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EFFECTS OF DIPHENYLHYDANTOIN AND PHENOBARBITAL ON PROTEIN METABOLISM IN THE RAT CEREBRAL CORTEX

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Abstract— The effects of the anticonvulsant drugs diphenylhydantoin and phenobarbital on the incorporation *in vivo* of L-[4.5-^{3}H]leucine into trichloroacetic acid-precipitable rat cerebral cortical protein were investigated. Protein specific activities were corrected for differences in precursor leucine specific activities. Plasma diphenylhydantoin levels of about 10 μg/ml were associated with a 41 per cent depression when radioisotope incorporation was conducted for 5 min; phenobarbital concentrations of approximately 28 μg/ml resulted in a 42 per cent depression after incorporation for the same period. A 3-fold increase in the diphenylhydantoin plasma levels and a 6-fold increase in the phenobarbital levels were found to invoke little, if any, additional effect. The induction period for the diphenylhydantoin effect was short (less than 1 hr), whereas a relative latency was associated with the phenobarbital effect. Simultaneous alterations in cortex and plasma concentrations of several amino acids were detected. Dose-related increases in cortex leucine, isoleucine and valine levels occurred after treatment with either drug. Cortex glycine concentrations were elevated only in response to the largest doses administered. Plasma leucine, isoleucine and valine levels were also elevated, but there was no change in plasma glycine concentration at any dose of either drug. The results indicate that diphenylhydantoin and phenobarbital inhibit the incorporation *in vivo* of radioactive leucine in the rat cortex when plasma drug levels have been attained that are therapeutically rational in the human, and substantial inhibitions would probably occur after much lower doses in the rat. The relationship of the altered amino acid levels to the effects on protein metabolism remains uncertain.

The involvement of protein synthesis as an integral process regulating the functional state of the nervous system is widely recognized [1–4]. The rate of protein synthesis in the brain is considerably greater than in most other organs, and it approximates that in secreting glandular tissue [5, 6]. The finished protein, however, is destined to be utilized and metabolized locally, having no egress to the periphery. The most likely explanation for such active protein metabolism concerns its involvement in the mechanisms of synaptic transmission [1]. Therefore, the functional state of the nervous system should be especially sensitive to agents which modify brain protein synthesis. The purpose of the present study was to evaluate the effects of the anticonvulsants diphenylhydantoin (DPH) and phenobarbital (PB) on the incorporation *in vivo* of radioactive leucine into rat cerebral cortical (total) protein. Protein specific activities were adjusted for differences in the precursor specific activity. Since fluctuations in the pool size of various amino acids have been strongly implicated in alterations of cerebral protein synthesis [7, 8], additional information was sought concerning changes in cerebral amino acid levels associated with the various treatment schedules.

The concurrent evaluation of DPH and PB was of interest because the two drugs have extensive application in the therapy of grand mal seizures, and a similarity with respect to their effects on cerebral protein metabolism could have practical as well as functional significance. Although a DPH-induced depression of cerebral protein synthesis has already been characterized in incubated tissue slice preparations [9–11], experiments *in vivo* have not been reported. The barbiturates have been shown to inhibit protein synthesis in numerous systems [12–15], but no information is available on the effects of PB on cerebral protein synthesis *in vivo*. Techniques *in vivo* avoid many of the uncertainties associated with the interpretation of data obtained from studies *in vitro* (e.g. the duplication of the complex aqueous environment and diffusion problems in cortical slices). The results indicate that both drugs, in therapeutic concentrations, inhibit the incorporation *in vivo* of radioactive leucine in the rat cortex.

MATERIALS AND METHODS

General experimental procedures. Male Sprague–Dawley rats, 28–30 days old (weight range 90–100 g), were used in all experiments. Animals for both the drug treatment and control groups within a given experiment were obtained simultaneously and kept in a room equipped with a diurnal lighting system and having free access to standard rat food and water. The sodium salts of DPH (Sigma Chemical Co.) and PB (Parke, Davis & Co.) were prepared fresh daily in aqueous 10% gum arabic, the control solution.

Each experiment consisted of a control group, a DPH-treated group and a PB-treated group. Animals received intraperitoneal injections according to one of three schedules. Schedule I animals received a single 100-mg/kg dose. Schedule II animals received three 100-mg/kg doses at 12-hr intervals. Schedule III animals received three injections at 12-hr intervals. DPH was administered in 100, 50 and 50 mg/kg doses and PB was administered in 50, 25 and 25 mg/kg doses. Plasma drug levels were determined by gas–liquid chromatography [16].

One hr after a single dose (schedule I) or 2 hr after the last of a multiple dose (schedules II and III), each

animal received intravenously 200 μCi/kg* of L-[4,5-^{3}H]leucine (ICN isotope and Nuclear Division; sp. ac., 47·6 Ci/m-mole) in isotonic saline. The injections were made with a 100-μl syringe via the right jugular vein while the animals were under light ether anesthesia. Radioisotope purity was routinely evaluated by paper chromatography using a butanol–acetic acid–water (4:1:1, v/v) solvent system and Whatman No. 41 paper. At various times after the radioisotope injection, the rats were sacrificed for subsequent biochemical analyses.

Measurement of radioactivity. Most samples, regardless of protein content, were prepared for counting with the Unisol, Unisol-Complement (Isolab Inc.) tissue solubilization system. (Exceptions are the effluent fractions collected from the amino acid analyzer; these were dissolved in Handifluor, a product of Mallinckrodt Chemical Works.) The radioactivity was measured with a Nuclear-Chicago PDS/3-ISOCAP/300 liquid scintillation data reduction system. Efficiency–quench correlation was accomplished by the external standard ratio method. Counting efficiency was 35–40 per cent and the cpm. were converted to dis/min using a suitable program for the PDS/3 computer. Samples were counted until the statistical uncertainty associated with the dis/min was less than 2 per cent.

Protein specific activity. Prior to decapitation, each animal was anesthetized lightly with ether and exsanguinated with a heparinized syringe via the dorsal aorta. Upon decapitation, the brain was removed and the cerebral cortices were dissected free on a cold plate, weighed and homogenized at 0–4° in 10 ml of 0·1 M Tris buffer, pH 7·5. Aliquots of cortical homogenate were treated with equal volumes of cold 10% (w/v) trichloroacetic acid (TCA) and centrifuged. The acid-soluble supernatants thus obtained were analyzed for radioactivity. The TCA-insoluble fractions were collected under vacuum on Whatman glass-fiber paper (GF/C grade, 2·4 cm diameter). The precipitates were washed on the filter three times with cold 5% TCA, extracted twice with ethanol–ether (1:1 v/v) and once with ether. The samples were then dried under an infrared lamp, transferred to a scintillation vial and processed for counting as described above.

The methanol solubility of TCA-precipitated liver proteins has been cited as a potential source of error when the precipitates are washed with this solvent [17]. However, a series of experiments designed to evaluate the magnitude of such a loss in the present system revealed virtually no decrement in the radioactivity associated with the TCA-precipitated cerebral proteins. (These data are not presented.)

The cortical homogenates were sampled directly for protein determination according to the method of Lowry *et al.* [18] with bovine serum albumin as a standard. Protein was collected (GF/C glass-fiber discs, 10 mm diameter) and processed as described above for radioactivity determinations. Results were expressed as dis./min/mg of protein.

Amino acid analyses and leucine specific activity. Plasma and cerebral cortical tissues were processed for amino acid extraction according to the perchloric acid procedure described by Saifer [19]. The amino acid analyses were performed with a Beckman model 121 amino acid analyzer, employing a single column and a programmed elution sequence designed to exclude resolution of the basic amino acids. The sodium citrate buffer elution procedure [20] employed failed to resolve adequately many of the acidic amino acids and their derivatives (particularly in the threonine to glutamic acid region of the chromatogram). Considerable improvement of resolution in this region has been claimed by others using a lithium citrate buffer gradient [21]. Amino acid concentrations were expressed as μmoles per g wet wt of cortex or per ml of plasma. The radioactivity in leucine and its metabolites was determined by collecting the column effluent at 2-min intervals and analyzing for radioactivity as described above. Specific activities were expressed as dis./min/μmole of amino acid.

Relative protein specific activity. Relative specific activities were derived by dividing the uncorrected values by the corresponding free leucine specific activities [22]. Although the free leucine specific activity is a complex function of cerebral blood flow, opposing transport rates of the label through brain cell membranes, fate of the label in other parts of the organism, etc., the assumption was made that the measured parameter accurately represents the isotope concentration at the site of protein synthesis.

Statistical analysis. Differences between control and experimental groups of animals were analyzed by one-way analysis of variance or the "Studentized" range test [23].

RESULTS

The plasma concentrations of DPH and PB at various times after intraperitoneal injection are presented in Table 1 for the specified treatment schedules. Substantial variability was observed in DPH concentrations after the administration of identical doses, particularly for the 'chronic' high dose experiments (schedule II). The overt symptoms of neurotoxicity (ataxia, graded on an arbitrary scale), which became apparent when plasma concentrations exceeded approximately 15 μg/ml, were also characterized by a large variability within a given treatment schedule. (These data are not presented.) PB plasma concentrations were much less variable, as were the overt symptoms of toxicity. Although schedule I PB levels are clearly within a range compatible with the comatose state in humans [24], the majority of rats used in these experiments were arousable upon stimulation. Schedule II levels, however, were consistent with a uniform production of coma. No overt symptoms of toxicity were associated with schedule III plasma levels at the time of measurement.

The therapeutic serum DPH concentration in humans is probably between 15 and 20 μg/ml; intoxication (ataxia, nystagmus, etc.) becomes frequent with concentrations greater than about 30 μg/ml [25]. Control of seizures with PB is generally thought to require serum concentrations greater than 10 μg/ml. Toxicity is usually not apparent at levels below 30 μg/ml and much higher levels have been compatible with

* Animals for schedule III experiments and leucine specific activity experiments were given 560 μCi/kg of L-[4,5-^{3}H]leucine.

Table 1. Plasma drug levels after specified treatment schedules*

	Diphenylhydantoin (μg/ml)				Phenobarbital (μg/ml)			
	Time after final injection (hr)							
Schedule†	1	2	3	4	1	2	3	4
I	17·0 ± 0·7	12·9 ± 2·0	16·1 ± 0·6		111·3 ± 3·8	92·8 ± 1·9	82·6 ± 1·0	
II		31·4 ± 5·6	27·6 ± 3·9	33·6 ± 0·4		162·0 (S)	186·2 ± 4·4	167·5 ± 6·0
III		10·2 ± 0·5	17·9 ± 0·6			28·2 ± 1·5	29·8 ± 0·1	

* Plasma drug levels were determined by gas chromatography [16]. Each value represents the mean ± S.E.M. of three to four determinations on separate animals, except where a single determination (S) is noted.

† Schedule I: single 100-mg/kg intraperitoneal injection; schedule II: three 100-mg/kg intraperitoneal injections at 12-hr intervals; schedule III: three intraperitoneal injections at 12-hr intervals; 100-, 50- and 50-mg/kg of diphenylhydantoin and 50, 25 and 25 mg/kg of phenobarbital.

a clear sensorium [24]. Thus, the DPH concentrations after the schedule I and III experiments were in a range clearly consistent with a therapeutic level in humans, whereas after schedule II treatments the plasma levels approached the range of minimal toxicity. The PB plasma levels were in a therapeutic range only after schedule III treatments; schedules I and II levels would produce variably light or deep coma, respectively, in humans.

The acute (schedule I) effects of DPH and PB on the specific activity (dis./min/mg of protein) of total cerebral cortical protein are depicted in Fig. 1a. These data are uncorrected for differences in the precursor leucine specific activity. The very rapid incorporation during the initial 15 min, and the gradual tapering off thereafter, are characteristic for such measurements in brain [26]. Specific activities in the drug-treatment groups exceeded those in the control groups when radioisotope incorporation was conducted for periods between 15 and 120 min. The 'chronic' (schedule II) effects illustrated in Fig. 1b, likewise uncorrected for differences in the free leucine specific activity, demonstrate an approximate reversal of the specific activities in the drug-treated groups relative to the control groups seen in Fig. 1a. Concurrent measurements of the cortex TCA-soluble radioactivity (Fig. 2, a and b) showed no such reversal. The drug-associated increase in radioactivity observed early in the acute experiments persists (and intensifies) in the 'chronic' experiments. Therefore, although the elevated protein specific activities observed acutely after drug treatment (Fig. 1a) might be due to an elevated free leucine specific activity, the lower drug-associated specific activities observed after 'chronic' administration (Fig. 1b) are unlikely to be due to a decrease in specific activity of the precursor.

The relationship between the precursor (free leu-

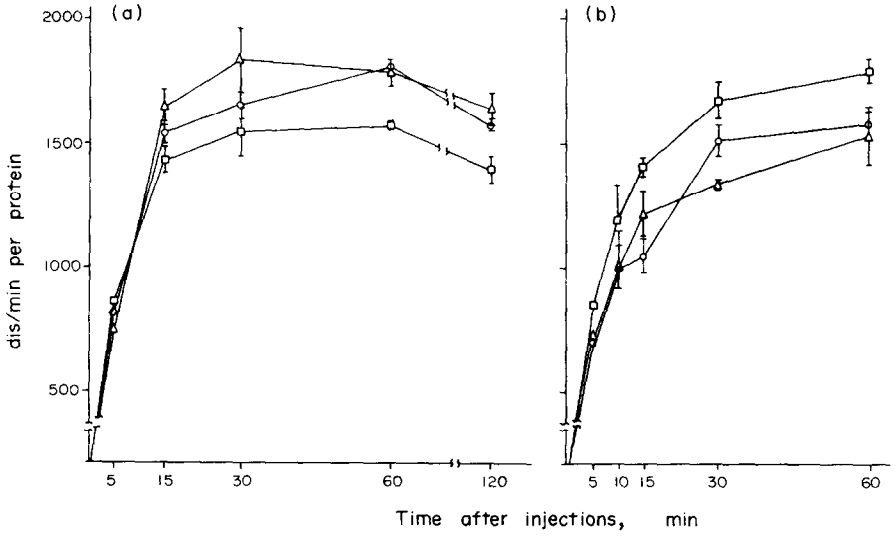

Fig. 1. Schedule I(a) and schedule II(b) effects of diphenylhydantoin (△) and phenobarbital (○) on the specific activity (dis./min/mg of protein) of the trichloroacetic acid-precipitable fraction of rat cerebral cortex measured at various times after L-[4,5-^{3}H]leucine injection. Values are not corrected for differences in the precursor leucine specific activity. Each point represents the mean of three to six determinations on separate animals. The vertical bars represent the standard errors of the mean. Control data (□).

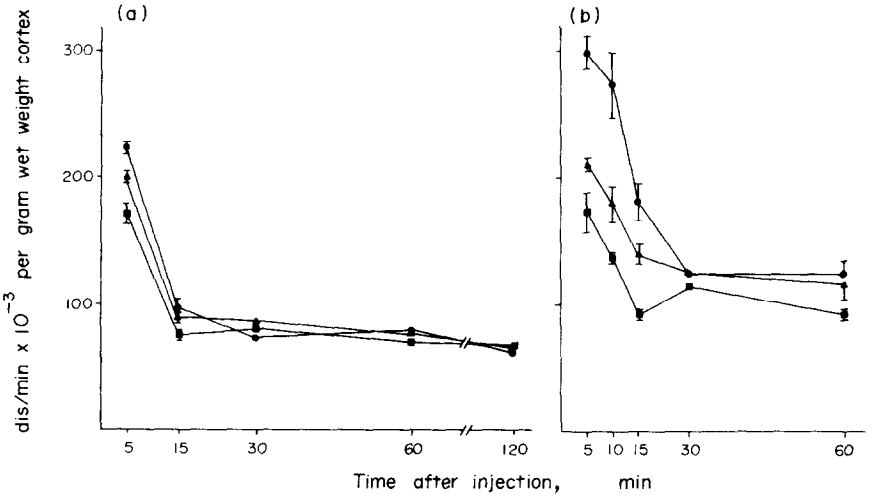

Fig. 2. Schedule I(a) and schedule II(b) effects of diphenylhydantoin (▲) and phenobarbital (●) on the radioactivity contained in the trichloroacetic acid-soluble fraction (dis./min/g wet wt) of rat cerebral cortex measured at various times after L-[4.5-^{3}H]leucine injection. Each point represents the mean of three to six determinations on separate animals. The vertical bars represent the standard errors of the mean. Control data (■).

cine) and product (total protein) specific activities was investigated directly by quantitative analyses of the perchloric acid-extractable cortical leucine and its associated radioactivity (see Materials and Methods). An apparent dose-related increase ($P < 0.01$) in cerebral leucine (μmoles/g wet wt of cortex) associated with the various treatments is shown in Table 2. When concentrations of the individual leucine fractions were related to the radioactivity contained therein, it was found that the specific activities of the amino acid were elevated by both drugs under all experimental conditions (Table 3). Thus, a portion of the increase in the total TCA-soluble radioactivity depicted in Fig. 2 might be due to whatever mechanisms are responsible for elevating cerebral leucine levels (*vide infra*); however, there is a net increase in the ratio of radioactive leucine to the unlabeled amino acid. The increase in free leucine specific activity was consistently greater than the increase in protein specific activity. Therefore, the elevated protein specific activities illustrated in Fig. 1a are a consequence of an elevated free leucine specific activity, whereas the lower drug-associated specific activities displayed in Fig. 1b are, in fact, overestimates of the actual values.

Numerical values for the uncorrected protein specific activities are presented for the 5- (or 7·5-) and 15-min incorporation periods in the upper half of Table 4. The lower half of Table 4 contains data derived by dividing the uncorrected values by the average of the corresponding leucine specific activities in Table 3 [22]. These corrected (relative) protein specific activities were consistently lower in the drug-treated groups than in the control groups. The maximum DPH-induced depressions associated with the various treatment schedules were quantitatively similar despite as much as a 3-fold difference in plasma drug levels (Tables 1 and 4). The inhibition by PB also appeared to be fairly insensitive to dose, since an approximate 6-fold increase in plasma drug concentration was associated with a relatively small increment of effect (schedules II and III). It is likely, therefore, that the observed effects approximate the efficacy of either drug as an inhibitor of cerebral protein synthesis. Experiments with lower doses are clearly warranted to establish a dose–response relationship.

Table 2. Effects of diphenylhydantoin and phenobarbital on free L-leucine levels in rat plasma and cerebral cortex*

	A. Plasma leucine (μmoles/ml)			B. Cerebral leucine (μmoles/g)			B/A†		
Schedule	Control	DPH	PB	Control	DPH	PB	Control	DPH	PB
I	0·068 ± 0·009	0·081 ± 0·010	0·059 ± 0·006	0·067 ± 0·002	0·091 ± 0·004‡	0·081 ± 0·003‡,§	0·99	1·12	1·37
II	0·072 ± 0·005	0·147 ± 0·012‡	0·104 ± 0·011‡,§	0·073 ± 0·007	0·126 ± 0·012‖	0·123 ± 0·013‖	1·01	0·86	1·18
III				0·055 ± 0·004	0·067 ± 0·004	0·063 ± 0·009			

* Animals were sacrificed for amino acid extraction approximately 1 hr after a single drug injection (schedule I) or approximately 2 hr after the last of three drug injections (schedules II and III). Extraction and analytical procedures are described in Materials and Methods. Abbreviations: DPH diphenylhydantoin; PB phenobarbital. Each value represents the mean ± S. E. M. leucine concentration from amino acid analyses of three to seven extracts involving four to five animals per extract.

† Cerebral leucine/plasma leucine.

‡ $P < 0.01$ for comparison of treatment value with control value.

§ $P < 0.05$ for comparison of phenobarbital value with diphenylhydantoin value.

‖ $P < 0.05$ for comparison of treatment value with control value.

Table 3. Effects of diphenylhydantoin and phenobarbital on L-leucine specific activity in rat plasma and cerebral cortex precursor pools*

	Control		Diphenylhydantoin		Phenobarbital	
	Incorporation period (min)					
Schedule	5 (7·5)†	15	5 (7·5)†	15	5 (7·5)†	15
	Plasma specific activity (dis./min × 10^{-3}/μmole leucine)					
I	212	52	266	71	145	41
II	187	54	216	65	269	81
	210	65	242	70	279	84
	Cortex specific activity (dis./min × 10^{-3}/μmole leucine)					
I	159	41	242	67	204	52
	204	51	298	91	233	76
	226	65		101	255	79
		70				
II	174	45	243	78	320	102
	195	60	262	85	350	122
III	119	61	141	85	151	85
	125	64	154	90	168	89

* Specific activities were measured as described in Materials and Methods. Values represent raw data for one to four determinations per experimental group. Plasma specific activities for schedule III experiments were not determined.

† The incorporation period for schedule III experiments was 7·5 min.

Patel and Balazs [27] have shown that an injected dose of radiolabeled leucine is extensively metabolized in brain tissue proper. Consequently, a general inhibition of cerebral protein synthesis should be consistent with the potential for inhibiting cerebral leucine metabolism. To evaluate this hypothesis, the radioactivity contained in the leucine fraction of the column effluent (see Materials and Methods) was expressed as a percentage of the total effluent radioactivity. Such data are presented in Table 5 for one to three determinations per experimental group. The apparent depression in leucine metabolism seems to correlate with the extent to which the incorporation of L-[4,5-^{3}H]leucine into cerebral protein was depressed. For example, approximately 26 per cent of the radioactivity remained in leucine in cerebral extracts from animals receiving control injections (schedule I, 5-min incorporation); an average 35 per cent remained in leucine in animals where incorporation was depressed approximately 19 per cent (PB), and an average 44 per cent remained in the amino acid when incorporation was depressed approximately 38 per cent (DPH).

Table 5. Effects of diphenylhydantoin and phenobarbital on L-[4,5-^{3}H]leucine metabolism in rat cerebral cortex*

	Control		Diphenylhydantoin		Phenobarbital	
	Incorporation period (min)					
Schedule	5 (7·5)	15	5 (7·5)	15	5 (7·5)	15
I	23·5	17·1	47·2	21·3	36·3	22·8
	28·3	7·4	46·2	20·1	28·0	14·2
	26·0	7·7	39·8	23·5	39·8	27·0
II	20·1	9·2	35·1	24·6	47·5	22·3
	27·0	6·8	35·0	15·7	41·7	35·0
III	15·6	7·3	29·4	20·4	38·4	25·0
	19·3		25·2		40·6	

* Each value is the per cent of the total acid-soluble radioactivity remaining in leucine at the specified time. The incorporation periods for schedule III experiments were 7·5 and 15 min. Raw data are presented for one to three determinations per experimental group.

Table 4. Effects of diphenylhydantoin and phenobarbital on incorporation *in vivo* of L-[4,5-^{3}H]leucine into rat cerebral protein*

	Control		Diphenylhydantoin		Phenobarbital	
	Incorporation period (min)					
Schedule	5 (7·5)	15	5 (7·5)	15	5 (7·5)	15
	Specific activity uncorrected (dis./min/mg protein)					
I	884 ± 62	1451 ± 57	751 ± 36	1666 ± 74	841 ± 44	1560 ± 48
II	864 ± 72	1403 ± 29	701 ± 89	1253 ± 97	683 ± 60	1092 ± 50
III	3943 ± 255	3973 ± 148	2824 ± 173	3487 ± 191	2979 ± 118	3542 ± 143
	Specific activity, corrected (dis./min/mg protein)					
I	451 ± 32	2553 ± 100	278 ± 13†	1927 ± 85†	364 ± 19‡,§	2254 ± 70‡,§
			(38·4)	(24·5)	(19·3)	(11·7)
II	469 ± 39	2657 ± 56	278 ± 35†	1532 ± 119†	204 ± 18†	972 ± 44†,‖
			(40·7)	(42·3)	(56·5)	(63·4)
III	3224 ± 209	6374 ± 238	1915 ± 118†	3984 ± 219†	1863 ± 74†	4077 ± 165†
			(40·6)	(37·5)	(42·2)	(36·0)

* Specific activities were measured as described in Materials and Methods. Schedule III experiments were conducted with an initial isotope radioactivity approximately three times that used in schedule I and schedule II experiments. The incorporation periods for schedule III experimants were 7·5 and 15 min. The corrected specific activities were derived by dividing the uncorrected values by the corresponding leucine specific activities in Table 3. Each value represents the mean ± S. E. M. specific activity determined on three to six animals. The numbers in parentheses beneath the corrected specific activities depict the per cent depression in that respective value relative to its control.

† $P < 0·01$ for comparison of treatment value with control value.

‡ $P < 0·05$ for comparison of treatment value with control value.

§ $P < 0·05$ for comparison of phenobarbital value with diphenylhydantoin value.

‖ $P < 0·01$ for comparison of phenobarbital value with diphenylhydantoin value.

Table 6. Effects of diphenylhydantoin and phenobarbital on free amino acid concentrations in rat plasma and cerebral cortex*

	Amino acid	Plasma (μmoles/ml)			Cerebral cortex (μmoles/g)		
		Control	DPH	PB	Control	DPH	PB
I	Glycine	0·295 ± 0·042	0·269 ± 0·031	0·256 ± 0·027	0·656 ± 0·019	0·644 ± 0·017	0·650 ± 0·014
	Alanine	0·432 ± 0·054	0·500 ± 0·058	0·386 ± 0·037	0·969 ± 0·028	1·074 ± 0·032†	0·834 ± 0·017†,‡
	Valine	0·091 ± 0·013	0·101 ± 0·015	0·084 ± 0·008	0·113 ± 0·004	0·129 ± 0·005§	0·133 ± 0·005§
	Methionine	0·018 ± 0·002	0·018 ± 0·002	0·019 ± 0·003	0·019 ± 0·001	0·019 ± 0·002	0·022 ± 0·001
	Isoleucine	0·046 ± 0·007	0·051 ± 0·007	0·042 ± 0·003	0·046 ± 0·003	0·057 ± 0·002§	0·054 ± 0·002§
	Tyrosine	0·044 ± 0·005	0·047 ± 0·008	0·045 ± 0·006	0·070 ± 0·005	0·065 ± 0·005	0·081 ± 0·008
	Phenylalanine	0·033 ± 0·001	0·035 ± 0·005	0·026 ± 0·003	0·042 ± 0·002	0·042 ± 0·003	0·046 ± 0·002
II	Glycine	0·260 ± 0·017	0·254 ± 0·019	0·269 ± 0·022	0·606 ± 0·018	0·791 ± 0·026†	0·807 ± 0·021†
	Alanine	0·463 ± 0·031	0·233 ± 0·048§	0·390 ± 0·061‖	0·850 ± 0·019	0·731 ± 0·035§	0·601 ± 0·034§,‖
	Valine	0·086 ± 0·005	0·167 ± 0·011†	0·122 ± 0·013§,‖	0·121 ± 0·008	0·181 ± 0·016§	0·212 ± 0·020†
	Methionine	0·022 ± 0·001	0·026 ± 0·003	0·027 ± 0·003	0·025 ± 0·002	0·023 ± 0·002	0·040 ± 0·005§,‖
	Isoleucine	0·041 ± 0·003	0·107 ± 0·007†	0·062 ± 0·008‡	0·044 ± 0·004	0·088 ± 0·009§	0·084 ± 0·012§
	Tyrosine	0·048 ± 0·004	0·052 ± 0·003	0·048 ± 0·003	0·068 ± 0·006	0·067 ± 0·002	0·089 ± 0·011
	Phenylalanine	0·039 ± 0·003	0·056 ± 0·004§	0·043 ± 0·003‖	0·051 ± 0·003	0·062 ± 0·002	0·069 ± 0·006§
III	Glycine				0·662 ± 0·036	0·667 ± 0·026	0·654 ± 0·042
	Alanine				0·891 ± 0·017	0·777 ± 0·001†	0·847 ± 0·016‖
	Valine				0·122 ± 0·005	0·138 ± 0·010	0·129 ± 0·010
	Methionine				0·015 ± 0·000	0·022 ± 0·002§	0·017 ± 0·002
	Isoleucine				0·040 ± 0·002	0·045 ± 0·002	0·042 ± 0·002
	Tyrosine				0·065 ± 0·009	0·064 ± 0·009	0·073 ± 0·008
	Phenylalanine				0·044 ± 0·003	0·056 ± 0·006	0·046 ± 0·002

* Animals were sacrificed for amino acid extraction as described in the legend to Table 2. Each value represents the mean ± S. E. M. amino acid concentration from analyses of three to seven extracts involving four to five animals per extract.

† $P < 0·01$ for comparison of treatment value with control value.

‡ $P < 0·01$ for comparison of phenobarbital value with diphenylhydantoin value.

§ $P < 0·05$ for comparison of treatment value with control value.

‖ $P < 0·05$ for comparison of phenobarbital value with diphenylhydantoin value.

By 15 min, the metabolites of L-[4,5-^{3}H]leucine accounted for approximately 91 per cent of the total acid-soluble radioactivity in control groups (Table 5). This is in close agreement with the data of Banker and Cotman [28], which indicated a 94–95 per cent metabolism in 16 min. The major radioactive metabolite eluted with the taurine-urea fractions and, as pointed out by Banker and Cotman [28], appears to be tritiated water. Substantial radioactivity was also found associated with the dicarboxylic amino acids (especially glutamic acid) and their derivatives. It is unlikely, however, that significant quantities of these labeled metabolites found their way into protein, since their specific activities were relatively low. (This is because the major dicarboxylic amino acids and derivatives thereof are present in exceptionally high concentrations in the brain.) The data reported by Roberts and Morelos [29] seem to bear this out. The radioactivity in a protein hydrolysate obtained 15 min after the injection of L-[U-^{14}C]leucine resided almost exclusively in leucine.

As already described, both DPH and PB appear to elevate cerebral leucine levels (Table 2). The concentrations of several other amino acids in the cerebral cortex of animals variably treated are presented in Table 6. The response of cerebral isoleucine and valine levels to drug treatment paralleled the leucine response, i.e. both drugs appeared to elevate both amino acids in the cerebral cortex. Glycine levels were elevated by both drugs after schedule II treatments ($P < 0·01$), but no change was apparent at lower plasma drug levels. The alanine response was not uniform among the various treatment schedules. A significant increase ($P < 0·01$) in cerebral alanine occurred after a single 100 mg/kg dose of DPH (schedule I), whereas after three such doses (schedule II), cerebral alanine levels appeared to be lower than those of controls ($P < 0·05$). Lower alanine levels ($P < 0·01$) were also found after schedule III DPH treatments. On the other hand, PB appeared to lower cerebral alanine levels regardless of the particular treatment schedule. Very large doses of PB might be responsible for a small elevation in cortex phenylalanine ($P < 0·05$, schedule II). Likewise, methionine levels appear to be elevated by high plasma PB levels ($P < 0·05$).

The alterations in plasma free amino acid levels did not uniformly parallel those in the cerebral cortex (Tables 2 and 6). Plasma leucine levels were significantly elevated after schedule II DPH ($P < 0·01$) and PB ($P < 0·05$) treatments; changes after schedule I experiments are less certain, and schedule III plasma amino acid levels were not determined. The response of plasma isoleucine and valine levels to drug treatment was similar to the leucine response. As in the cerebral cortex, plasma levels of both amino acids appeared to be elevated by both drugs. Plasma glycine levels remained constant (unlike the cerebral levels) even after schedule II treatments. The alanine response was again not uniform among the various treatment schedules. Plasma alanine levels might have increased slightly after a single injection of DPH (schedule I), but 'chronic' administration (schedule II) was associated with a significant decrease ($P < 0·05$). Plasma alanine concentrations were lower after PB treatment regardless of the particular treatment sche-

dule. High doses of DPH appear to be responsible for a small rise in plasma phenylalanine levels ($P < 0.05$, schedule II).

DISCUSSION

Protein synthesis is operationally defined for this investigation as the incorporation of L-[4,5-^{3}H]leucine into the fraction of the cerebral cortex which is precipitated in cold 5 per cent TCA. Several assumptions are implicit in the interpretation of the data. The rates of protein synthesis and degradation are in a steady state during the experimental period; no significant recycling of the label occurs; the protein-bound radioactivity resides exclusively in leucine; and the drug treatments do not alter the subcellular distribution of free leucine in the cortex [30]. The contribution to error in the first three assumptions is probably minimized by experimental design (short incorporation periods and low specific activities of leucine metabolites in the brain). It is not possible at present to estimate the error inherent in the fourth assumption. Compartmentalization of free leucine would hinder the equilibration of the labeled and the unlabeled molecules; and since computation of precursor specific activities is normally based upon the total extractable tissue amino acid, local differences could be overlooked. Thus, the observed precursor specific activities might not represent those at the actual site of protein synthesis. Problems of interpretation would arise in comparative studies if the experimental treatment altered the specific activities of the precursor pool in equilibrium with protein synthesis.

The present results provide evidence that DPH and PB inhibit cerebral protein synthesis *in vivo*. The induction period for the DPH effect is relatively short, since an approximate 38 per cent depression in isotope incorporation was observed in schedule I only 1 hr after a single dose. This is consistent with the rapid onset of anticonvulsant action produced by the drug. The time of peak effect is about 15 min in the rat [31] and is probably related to the facility with which it enters the central nervous system (see below).

The induction period for the PB effect is apparently longer than that for DPH. An approximate 19 per cent depression was observed 1 hr after a single dose (schedule I) but a 42 per cent depression occurred after multiple (schedule III) dosage when plasma drug levels were one-fourth those after the single dose (Tables 1 and 4). The relative latency of the PB effect can likely be explained in terms of two physical properties: the dissociation constant and lipid solubility. Because of its relatively high degree of dissociation at pH 7·4 and its very low partition coefficient, PB probably attains a steady state brain concentration much more slowly than DPH [32, 33].

Yanagihara and Hamberger [10] have proposed that the DPH-induced inhibition may be due partially to a decreased tissue uptake of leucine. However, the data presented in this paper reveal a net increase in total cerebral free leucine after DPH (or PB) treatment (Table 2). Thus, it appears unlikely that the inhibition of cerebral protein synthesis by these drugs is secondary to a lower free leucine concentration.

Nevertheless, studies *in vitro* [7, 8] and *in vivo* [29] have indicated that protein synthesis in the brain may be unusually sensitive to alterations in the levels of various amino acids. Appel [8] has shown *in vitro* that increasing concentrations of leucine were inhibitory for the incorporation of several unrelated protein precursors. In addition, leucine incorporation itself was inhibited by increasing valine or isoleucine concentrations in the incubation medium. This is particularly interesting in the context of the present results since, apart from the elevated leucine levels, valine and isoleucine were the only amino acids which appeared to be consistently elevated after the various treatment schedules (Table 6). Thus, the inhibition by DPH and PB of cerebral protein synthesis *in vivo* might be due in part to higher concentrations of valine and isoleucine. However, it is unlikely that such an effect can explain the inhibition *in vitro* described by Yanagihara and Hamberger [10] (since the higher brain levels of these amino acids are probably secondary to elevated peripheral concentrations; see below). Furthermore, the valine and the isoleucine increases, like those of leucine, appeared to be dose related, whereas the effects on protein synthesis were not.

The origin of the elevated cerebral amino acid levels is uncertain. Since increases in plasma concentrations also occurred (Tables 2 and 6), it is possible that the elevated cerebral levels were secondary to higher plasma levels, but Lajtha and Toth [34] have observed that brain levels of most amino acids are not altered in response to elevated plasma concentrations. Therefore, the mechanism probably involves a specific effect on amino acid transport. Since the transport of L-leucine, L-isoleucine and L-valine is presumably mediated by the same system (the L-system proposed by Oxender and Christensen [35]), the spectrum of changes described presently is consistent with a specific transport effect.

Although specific mechanisms have been demonstrated for the transport of nonmetabolized amino acids from the cerebrospinal fluid to plasma [36], the locus of this process *in vivo* has not been clarified; both the choroid plexus [37] and brain slices [38] have been shown to concentrate amino acids. Leucine is very rapidly metabolized in the brain (this paper and Refs. 28 and 29), and if a significant proportion of this amino acid is transported into the brain, its metabolism there may interfere with its further transport. The studies of Lajtha and Toth [34] did not account for this leucine 'sink' effect. Thus, an inhibition of cerebral protein synthesis might account for the elevated levels of this amino acid by inhibiting its metabolism.

The elevated cerebral glycine levels after schedule II treatments (Table 6) are probably not related to peripheral effects, since plasma concentrations were unaltered. This is in agreement with available evidence that cerebral glycine is not derived from the blood but rather from *de novo* synthesis in the cortex [39]. It is doubtful that functional [40, 41] significance can be attributed to the elevated glycine levels, since the concentrations apparently do not change after therapeutically more reasonable doses of either drug (schedules I and III, Table 6).

Van Gelder *et al.* [42, 43] have correlated regions of epileptogenicity in the cortex with substantial increases in glycine concentration. The observation that seizure activity is apparently associated with an inhi-

bition of protein synthesis in the affected tissue [44, 45] has led these investigators to suggest that the accumulation of glycine in these tissues is due to a diminished reutilization of this amino acid under these circumstances. The apparent refractoriness of glycine to metabolic degradation at the synaptic cleft [39] might contribute to its accumulation. Since particularly high concentrations of glycine occur in the more slowly metabolized brain proteins [6], the elevated levels of this amino acid possibly reflect inhibition in this fraction.

Very large doses of DPH can elicit convulsive behavior in the rat; and the schedule II dosage is similar to that at which clonic seizure activity has developed [11]. Therefore, the increase in cerebral glycine levels after schedule II drug treatments (Table 6) might be related not to the anticonvulsant effects, but rather to the convulsive effects of very large doses. Furthermore, whereas the lower doses of either drug appear to inhibit primarily the more rapidly metabolized proteins (schedules I and III, 5-min incorporation, Table 4), the higher plasma levels associated with schedule II treatments appear to inhibit the more slowly metabolized proteins to an equal or greater extent (15-min incorporation, Table 4). On the basis of these data, it seems possible that seizure activity is related to the deficiency of a particular protein with a relatively long half-life. The inhibition of this protein after large doses of either drug could result in the accumulation of glycine for the reason noted above.

DPH and PB might conceivably alter cerebral protein synthesis in a more direct way. Recent work has shown that certain hydantoins [46] and barbiturates [47] form highly specific hydrogen-bonded dimers with adenine, or with the adenine moiety of FAD and NAD [48]. The affinities of DPH and PB for adenine are both greater than those of uracil and thymine for adenine [49]. Thus, a preferential assiciation of these drugs with the recently discovered poly A-rich segments of a particular messenger RNA might effectively interfere with the transcription of protein.

Direct evidence for such a phenomenon, however, is lacking. Kemp and Woodbury [50] have demonstrated that the microsomal fraction is the major binding site for DPH in rat cerebral cortex. Accumulation in this fraction was noted for periods up to 12 hr after a single intracisternal injection; indirect evidence was provided that this accumulation might occur as a result of an interaction with membrane-bound ribonucleoprotein. Yanagihara and Hamberger [10] have reported a prolonged inhibition of leucine incorporation into microsomal proteins after DPH treatment in the rat. Thus, DPH accumulates and appears to inhibit protein synthesis in those subcellular structures with which the majority of cellular RNA is normally associated.

A correlation of the results from the present investigation with a relevant (anticonvulsant) alteration of the functional state of the central nervous system must remain entirely speculative. Since the most active brain protein metabolism appears to be related to synaptic function [1], metabolic alterations of the proteins involved therein might conceivably be the basis for an anticonvulsant effect. However, the involvement of such an inhibition in the mediation of an anticonvulsant effect must be considered in relationship to the latency of such an effect. For example, the time of peak effect of DPH for protection against maximal electroshock is about 15 min in rats [31]. Thus, the relevant alterations must occur within this time. Although the neuron is a cell with a high capacity for protein synthesis, the preponderance of evidence indicates that most of the synthesis occurs in the soma [51]. Axoplasmic flow is thought to provide the bulk of the distal protein requirements, but the most rapid flow rates are probably too slow to account for a distal protein deficiency within 15 min of somal inhibition [52]. An effect on local (synaptic) synthesis [53, 54], therefore, may be more consistent with the latency of the anticonvulsant effect. Since the prolonged inhibition of protein synthesis in the synaptosomal fraction by DPH appears to be correlated with the duration of its anticonvulsant effect in rats [10], further study of the actions of the drug on synaptic protein metabolism appears warranted.

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