

## Effect of diphenylhydantoin on synaptosome sodium-potassium-ATPase

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### Research Article

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Rat cerebral cortical synaptosomes isolated in Ficoll gradients were employed as the source for Na-K-ATPase. With 50 mM Na, 10 mM K, 7.5 mM Mg, and 1.8 mM ATP, the specific activity of the preparation was 70  $\mu$ moles  $P_i$  released/mg synaptosomal protein per 30 min. The ionic and substrate concentrations yielding one-half maximal velocity were 0.5 mM K, 5 mM Na, and  $8.5 \times 10^{-5}$  M ATP, respectively.

At 50 mM Na and 0.2 mM K, DPH produced an average of 92% stimulation of  $P_i$  release above control. The ratio of Na:K rather than the absolute levels of the ions was critical in determining the effect of DPH. DPH produced significant stimulation of enzyme activity under conditions of a high Na:K ratio (25-50:1). At ratios of 5-10:1, DPH produced little or no effect, and at low Na:K ratios (less than 5:1), DPH was inhibitory. Under all ionic conditions examined, DPH produced no apparent change in enzyme affinity for ATP.

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# Effect of Diphenylhydantoin on Synaptosome Sodium-Potassium-ATPase

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**ABSTRACT** Previous studies have demonstrated that electrically induced seizures in rat result in an increased brain intracellular sodium which can be decreased by treatment with sodium diphenylhydantoin (DPH). The correlation of cation transport with membrane-oriented sodium-potassium-adenosine triphosphatase (Na-K-ATPase) prompted an investigation of the effect of DPH upon ATPase enzyme activity.

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Assuming the proposed association of Na-K-ATPase with cation transport in brain, the data suggest the possibility that DPH may control seizures by its stimulation of Na-K-ATPase activity.

## INTRODUCTION

Diphenylhydantoin sodium (DPH) has been effective in the treatment and management of major motor and psychomotor seizures as well as in the control of certain cardiac arrhythmias (1–4). Our knowledge of the distribution, metabolism, and excretion of the drug in man and experimental animals is extensive (5), but its mechanism of action on excitable membranes has not yet been clearly defined.

In rats in which seizures were electrically induced, Woodbury noted an increase in the brain intracellular sodium (6). Diphenylhydantoin decreased the brain intracellular sodium and increased the turnover of  $^{22}\text{Na}$  (6). The action of DPH was thus related to its enhancement of “sodium pump” action.

Recently a close link has been established between sodium-potassium-adenosine triphosphatase (Na-K-ATPase) activity and cation transport in many tissues including brain (7–8). The presence of the Na-K-ATPase in synaptic endings has further suggested the potential importance of this enzyme system in regulating ionic and electrical activity at neuronal membranes (9–11). It is, therefore, reasonable that attempts would be made to explain the action of DPH in terms of a stimulation of ATPase activity which might enhance Na efflux and K influx in epileptic neurons. How-

ever, previous studies with brain microsomal ATPase assayed in the presence of Na:K ratios of 10:1 or less have not been able to demonstrate such a stimulation (12).

In the present experiments Na-K-ATPase activity has been examined in rat cerebral cortex synaptosomes over a range of ionic conditions. DPH produced significant stimulation of enzyme activity only under conditions of a high Na to K ratio (50:1). At more physiological ratios (5–10:1), DPH produced little or no effect, and at low Na to K ratios (less than 5:1), DPH was inhibitory.

## METHODS

*Preparation of synaptosomes.* Synaptosomes were isolated from rat cerebral cortices as reported previously (13). Synaptosomal fractions, after washing to remove the Ficoll, were immediately flash-frozen and thawed four times before use in the experiments.

*Na-K-ATPase assay.* Disodium adenosine triphosphate (P-L Laboratories) was converted to the Tris form as described by Post and Sen (14). All solutions were examined for sodium and potassium contamination in an atomic absorption spectrophotometer (model 303 Perkin-Elmer Corp., Norwalk, Conn.) and found to contain concentrations of ions less than  $10^{-5}$  M. Cellulose nitrate tubes were used in the incubation to reduce contaminating phosphate adsorption.

In a final volume of 1.5 ml, the assay media had the following concentrations: 7.5 mM  $MgCl_2$ ; 0.75 mM EDTA; 30 mM imidazole; 30 mM glycylglycine, (pH 7.4). Tris-ATP, sodium, and potassium of varying concentrations were added appropriately. DPH (Dilantin, Parke, Davis & Co., Detroit, Mich.)<sup>1</sup> was preincubated for 10 min with synaptosomal protein and appropriate ions before starting the reaction with ATP. Incubation was at 37°C in a reciprocating water bath (Warner-Chilcott Laboratories, Morris Plains, N. J.) for 30 min. The reaction was terminated with the addition of 0.5 ml of ice-cold 1.2 N perchloric acid (containing 8% silicotungstic acid), and tubes were immediately placed in an ice bath.

*Inorganic phosphate assay.* A modification of the methods of Post and Sen, and Wahler and Wollenberger was used (14, 15). After the reaction was terminated with perchloric acid, protein was removed by centrifugation, 1.5 ml of the supernatant was added to 1.5 ml of ice-cold sodium molybdate solution (60 mM sodium molybdate, 2.25 M sodium chloride), and tubes were briefly swirled. 4 ml of redistilled butyl acetate was then added at room temperature, and the tubes were vigorously shaken on a Vortex mixer. The emulsion was separated by brief centrifugation, and 2 ml of the upper layer was added to 4 ml of a waiting isopropanol solu-

tion (redistilled isopropyl alcohol, 0.06 mM  $CuCl_2$ , and 0.27 N  $H_2SO_4$ ). 5  $\mu$ l of beta mercaptoethanol was added to this mixture. The development of the bright blue color was allowed to stabilize over 20 min and was then read in a Zeiss spectrophotometer at 625 m $\mu$ .

*Specific activity.* Specific activity is defined as  $\mu$ moles  $P_i$  released/mg synaptosome protein per 30 min in the presence of Na-K-Mg less  $P_i$  released in the presence of Mg plus ouabain.

*Protein determination.* Protein determination was performed according to the method of Lowry, Rosebrough, Farr, and Randall (16).

## RESULTS

*Kinetic parameters of Na-K-ATPase.* In the presence of 50 mM sodium and 10 mM potassium the liberation of inorganic phosphate from ATP was linear with time and increments of synaptosomal protein. To determine the effective concentration ranges of each ion, we made activity measurements over a range of concentrations of both sodium and potassium. These studies indicated that the concentration of potassium giving one-half maximal velocity was 0.4–0.6 mM (at 50 mM sodium). The sodium concentration yielding one-half maximal velocity was 5 mM (at 10 mM potassium). These values are in accord with the apparent  $K_m$  values recorded for Na-K-ATPase values in other systems (17).

The concentrations of ions yielding maximal activity were 50 mM sodium and 10 mM potassium. Under these conditions the specific activity of the preparation was approximately 70  $\mu$ moles  $P_i$ /mg synaptosomal protein per 30 min. When the specific activity is expressed as  $\mu$ mole  $P_i$ /min per g protein, the obtained value of 2000 is among the most active preparations reported (18). This maximal activity was 98% inhibited by  $10^{-4}$  M ouabain. With optimal ionic conditions, the ATP concentration yielding one-half maximal velocity was calculated to be  $8.5 \times 10^{-5}$  M. Evidence of substrate inhibition was present at ATP concentrations greater than 3 mM.

*Effect of diphenylhydantoin on Na-K-ATPase activity.* The effect of  $10^{-4}$  M DPH on Na-K-stimulated ATPase activity was assessed under conditions in which one of the three variables (sodium, potassium, or ATP) was manipulated while the other two were held constant. No effect of DPH was noted on the Mg-ATPase in the absence of sodium and potassium. At the concentrations of DPH employed, there was no change in

<sup>1</sup> We are grateful to Dr. A. C. Bratton, Jr., for a generous supply of diphenylhydantoin sodium.

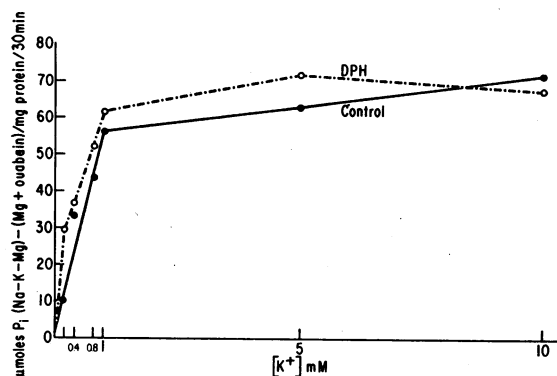


FIGURE 1 Effect of diphenylhydantoin on K-activation of Na-K-ATPase.  $10^{-4}$  M DPH was preincubated with 125  $\mu$ g of synaptosomal protein, 50 mM Na, 7.5 mM  $Mg^{++}$ , and varying concentrations of K for 10 min before initiating reaction with 1.8 mM Tris-ATP. Incubation was at 37°C for 30 min in a shaking water bath. The addition of 0.5 ml ice-cold perchloric acid (1.2 N) terminated the reaction. Specific activity was as defined in Methods.

the pH of the incubation mixture. Fig. 1 demonstrates the effects of DPH with varying potassium and constant sodium and ATP concentrations. In this experiment with 50 mM sodium and 0.2 mM potassium there was a 180% en-

TABLE I  
Effect of DPH on the Specific Activity of Na-K-ATPase

Experiment No.	Control	DPH ( $10^{-4}$ M)	Stimulation over control
	$\mu$ moles $P_i$ /mg protein per 30 min		%
1	16.07	32.14	100
2	22.98	37.36	63
3	20.11	34.48	71
4	20.61	41.24	100
5	33.33	52.38	57
6	30.37	51.40	69
7	25.0	36.76	47
8	10.0	28.0	180
9	19.77	32.49	64
10	21.08	57.22	171
Mean			$92.2 \pm 14.9$

Incubation was performed at 37°C for 30 min. 125  $\mu$ g of synaptosomal protein was preincubated with DPH ( $10^{-4}$  M) for 10 min in the presence of 50 mM Na, 0.2 mM K, and 7.5 mM  $Mg^{++}$ . Tris-ATP was added to start the reaction. Specific activity of Na-K-ATPase is defined as  $\mu$ moles of  $P_i$  formed in the presence of Na, K, and  $Mg^{++}$  minus  $\mu$ moles of  $P_i$  liberated in the presence of  $Mg^{++}$  and ouabain ( $10^{-4}$  M). Each experiment was performed with a separate synaptosome preparation from 18–20-day-old white Wistar rat cerebral cortices.

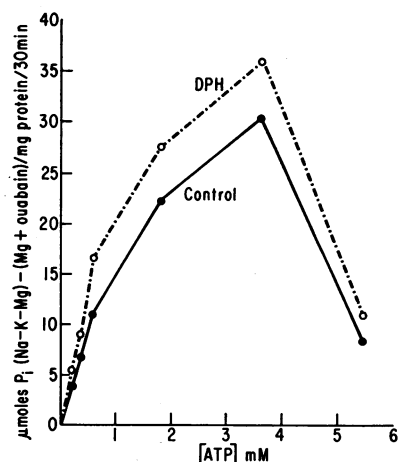


FIGURE 2 Effect of diphenylhydantoin on enzyme activity at varying ATP concentrations. Concentrations of ions which gave maximal stimulation (50 mM Na, 0.2 mM K) were incubated with 7.5 mM  $Mg^{++}$ ,  $10^{-4}$  M DPH, and varying ATP concentrations. The reaction was initiated by the addition of synaptosomal protein and  $P_i$  released as assessed as described in Methods.

hancement of enzyme activity above control. In 10 similar experiments (Table I) with 50 mM sodium and 0.2 mM potassium, the stimulation averaged 92.2% with a range of 47–180% over control values. In separate experiments the stimulation was found to reflect an increase in the initial velocity of the enzyme reaction as evidenced by the linearity over the 30 min incubation in the presence of DPH.

At 50 mM sodium, 0.2 mM potassium, and varying ATP concentrations (Fig. 2), stimulation was noted over the entire range of ATP employed. This stimulation over control was also present with ATP concentrations which were inhibitory.

TABLE II  
Effect of DPH Concentration on Na-K-ATPase Activity

	$\mu$ moles $P_i$ /mg protein per 30 min	Stimulation %
Control	30.74	—
DPH		
$10^{-4}$ M	45.08	47
$10^{-6}$ M	40.98	33
$10^{-8}$ M	36.89	20
$10^{-10}$ M	32.79	6.6

Incubation was performed as described in Table I with varying concentrations of DPH.

There was no change in the concentration of ATP yielding one-half maximal velocity. DPH, therefore, appeared to have little effect on the interaction of ATP with the enzyme.

The stimulatory effects noted with 50 mM sodium and 0.2 mM potassium were seen over a wide range of DPH concentrations. The studies indicated above were performed with  $10^{-4}$  M DPH. Concentrations of DPH as low as  $10^{-8}$  M were still effective in bringing about a 20% stimulation of ATPase activity (Table II).

With potassium concentrations of 10 mM, inhibition of enzyme activity with DPH was noted over the entire range of sodium concentrations from 2.5 to 50 mM (Fig. 3). This inhibition was also present at varying ATP concentrations in the presence of 10 mM potassium and 5 mM sodium. Rawson and Pincus have noted a similar inhibitory effect of DPH on brain microsomal Na-K-ATPase activity at optimal sodium and potassium and at low sodium but optimal potassium concentrations (12).

Our data could not resolve whether the observed stimulatory effect of DPH on the ATPase was determined by (a) an absolute critical level of potassium, (b) the ratio of sodium to potassium, or (c) a combination of both. In the presence of low potassium (0.2 mM) Fig. 4 demonstrates the stimulatory effect of DPH over a wide range of sodium concentrations. The stimulation was pres-

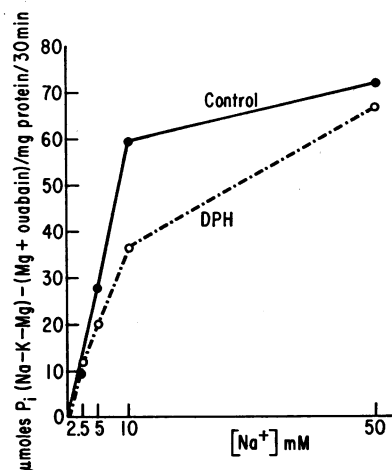


FIGURE 3 Effect of diphenylhydantoin on Na-activation of Na-K-ATPase.  $10^{-4}$  M DPH was preincubated for 10 min with 125  $\mu$ g synaptosomal protein, 10 mM K, 7.5 mM  $Mg^{++}$ , and varying Na concentrations. 1.8 mM Tris-ATP initiated the reaction as in Fig. 1.

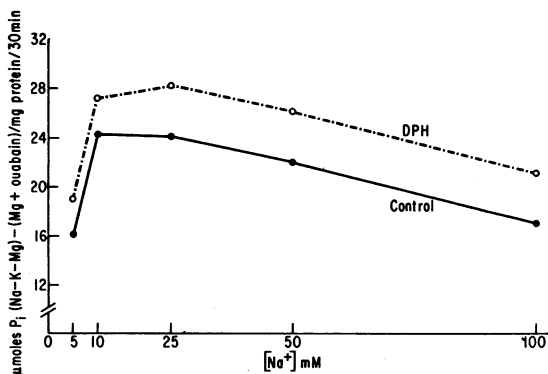


FIGURE 4 Diphenylhydantoin stimulation of Na-K-ATPase at 0.2 mM K and varying Na concentrations. At constant K (0.2 mM) 125  $\mu$ g synaptosomal protein was preincubated with  $10^{-4}$  M DPH, 7.5 mM  $Mg^{++}$ , and varying Na concentrations. Enzyme assay was carried out as described in Fig. 1.

ent throughout the range of sodium employed, with a peak at 10–25 mM or a sodium to potassium ratio of 50:1. When this ratio of 50:1 (sodium:potassium) was maintained over a wide range of ionic concentrations, similar stimulation was noted (Table III). Therefore, with these ionic concentrations, the ratio of sodium to potassium appeared critical in determining the stimulatory effect.

## DISCUSSION

In the present experiments, DPH altered the activity of synaptosome sodium-potassium-ATPase with the particular effect being determined primarily by the ratio of sodium to potassium present

TABLE III  
Influence of the Na/K Ratio on DPH Stimulation

Na	K	Control	DPH ( $10^{-4}$ M)	Stimulation*
mM	mM	$\mu$ moles $P_i$ /mg protein per 30 min		%
10	0.2	18.51	27.77	50
20	0.4	25.92	37.04	43
40	0.8	31.48	46.30	47
50	1.0	29.63	44.89	51
100	2.0	37.03	55.56	50

Incubation was performed as described in Table I. Specific activity is expressed as  $P_i$  released in the presence of Na + K less that in the presence of  $Mg^{++}$  + ouabain.

\* The per cent stimulation observed in this experiment at DPH  $10^{-4}$  M represents the lower limit of the range as seen in Table I.

in the incubation medium. At sodium to potassium ratios of 25–50:1, DPH stimulated; at ratios of 5:1 or less, DPH inhibited; and at ratios of 5–10:1, DPH had little effect on sodium-potassium-ATPase activity.

The most critical question is whether these data help explain the effectiveness of DPH in controlling seizures. Seizures may result from a number of causes, among which are processes which interfere with brain oxidative metabolism or the maintenance of high energy phosphates (19). The common denominator of these epileptogenic processes is the alteration of ionic constituents. In electrically excited brain slices, intracellular potassium is decreased (20, 21). After electroconvulsive shock, brain intracellular sodium is increased, and the turnover of  $^{22}\text{Na}$  decreased (6). Similarly, arrhythmias induced in myocardial tissue with cardiac glycosides appear to be related to the loss of potassium from myocardial cells (22, 23).

The underlying basis for membrane irritability and focal depolarization may thus relate to (a) increased intracellular sodium and (b) depletion of intracellular potassium. These ionic alterations may be due to a diminished active transport or increased passive permeability (19, 25). Agents which correct this membrane irritability would then be expected to act on either the active or passive components of ionic flux across membranes.

DPH has been found to correct the ionic imbalances associated with cellular irritability. DPH prevented the efflux of potassium from cardiac tissue pretreated with toxic doses of digitalis and reversed digitalis-induced ventricular arrhythmias (24). DPH increased the rate of transfer of  $^{22}\text{Na}$  and decreased the intracellular to extracellular sodium ratios which occurred following electroconvulsive treatment (6). Further, DPH has a number of electrophysiologic effects which are consonant with its restitution of normal intracellular ionic constituents (Table IV).

However, these cited experiments do not specify the mechanism by which DPH exerts its normalizing effect. The data of Korey supported a stabilizing effect of DPH on membrane function (26). The experiments of Woodbury suggested an enhancement by DPH of "sodium pump" action (6). Our results suggest the possibility that the

TABLE IV  
*Known Electrophysiologic Effects of Diphenylhydantoin*

1. Threshold stabilizing action (peripheral nerve, invertebrate nerve, cardiac muscle) (4, 5, 25).
2. Abolishes post-tetanic potentiation (PTP), the increase in synaptic excitation resulting from repetitive stimulation (32).
3. No effect on interseizure EEG (5).
4. Most effective (in animals) against tonic extensor component of maximal seizure—relatively ineffective against focal or generalized clonic seizure (19, 25).
5. Clinically, most effective in controlling major motor tonic seizures (suggests protection of surrounding normal neurons against invasion by seizure activity from discharging foci) (19, 25).
6. Can rapidly reverse digitalis-induced ventricular tachyarrhythmias clinically and experimentally (2–4, 24).\*

\* Zeft, H., Whalen, R., Morris, J., Rumo, N., and H. D. McIntosh. Prophylaxis versus treatment of acetyl strophanthidin intoxication. *Am. Heart J.* In press.

effectiveness of DPH is related to its action on the Na-K-ATPase. With red blood cell Na-K-ATPase, the enzyme activity is determined by the sodium concentration internal to the membrane and the potassium concentration external to the membrane (17, 18). In cat brain, neuronal intracellular sodium has been estimated at 28 mEq/liter (27). No precise estimate of extracellular potassium is available; but assuming a close relationship to cerebrospinal fluid (CSF) concentrations, an extracellular potassium of 2.5 mEq/liter may be assumed. The normal intracellular sodium to extracellular potassium ratio would then be approximately 10:1; and it is of interest that DPH has little effect under normal conditions (5, 25). In seizures the intracellular sodium is increased (6); and it is possible that intracellular sodium to extracellular potassium ratios may reach 20–25:1, or levels where DPH was found to have a stimulatory effect on Na-K-ATPase activity, in vitro. Enhancement of ATPase activity may indirectly promote respiration and increased energy availability for other critical processes important to the integrity of membrane and cellular function (28).<sup>2</sup> In this regard, it is relevant that after administration of large doses of DPH, the

<sup>2</sup> Appel, S. H., L. A. Autilio, B. W. Festoff, and A. V. Escueta. Biochemical studies of synapses in vitro: III. Relation of ion flux to protein synthesis. In preparation.

concentration of creatine phosphate increased in whole brain slices (29).

However, several factors are against interpreting the action of DPH simply in terms of its stimulation of Na-K-ATPase activity. If DPH does act by stimulating Na-K-ATPase, intracellular potassium would be expected to be increased. However, Woodbury found that intracellular potassium was not decreased during seizures, nor did DPH cause any increase in potassium (6). These results of Woodbury, however, are not what would be expected from the threshold stabilizing effects indicated in Table IV. It is thus possible that analyses of whole brain tissue, including neurons and glia, prevent adequate assessment of lowered potassium seen with electrically excited brain slices or digitalis-treated cardiac tissue. In preliminary results in our laboratory we find that under the ionic conditions in which DPH enhances ATPase activity, DPH also enhances the uptake of potassium into synaptosomes.<sup>8</sup>

Another present limitation of ascribing the action of DPH to its stimulatory effect on Na-K-ATPase activity exists. The neuronal intracellular sodium concentration in the seizure state is not known; and at present there is no evidence that the ratios which cause stimulation in vitro exist in the seizure state in vivo. Our observations do not eliminate the possibility that the drug is exerting its effect on some other parameter of neuronal metabolism in ameliorating seizure conditions (30-32). Furthermore, they do not prove the basic assumption that the sodium-potassium-ATPase in synaptosomes mediates the active transport of potassium and sodium, although much recent data from the literature as well as from our own laboratory are consistent with this point of view (7, 8, 18).<sup>8</sup> Further work is necessary to determine whether DPH brings about the enhancement of sodium efflux and potassium influx in synaptosomes predicted by its alteration of fluxes in whole brain and its stimulation of synaptosomal Na-K-ATPase activity.

#### ACKNOWLEDGMENTS

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<sup>8</sup> Escueta, A. V., and S. H. Appel. Biochemical studies of synapses in vitro: II. Potassium transport. In preparation.

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