IN VIVO AND IN VITRO EFFECTS OF PHENYTOIN ON ANION AND CATION TRANSPORT SYSTEMS OF THE RAT BRAIN

by

H. Steve White

A dissertation submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Pharmacology

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March 1984

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

FINAL READING APPROVAL

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This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

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Date

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THE GRADUATE SCHOOL

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FOR THE Ph.D. X, Ed.D. _____ OR M.Phil. _____ DEGREE

Date December 21, 1983

Return one copy signed by committee to:
Graduate School
402 Park Building

Name of Student Harold Steve White

Major Field Pharmacology Minor Field _____
(For Ed.D Degree)

Date of Examination December 22, 1983

The student's examination was evaluated as follows by the Committee:

Passed X Failed _____

The student's dissertation was evaluated as follows by the Committee:

Passed X Failed _____

Signatures of the Examining Committee: Dixon M. Woodbury Chairman

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ABSTRACT

Various parameters of anion and cation transport were measured in the cerebral cortex of neonatal (3 days old) and adult rats following acute and chronic treatment with PHT. Acutely, phenytoin (PHT) significantly inhibited the enzyme Na^+, K^+-ATPase in both neonatal and adult rats. This effect was accompanied by a significant increase in cerebral cortical sodium (Na^+) content and a decrease in potassium (K^+) content only in neonatal animals. Chronic treatment (bid and qid for 7 days) of adult rats with PHT significantly reduced the Na^+ content without affecting the whole homogenate Na^+, K^+-ATPase activity. The activity of this enzyme was markedly increased in the myelin- (glial product) and slightly decreased in the synaptosomal- (neuronal) fractions following chronic (qid for 7 days) PHT treatment. These results suggest that PHT differentially affects the two forms (neuronal and glial) of the enzyme Na^+, K^+-ATPase. The possible relevance of this hypothesis in relationship to the anticonvulsant and excitatory properties of PHT is discussed.

Chronic (bid and qid for 7 days) PHT treatment increased both DNA content and activity of the glial marker enzyme carbonic anhydrase. Activity of the mitochondrial enzyme HCO_3^--ATPase was also increased following chronic PHT treatment. These two enzymes are intimately involved in the regulation of HCO_3^--Cl^- transport across glial cell and mitochondrial membranes, and these results suggest that PHT is able to beneficially effect glial regulatory processes. The ability to enhance glial regulation of extracellular fluid anions and cations provides new and important insights into the mechanism of the anticonvulsant action of PHT.

The activity of enzymes involved in anion and cation transport, the concentration of intracellular potassium (K^+_i), and the trans-membrane potential (E_m) was determined following acute and chronic exposure of primary astroglial cultures to micromolar concentrations of phenytoin (PHT). Na^+, K^+-ATPase activity of homogenates of cultured glial cells was determined in the presence of increasing K^+ concentration (1-20 mM). Acutely, PHT had little effect on the K^+-activation pattern of Na^+, K^+-ATPase. In contrast, the percent of Na^+, K^+-ATPase activated by elevating the K^+ concentration was dose-dependently increased by chronic PHT treatment. This effect was accompanied by a marked increase in K^+_i and a significant membrane hyperpolarization. The acute effect of PHT on the E_m was biphasic, characterized by membrane hyperpolarization at concentrations of 1×10^{-6} to 1×10^{-5} M. At concentrations between 1×10^{-5} M and 1×10^{-4} M, the E_m progressively returned to control values. These results suggest that glial cells acutely and chronically treated with therapeutic concentrations of PHT have an enhanced capacity to control elevated extracellular potassium. Return of the E_m to control values at PHT concentrations greater than 1×10^{-5} M suggest that these cells are less able to regulate extracellular potassium. These data can partially explain the excitatory effects of PHT at high therapeutic concentrations.

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PART ONE

EFFECTS OF ACUTE AND CHRONIC PHENYTOIN ON THE ELECTROLYTE CONTENT AND THE ACTIVITIES OF NA^+, K^+-, CA^{++}, MG^{++}-, HCO_3^--ATPase AND CARBONIC ANHYDRASE OF NEONATAL AND ADULT RAT CEREBRAL CORTEX

INTRODUCTION

The available experimental evidence suggests that one of the major actions of PHT is on the movement of sodium (Na^+) (Lipicky, et al., 1972; Schwartz and Vogel; 1977) and calcium (Ca^{++}) (Pincus, 1972; Sohn and Ferrendelli, 1973 & 1976) across biological membranes. Through an interaction with either passive or active transport mechanisms, it has been observed to decrease both Na^+ and Ca^{++} influx and consequently, it decreases intracellular Na^+ and Ca^{++} concentrations in the cytosol. These effects tend to stabilize neuronal membranes and have been postulated to be the basis for its anticonvulsant activity. A decrease in Na^+ and/or Ca^{++} permeability cannot explain PHT's excitatory effect in neonatal animals and in adults with high doses unless it selectively affects inhibitory neurons (for discussion, see Woodbury 1982). In neonatal animals, however, this is inconsistent with both anatomical and biochemical evidence suggesting that cerebral and cerebellar cortical inhibitory circuits are poorly developed. Under normal circumstances inhibitory output predominates over excitatory output in the adult central nervous system (CNS), as suggested by the observation that blockade of GABA mediated inhibition with bicuculline produces intense seizure activity. Therefore, a decrease in Na^+ and Ca^{++} permeability would tend to stabilize inhibitory neurons, and produce excitatory rather than anticonvulsant effects through a disinhibition of excitatory pathways. This discrepancy between anticonvulsant action and these proposed mechanisms has led us to investigate the hypothesis that PHT is primarily an excitatory drug that possesses anticonvulsant activity by virtue of its ability to stimulate predominating inhibitory pathways of the adult CNS. Previous reports have demonstrated that PHT is capable of

facilitating polysynaptic inhibitory systems (Davidoff, 1972; Halpern and Julian, 1972; Raabe and Ayala, 1976; Fromm, et al., 1981a, 1981b, and 1982). From this, it would appear that the net effect of PHT administration depends on the predominating system (excitatory or inhibitory) at the time it is given. In neonatal animals for example, stimulation of predominating excitatory pathways by PHT produces an enhanced excitatory CNS output. In contrast, PHT stimulates both excitatory and inhibitory CNS pathways, in the adult CNS, but because inhibitory pathways predominate, the end result, at least in low doses, is one of inhibition (anticonvulsant effect) rather than excitation, whereas in high doses excitatory effects appear.

The CNS of the adult rat is comprised of two functional cell types (i.e., the neuron and the glia). In light of increasing evidence supporting a regulatory role for glial cells in maintaining ionic and acid-base homeostasis, it seems reasonable to expect that they would be involved not only in epileptogensis (Pollen and Trachtenberg, 1970; Grisar et al., 1983), but also in the prevention of seizure spread. Furthermore, it follows, that PHT through an interaction with glial regulatory processes, can provide the CNS with an enhanced ability to regulate the ionic and acid-base homeostasis within the brain extracellular fluid (ECF), thereby limiting the spread of seizure activity. Thus, by identifying specific biochemical alterations attributable to acute and chronic administration of PHT, we attempted in the present investigation to characterize both the neuronal and the glial components that contribute to PHT's antiepileptic, excitatory and toxic effects. The cellular heterogeneity of the adult CNS, however, makes it difficult to draw associations between a specific drug effect and a particular cell type. To circumvent this problem, the studies described in the present report were conducted in both neonatal and adult rats. At postnatal day 3, the neuropil of the CNS is comprised primarily of nerve cells and effects observed following

treatment with PHT at this age, reflect a direct interaction between PHT and neuronal cells. On the other hand, the adult CNS is comprised of functional neuronal and glial cells, and effects observed following treatment with PHT at this age reflect a direct or indirect effect on neuronal and/or glial cells.

In addition to the glial marker enzyme carbonic anhydrase, the activities of Na^+ K^+-, Ca^{++}, Mg^{++}-, and HCO_3^--ATPase and the electrolyte (Na^+, K^+ & Cl^-) content of the cerebral cortex were determined following acute and chronic PHT treatment of neonatal and adult rats. Because changes in the activities of these transport enzymes can have a significant influence on the level of CNS excitability by altering electrolyte (Na^+, K^+, and Cl^-) homeostasis, it is important to define and characterize the effect of PHT on these processes.

METHODS AND PROCEDURES

Animals

Neonatal (postnatal day 3, males and females) and adult (250 gm-males) Sprague-Dawley rats were used in all experiments. Pregnant females were obtained from an in-house breeding colony, and served as the source for all neonatal pups. Adult males were purchased from Simmonsen Laboratories, Gilroy, California. Upon shipment, animals were allowed 7-10 days to acclimatize. All rats were housed in a temperature and humidity controlled environment, and allowed free access to Purina Rat Chow and water ad libitum. Lighting was maintained on a 12-hour on, and a 12-hour off cycle (on 0800, off 2000 hours).

Drug Treatment

Time-Response. Neonatal (n = 5-8 per group) and adult rats (n = 8 per group) received an anticonvulsant dose of PHT (20 mg/kg, ip) at various times (15, 30, 60, 120, and 240 min) prior to electroshock testing and/or sacrifice. Control animals received an equivalent volume of vehicle (5% gum Arabic) at similar time intervals prior to sacrifice.

Dose-Response. Varying doses of PHT (10, 20, 40, and 80 mg/kg) were administered intraperitoneally to adult rats (n = 8 per group) two hours prior to electroshock testing.

Acute and Chronic Effect. Two hours prior to sacrifice, neonatal (n = 6 per group) and adult animals (n = 7-8 per group) received one acute dose of PHT (20 mg/kg, ip). To evaluate the chronic effects of PHT on various biochemical parameters, two control and two treated groups were employed. One group of

adult male rats was given PHT (20 mg/kg, ip) twice daily for 7 days, while the other treatment group received an equivalent dose (20 mg/kg) four-times-a-day for 7 days. The two control groups received a similar volume of vehicle twice-a-day and four-times-a-day for 7 days. Animals were sacrificed two hours after the last dose.

Anticonvulsant Testing

Maximal electroshock seizures (MES) were elicited with a current intensity of 150 ma for 0.2 sec delivered through corneal electrodes at 60 Hz. Endpoint for anticonvulsant effect was taken as the abolition of hindlimb extension. The total length of the tonic phase (flexion + extension) of the seizure, the extension and flexion time, and the extension/flexion (E/F) ratio of those animals not protected against a MES stimulus was also recorded. This method has been previously described by Swinyard (1949).

Minimal electroshock seizure threshold (EST) was determined according to the "staircase" procedure of Finney (1952), and described in detail by Swinyard (1972). Increasing and decreasing increments of current (60 Hz) are delivered for 0.2-sec stimulus duration through corneal electrodes. The presence or absence of a minimal seizure characterized by clonic activity of the vibrissae, lower jaw, or forelimbs was taken as the endpoint. An estimation of the convulsive current fifty (cc_{50}) and calculation of the 95% confidence limits were carried out using the graphic method of Litchfield and Wilcoxon (1949). These methods and their interpretation have been discussed in detail by Swinyard (1972).

MES and EST patterns of adult rats were determined, using an electroshock apparatus designed by Woodbury and Davenport (1952).

Sample Collection and Biochemical Assay Procedures

Just prior to sacrifice, each animal was anesthetized with ether. Within 2 minutes of exsanguination, a 25-100 microliter (ul) sample of cerebrospinal fluid (CSF) was taken from the cisterna magna with a 0.1 ml tapered capillary tube, and weighed. Animals were perfused through the left ventricle of the heart with ice-cold isotonic sucrose. The whole brain was removed, and carefully dissected on a ice-cold aluminum plate. One-half of the cerebral cortex was placed on a preweighed parafilm, reweighed and frozen (-20°C) until further analysis could be performed. The remaining tissue was placed into a preweighed vial for determination of water and electrolyte (Na^+, K^+, and Cl^-) content.

Enzymatic Assays. On the day of analysis, tissues were homogenized in ice-cold distilled water (final concentration of 25 mg tissue/ml water) for determination of carbonic anhydrase activity and to 1 mg/ml for determination of Na^+, K^+-,HCO_3^-, and Ca^{++}, Mg^{++}-ATPase enzymic activity. Carbonic anhydrase activity was estimated according to the method of Maren (1960). Na^+, K^+-, HCO_3^-, and Ca^{++}, Mg^{++}-ATPase enzymic activities were determined by our modifications of the procedures of Bonting (1970), Kimelberg and Bourke (1973), and Stekhoven and Bonting (1981), respectively. Shown in Table 1 are the concentrations and amounts of the solutions used in each assay for the determination of Na^+,K^+- and HCO_3^--ATPase activity.

After a 6 to 10 min preincubation, Na_2ATP (5 mM final concentration) was added. The reaction was terminated by the addition of 0.02 ml of 2.5 M $HClO_4$ after a 6 min incubation. The inorganic phosphate released was determined by a modification of the method of Itaya and Ui (1966) using malachite green dye. The optical density was read at a wavelength of 640 nm after 6 minutes of color development. The amount of phosphate was determined from the regression equation of a phosphate standard curve, and the activity of Na^+, K^+-ATPase (nM

Table 1. COMPOSITION OF BUFFERS USED FOR THE DETERMINATION OF Na^+,K^+- and HCO_3^--ATPase ACTIVITY.

	Na^+, K^+-ATPase	Mg^{++}-ATPase	HCO_3^--ATPase	Mg^{++}-ATPase
	ml	ml	ml	ml
Tissue homogenate	0.1	0.1	0.1	0.1
Buffer	0.1 pH 7.4	0.1 pH 7.4	0.1 pH 8.7	0.1 pH 8.7
$MgSO_4$(5mM)	+	+	+	+
K_2SO_4(5mM)	+	-	-	-
Na_2SO_4(60mM)	+	+	+(40mM)	+
EDTA(0.1mM)	+	+	+	+
TRIS (92mM)	+	+	+	+
$NaHCO_3$(20mM)	-	-	+	-
ATP(60mM)	0.02	0.02	0.02	0.02
Ouabain (1mM)	-	0.02	-	0.02
Water	0.02	-	0.02	-
Total Volume	0.24	0.24	0.24	0.24

(+)-present in buffer system.
(-)-absent in buffer system.

of Pi/mg protein/min) was that amount inhibited by 1.0 mM ouabain. This method has been determined to be sensitive to 6 nmoles of phosphate and is somewhat more sensitive than methods which detect p-nitrophenol (Anderson and Kemp, unpublished observations).

Ca^{++}-activated, Mg^{++}-dependent enzyme activity was also determined according to our modification of the method of Stekhoven and Bonting (1981). One hundred microliters of tissue homogenate was incubated under identical conditions as described above, in a buffer containing 48 mM Histidine-HCl, 12 mM $MgCl_2$, 1.2 mM EGTA, and 1.2 mM $CaCl_2$ (pH 7.0). The Mg^{++}-activated ATPase activity was determined in a buffer composed of 48 mM Histidine-HCL, 12 mM $MgCl_2$, and 1.2 mM EGTA (pH 7.0).

Subcellular Fractionation. Subcellular fractionations were performed on cerebral cortical tissue obtained from vehicle-treated controls and chronic PHT-treated animals (20 mg/kg, ip, qid for 7 days). Fractionation procedures were conducted at 4°C in a Beckman L5-65B centrifuge according to the method of Gray and Whitaker (1962). Whole homogenate, microsomal, synaptosomal, myelin and mitochondrial fractions were assayed for Na^+, K^+-, Ca^{++}, Mg^{++}- and HCO_3^--ATPase and carbonic anhydrase activity.

Electrolyte Content. Measurement of brain tissue extracellular space (ECS) from $^{36}Cl^-$ uptake curves (Smith et al., 1981), total H_2O, and total electrolyte content provides the necessary data for adequately estimating the intracellular electrolyte concentration. Intracellular concentrations of Na^+ (Na^+_i), and K^+ (K^+_i) were estimated by subtracting the extracellular electrolyte content from the total electrolyte content and correcting for water content. Extracellular electrolyte content was obtained from the product of the fractional ECS times the CSF electrolyte concentration. This method has been described previously by Smith et al. (1981). CSF electrolyte (Na^+, K^+ and Cl^-) content was determined on

the day of sacrifice. CSF was diluted 1:200 with a solution containing 15 meq/l Lithium (Li^{++}). The concentrations of Na^+ and K^+ (meq/l) were determined directly on this solution with an Instrumentation Laboratory, Inc. flame photometer. Chloride content was estimated with a Buchler-Cotlove chloridometer after mixing 1 ml of the above solution and 3 ml nitric acid reagent (containing 0.1N HNO_3 and 1.8N acetic acid).

Tissue water content was determined by dessication at 60°C for 24-48 hours. Tissue Na^+ and K^+ content was determined by flame photometry. Fifty milligrams of dry tissue was first extracted in 8.0 ml of a 1.0 N HNO_3 solution containing 15 meq/l Li^{++} for 18-24 hours. On the day of analysis, a 1:5 dilution of tissue extract with acid Li^{++} was made, and the content of Na^+ and K^+ determined. Tissue Cl^- content was estimated by a Buchler-Cotlove chloridometer after mixing 0.5-1.0 ml of tissue extract with 3.5-4.0 ml nitric acid reagent (containing 0.1N HNO_3 and 1.8 N acetic acid).

DNA and Protein Assays. The DNA content of the cerebral cortex was determined in triplicate according to our modifications of the method of Hinegardner (1971). One hundred microliters (0.1 ml) of tissue homogenate (1mg/ml) was placed into a 6 x 50 mm disposable glass culture tube, and centrifuged at 4000 rpm for 20 min. The supernatant was carefully aspirated with a tapered 0.1 ml capillary tube attached to the house vacuum line, and discarded. The remaining pellet was incubated with 0.020 ml of diaminobenzoic acid (33% w/v) at 60°C in a temperature controlled aluminum block for 45 min. At the end of this time, the reaction was stopped by adding 0.3 ml of 1.0 N HCl to each tube. Samples were vortexed to insure adequate mixing and the relative fluorescence of each sample was determined with an Aminco-Bowman Spectrophotofluorometer (excitation wavelength-410 nm; emission wavelength-510 nm). DNA content was estimated from a standard curve using Calf Thymus as a

standard. By using 6 x 50 mm culture tubes and reducing the sample volume, we have found the sensitivity and linearity of this method to be in the range of 10 - 200 nanograms of total DNA.

Protein content of individual cultures was determined in triplicate by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical Analysis

Six or more animals were used for each experimental group. Data analysis involved calculation of means, standard deviations, and standard errors of the means for each experimental group. The unpaired student's t-test was used to determine significant differences in enzyme and electrolyte contents between controls and treated groups. Unless otherwise stated, differences between means were considered significant at the 0.05 or lower probability level using the one-tail t-table.

RESULTS

Anticonvulsant and Excitatory Effects

The peak anticonvulsant effect of PHT (20 mg/kg), as measured by changes in MES patterns was observed 2-hours after a single dose (Fig. 1). At this time, 67% of the animals tested were protected against a maximal electroshock seizure (A). In unprotected animals PHT decreased seizure duration (B), decreased total time of hind-limb extension (C), and decreased the extension to flexion ratio (E/F ratio) (D). As illustrated in Fig. 2, the dose producing peak protection against MES was 20 mg/kg. Above this dose (20 mg/kg), PHT increased the seizure duration (A), and the extensor component (B) progressively to above control levels, decreased the flexor component (B) above 40 mg/kg, and increased the E/F ratio (C) to pre-drug control values.

As demonstrated previously by other investigators, PHT (20 mg/kg) did not significantly affect the EST (Fig. 3-top). A significant regression between PHT dose and EST was, however, observed at 30 min as illustrated in Fig. 3-bottom.

Effect of PHT on Cerebral Cortex Electrolytes and Enzymic Activity

The effects of acute and chronic PHT administration on neonatal and adult rat cerebral cortical Na^+ and K^+ content, and the activity of Na^+, K^+-ATPase (whole homogenate activity) are summarized in Fig. 4. Acute PHT treatment of neonatal animals significantly inhibited cerebral cortical Na^+, K^+-ATPase. Consistent with this effect, a significant increase in total tissue Na^+, and a significant decrease in total tissue K^+ was observed. Na^+, K^+-ATPase activity was markedly attenuated following acute PHT treatment of adult animals (Fig. 4),

Fig. 1. Acute anticonvulsant profile of PHT in adult rats. At various times after PHT administration (20 mg/kg, ip), animals were tested for protection (%) (A) against maximal electroshock induced seizures as described by Swinyard (1949). A 60 hz, 150 ma stimulus (0.2 sec duration) was delivered to treated and control animals through corneal electrodes. The total length (sec) of the tonic phase (flexion + extension) of the seizure (B), the extension and flexion time (sec) (C), and the E/F ratio (D) of animals not protected against a MES stimulus were also recorded. The results represent the mean ± SEM. (* $p < 0.05$).

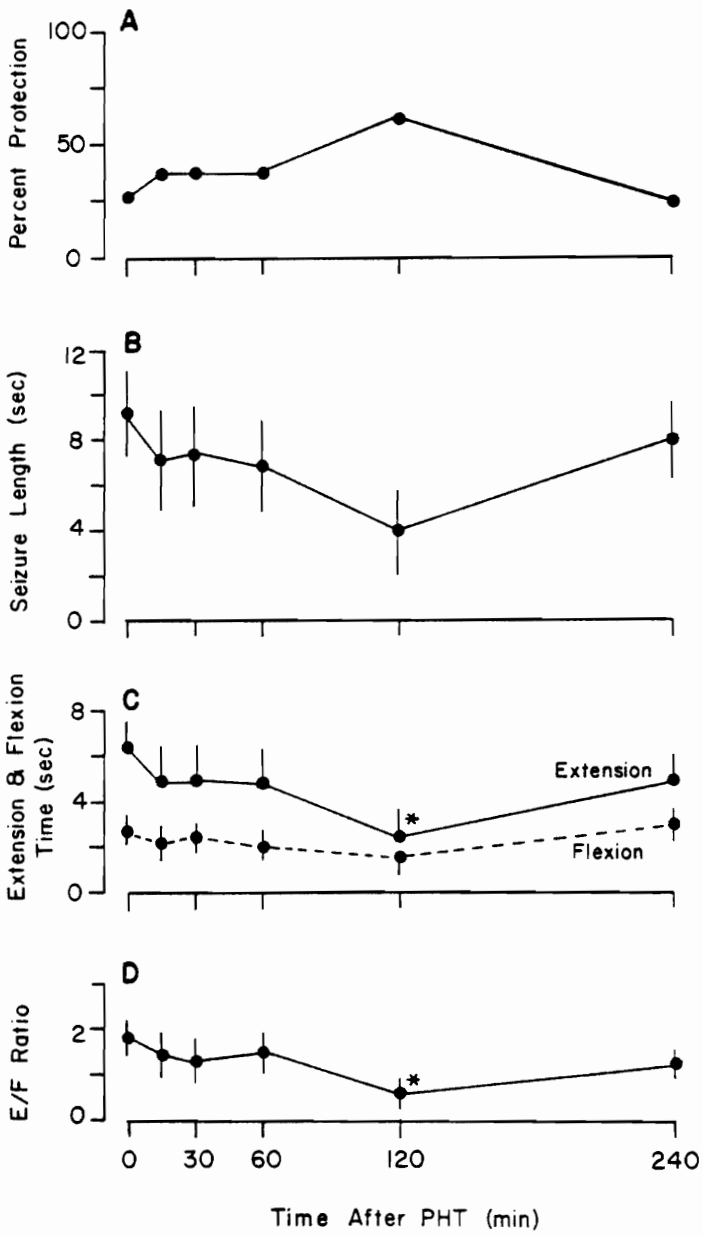

A
100
50
0
Percent Protection
B
12
8
4
0
Seizure Length (sec)
C
8
4
0
Extension & Flexion Time (sec)
Extension
Flexion
*
D
2
0
E/F Ratio
*
0
30
60
120
240
Time After PHT (min)

Fig. 2. The effect of increasing PHT dose on various anticonvulsant indices. Increasing doses (10, 20, 40, and 80 mg/kg) were administered to adult male Sprague-Dawley rats two hours prior to anticonvulsant testing (see legend to Fig. 1, and methods for explanation). Twenty-four hours prior to PHT treatment, each animal received a MES stimulus and the seizure length (A), the extension and flexion times (B), and the E/F ratio were recorded. In this way each animal served as its own control. The results are thus expressed as a percent of these pre-drug control values and represent the mean ± the SEM. (* $p < 0.05$).

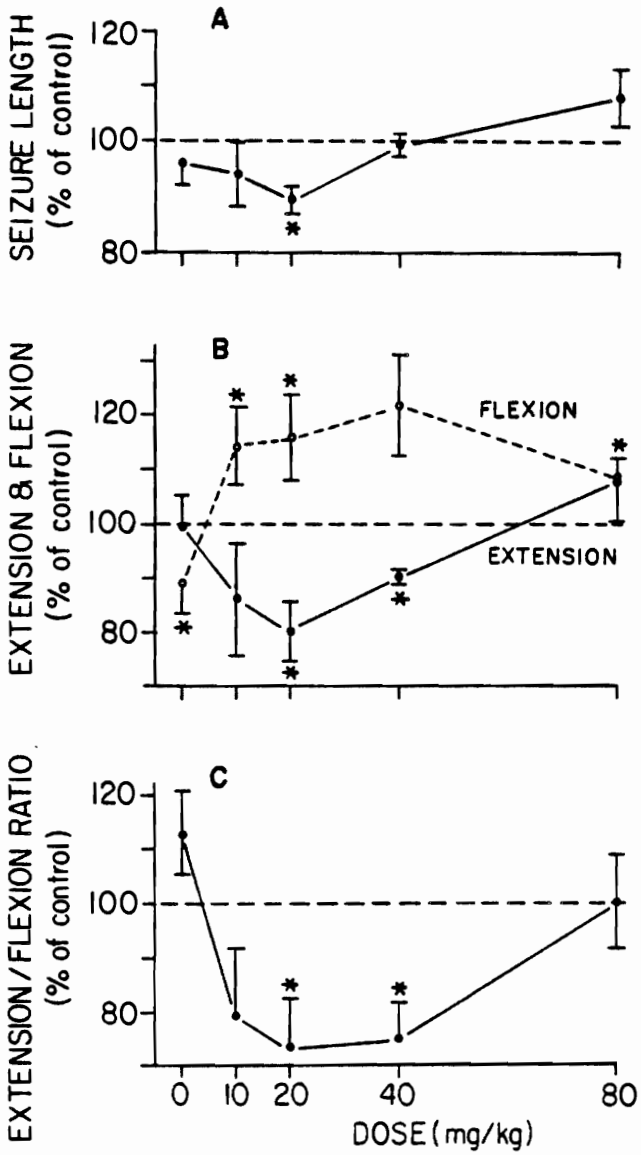

A
SEIZURE LENGTH (% of control)
120
100
80
B
EXTENSION & FLEXION (% of control)
FLEXION
EXTENSION
C
EXTENSION/FLEXION RATIO (% of control)
0
10
20
40
80
DOSE (mg/kg)

Fig. 3. Time- and dose-effect of PHT on the electroshock seizure threshold of adult rats. At various times (top) after a single dose (20 mg/kg, ip) of PHT, the seizure threshold of control and treated rats was determined according to the staircase procedure of Finney (1952). The acute dose-effect of PHT (bottom) on the EST was determined 30-minutes after PHT treatment. Each point represents the current (ma) necessary to produce a minimal seizure in 50% of the animals tested. Ninety-five percent confidence limits are shown for the top curve.

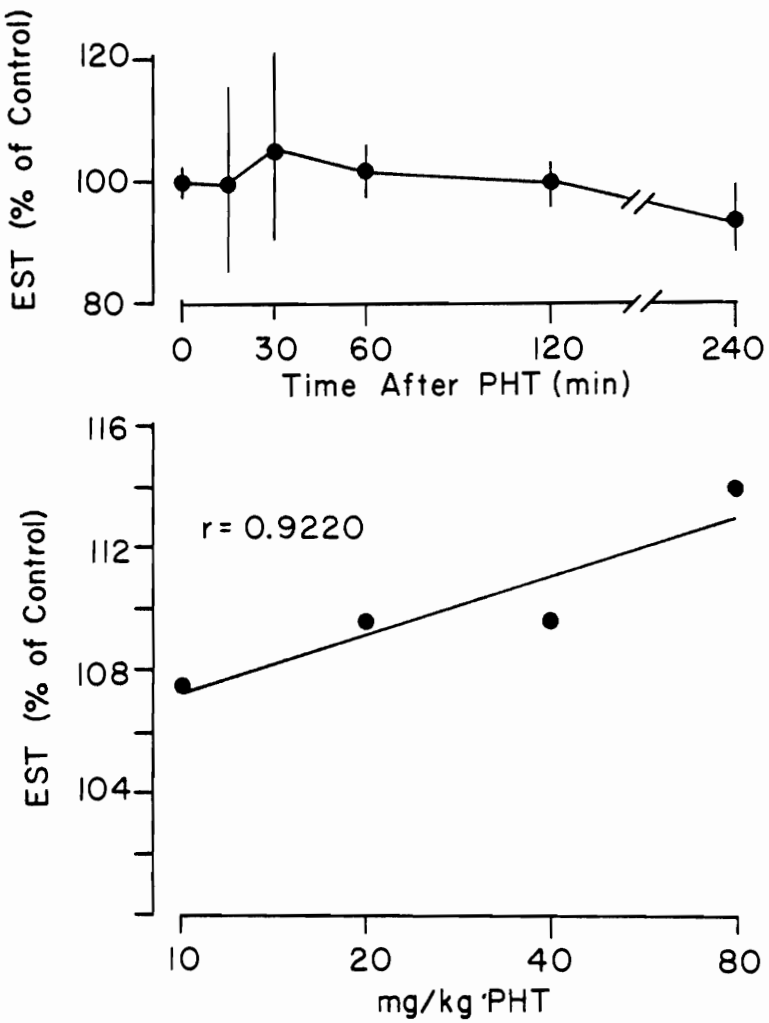
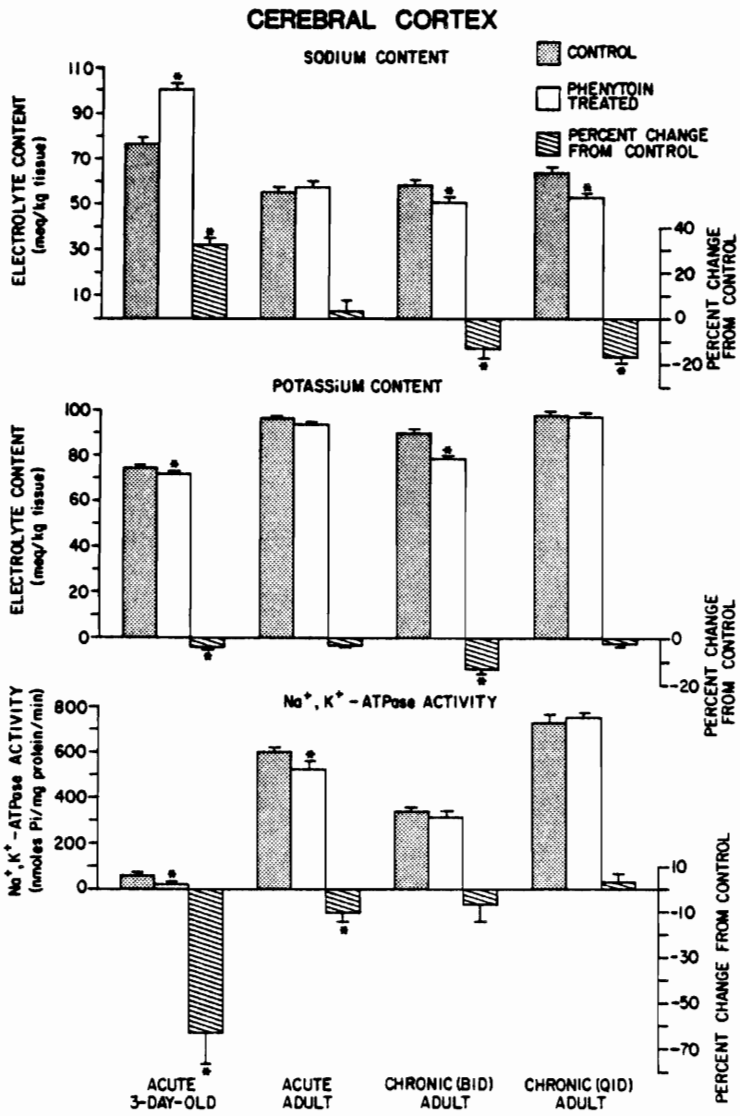

EST (% of Control)
120
100
80
0
30
60
120
240
Time After PHT (min)
116
112
108
104
r = 0.9220
EST (% of Control)
10
20
40
80
mg/kg PHT

Fig. 4. Effect of acute and chronic PHT treatment on neonatal and adult rat cerebral cortical Na^+ and K^+ content and Na^+, K^+-ATPase activity. All values are expressed as the mean ± SEM. The total cerebral cortical Na^+ and K^+ content and Na^+, K^+-ATPase activity of vehicle-treated controls and PHT-treated animals is represented by the stippled (controls) and open (PHT-treated) bars. Hatched bars represent the percent change from control following PHT-treatment. (* $p < 0.05$).


CEREBRAL CORTEX
SODIUM CONTENT
CONTROL
PHENYTOIN TREATED
PERCENT CHANGE FROM CONTROL
ELECTROLYTE CONTENT (meq/kg tissue)
PERCENT CHANGE FROM CONTROL
POTASSIUM CONTENT
Na+, K+ - ATPase ACTIVITY
Na+,K+-ATPase ACTIVITY (nmoles Pi/mg protein/min)
PERCENT CHANGE FROM CONTROL
ACUTE 3-DAY-OLD
ACUTE ADULT
CHRONIC (BID) ADULT
CHRONIC (QID) ADULT

and although enzymic activity was still inhibited, no significant changes in tissue electrolytes (Na^+, and K^+) were observed. Na^+, K^+-ATPase activity was not significantly affected following chronic PHT treatment (bid and qid for 7 days). In contrast to the acute effect, total tissue Na^+ was significantly reduced by both twice daily and four times daily PHT administration. These effects were observed in the absence of any measurable effect on whole homogenate Na^+, K^+-ATPase activity.

As shown in Fig. 5, the cerebral cortical Na^+, K^+-ATPase activity of neonatal rats was maximally inhibited 2 hours after a single dose of PHT (20 mg/kg, ip). This figure also demonstrates the inverse relationship between Na^+, K^+-ATPase activity, and Na^+_i concentration. The concentration of Na^+_i increased rapidly with increasing inhibition of Na^+, K^+-ATPase. Four hours after a single dose, the activity of Na^+, K^+-ATPase and the concentration of Na^+_i were approaching pre-drug levels. As also shown in Fig. 5, the activity of Ca^{++}, Mg^{++}-ATPase was increased over 2-fold 15 min after a acute dose of PHT (20 mg/kg). The activity of this enzyme was also found to be elevated 240 min after a similar dose. Between these two extremes of the time curve, Ca^{++}, Mg^{++}-ATPase activity remained elevated but was not statistically different from vehicle-treated controls as shown in Fig. 5.

The activity of Na^+, K^+-ATPase was determined in several subcellular fractions of the cerebral cortex following chronic PHT treatment of adult rats (20 mg/kg, ip, qid for 7 days). As shown in Fig. 6, no change in whole homogenate or microsomal enzymic activity was observed with this treatment. A significant increase in both myelin (glial product) and mitochondrial Na^+, K^+-ATPase activity was observed, and although not statistically different from controls, a 20% inhibition of the synaptosomal (neuronal) enzyme was seen following this treatment (20 mg/kg, ip, qid for 7 days).

Fig. 5. Time-effect of PHT on the activities of cerebral cortical Na^+, K^+- and Ca^{++}, Mg^{++}-ATPase, and the concentration of Na^+_i in the neonatal rat. At various times after a single dose of PHT (20 mg/kg, ip), neonatal animals were sacrificed, and the cerebral cortex removed. Enzymic activity (nM Pi/mg Protein/min) and Na^+_i (meq/l cell water) concentration was determined according to the procedures outlined in the methods.

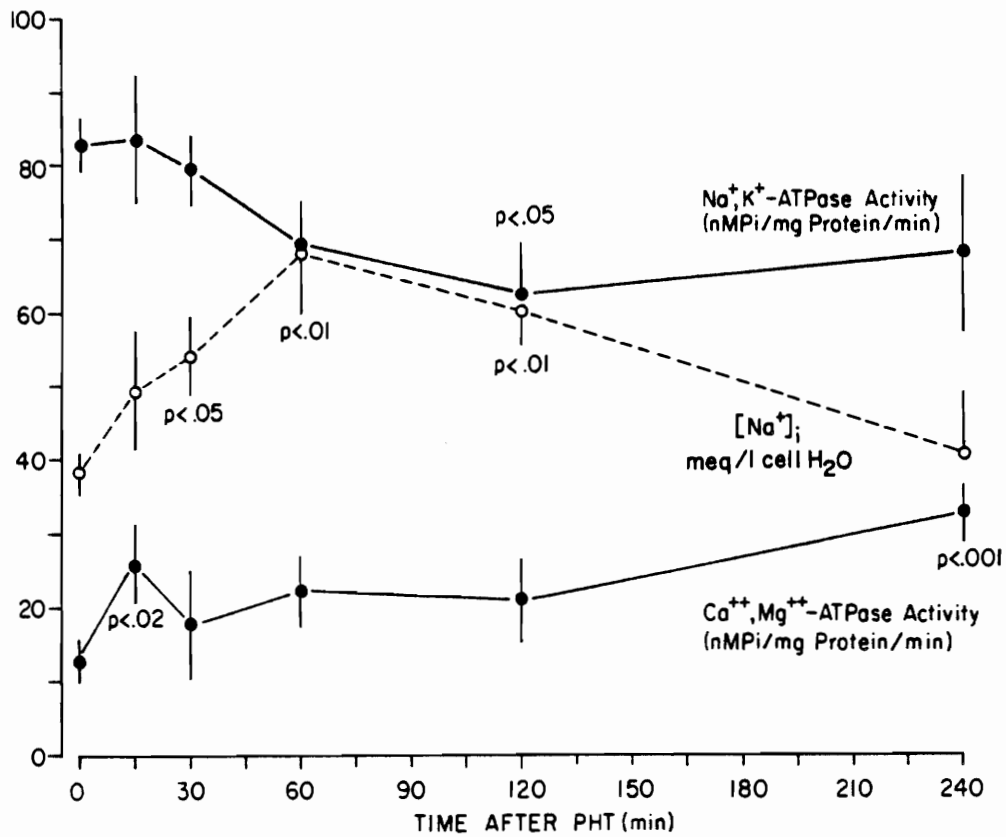
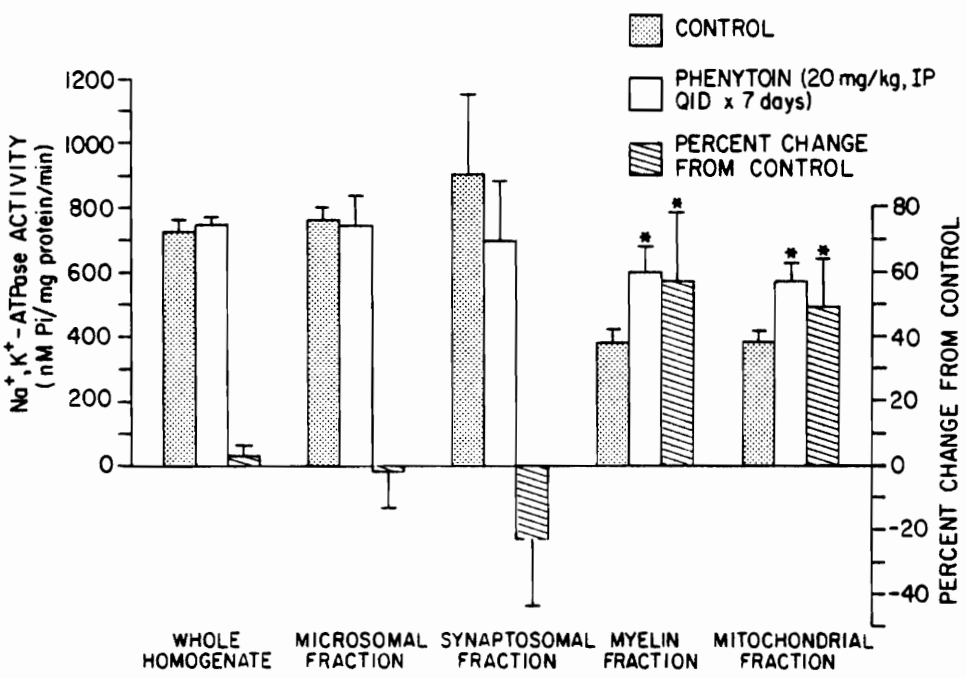

100
80
60
40
20
0
0
30
60
90
120
150
180
210
240
TIME AFTER PHT (min)
p<.05
p<.01
p<.01
p<.05
p<.02
p<.001
Na+,K+-ATPase Activity
(nMPi/mg Protein/min)
[Na+]i
meq/l cell H2O
Ca++,Mg++-ATPase Activity
(nMPi/mg Protein/min)

Fig. 6. Effect of chronic PHT treatment on the subcellular activity of Na^+, K^+-ATPase. Adult male rats were administered PHT (20 mg/kg, ip) four times a day for 7 days. Two hours after the last dose, animals were sacrificed, perfused with 0.32 M sucrose, and cerebral cortex removed as outlined in the methods. Subcellular fractions obtained by sucrose density centrifugation (Gray and Whitaker, 1972) were assayed for Na^+, K^+-ATPase activity according to the procedures outlined in the methods. All values are expressed as the mean ± SEM. Subcellular Na^+, K^+-ATPase activity vehicle-treated controls and PHT-treated animals is represented by the stippled (controls) and open (PHT-treated) bars. Hatched bars represent the percent change from control following PHT-treatment. (* $p < 0.05$).

EFFECT OF CHRONIC PHENYTOIN TREATMENT ON THE SUBCELLULAR ACTIVITY OF Na^+, K^+-ATPase



As illustrated in Fig. 7, the cerebral cortex subcellular fractions of untreated adult rats contained measurable Ca^{++}, Mg^{++}-ATPase activity. In contrast to its acute effect at postnatal day 3 (Fig. 5), chronic PHT (20 mg/kg, ip, qid for 7 days) significantly reduced the activity of this enzyme in all subcellular fractions except the mitochondrial, in which no change in enzymic activity was observed.

The effects of PHT on anion homeostasis as measured by changes in Cl^- content, HCO_3^--ATPase activity, and carbonic anhydrase activity of the cerebral cortex are summarized in Fig. 8. The acute administration of PHT to neonatal rats produced a significant increase in cerebral cortical Cl^- content (Fig. 8A). The Cl^- content of adult rat cerebral cortex was not significantly affected by either acute or chronic (bid or qid) PHT administration. Cerebral cortical HCO_3^--ATPase activity was increased 2-fold following acute administration of PHT to neonatal animals (8B), and was increased 58% above control values following chronic (bid for 7 days) PHT-treatment of adult rats. No change in the activity of HCO_3^--ATPase was observed in the whole homogenate fractions of cerebral cortices obtained from animals treated chronically (20 mg/kg, ip, qid for 7 days). In these same animals, however, a significant increase in mitochondrial- (72%) and myelin- (378%) associated HCO_3^--ATPase activity was observed (Fig. 9).

The effect of PHT on the activity of carbonic anhydrase was not determined in neonatal animals since little carbonic anhydrase activity is present at this age. The effect of PHT on the carbonic anhydrase activity of the cerebral cortex was dependent in-part on the duration of exposure as shown in Fig. 8C. Acutely, PHT significantly decreased the activity of this enzyme whereas, chronically PHT increased enzymic activity in a dose-related manner.

The effects of chronic PHT-treatment on glial cell proliferation (DNA content) and carbonic anhydrase activity are summarized in Fig. 10. In a dose-related manner, the activity of the glial enzyme carbonic anhydrase (top) was

Fig. 7. Effect of chronic PHT-treatment on the subcellular activity of Ca^{++}, Mg^{++}-ATPase. See legend to Fig. 6 for explanation.

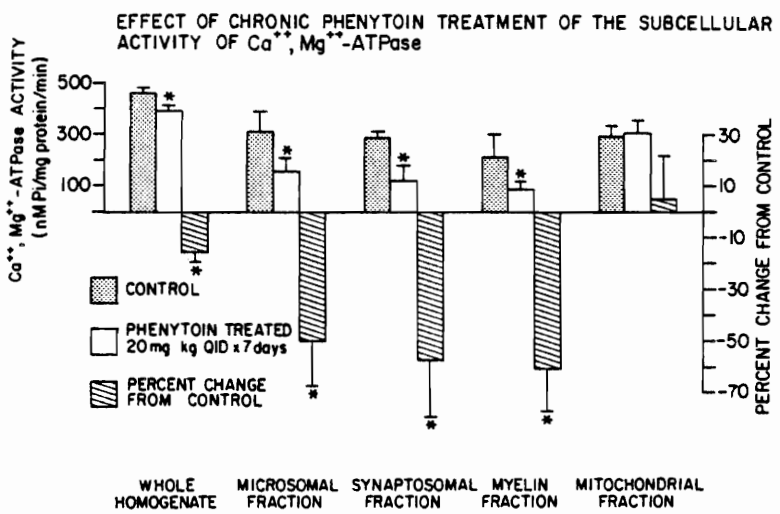

EFFECT OF CHRONIC PHENYTOIN TREATMENT OF THE SUBCELLULAR ACTIVITY OF Ca++, Mg++-ATPase
Ca++, Mg++-ATPase ACTIVITY (nM Pi/mg protein/min)
500
300
100
30
10
-10
-30
-50
-70
PERCENT CHANGE FROM CONTROL
CONTROL
PHENYTOIN TREATED 20 mg kg QID x 7 days
PERCENT CHANGE FROM CONTROL
WHOLE HOMOGENATE
MICROSOMAL FRACTION
SYNAPTOSOMAL FRACTION
MYELIN FRACTION
MITOCHONDRIAL FRACTION

Fig. 8. Effect of acute and chronic PHT treatment on neonatal and adult rat cerebral cortical Cl^-content, and the activity of HCO_3^--ATPase and carbonic anhydrase. All values are expressed as the mean ± SEM. The cerebral cortical Cl^- content, HCO_3^--ATPase, and carbonic anhydrase activity vehicle-treated controls and PHT-treated (20 mg/kg, ip) animals are represented by the open (controls) and hatched (PHT-treated) bars. Stippled bars represent the percent change from control following PHT-treatment. (* $p < 0.05$).

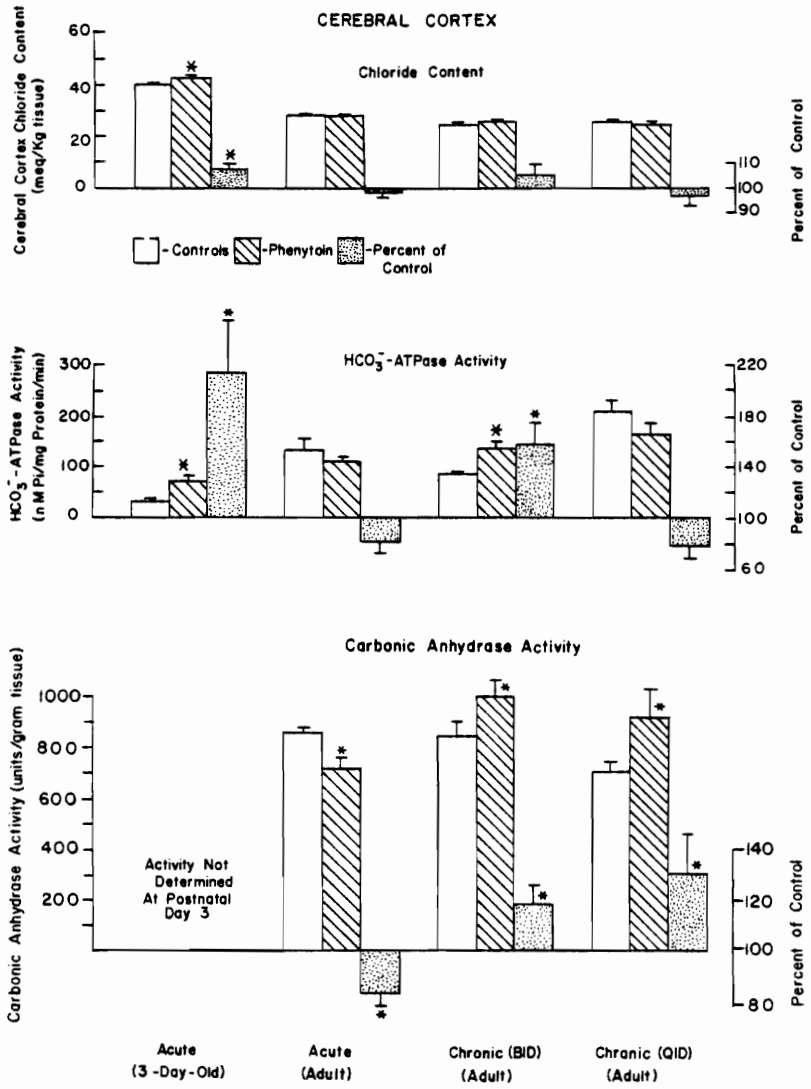

CEREBRAL CORTEX
Chloride Content
Cerebral Cortex Chloride Content (meq/Kg tissue)
60
40
20
0
110
100
90
Percent of Control
- Controls
-Phenytoin
-Percent of Control
HCO3- -ATPase Activity
HCO3- -ATPase Activity (n M Pi/mg Protein/min)
300
200
100
0
220
180
140
100
60
Percent of Control
Carbonic Anhydrase Activity
Carbonic Anhydrase Activity (units/gram tissue)
1000
800
600
400
200
Activity Not Determined At Postnatal Day 3
140
120
100
80
Percent of Control
Acute (3 -Day-Old)
Acute (Adult)
Chronic (BID) (Adult)
Chronic (QID) (Adult)

Fig. 9. Effect of chronic PHT treatment on the subcellular activity of HCO_3^--ATPase. See legend to Fig. 6 for explanation.

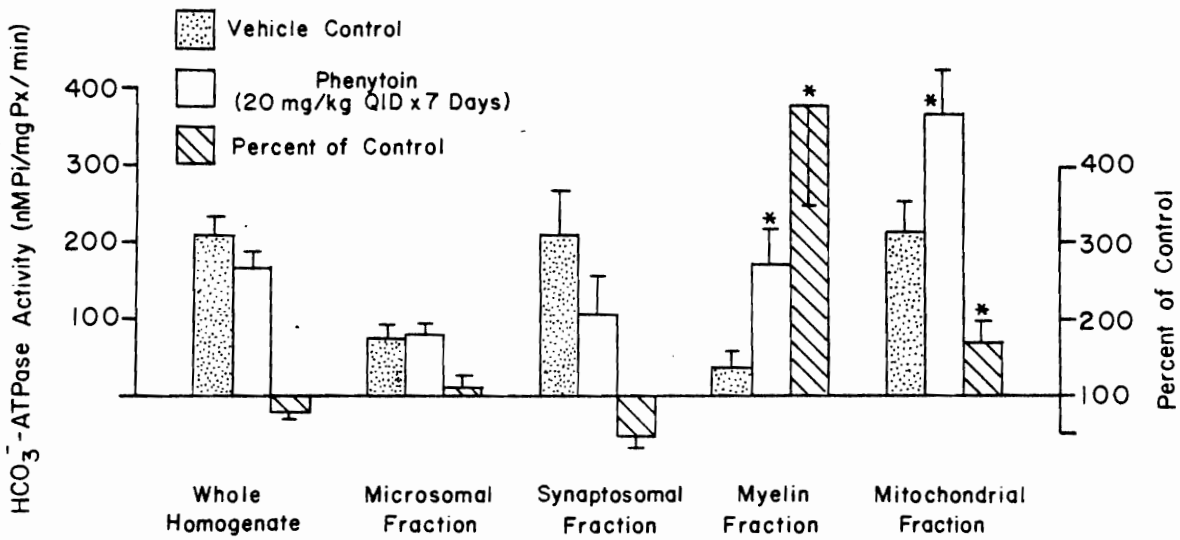
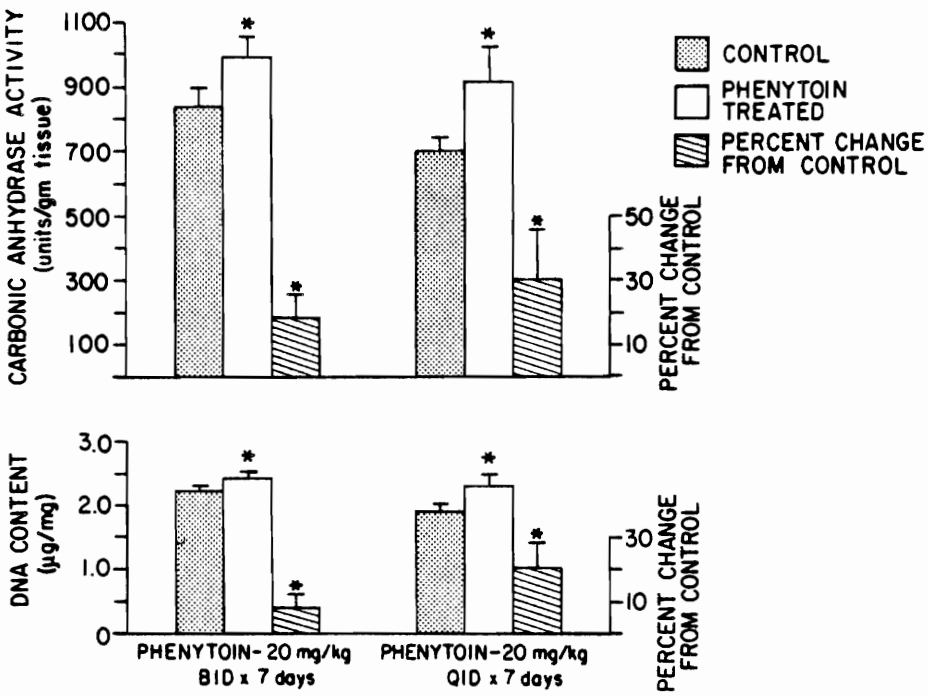

Vehicle Control
Phenytoin
(20 mg/kg QID x 7 Days)
Percent of Control
HCO_3^--ATPase Activity (nMPi/mgPx/min)
400
300
200
100
400
300
200
100
Percent of Control
*
Whole Homogenate
Microsomal Fraction
Synaptosomal Fraction
Myelin Fraction
Mitochondrial Fraction

Fig. 10. Effect of chronic PHT treatment on cerebral cortical carbonic anhydrase activity and DNA content of the adult rat. All values are expressed as the mean ± SEM. The absolute activity of CA (units/gm tissue) (top) and DNA content (ug/mg tissue) (bottom) for vehicle treated controls and PHT-treated animals is represented by the stippled (controls) and open (PHT-treated) bars. Hatched bars represent the percent change from control following PHT-treatment. (* $p < 0.05$).

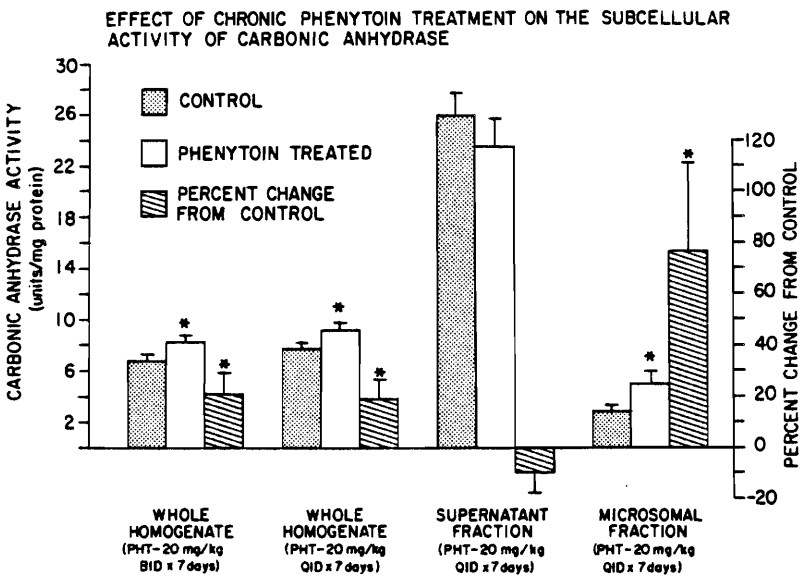
EFFECT OF CHRONIC PHENYTOIN TREATMENT ON CEREBRAL CORTEX CARBONIC ANHYDRASE ACTIVITY AND DNA CONTENT



increased 20% (bid) and 30% (qid) following chronic PHT-treatment. In a similar manner, the cerebral cortex DNA content (bottom) was increased 10% (bid) and 20% (qid) with chronic PHT-treatment.

The effect of chronic PHT-treatment on the subcellular activity of carbonic anhydrase is summarized in Fig. 11. As shown in this figure, the supernatant fraction contained the highest, and the microsomal fraction the lowest activity of carbonic anhydrase (units/mg protein). Both treatment schedules (bid and qid for 7 days) increased carbonic anhydrase activity of the whole homogenate, and this increase was apparently the result of a specific increase (75%) in the membrane bound (microsomal) fraction. PHT produced no change in the activity of the supernatant fraction of carbonic anhydrase.

Fig. 11. Effect of chronic PHT treatment on the subcellular activity of carbonic anhydrase. See legend to Fig. 6 for explanation.


EFFECT OF CHRONIC PHENYTOIN TREATMENT ON THE SUBCELLULAR ACTIVITY OF CARBONIC ANHYDRASE
CARBONIC ANHYDRASE ACTIVITY (units/mg protein)
30
26
22
18
14
10
6
2
CONTROL
PHENYTOIN TREATED
PERCENT CHANGE FROM CONTROL
120
100
80
60
40
20
0
-20
PERCENT CHANGE FROM CONTROL
WHOLE HOMOGENATE (PHT-20 mg/kg BID x 7 days)
WHOLE HOMOGENATE (PHT-20 mg/kg QID x 7 days)
SUPERNATANT FRACTION (PHT-20 mg/kg QID x 7 days)
MICROSOMAL FRACTION (PHT-20 mg/kg QID x 7 days)

DISCUSSION

Three-day old rats are an excellent model for studying in vivo the effects of a drug on neurons, since the brain at this age is comprised primarily of neurons and essentially no functional glial cells. The marked inhibition of Na^+, K^+-ATPase and the subsequent increase in total Na^+, and decrease in total K^+ (Fig. 4) after an acute dose of PHT given at this age reflects a direct action on neurons. The time course of the effect of PHT on Na^+_i concentration and Na^+, K^+-ATPase activity (Fig. 5) clearly illustrates that its inhibitory effect on the Na^+, K^+-ATPase enzyme was not compensatory to a decrease in Na^+_i, since at no time was the concentration of Na^+_i found to be lower than control. An increase in total Na^+ of the magnitude shown in this figure, accompanied by a decrease in total K^+, would be expected to produce a substantial depolarization of the neuronal membrane. Because both anatomical and biochemical evidence suggest that cerebral and cerebellar inhibitory circuits are poorly developed at this age, depolarization of neurons would lead not to protection against, but enhancement of seizure activity, whereas in adult animals, PHT possesses anticonvulsant activity by virtue of its ability to depolarize (stimulate) the now predominating inhibitory pathways. These data provide not only an explanation of PHT's excitatory effects that have been observed in neonatal animals (Vernadakis and Woodbury, 1969) and adult animals (Gruber et al., 1940) and human patients (Glaser, 1974; Lascelles et al., 1970; Levy, 1965; Patel and Crichton, 1968; Roseman, 1961; Schreiner, 1958; Troupin and Ojemann, 1975) with high doses, but when extrapolated to adults, provides new information pertaining to its often controversial mechanism of action.

Support for this conclusion comes in part from studies described in the present report demonstrating that PHT when administered to adult rats in doses greater than 40 mg/kg, increased the seizure duration and the time spent in tonic hindlimb extension relative to the effects of PHT observed at 20 mg/kg (Fig. 2). Furthermore, doses greater than 40 mg/kg decreased the time spent in hindlimb flexion and increased the E/F ratio. These effects are all consistent with enhanced excitability. In addition, it was demonstrated as early as 1941 by Goodman and Lih that PHT in large acute doses intensified the convulsive response of rodents to pentylenetetrazol. This observation has been subsequently confirmed and extended by Stone and Javid (1982). These investigators, reported that at 40 mg/kg, PHT enhanced the convulsive actions of both picrotoxin and bicuculline. That PHT is an excitatory drug is further suggested by the findings that it enhances the seizure activity of animals given strychnine, and of animals undergoing CO_2 withdrawal seizures (see Woodbury 1980a and 1982 for discussion).

PHT has been previously demonstrated to increase epithelial cell (toad bladder, frog skin, choroid plexus and glial cells) Na^+- K^+-transport (Watson and Woodbury, 1972 and 1973; Carroll and Pratley, 1970; DeSousa and Grosso, 1973; Riddle et al., 1975; and Johanson and Smith 1977) and thus decrease Na^+_i and increase K^+_i. Since this effect is opposite that described above for neurons, it might be anticipated that the effect of PHT, when administered to an adult rat whose neuropil is comprised of both functional neurons and glia would be lessened or even completely attenuated. Although the acute administration of PHT to adult rats was found to inhibit Na^+, K^+-ATPase (Fig. 4), the magnitude of its effect was much less than that observed in 3-day-old animals. The effect of PHT to inhibit Na^+, K^+-ATPase was completely attenuated by prolonged administration (twice daily and 4 times a day treatment for 7 days). Despite no observable change in the activity of Na^+, K^+-ATPase, both chronic treatment regimens

significantly reduced the Na^+ content of the CNS (Fig. 4). A decrease in total Na^+ without any change in enzyme activity would suggest that PHT is decreasing membrane permeability to Na^+ by blocking Na^+ channels. This is however, unlikely in the brain, since radioactive sodium turnover has been previously reported to be increased rather than decreased, a finding suggesting that Na^+ transport or efflux was increased (Woodbury, 1955). Thus, in adult rats, it would appear that chronic PHT enhances the activity or increases the synthesis of Na^+, K^+-ATPase in glial cells, and this increased activity masks the continuing inhibition of this enzyme in neurons. The end effect of this apparent differential action on neuronal and glial cell Na^+, K^+-ATPase, is refected by no net change in whole homogenate enzymic activity.

That PHT is differentially affecting neuronal and glial cells is supported by enzyme studies conducted on subcellular cerebral cortical fractions obtained from rats treated chronically 4 times a day for 7 days (Fig. 6). Although there was no change in whole homogenate or microsomal Na^+, K^+-ATPase activity, a significant increase in myelin (a glial product) ATPase activity was observed. In contrast, a 20% decrease in the activity of this enzyme was observed in the synaptosomal (neuronal) fraction. These results suggest that even with chronic treatment, PHT continues to inhibit the neuronal enzyme but that this effect can be masked in the whole homogenate by its effect to enhance differentially the glial Na^+, K^+-ATPase.

PHT's ability to enhance glial cell Na^+, K^+-ATPase activity could result from either a direct effect on the glial enzyme or indirectly through its action to depolarize neuronal cells. As discussed in the following report (this issue) we have demonstrated that both acute and chronic PHT treatment of primary glial cultures directly increased glial Na^+, K^+-ATPase activity. However, it is very probable that an increase in ECF K^+ (K^+_o) concentration resulting from PHT's direct

inhibitory effect on neuronal Na^+, K^+-ATPase is stimulating the ATPase enzyme of glial cells. Ultimately, by increasing the activity of glial Na^+, K^+-ATPase, PHT provides the CNS with an enhanced ability to regulate K^+_o. As discussed in the following report, this effect may prove to be very important in understanding the ability of PHT to prevent the spread (ictal transition) from a seizure focus. From these studies, it is apparent that a majority of the discrepancies in the literature with regard to the effects of PHT on Na^+, K^+-ATPase activity in adults (see Woodbury, 1980a, for review) can be explained by a paucity of studies on both subcellular fractions of brain and isolated glia, and during ontogenesis of the nervous system.

Carbonic anhydrase has a central role in the co-operative metabolism of neurons and closely contacting glia during regulation of anion and acid-base metabolism of brain cells, interstitial and cerebrospinal fluids. It is located exclusively in glia and choroid plexus (Giacobini, 1961; Korhonen et al., 1964; Rose, 1967; Tower and Young, 1973) but depends for its substrates on metabolites that are mostly generated and released during neuronal discharge. Bicarbonate-stimulated ATPase in a similar manner depends for its substrate on the activity of carbonic anhydrase (CA) which catalyses the following reation: ($CO_2 + H_2O \overset{CA}{\rightleftharpoons} H_2CO_3 \rightleftharpoons HCO_3^- + H^+$). Since both enzymes are dependent in part on neuronal activity, it reasons that a change in neuronal excitability would be reflected biochemically by a change in their activities.

In the present report, cerebral cortical carbonic anhydrase activity was significantly reduced by acute PHT-treatment (Fig. 8). In contrast, chronic administration of PHT increased the activity of carbonic anhydrase in the whole homogenate of the cerebral cortex (Fig. 8). Bicarbonate-ATPase activity was also increased in the whole homogenate of cerebral cortex following twice a day treatment for 7 days, but was not affected by the 4 times a day dosing schedule

(Fig. 8). Although no change was observed in the whole homogenate, a significant increase was found in the myelin and mitochondrial fractions (Fig. 9). These results illustrate the integral relationship that exists between carbonic anhydrase and HCO_3^--ATPase. The decreased carbonic anhydrase activity observed following acute PHT administration would, like acetazolamide, cause CO_2 to back up in neurons and produce an anticonvulsant effect. Carbonic anhydrase activity in several systems appears to be mediated at least in part by a cyclic AMP induced phosphorylation of carbonic anhydrase (Narumi and Miyamoto, 1974; Narumi et al., 1978; Church et al., 1980). From this it follows that the acute effect of PHT on this enzyme is probably related to its well-established ability to inhibit calcium-dependent phosphorylation of specific brain proteins (DeLorenzo and Glaser, 1976; DeLorenzo et al., 1977). As with acetazolamide, tolerance to the inhibitory effect of PHT on carbonic anhydrase develops as suggested by the significant increase in activity of this enzyme following chronic PHT-treatment (Figs. 8 and 10). This effect was accompanied by increased HCO_3^--ATPase activity in the whole homogenate, and in the myelin and mitochondrial subcellular fractions following either twice- or four-times daily treatment. These results further illustrate the intimate relationship that exists between these 2 enzymes.

Carbonic anhydrase, along with the mitochondrial enzyme HCO_3^--ATPase (Kimelberg and Bourke, 1973) are both intimately involved in the regulation of HCO_3^--Cl^- transport across glial cells and mitochondrial membranes. Intracellular Cl^- is also thought to be regulated by a Cl^--HCO_3^- exchange mechanism in which extracellular Cl^- exchanges with intracellular HCO_3^-- (Kimelberg et al., 1979), and it is through this mechanism that the stilbene derivative SITS inhibits the uptake of Cl^- into glial cells. Inhibition of Cl^- transport into glial cells by low doses of the monovalent anions, perchlorate and thiocyanate which inhibit HCO_3^--ATPase and carbonic anhydrase, results in an

increased brain excitability (increased E/F ratio). Furthermore, in higher doses it cause seizures, that can be enhanced by acetazolamide (Woodbury and Kemp, 1977). Thus, it is evident that glial cell active Cl^- transport is involved in regulation of brain excitability and that carbonic anhydrase and HCO_3^--ATPase play an important role in mediation of this process. Indeed the data in Fig. 8 suggest a relationship between cerebral cortical Cl^- content and the activities of these two enzymes. Experiments to document this are now being conducted in this laboratory. That carbonic anhydrase activity is related to brain excitability is demonstrated by the data on audiogenic seizure mice (Woodbury et al., 1983). As illustrated in this report, the intensity of the audiogenic seizure, was directly related to the activity of carbonic anhydrase. Furthermore, Engstrom et al. (1983) have recently demonstrated that in audiogenic seizure susceptible DBA mice, the cerebral and cerebellar cortical carbonic anhydrase activity is highest at postnatal day 21, the period when they are maximally susceptible to audiogenic seizures. Thus, it appears that the increased activity of these two enzymes observed in various cerebral tissues of the audiogenic susceptible mouse were compensatory to increased levels of neuronal activity.

In neonatal animals, a significant increase in the cerebral cortical activity of HCO_3^--ATPase was observed 2 hours after an acute dose of PHT (Fig. 8). This observation, adds further support to the hypothesis that PHT is enhancing neuronal activity, since, (as discussed above) the substrate for this enzyme (HCO_3^-) is primarily derived from conversion of the neuronal waste product CO_2 to HCO_3^- and H^+. In the absence of any significant carbonic anhydrase activity in the cerebral cortex of control animals at postnatal day 3, CO_2 would build up and HCO_3^- would be formed through the much slower uncatalyzed conversion of CO_2.

Bicarbonate ATPase has also been postulated to be an important enzyme in biotin catalyzed "CO_2"-fixation reactions of the mitochondria. These reactions

are important for the synthesis of fatty acids for myelin formation, synthesis of neurotransmitter precursors, such as oxaloacetate, a precursor of aspartate and which is also converted in the Kreb's cycle to alpha-ketoglutarate, a precursor of glutamate (Woodbury, 1980b). Therefore, through an activation of both HCO_3^--ATPase and carbonic anhydrase, PHT enhances the ability of the CNS to regulate anion homeostasis, cell pH, and "CO_2"-fixation reactions, all metabolic functions maintained under tight control in order that optimal conditions for proper enzymic functioning be maintained.

Calcium concentration in the cell is regulated through both a Ca^{++}:Na^+ counter-transport process and the enzyme Ca^{++}, Mg^{++}-ATPase (for discussion see Latzkovits and Fajszi, 1982). Thus, when the concentration of $Ca^{++}{}_i$ increases above physiological levels, cytosolic Ca^{++} exchanges with extracellular Na^+, and also activates an active extrusion mechanism (Ca^{++}, Mg^{++}-ATPase) in a compensatory attempt to decrease $Ca^{++}{}_i$ concentrations. When $Ca^{++}{}_i$ is decreased, the reverse is true, that is, exchange of $Na^+{}_i$ for $Ca^{++}{}_o$ and a inhibition of the Ca^{++}, Mg^{++}-ATPase. Therefore, it follows that the increased activity of Ca^{++}, Mg^{++}-ATPase observed after acute treatment of neonatal animals (Fig. 5) is compensatory to an increased $Ca^{++}{}_i$. It can be further reasoned that the increase in $Na^+{}_i$ observed in these same animals activates a compensatory Na^+:Ca^{++} counter-transport process which would decrease $Na^+{}_i$. This has the effect to increase $Ca^{++}{}_i$, which also activates Ca^{++}, Mg^{++}-ATPase. In contrast to the acute effect of PHT, the data presented in Fig. 7 suggest that chronic PHT-treatment of adult rats decreases the $Ca^{++}{}_i$ concentration of the cerebral cortex. This conclusion is supported by the observation that Ca^{++}, Mg^{++}-ATPase activity was decreased in all subcellular fractions except the mitochondria. Additional studies are being conducted to determine whether this enzymatic decrease is secondary to a decreased Ca^{++}-flux (Sohn and Ferrendelli, 1973 and

1976), or is a direct effect of PHT to inhibit this enzyme.

In the present report, we have examined the acute and chronic effects of PHT on several parameters of cation and anion homeostasis in the intact cerebral cortex of neonatal and adult rats. In the neonate (postnatal day 3) PHT produced marked and significant changes in Na^+, K^+-, Ca^{++}, Mg^{++}-, and HCO_3^--ATPase. These effects when correlated with tissue electrolyte content are consistent with the hypothesis that PHT is an excitatory drug. In the adult rat, when the CNS is comprised of both functional neurons and glia, similar changes of a lesser magnitude were observed following acute PHT administration. These changes were further attenuated by chronic PHT-treatment, and discernable changes in enzymic activity could only be detected in the subcellular fractions of the cerebral cortex. Inhibition of the synaptosomal-, and stimulation of the myelin-associated Na^+, K^+-ATPase strongly suggest that chronic PHT is differentially affecting neuronal and glial cell membranes. In addition, the present results suggest that PHT by increasing neuronal activity, indirectly enhances both anion and acid-base metabolism within the CNS.

The studies conducted in the present report support the hypothesis that PHT possesses anticonvulsant activity by virtue of its ability to stimulate predominating inhibitory systems of the mature brain. Furthermore, by stimulating glial cell cation and anion systems, PHT provides the CNS with an enhanced ability to regulate the ionic and acid-base environment of the intact CNS, thereby limiting the spread of seizure activity. These effects on active transport processes of neuronal and glial cells were observed at the anticonvulsant dose of 20 mg/kg, which produces blood levels of approximately 10 ug/ml (unpublished observations). Additional <u>in vitro</u> studies conducted on primary astroglial cultures have demonstrated significant effects of PHT on the activity of Na^+, K^+-ATPase and the membrane potential at concentrations of 10^{-6} and 10^{-5}

M PHT (for discussion see following report). MacDonald et al. (1983) have recently demonstrated that these concentrations possess significant anticonvulsant activity in cultured spinal and cerebral cortical neurons, and are much lower than the levels required to block both Na^+ and Ca^{++} influx. Since the effects on neuronal and glial active transport processes described in the present report were observed at similar concentrations, they appear to be equally important to the mechanism of the anticonvulsant action of PHT.

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PART TWO

EFFECTS OF PHENYTOIN ON PRIMARY GLIAL CELL CULTURES

INTRODUCTION

The mechanism of action of PHT, as with most anticonvulsant agents, remains uncertain despite its continued wide-spread use in the treatment of convulsive disorders. PHT has been observed to exert an effect on many different systems thereby making it difficult to formulate a single mechanism of action. For example, it decreases both inward sodium (Na^+) (Lipicky et al., 1972) and calcium (Ca^{++}) (Sohn and Ferrendelli, 1973; Sohn and Ferrendelli, 1976) fluxes across biological membranes as well as increases mucosal membrane permeability of epithelial cells to Na^+ (Watson and Woodbury, 1972; Watson and Woodbury, 1973). In addition, PHT exerts numerous effects on neurotransmitter synthesis and release most of which are presumably the result of its predominate action to alter Na^+ and Ca^{++} homeostasis (For reviews of PHT's proposed mechanism of action see Woodbury, 1982 and Woodbury et al., 1983)

Previous in vivo studies (see preceeding paper) have demonstrated that chronic administration of PHT to adult rats increases cerebral cortical DNA content, carbonic anhydrase activity, and myelin-associated Na^+, K^+-ATPase activity (White et al., 1983). These observations suggest that PHT is capable of significant interaction with glial cells. That this may be functionally important in understanding PHT's mechanism of action is suggested by a growing body of evidence supporting a primary role for glial cells in the homeostatic regulation of extracellular K^+ (K^+_o) as well as other neuroactive substances including glutamate, aspartate, and GABA (Hertz, 1979 and 1982). As originally proposed in 1970 by Pollen and Trachtenberg, disease states, such as epilepsy, that are characterized by abnormal neuronal discharges might result from, or be aggravated by the absence or failure of the glial buffering system.

With cell culture techniques, it has become possible to grow various CNS cell types for long periods of time. This allows the investigator to study in isolation not only the effects of drugs on neuronal and glial cells, but also provides insight into the compartmentation of electrolytes, enzymes and amino acid metabolism. Extensive biochemical, morphological and histochemical studies suggest that primary cultures of newborn rat brain retain a remarkable capability to differentiate into cells closely resembling astrocytes in vivo. In addition, numerous examples exist in which in vitro development of glial cells closely parallels in vivo development. For example, three enzymes involved in cation and anion transport in the CNS (Na^+, K^+-ATPase, HCO_3^--ATPase, and carbonic anhydrase) have all been demonstrated to exist and develop in vitro in a manner approximating in vivo maturation (Kimelberg et al., 1978). Although drawbacks do remain, the use of primary cultures of new born rat brain as a model for normal astrocytes does provide obvious advantages when studying in isolation, the effects of drugs for the purpose of identifying and separating specific drug actions attributable to a particular cell type.

The studies conducted in the present investigation were designed to examine the hypothesis that PHT, by increasing the responsiveness and/or proliferation of glial cells is capable of providing the CNS with an enhanced ability to regulate ionic and acid-base homeostasis within the brain ECF, thereby limiting the spread of seizure activity. We have attempted to provide direct evidence for this hypothesis by examining changes in the concentration of intracellular K^+, the activity of enzymes involved in cation and anion transport (Na^+, K^+-ATPase, Ca^{++}, Mg^{++}-ATPase, and HCO_3^--ATPase) and the transmembrane potential of primary astroglial cultures following acute and chronic exposure to micromolar concentrations of PHT.

METHODS AND PROCEDURES

Animals

Pregnant female rats of the Sprague-Dawley strain were obtained from an in-house breeding colony, and served as the source for neonatal pups used in all experiments. All rats were housed in individual cages in a temperature and humidity controlled environment, and allowed free access to Purina Rat Chow and water ad libitum. Lighting was maintained on a 12 hour on and a 12 hour off cycle (on 0800, off 2000 hours).

Cell Culture

Primary cell cultures of non-neuronal cells were started from cerebral cortical tissue of 3-day-old rat pups using a method modified from Booher and Sensenbrenner (1972) and Kimelberg et al. (1978). Briefly, under sterile conditions, the brains of 3-day-old Sprague-Dawley rats were removed, the cerebral cortex dissected free, and the meninges carefully removed. Tissues were then minced by razor blades on a teflon plate and placed in prewarmed (37°C) Ca^{++}-free Dulbecco's phosphate buffered saline (PBS) containing 0.25% trypsin (10cc/8 brains). Tissues were digested by stirring with trypsin for 10 min in a sterile polyethylene beaker. One ml of rabbit serum was then added to 15 ml of digest fluid to inactivate the trypsin. At this time, undigested debris was removed with a pipette, and separated cells were pelleted and resuspended 3 times in basal minimal essential (Eagle's) medium supplemented with 10% fetal bovine serum, Penicillin (100,000 U/l), Streptomycin (100,000 mcg/l), and Fungizone (250 mcg/l). Cells obtained from 8 cortices were then plated into thirty 60 x 15 mm

Falcon disposable plastic culture dishes. Culture medium was changed 24 hours after the initial plating, and twice a week thereafter. Cultures were grown at 37°C in a 95% air/5% CO_2 humidified incubator. Cultures were maintained for 14 days under these conditions. Beginning on day 14, one-half of the cultures were exposed to medium supplemented with 0.25 mM Dibutyryl cyclic Monophosphate (DBcAMP) for an additional 1-2 weeks. Chronic treatment with DBcAMP transforms normal flat polygonal cells into cells possessing abundant processes that morphologically resemble mature astrocytes (Lim et al., 1973; Moonen et al., 1975; Sensenbrenner et al., 1977; and Walz and Hertz, 1982). In light of this, it was of interest to examine the effect of PHT on both "undifferentiated" flat cells and "DBcAMP-differentiated" cells. Medium supplemented with DBcAMP was prepared fresh at the time of each medium change. At the completion of the experiment, cells were washed 3 times with 0.32 M sucrose and placed in the freezer (-20°C) until further analysis could be performed.

Drug Treatment

Stock drug solutions were made by dissolving sufficient PHT into either 95% ETOH or Dimethyl Sulfoxide (DMSO) to give concentrations of 0.5, 5.0, and 50 mM PHT in vehicle. At the time of the experiment, these solutions were then diluted 1:5000 with culture medium to produce a final concentration of 0.1, 1.0, and 10 uM PHT in medium containing 0.02% vehicle.

Cultures grown in the presence or absence of DBcAMP were treated acutely for 2 hours and chronically for 4 days with various concentrations of PHT. Results of these studies were compared to control cells which were exposed for a similar period of time to medium plus vehicle (0.02% ETOH or DMSO).

Enzyme Assays

On the day of determination, cells still attached to the dish were covered with 2 ml of ice cold distilled water, scraped free from the plastic surface with a rubber policeman, and transferred to a glass homogenizing tube fitted with a teflon pestle. After homogenizing (ten strokes of the pestle), cells were diluted to a final volume of 5 to 10 ml with ice cold distilled water. This solution was then used for determination of enzyme activities, DNA and protein contents as described in the preceeding paper.

The effect of K^+_o concentration on Na^+, K^+-ATPase activity was determined following both acute and chronic exposure of glial cells to various doses of PHT. In these studies, Na^+, K^+-ATPase activity was determined in the presence of buffer containing different K^+_o (1-20 mM) concentrations.

Determination of Intracellular K^+ and P_{Na}/P_K Ratio.

Concentration of K^+_i and the ratio of P_{Na}/P_K following various treatments were estimated from a plot of the exponent of membrane potential/60 against K^+_o concentration as described by J. W. Woodbury (1982). The P_{Na}/P_K ratio obtained from the potassium depolarization curve is a reliable estimate of Na^+ and K^+ permeability across cell membranes.

Measurement of transmembrane potentials

Technical difficulty in measuring the membrane potential of flat monolayer cells grown on plastic culture dishes necessitated that cells be replated onto collagen-coated culture dishes three days prior to measurement. This period of time allowed the transferred cells to regain their normal morphology. The presence of the collagen layer also permitted greater numbers of cells to be impaled with less breakage of microelectrode tips. With this method, the effects

of acute and chronic PHT on the E_m of both flat and morphologically differentiated glial cells (DBcAMP-treated) were investigated.

Collagen-coated dishes were prepared by mixing 0.5 ml of sterile purified collagen solution (Vitrogen 100 from Flow Laboratories, McLean, Virginia) and 1 ml of culture medium in a 35 x 10 mm Falcon tissue culture dish. Dishes were warmed at 37°C for approximately 30 min until a collagen gel layer securely attached to the bottom of the culture dish was formed. Glial cells that were successfully grown in culture for various periods of time (14-28 days) were then harvested by adding 2 ml of 0.125% trypsin in Ca^{++}-free PBS to one 60 x 15 mm culture dish. After 30 min, the cells were scraped off the dish with a rubber policeman, transferred to a sterile centrifuge tube containing 0.5-1.0 ml of rabbit serum, and centrifuged at 1500 rpm for 5 min. The cell pellet was washed 3 times with culture medium to eliminate the inactivated trypsin and rabbit serum. The final pellet was resuspended in 4 ml of culture medium. One ml of the final cell suspension and 1 ml of culture medium were then plated directly onto a collagen-gel-coated culture dish and mixed well before placing in the incubator. Membrane potentials were measured 3 days after replating onto collagen.

Microelectrodes were constructed from Corning 7740 capillary tubing (o.d. 1.2 mm, i.d. 0.9 mm) and were drawn by a Narishige PE-2 vertical electrode puller. The tip diameter was approximately 1 um with a resistance of 10 to 20 megohms when filled with 3 M KCl. Microelectrodes were connected to an electrometer with negative capacitance compensation, and the output was simultaneously displayed on a Hewlett-Packard 132H oscilloscope and a Beckman dynograph. The microelectrode was gradually advanced into the cell body of each cultured glial cell with a Brinkman micromanipulator attached to the slide stage of an Olympus phase-contrast microscope.

On the day of the experiment, culture medium was replaced with a HEPES

buffered iso-osmotic medium, consisting of the following components (in mmol/l): NaCl 122; KCl 3; $CaCl_2$ 1.3; $MgSO_4$ 1.2; KH_2PO_4 1.2; HEPES (titrated to 7.4 with NaOH) 20; $NaHCO_3$ 10; glucose 10, and sucrose 60.

The effects of acute and chronic PHT treatment on the membrane potential and the P_{Na}/P_K ratio of cultured glial cells grown in the presence and absence of DBcAMP were determined. The effects of K^+_o concentrations (3-90 mmol/l) on the potential of chronic PHT-treated glial cells were also determined. In these experiments, Na^+_o was reciprocally adjusted to maintain the osmolality when K^+_o concentration was varied.

The acute effect of PHT on the membrane potential of 17-day cultures grown in the absence and presence of DBcAMP (0.25 mM for 3 days) was determined. Cells were incubated at 37°C for 60 min with HEPES buffer containing a final K^+_o concentration of 5.4 mM. After several control impalements were made, the same dish was incubated for an additional 60 min with HEPES buffer containing various concentrations (1×10^{-6} to 1×10^{-4} M) of PHT. The E_m of cells chronically exposed to PHT (10^{-5} M) for 4 days was also determined at several K^+_o concentrations (3-90 mM).

RESULTS

Culture Growth and Morphology.

Primary cultures of glial cells grown in the absence of DBcAMP for 28 days were composed of a monolayer of flat polygonal cells that exhibited little or no process extension, and reached confluency at or near 2 weeks. In addition to the predominate flat cell, it was common to find 3 additional cell types firmly attached to the dish. These included, large phase-bright cells with round cell bodies, small phase-dark process-bearing cells, and ciliated epithelial cells. Figure 12A represents an average culture with a few dispersed phase-bright cells on a monolayer of predominate flat cells.

Figure 12B illustrates the marked morphological changes induced in primary cerebral cultures when they were chronically exposed (14 days) to medium containing 0.25 mM DBcAMP beginning on culture day 14. It can be seen from this figure that process extension and rounding of the cell body are characteristic features produced by this cyclic nucleotide derivative. No differences in morphology between control and PHT-treated (either acute or chronic) cells were observed under phase contrast microscopy.

Chronic exposure to 0.25 mM DBcAMP reduced the total protein (Fig. 13-top) and DNA content (Fig. 13-middle) of cultured glial cells by 14% and 34% respectively. These large decreases in protein and DNA content were accompanied by significant cell differentiation as suggested by the 37% increase in total protein/DNA ratio (Fig. 13-bottom).

Chronic exposure to 10^{-5} M PHT for 4 days had little effect on the total protein and DNA content of cultured glial cells grown either in the presence or

Fig. 12. Phase contrast micrograph of cultured rat glial cells (x100).

a. Control cells after 28 days in culture. This photomicrograph illustrates the cellular heterogeneity often found in primary glial cultures. Besides the predominate flat cell, numerous phase bright and phase dark cells can also be seen in the average culture.

b. Cultured glial cells cultivated in the presence of 0.25 mM DBcAMP for 14 days. Note the extensive process extension and rounded cell body. These features are characteristically produced by this cyclic nucleotide derivative.

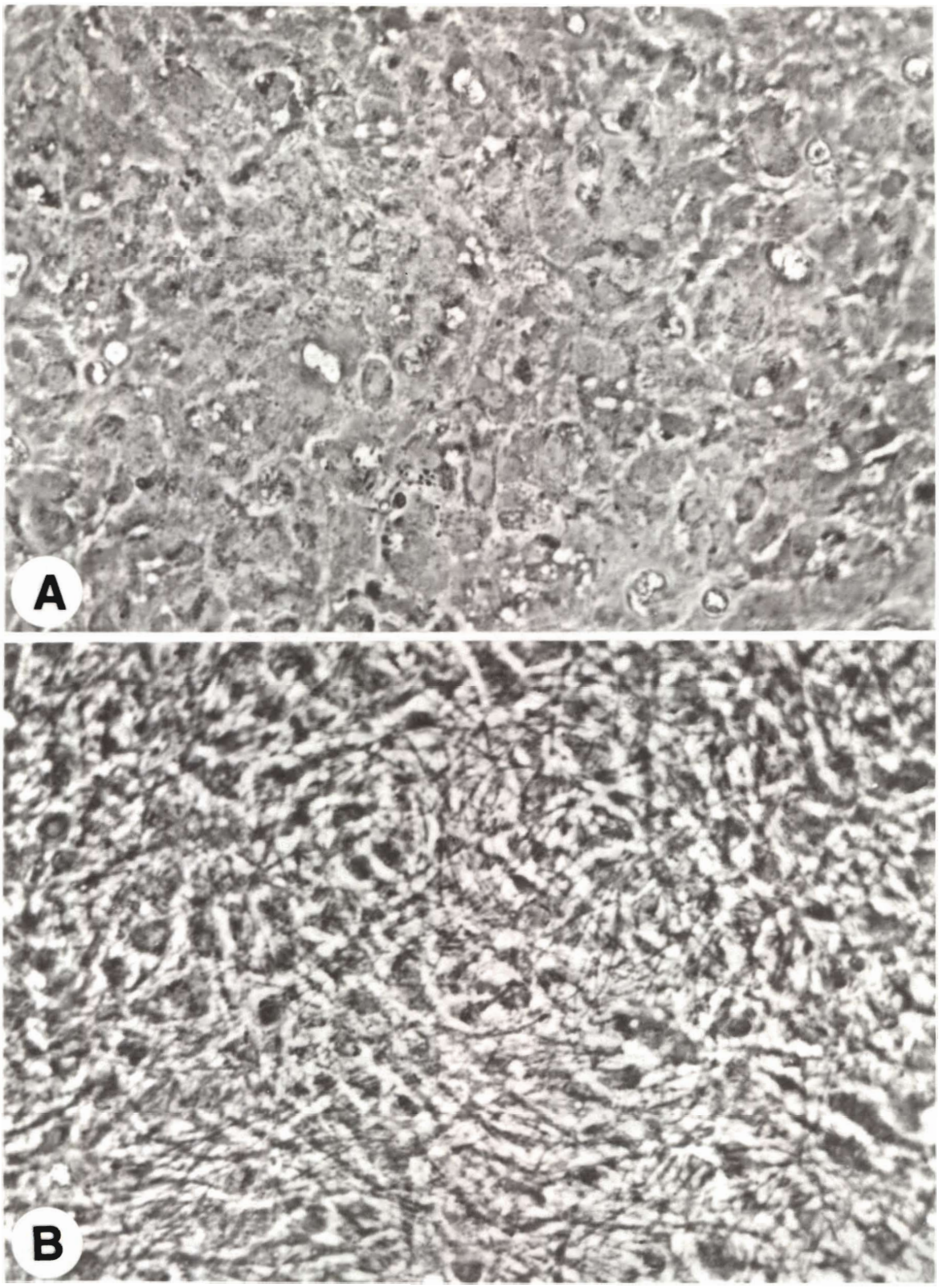
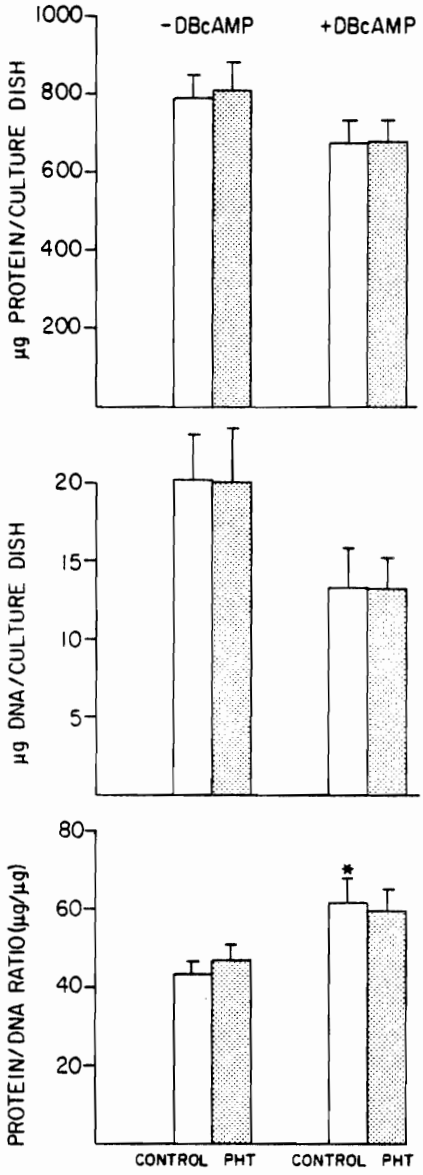

A
B

Fig. 13. The effect of chronic PHT treatment on the total protein (top) and DNA content (middle) and the protein/DNA ratio (bottom) of 4 week glial cultures. Beginning on culture day 24, cells grown in the absence (left) and presence (right) of 0.25 mM DBcAMP were treated with 1 x 10^{-5} M PHT for 4 days. Results represent the mean ± SEM of 10-11 samples obtained from 3 independent experiments. * indicates statistical significance ($p<0.05$) from vehicle treated controls.


-DBcAMP
+DBcAMP
µg PROTEIN/CULTURE DISH
1000
800
600
400
200
µg DNA/CULTURE DISH
20
15
10
5
PROTEIN/DNA RATIO(µg/µg)
80
60
40
20
*
CONTROL
PHT
CONTROL
PHT

absence of DBcAMP (Figs. 13-top and 13-middle). In addition, PHT had no significant effect on cell growth as estimated by the protein/DNA ratio shown in Fig. 13-bottom.

Enzymic Activity

The Na^+, K^+-ATPase activity of cultured cells was measured in the presence of increasing K^+_o concentration following acute and chronic treatment with PHT. The results are expressed as a percentage of the enzyme activity obtained at a K^+_o of 10 mM and are summarized in Fig. 14. Following acute exposure to 10^{-6} M PHT (Fig. 14A), a significant activation of Na^+, K^+-ATPase was observed at 20 mM K^+_o, but at all other K^+_o concentrations, the activation pattern of control and PHT-treated cells was not statistically different. However, it is of interest to point out that in the presence of 10^{-6} M PHT, the resulting K^+_o activation curve was defined by two distinct peaks in enzyme activity. These peaks at 5 and 15 mM K^+_o were markedly higher than were the corresponding peaks of control cells. In sharp contrast, the K^+_o activation pattern of cells acutely exposed to 10^{-5} M PHT was characterized by one broad peak in enzyme activity that extended over a wide range of K^+_o concentrations (5-15 mM).

Na^+, K^+-ATPase activity of cultured glial cells was also measured in the presence of increasing K^+_o following chronic exposure to similar concentrations of PHT for 4 days (Fig. 14B). In both control and treated cells, activation curves were characterized by 2 peaks in enzyme activity. The percent of enzyme activated at each K^+_o concentration examined was higher in cells chronically exposed to PHT as compared to control cells.

Membrane Potentials.

The tracings in Fig. 15 illustrate the successful impalements of several 28-

Fig. 14. Infuence of K^+ on the Na^+, K^+-ATPase activity of 22-26 day glial cultures acutely (A) and chronically (B) exposed to micromolar concentrations of PHT. Data are expressed in percent of activation of activities measured in the presence of 10 mM K^+ (considered 100%). * $p<0.05$ when compared to vehicle treated controls.

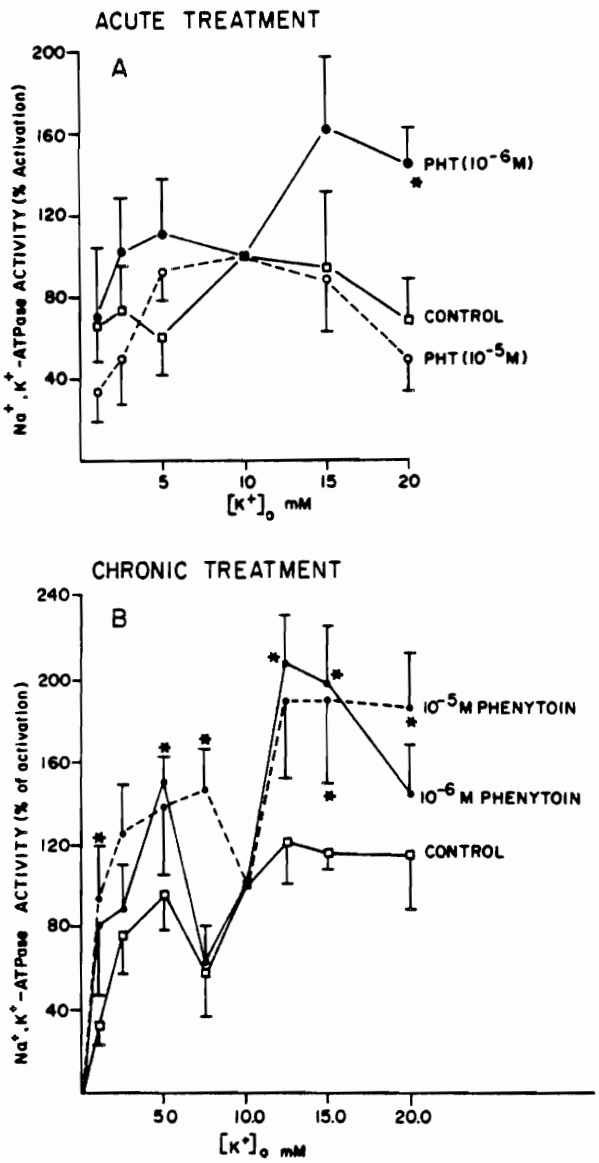
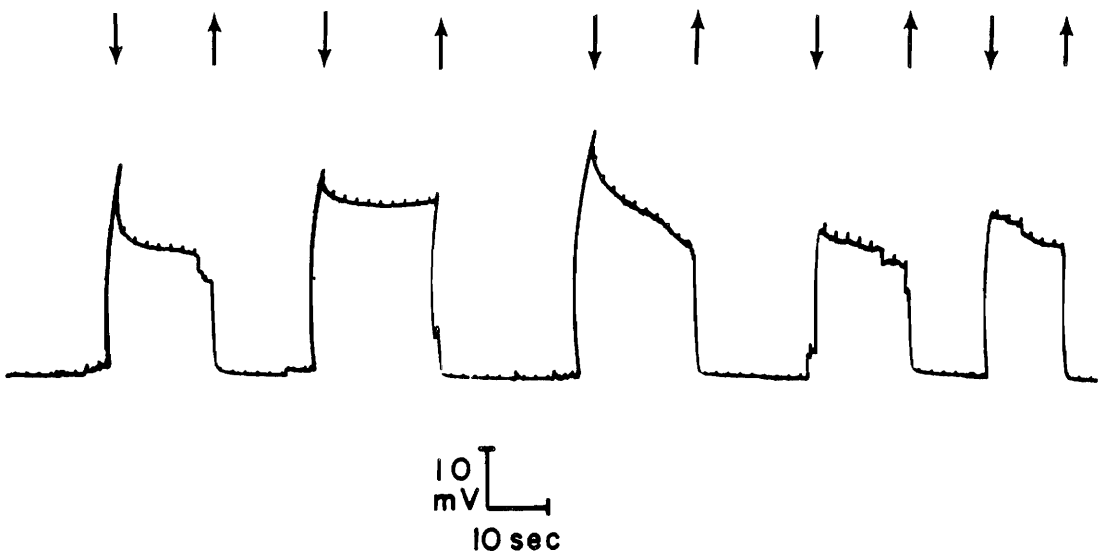

ACUTE TREATMENT
A
200
160
120
80
40
Na+,K+-ATPase ACTIVITY (% Activation)
PHT(10-6M)
CONTROL
PHT(10-5M)
5
10
15
20
[K+]o mM
CHRONIC TREATMENT
B
240
200
160
120
80
40
Na+,K+-ATPase ACTIVITY (% of activation)
10-5M PHENYTOIN
10-6 M PHENYTOIN
CONTROL
5.0
10.0
15.0
20.0
[K+]o mM

Fig. 15. Membrane potential recordings from the successful impalement of control glial cells in culture. Three days prior to E_m measurement, cells initially plated onto plastic culture dishes were replated onto collagen-coated culture dishes. Cells were impaled with 3 M KCl microelectrodes at the points indicated by the downward arrows. The upward arrows indicates the point of electrode withdrawal. All impalements were made at room temperature (24-25°C) at a K^+_o of 5.4 mM. The membrane potentials of the the 5 cells shown here were -33, -32, -40, -23, -25 mV, respectively.


10
mV
10 sec

day-old cells. Upon impalement, stable negative potentials were recorded. In Fig. 16, histograms of the membrane potentials of a group of 28-day-old cerebral cultures grown in the absence (16A) and presence (16C) of 0.25 mM DBcAMP are shown. Membrane potentials were recorded at room temperature at a K^+_o concentration of 5.4 mM. The mean potential for undifferentiated controls was -24 mV with a range of 57 mV (-4 to -61 mV) as shown in Fig. 16A. In addition to the marked morphological changes associated with chronic exposure to DBcAMP, a significant hyperpolarization (-13 mV) was also observed (Fig. 16C).

The membrane of cultured glial cells grown in the absence and presence of DBcAMP and chronically exposed (4 days) to 10^{-5} M PHT was also hyperpolarized as compared to controls. Chronic PHT treatment of flat undifferentiated cells resulted in a -12 mV increase in their E_m as shown in Fig. 16B. Chronic PHT treatment also increased the E_m of DBcAMP differentiated cells (Fig. 16D). However, its effect on these cells was of a lesser magnitude (-5 mV) than that observed when undifferentiated cells were exposed to a similar concentration of PHT (Fig. 16B).

Effect of Acute Phenytoin on the Em of Glial Cells

The acute (1 hour) effect of various concentrations of PHT on the E_m of 17-day-old cultured glial cells was determined at a K^+_o of 5.4 mM. As shown in Fig. 17, the effect of PHT on the E_m was biphasic, characterized by membrane hyperpolarization at concentrations up to 1 x 10^{-5} M. Above this concentration, the membrane was depolarized (relative to the E_m at 10^{-5} M PHT). When cells were exposed for 3 days to 0.25 mM DBcAMP, a similar biphasic effect of PHT was also observed (Fig. 17). In both control and DBcAMP treated cells, a peak hyperpolarization of 24 and 22.5% respectively was observed at 1 x 10^{-5} M PHT. Above this concentration, E_m progressively returned to control values (Fig. 17).

Fig. 16. Histograms illustrating the distribution of cultured glial cell membrane potentials grown in the absence (A) and presence (C) of DBcAMP and chronically exposed to 10^{-5} M PHT (B and D). Membrane potential were recorded with 3 M KCl microelectrodes at room temperature (24-25°C) at a K^+_o of 5.4 ent of activation of activities measured in the presence of 10 mM K^+ (considered 100%). * $p<0.05$ when compared to vehicle treated controls.

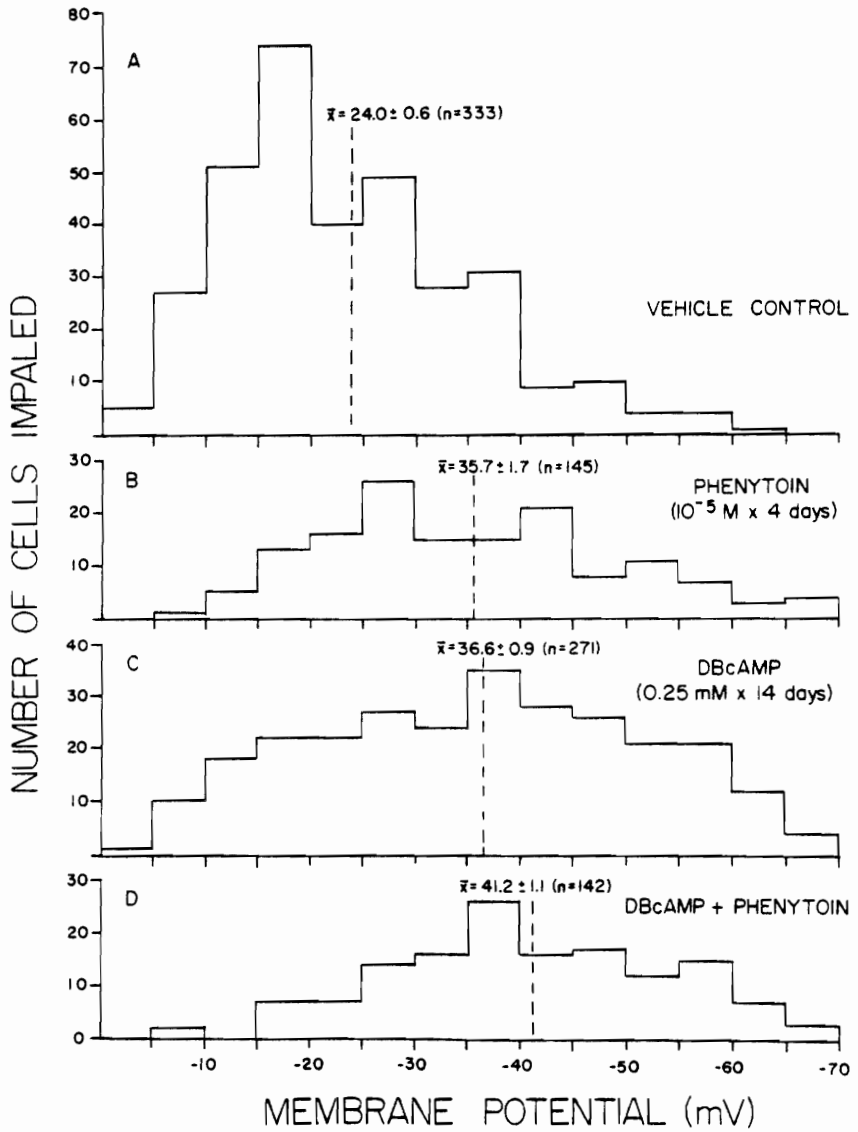
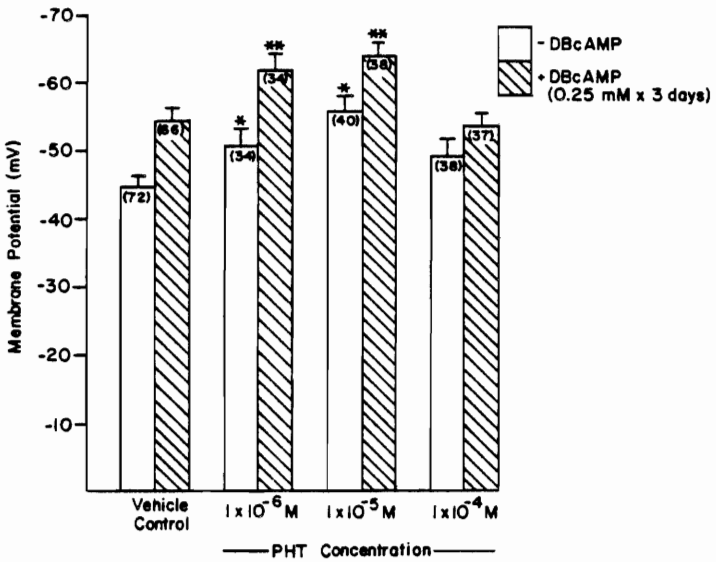

A
$\bar{x}$ = 24.0 ± 0.6 (n=333)
VEHICLE CONTROL
B
$\bar{x}$ = 35.7 ± 1.7 (n=145)
PHENYTOIN
(10^{-5} M x 4 days)
C
$\bar{x}$ = 36.6 ± 0.9 (n=271)
DBcAMP
(0.25 mM x 14 days)
D
$\bar{x}$ = 41.2 ± 1.1 (n=142)
DBcAMP + PHENYTOIN
NUMBER OF CELLS IMPALED
MEMBRANE POTENTIAL (mV)
-10
-20
-30
-40
-50
-60
-70

Fig. 17. Effect of acute PHT on the membrane potential of 17-day old cultured glial cells grown in the absence and presence of DBcAMP (0.25 mM for 3 days). Cells plated on collagen-coated culture dishes were exposed to various concentrations of PHT for 1 hour prior to E_m measurement. Open bars represents cells grown in the absence of DBcAMP, and hatched bars represent cells grown in the presence of 0.25 mM DBcAMP for 3 days. Number in parenthesis represents the number of individual cells impaled at each point. * $p < 0.05$ when compared to open bar control. ** $p < 0.05$ when compared to hatched bar control.


-70
-60
-50
-40
-30
-20
-10
Membrane Potential (mV)
(72)
(56)
(34)
(34)
(40)
(58)
(38)
(37)
*
**
*
**
- DBcAMP
+ DBcAMP
(0.25 mM x 3 days)
Vehicle Control
1 x 10⁻⁶ M
1 x 10⁻⁵ M
1 x 10⁻⁴ M
PHT Concentration

Effect of K^+ Depolarization on the Em of cultured Glial Cells

The effects of increasing K^+_o on the E_m of 29-day-old cultured glial cells are shown in Fig. 18. In order to maintain a constant osmolality in the bathing solution and prevent swelling of cells, the Na^+_o of the buffer was reciprocally lowered as the K^+_o was increased. At all K^+_o concentrations, the membrane potentials of cultured glial cells demonstrated a divergence from the 59 mV slope that is predicted by the Nernst equation for a perfect K^+ electrode.

Chronic PHT treatment produced no significant change in the slope of the K^+_o depolarization curve when compared to cells grown in the absence (Fig. 18A) and presence of DBcAMP (Fig. 18B). However, as already demonstrated, the E_m of cells chronically exposed to PHT was consistently higher (hyperpolarized) than that of the corresponding control at each K^+_o concentration.

Effect of DBcAMP on the Intracellular K^+ Content and Enzyme Activity of Cultured Glial Cells

In addition to the marked morphological and electrophysiological changes that were seen in primary glial cultures chronically exposed to DBcAMP, significant alterations in the activities of 3 major transport enzymes were observed (Figs. 19 and 20). However, the K^+_i concentration and the P_{Na}/P_K ratio were not significantly changed (Figs. 21-top and 21-bottom). DBcAMP caused a 2-fold increase in the activities of HCO_3^- (Fig. 19), and Na^+, K^+-ATPase (Fig. 20A), and a 2.5-fold increase in the activity of Ca^{++}, Mg^{++}-ATPase (Fig. 20B), an enzyme involved in the translocation of Ca^{++}_i.

Fig. 18. Effect of chronic PHT treatment on the K^+_o-induced depolarization of 28-day old glial cells grown in the absence (A) and presence (B) of DBcAMP. Culture medium was replaced with HEPES buffered medium containing varying K^+ concentrations. Na^+ concentration of the buffer was reciprocally changed as the K^+_o was increased in order to maintain osmolality. The number of cells impaled at each K^+_o concentration ranged between 23 and 41. Each value shown is the mean ± SEM. * $p < 0.05$ when compared to vehicle treated controls.

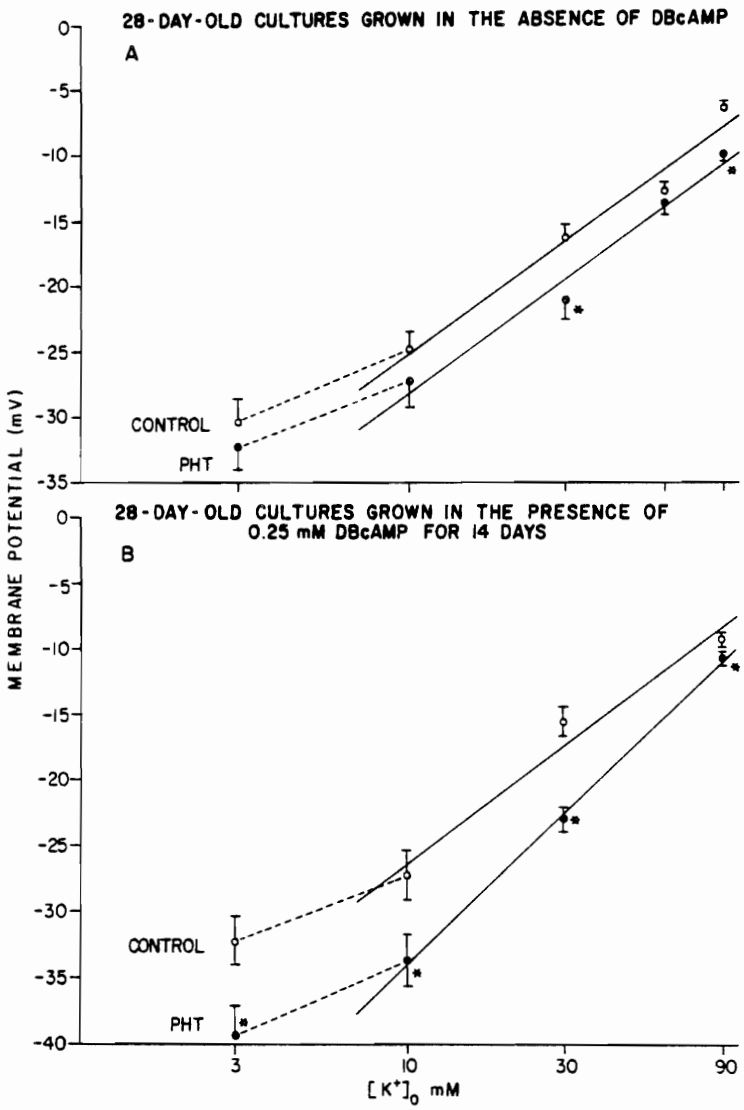

28-DAY-OLD CULTURES GROWN IN THE ABSENCE OF DBcAMP
A
28-DAY-OLD CULTURES GROWN IN THE PRESENCE OF 0.25 mM DBcAMP FOR 14 DAYS
B
MEMBRANE POTENTIAL (mV)
0
-5
-10
-15
-20
-25
-30
-35
-40
CONTROL
PHT
3
10
30
90
[K+]o mM

Fig. 19. Effect of chronic PHT treatment on the activity of HCO_3^--ATPase. Beginning on culture day 24, glial cells grown in the absence (left) and presence (right) of DBcAMP were treated for 4 days with 1 x 10^{-5} M PHT. Chronic DBcAMP increased the activity of HCO_3^--ATPase 2-fold when compared to vehicle treated controls. PHT did not significantly affect the enzymic activity of either undifferentiated controls or DBcAMP-differentiated cells. Results represent the mean ± SEM of 10-11 samples obtained from 3 independent experiments. ** $p < 0.05$ when compared to controls.

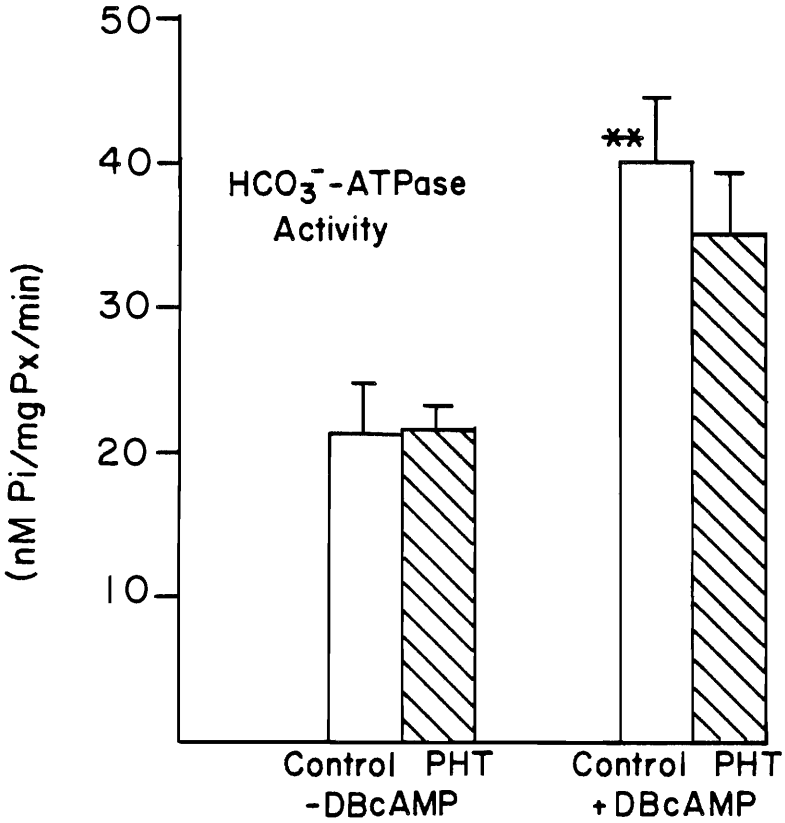
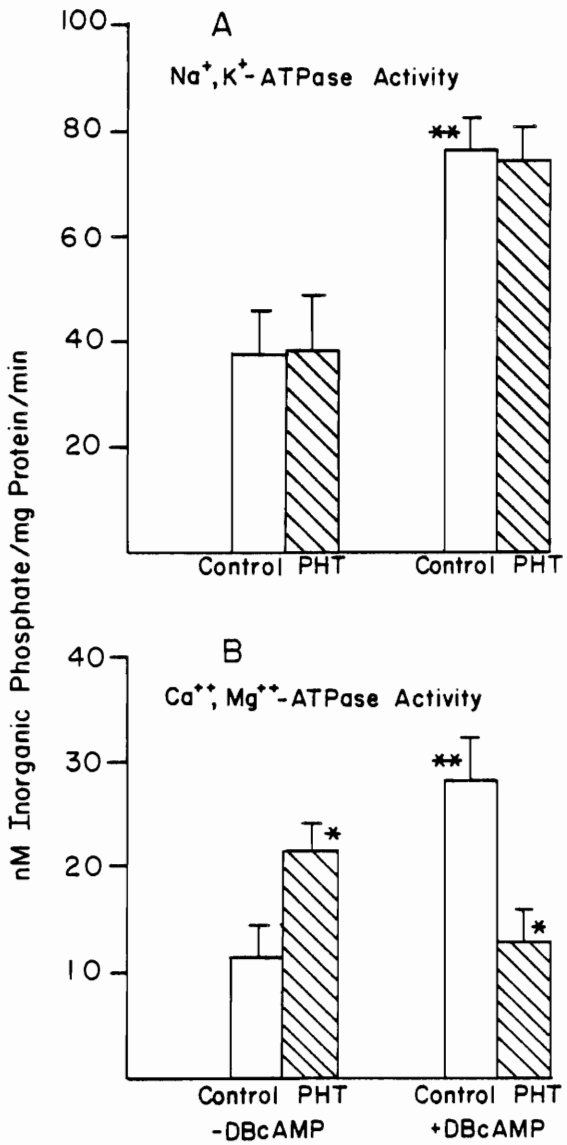

50
40
30
20
10
(nM Pi/mg Px/min)
HCO_3^--ATPase
Activity
**
Control
PHT
-DBcAMP
Control
PHT
+DBcAMP

Fig. 20. Effect of chronic PHT treatment on the activities Na^+, K^+- and Ca^{++}, Mg^{++}-ATPase. Four-week cultured glial cells grown in the absence (left) and presence (right) of DBcAMP were treated for 4 days with 10^{-5} M PHT beginning on culture day 24. The activity of both Na^+, K^+- (A) and Ca^{++}, Mg^{++}-ATPase (B) was increased 2- and 2.5-fold, respectively with DBcAMP treatment. In morphologically undifferentiated control cultures (left), chronic PHT treatment produced a 2-fold increase in the activity of Ca^{++}, Mg^{++}-ATPase, whereas in DBcAMP-differentiated cultures (right), chronic PHT treatment produced a 2-fold decrease in the activity of this enzyme. Results represent the mean ± SEM of 10-11 samples obtained from 2-3 independent experiments. * $p < 0.05$ when compared to respective controls. ** $p < 0.05$ when compared to morphologically undifferentiated (left) controls.

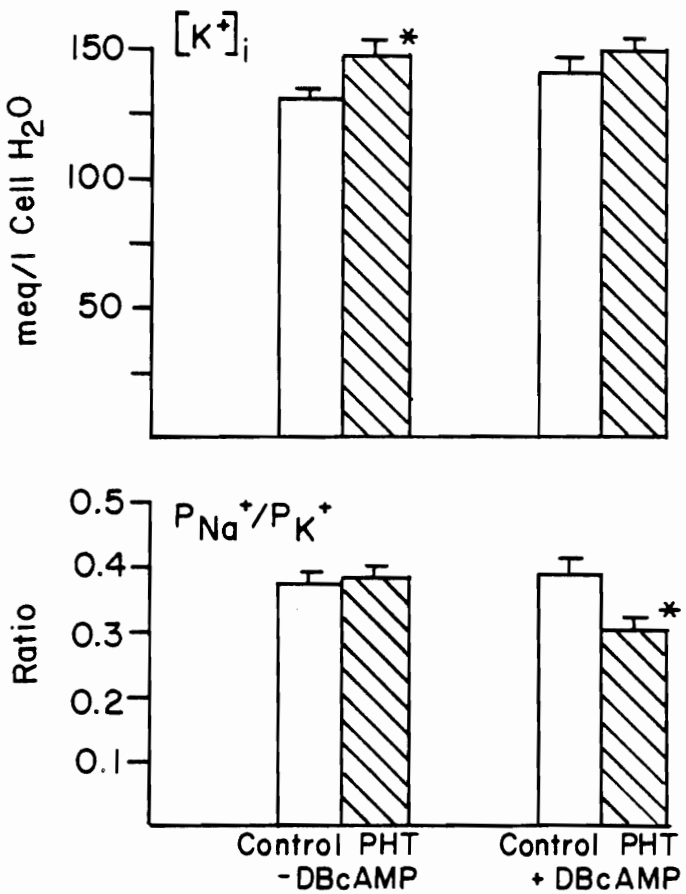

A
Na+, K+-ATPase Activity
100
80
60
40
20
**
Control PHT
Control PHT
B
Ca++, Mg++-ATPase Activity
40
30
20
10
**
*
*
Control PHT
-DBcAMP
Control PHT
+DBcAMP
nM Inorganic Phosphate/mg Protein/min

Effect of PHT on the Intracellular K+ Content and Enzyme Activity of Cultured Glial Cells

As shown in the top portion of Fig. 21, the K^+_i concentration of undifferentiated flat cells was significantly elevated following their chronic exposure to 10^{-5} M PHT for 4 days; however, the P_{Na}/P_K ratio was not significantly altered (Fig. 21-bottom). There was no significant difference in the concentration of K^+_i, but the P_{Na}/P_K ratio was significantly reduced following the chronic treatment of DBcAMP differentiated cells with 10^{-5} M PHT for 4 days.

Ca^{++}, Mg^{++}-ATPase was the only enzyme significantly affected by chronic PHT treatment (Fig. 20B). When compared to undifferentiated controls, its activity was increased almost 2-fold (89%) in PHT treated cells. In contrast, chronic PHT treatment of DBcAMP differentiated cells significantly inhibited Ca^{++}, Mg^{++}-ATPase (Fig. 20B). No change in the specific activity (nM inorganic phosphate/mg protein/min) of Na^+, K^+- (Fig. 20A) or HCO_3^--ATPase (Fig. 19) was observed. Because the activity of Na^+, K^+-ATPase in these experiments was determined at only one K^+_o concentration (K^+_o = 5.0 mM), this figure is not directly comparable to Fig. 14 which illustrates the effect of PHT on enzyme activation rather than on specific activity.

Fig. 21. Effect of chronic PHT treatment on the K^+_i concentration (A) and the P_{Na}/P_K ratio (B) of 28-day glial cultures grown in the absence and presence of DBcAMP. The K^+_i (A) and P_{Na}/P_K (B) ratio were both determined from a plot of the exponent of $-E_m/60$ against Na^+_o concentration as described by J. W. Woodbury (1982). Chronic PHT increased the K^+_i concentration (A) of undifferentiated controls without significantly affecting the P_{Na}/P_K ratio (B). Treatment of DBcAMP-differentiated cultures (right) significantly reduced the P_{Na}/P_K ratio (B-right) while only increasing the K^+_i concentration slightly (A-right). * $p < 0.05$ when compared to each respective control.


[K+]i
150
100
50
meq/l Cell H2O
*
PNa+/PK+
0.5
0.4
0.3
0.2
0.1
Ratio
*
Control PHT
-DBcAMP
Control PHT
+ DBcAMP

DISCUSSION

Previously, we have demonstrated that the acute administration of PHT (20 mg/kg) to 3-day-old rats significantly lowered Na^+, K^+-ATPase activity, and consequently increased Na^+_i, and decreased K^+_i of the cerebral and cerebellar cortices (White et al., 1983). Since the neuropil of the developing CNS is comprised primarily of nerve cells at this age, this observation reflects a direct interaction between PHT and neuronal cells. Several other in vivo observations including significant increases in cerebral cortex carbonic anhydrase activity, myelin-associated Na^+, K^+-ATPase activity, and DNA content have suggested that chronic PHT treatment of adult rats is capable of significant interaction with the glial regulatory system. In the present study, we have attempted to identify and characterize the in vitro effects of PHT on cultured neuroglial cells. In vitro studies on cultured glial cells provide one means whereby certain interpretational problems associated with the cellular heterogeneity of the intact CNS can be circumvented.

Under two distinct culture conditions (medium supplemented with or without DBcAMP), we investigated the effect of acute and chronic PHT treatment on several biochemical and electrophysiological parameters of cultured glial cells. The total protein content of cultures exposed to 0.25 mM DBcAMP for 2 weeks beginning on culture day 14 was reduced (Fig. 13-top). Since cell division was reduced even further (Fig. 13-middle), the protein/DNA ratio which is an indirect measure of cell differentiation was significantly elevated above control cultures (Fig 13-bottom). However, it has been suggested by Sensenbrenner et al. (1980) that changes induced by DBcAMP represent a pathological phenomena rather than

a maturation process. Although it is yet to be resolved how closely DBcAMP-differentiated cells resemble normal astrocytes, it was of interest to examine the biochemical and the electrophysiological effects of PHT on two distinct morphological cell types.

In the present report, Na^+, K^+-ATPase activity was measured in the presence of varying concentrations of K^+_o (Figs. 14A and 14B). The presence of 2 peaks in enzyme activity at 5 and 12.5 mM K^+_o suggest that 2 distinct enzyme forms with different kinetic characteristics are present in these cultures. Although the predominate cell type of primary astroglial cultures is the morphologically flat polygonal cell, considerable heterogeneity does exist, and it is quite common to find small phase-dark process-bearing cells growing in the average culture. These cells have been frequently observed by others, and have tentatively been identified both morphologically (McCarthy and DeVillis, 1980; Bhat et al., 1981) and immunocytochemically (Bhat et al., 1981) as oligodendrocytes. Although additional studies are needed, it is quite possible that the lower enzyme peak at 5 mM K^+_o reflects the activation pattern of oligodendrocytes, whereas the higher peak between 12.5 and 20 mM is primarily astrocytic in origin. Consequently, such cell heterogeneity makes any conclusion regarding the possible presence of 2 enzyme forms extremely tenuous.

More importantly, our present results indicate that glial cultures chronically treated with micromolar concentrations of PHT have increased their capacity to control elevated K^+_o. As illustrated in Fig. 14B, the efficiency of glial Na^+, K^+-ATPase was substantially enhanced over a wide-range of K^+_o. The enhanced efficiency of PHT-treated cells at low K^+_o would presumably prevent ECF K^+ concentrations from rising much above normal levels during excessive neuronal firing. In addition, these cells are also capable of enhanced regulation at high K^+_o in the event that levels should exceed the normal physiological range.

Mechanistically, these results suggest that through an enhancement of glial regulatory processes, PHT may prevent the transition of interictal discharges to ictal episodes, an event that as suggested by Dichter et al. (1972) results from elevated K^+_o. Furthermore, these results are consistent with the clinical ability of PHT to prevent the spread (ictal transition) from a seizure focus, but not the initiation process (interictal discharge). Because focal discharge is not prevented, it might be further reasoned that PHT is only able to enhance K^+_o regulation in perifocal areas and not influence the regulatory ability of glial cells in the primary focus.

Results of electrophysiological studies also support the hypothesis that PHT enhances regulation of K^+_o. The data in Fig. 18 clearly demonstrate that for any given level of K^+_o, cells chronically exposed to PHT are likely to be less depolarized than controls. These data, therefore, fit with the expectation that protected tissues are less depolarized than susceptible ones for an equivalent concentration of K^+_o (Somjen, 1975). During normal physiological activity, ECF K^+ is maintained between 3 and 4 mM (see Prince et al., 1978); however, it can rise as high as 12 mM during seizures and has been observed to rise even higher (greater than 20 mM) during spreading depression (Viskocil et al., 1972; Futamachi et al., 1974; Somjen et al., 1976; Somjen, 1979).

In light of evidence suggesting that increases in K^+_o could affect neuronal firing, Pollen and Trachenberg in 1970 proposed that epilepsy could be the result of inadequate spatial buffering of K^+_o by glial cells. Numerous studies have suggested that the subsequent removal of elevated K^+_o is via an active accumulation into adjacent astrocytes. However, following the failure of several electrophysical studies to demonstrate any difference in baseline K^+_o between normal and epileptogenic brains (Lux, 1974; Pedley et al., 1976) it was concluded that an impairment of glial buffering was an unlikely cause of paroxymal

depolarization shifts. However, as pointed out by Lewis et al., (1977), the highly localized and transient nature of relatively large K^+_o electrodes could miss transient and localized increases in K^+_o induced by bursts of high frequency firing associated with alumina gel foci (Lewis et al., 1977). These authors have also demonstrated, that within gliotic scars induced by local application of alumina gel, clearance of K^+_o was slowed despite no observable difference in the base-line potassium level of epileptogenic and normal brain areas. The insensitivity of the K^+ electrode in detecting fine kinetic characteristics of cation movement has been further discussed by Grisar et al., (1983). This recent report has examined in some detail the kinetic characteristics of Na^+, K^+-ATPase of synaptosomal and glial fractions isolated from normal and focal areas of epileptogenic brains. Results reported therein demonstrate that a significant defect in the ability of glial cells isolated from focal lesions to regulate elevated K^+_o does exist. However, glial cells isolated from brain areas surrounding the lesion appeared to have an increased ability to regulate K^+_o during periods of epileptiform discharges. The demonstration that exogenously administered compounds (i.e., PHT) are capable of enhancing the ability of the CNS to regulate K^+_o (Figs. 14B and 18), through an interaction with glial cells, provides important insight into the etiology and the arrest of seizures.

The average E_m of undifferentiated control cells in the present study was 30 to 60 mV less than that commonly reported for primary astroglial cultures (Kimelberg, et al., 1979; Bowman, et al., 1983; Walz and Hertz, 1983). Our methods for E_m recording differ in several respects from those used in other investigations, and could contribute to the lower membrane potentials reported in this study. For example, our potentials were recorded from cells replated onto collagen-coated culture dishes at a K^+_o of 5.4 mM. When confluent cultures are replated onto collagen-coated dishes, cells are removed from the forces of

contact inhibition, and begin to divide. That dividing cells have different electrophysiological properties is suggested by a previous observation illustrating the dependency of the E_m on the age of the culture (data not shown). The E_m of cells replated onto collagen has been observed to be significantly lower than that measured from cells still attached to plastic culture dishes (C. Bowman-personal communication). One factor that contributes to the lower E_m reported in this study is the higher K^+_o (5.4 mM) of the HEPES buffer as compared to other studies (3.0 to 4.5 mM). Furthermore, since no deviation from a normal distribution could be demonstrated (Fig. 16), all stable membrane potentials were accepted regardless of potential.

Acutely, concentrations of PHT greater than 10^{-5}M (Figs. 14A and 17) appeared to decrease the efficiency of glial Na^+, K^+-ATPase. One possible explanation for this apparent discrepancy is that PHT increases membrane permeability to Na^+ in glial cells. In so doing, Na^+_i increases and subsequently depolarizes the cell membrane. Previously, it has been demonstrated that PHT is capable of increasing the short-circuit current (SCC) across frog skin and toad bladder (Watson and Woodbury, 1972; Watson and Woodbury, 1973). In these models, the SCC is a direct measure of active Na^+ transport, and the increase in SCC is the result of enhanced permeability of mucosal-side membrane to Na^+ (for discussion see Woodbury et al., 1983). The link between glial cells, frog skin and toad bladder is that all 3 are epithelial cells and would, therefore, be expected to respond to PHT in a similar manner. Thus, as the Na^+_i concentration increases with increasing PHT concentrations, the cell membrane is depolarized. Acutely, at concentrations greater than 1×10^{-5} M, E_m progressively returned to control values (Fig. 17). This observation provides one explanation for the excitatory effects often observed clinically when plasma PHT concentrations become too high (Glaser, 1972; Lascelles et al., 1970; Levy, 1965; Patel & Crichton, 1968;

Roseman, 1961; Schriener, 1958; and Troupin and Ojemann, 1975). For example, in the presence of high therapeutic concentrations of PHT, glial cells may have a reduced ability to regulate K^+_o. The resultant increase in K^+_o concentrations leads to a nonspecific depolarization of surrounding neurons and a possible reduction in seizure control. However, PHT's excitatory effects could also be the result of its direct action to inhibit neuronal Na^+, K^+-ATPase (For discussion, see preceeding report).

That chronic PHT treatment of cultured glial cells did not affect the activity of HCO_3^--ATPase (Fig. 19) is of interest considering our previous observations in vivo (White et al., 1983; and preceeding paper-this issue). In these studies, a significant increase in cerebral cortex HCO_3^--ATPase activity was observed following chronic PHT treatment of adult rats (20 mg/kg, ip, bid for 7 days). Considering the postulated relationship between this enzyme and CO_2-fixation reactions of the mitochondria (for discussion, see Woodbury, 1980), an increase in enzymic activity indirectly reflects an increase in neuronal activity and thus CO_2 production. Our present results, therefore, do not necessarily constitute a conflict between in vivo and in vitro observations but instead suggest that the increased enzymic activity observed in vivo is indirectly related to PHT's direct stimulatory effect on neurons (White et al., 1983; and preceeding report, this issue), and dependent in part on the intimate relationship between neurons and glia found only in vivo. We are currently investigating the relationship between the activities of HCO_3^--ATPase and carbonic anhydrase and anion transport in primary glial cultures and their respective contribution to the maintenance of the E_m.

The increase in Ca^{++}, Mg^{++}-ATPase activity (Fig. 20) observed following chronic PHT-treatment of undifferentiated cells indirectly suggests that Ca^{++}_i concentrations are elevated. This conclusion is further supported by a previous

study wherein similar concentrations of PHT were observed to decrease Ca^{++}-uptake into mitochondria, and hence increase $Ca^{++}{}_i$. A decrease in the Ca^{++}, Mg^{++}-ATPase activity (Fig. 20) of DBcAMP-differentiated cells was seen following chronic treatment with PHT. This result suggest that the $Ca^{++}{}_i$ concentration was decreased. Such an effect could be the indirect result of the decreased P_{Na}/P_K ratio (Fig. 20) also observed with chronic PHT-treatment of DBcAMP-differentiated cells. As the membrane permeability to sodium is decreased, less $Na^+{}_o$ leaks across the membrane. One means whereby the cell regulates $Na^+{}_i$ is through a Ca^{++}/Na^+ counter-transport process in which $Ca^{++}{}_i$ is exchanged for $Na^+{}_o$ (Latzkovits and Fajszi, 1982). Thus in a compensatory attempt to regulate $Na^+{}_i$, the cell also decreases $Ca^{++}{}_i$. We are currently investigating why the response of the P_{Na}/P_K ratio and the Ca^{++}, Mg^{++}-ATPase enzyme to PHT is different in DBcAMP-differentiated cells as compared to undifferentiated flat cells.

The studies presented in this report provide important new biochemical and electrophysiological data demonstrating that both acute and chronic PHT treatment can have profound effects on isolated cultures of glial cells. Through an interaction with glial regulatory processes, PHT in therapeutic concentrations appears to provide the CNS with an enhanced ability to regulate $K^+{}_o$ homeostasis within the brain ECF thereby limiting the spread of seizure activity. Furthermore, it appears that its ability to interact with glial cells can contribute to the acute and chronic CNS toxicity associated with high doses of PHT.

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VITA

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