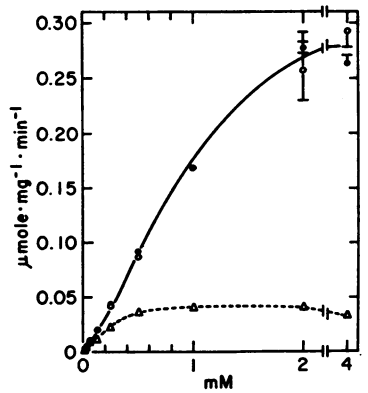

FIGURE 3 Activation of rat brain ATPase by ATP plus $MgCl_2$. Tris-ATP and $MgCl_2$ were varied in equimolar proportions. Other conditions as described in Methods. △ - - △, no univalent cations added; solid line, 80 mM NaCl plus 5 mM KCl added; ○, control; ●, DPH 22 $\mu g \cdot ml^{-1}$.

maximum rates are obtained at the optimal Na : K ratio. The absence of any significant DPH effect in the presence of 80 mM Na^+ plus 5 mM K^+ is confirmed in Fig. 3. Thus, the principal effect of DPH is not on the maximum velocity but rather on the inhibition seen at high Na : K ratios. This may be manifested as an increase in the observed velocity which is a resultant of both the stimulatory and inhibitory cationic effects. The DPH effect is most reproducible under conditions of 150 mM Na^+ and 1 mM K^+, taking into account the amount of enzyme activity required for reliable measurements under these conditions.

HPPH has the same effect as DPH in reducing the sodium ion inhibition at high Na : K ratios (Fig. 4). No effect is seen on the activation phase of the Na^+ curve. Table II compares the effects of DPH and HPPH with phenobarbital and diazepam in the same experiment under identical conditions and shows that both HPPH and DPH produce an apparent stimulation

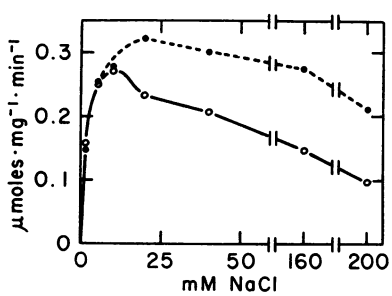

FIGURE 4 Effect of HPPH on rat brain $(Na^+ + K^+)$-ATPase under varying Na^+ concentration in 0.5 mM KCl. Assay media contained 1 : 1750 dilution of Parke, Davis Solvent. Other conditions as described in Methods. ○—○, control; ● - - ●, HPPH 25 $\mu g \cdot ml^{-1}$.

TABLE II

*Effects of Hydantoins on Rat Brain $(Na^+ + K^+)$-ATPase at High $Na^+ : K^+$ Ratio**

Addition	Mg^{++}	$Mg^{++}+Na^+ + K^+$	$\Delta(Na^+ + K^+)$	% Control
0.1 mmole/liter	*μmoles·mg⁻¹·min⁻¹ (SEM‡)*			
Control	0.096 (0.003)	0.230 (0.003)	0.134	
DPH	0.092 (0.002)	0.357 (0.012)	0.265	198
HPPH	0.094 (0.003)	0.366 (0.007)	0.272	203
Phenobarbital	0.095 (0.001)	0.239 (0.014)	0.144	107
Diazepam	0.095 (0.002)	0.230 (0.011)	0.135	101

Other conditions as described in Methods. DPH and diazepam were dissolved in Parke, Davis Solvent (see Methods). The final dilution of this solvent in the assay media was 1750-fold. The same amount was added to control tubes and those with HPPH. It was ascertained that this amount of solvent did not alter the enzyme activity or DPH effect. The sodium salt of phenobarbital was used as described in Methods.

* 150 mM NaCl plus 1 mM KCl.

‡ N = 3.

under these conditions of 103% and 98%, respectively. Phenobarbital and diazepam have no significant effect under these conditions.

Microsome preparations from cat brain yield results qualitatively similar to those obtained with rat brain. In the presence of 150 mM Na^+ and 1 mM K^+, DPH stimulates the $(Na^+ + K^+)$-ATPase by 44% and does not affect the Mg^{2+}-ATPase activity. The inhibition produced by pretreatment with ouabain is not altered by DPH under these conditions (Table III). Ouabain inhibition was also unaffected when microsomes were treated first with DPH in an experiment otherwise similar to that of Table III. Fig. 5 shows that in 0.5 mM K^+, NaCl activation is not altered by DPH, whereas the inhibition in high Na^+ is reduced. Fig. 6 shows

TABLE III

Effects of DPH plus Ouabain on Cat Brain $(Na^+ + K^+)$-ATPase Activity

Additions	ΔMg^{2+}	$\Delta(Na^+ + K^+)$*
	% of control	
Ouabain (0.05 mM)	96	3.9
DPH (20 $\mu g \cdot ml^{-1}$)	97	144.
Ouabain plus DPH		5.9

Microsomes were pretreated in 0.05 mM ouabain, 25 mM Tris-HCl (pH 7.4), and 1.5 mM $MgCl_2$ at 0°C for 30 min. The ouabain-treated microsomes were then preincubated an additional 15 min at 0°C with and without DPH and finally assayed for ATPase activity for 30 min at 37°C as described in Methods. Ouabain concentrations in the pretreated samples were constant throughout the assay. Controls were preincubated for identical periods.

Control values (nmoles·mg^{-1}·min^{-1}): ΔMg^{2+} = 10.2, $\Delta(Na^+ + K^+)$ = 20.3.

* 150 mM NaCl plus 1 mM KCl.

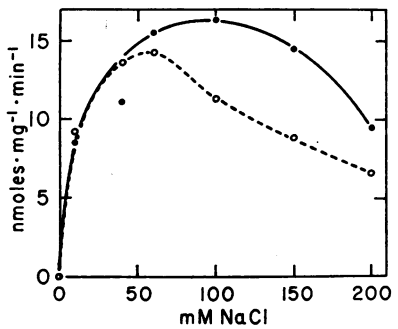

FIGURE 5 Effect of DPH on cat brain $(Na^+ + K^+)$-ATPase under varying Na^+ concentration in 0.5 mM KCl. Other conditions as described in Methods. O - - O, control; ●—●, DPH 20 $\mu g \cdot ml^{-1}$.

that in 150 mM NaCl, the K^+ activation is further increased by DPH where the Na:K ratio is large, as in the case of rat brain. No significant DPH effect was found at optimal cation ratios. In a separate experiment performed with 150 mM NaCl as in Fig. 6, but with higher KCl concentrations, the maximal observed rates were obtained with 12.5 mM KCl: 31.2 and 34.9 $nmoles \cdot mg^{-1} \cdot min^{-1}$ for control and DPH treated samples, respectively. These rates did not vary significantly at KCl levels from 12.5 to 100 mmoles/liter. Their averages over this range were, in $nmoles \cdot mg^{-1} \cdot min^{-1}$: 30.3 (SEM 0.37) for control and 31.5 (SEM 1.25) for DPH samples (n = 4).

The effects of high KCl concentrations in low [NaCl] are shown in Fig. 7. In 10 mmoles/liter of NaCl inhibition by K^+ occurs in concentrations of 10 mmoles/liter or higher and this action of K^+ is not appreciably altered by DPH. Hence, the inhibition due to high K:Na ratios is not affected by DPH, in contrast to inhibition

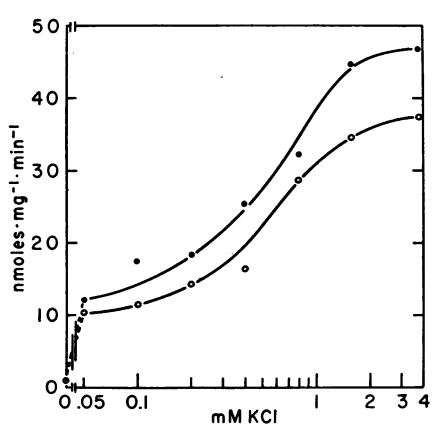

FIGURE 6 Effect of DPH on cat brain $(Na^+ + K^+)$-ATPase under varying K^+ concentration in 150 mM NaCl. Other conditions as described in Methods. O—O, control; ●—●, DPH 20 $\mu g \cdot ml^{-1}$.

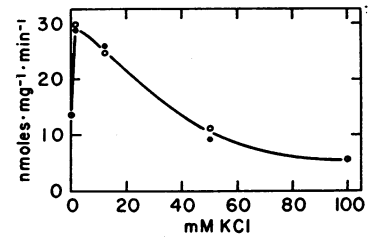

FIGURE 7 Inhibition of cat brain $(Na^+ + K^+)$-ATPase by excess K^+ in 10 mM NaCl. Other conditions as described in Methods. O, control; ●, DPH 20 $\mu g \cdot ml^{-1}$.

by high Na:K ratios. This observation is shown also when Na^+ is varied; i.e., no effect of DPH is seen on the activation by low Na^+ concentrations where the K:Na ratio is high (Figs. 1, 4, and 5). When the sodium and potassium ion concentrations are varied simultaneously to maintain a constant ratio of Na:K = 150, DPH produces an average apparent stimulation of about 45% in the $(Na^+ + K^+)$ increments throughout the total cation concentration range (Fig. 8). Thus, the DPH effect is independent of total ionic strength and of the concentration of either cation alone.

DISCUSSION

There is evidence that the $(Na^+ + K^+)$-activated ATP hydrolysis proceeds through a multi-stage reaction mechanism in which Na^+ activates enzyme phosphorylation and K^+ activates enzyme dephosphorylation (3, 11). It is well known that excesses of either Na^+ or K^+ exert inhibition of a mutually competitive nature (12, 13), but there is disagreement on the basis of

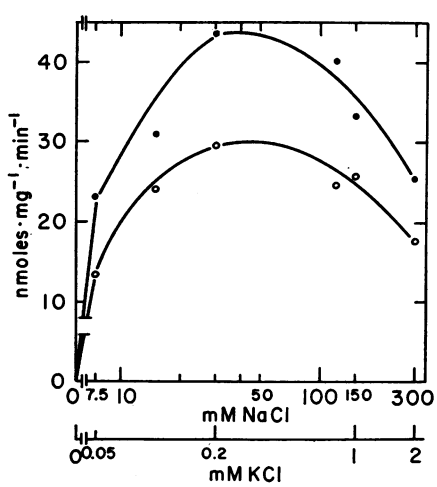

FIGURE 8 Effect of DPH on cat brain $(Na^+ + K^+)$-ATPase. NaCl and KCl were varied simultaneously in the proportion of 150:1. Other conditions as described in Methods. O—O, control; ●—●, DPH 20 $\mu g \cdot ml^{-1}$.

kinetic data as to whether the respective binding sites are necessarily separate or identical. In the present study it is observed that DPH decreases the inhibition due to high Na:K ratios but has no effect on inhibition due to relative excess of K^+.

The reduction of Na^+ inhibition may be explained by assuming that DPH antagonizes the Na^+ effect relative to K^+ at a site which when occupied by Na^+ is inhibitory. This site might be either identical with or interacting with a K^+ site, for example, the site involved in dephosphorylation. Conversely, if DPH were also to antagonize Na^+ activation of enzyme phosphorylation, then one would expect to observe an apparent inhibition by DPH at low Na:K ratios. This was not observed in the present study. In addition, it has been reported that DPH has no effect on myocardial $(Na^+ + K^+)$-ATPase activity in the presence of 100 mM Na^+ plus 10 mM K^+ (14). The fact that such inhibition by DPH was, however, reported at low Na:K ratios in other types of brain enzyme preparations (4–6) might indicate that under certain conditions more of the cation binding sites become susceptible to the DPH action. Further information on the mechanism of the DPH effect may be obtained from direct studies of the phosphorylation and ATP-ADP transphosphorylation reactions (11).

An important question is whether the observed DPH effect on $(Na^+ + K^+)$-ATPase can be related to the drug's anti-seizure activity. Woodbury observed a direct correlation between the ratio of extracellular to intracellular brain sodium ion concentration and the electroshock seizure threshold in rats. Based on the observations that DPH increased the rate of sodium flux in normal rat brains and decreased the intracellular brain sodium concentrations in normal rats and in rats subjected to electroshock seizures, he proposed that the anti-seizure activity results from stimulation of active sodium extrusion (1). Festoff and Appel (5) found that DPH appears to stimulate $(Na^+ + K^+)$-ATPase activity in synaptosomes from rat cerebrum when the Na:K ratio is in the range 50:1. The experiments reported here with NaI-extracted brain microsomes confirm the apparent stimulatory effect of DPH at high Na:K ratios and show that this effect results from a decrease in Na^+ inhibition. No qualitative difference between rat and cat brain preparations regarding this effect was noted under the conditions tested. Although the optimal condition for the effect in this study is a Na:K ratio of 150:1, this value is quite broad and differences in this ratio may be due to differences in membrane preparations used, extraction procedures, or relative extents of Na^+ inhibition. Of greater significance is the fact that the DPH effect depends on specific ionic proportions in the range which can regulate the enzyme activity.

Regulation of $(Na^+ + K^+)$-ATPase is complicated; it depends on a balance among both activation and inhibition effects by both sodium and potassium. It has been found in studies of red cells that activation of the sodium pump and $(Na^+ + K^+)$-ATPase is due to intracellular Na^+ and extracellular K^+ while, on the other hand, extracellular Na^+ is inhibitory (15, 16). It is plausible that in vivo DPH might produce an increased rate of transport under a given set of conditions through a reduction in the relative inhibition due to extracellular Na^+ similar to its effect on $(Na^+ + K^+)$-ATPase in vitro. However, as described above, other studies have indicated an inhibitory effect of DPH at low Na:K ratios which suggests that DPH under some conditions may also decrease Na^+ activation. Therefore, the net result of the DPH action will depend on its relative effects on the two Na^+ actions.

It is difficult to draw comparisons on a molecular basis between actions on $(Na^+ + K^+)$-ATPase in vitro and anti-seizure activity because other factors such as absorption, metabolism, excretion, and permeability might limit a drug's potency in vivo. HPPH in very high doses was reported ineffective against electroshock-induced seizures in rats (17) yet it exhibits the same potency as DPH in its effects on $(Na^+ + K^+)$-ATPase. HPPH is the main excretory product of DPH (18) and is found conjugated with glucuronic acid in the urine (19). The liver is an important site for the formation of HPPH (8). If the action on $(Na^+ + K^+)$-ATPase is critical for the anti-seizure activity of the hydantoins, then it might be supposed that the nervous system action of HPPH is limited by a more rapid rate of excretion or decreased permeation into brain in the conjugated form rather than by hydroxylation per se. Alternatively, it remains possible that the hydantoin action as measured on $(Na^+ + K^+)$-ATPase in vitro is itself not sufficient to account for the antiseizure activity of the hydantoins. Studies of HPPH metabolism and concentration within the brain may help answer these questions.

The observation that DPH stimulates the $(Na^+ + K^+)$-ATPase, although consistent with Woodbury's hypothesis, is not confirmatory, as discussed by several authors (5, 20). In addition, recent studies with lobster nerves have shown that DPH is able to lower the intraneuronal Na^+ concentration without affecting K^+ in the presence of ouabain or cyanide inhibition (21). Thus, DPH has multiple actions that may modify Na^+ translocation and it is not known which of these, if not a combination, is important physiologically in the control of seizures. Further physiologic studies comparing HPPH and DPH effects on seizures and on

membrane properties in vitro will be pertinent to this issue.

ACKNOWLEDGMENTS

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