EFFECTS OF DIPHENYLHYDANTOIN AND PHENOBARBITAL

ON PROTEIN METABOLISM IN THE RAT CEREBRAL CORTEX

by

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ABSTRACT

The effects of the anticonvulsant drugs diphenylhydantoin and

phenobarbital on the in vivo incorporation of L-[4,5-^{3}H] leucine into

trichloroacetic acid-precipitable rat cerebral cortical protein were inves-

tigated. Protein specific activities were corrected for differences in

precursor leucine specific activities. Incorporation was substantially

depressed following the establishment of plasma concentrations of

either drug which approximate therapeutic levels in humans. Plasma

diphenylhydantoin levels of about 10 μg/ml were associated with a 41%

depression when radioisotope incorporation was conducted for 5 min;

phenobarbital concentrations of approximately 28 μg/ml resulted in a

42% depression following incorporation for the same period. A three-

fold increase in the diphenylhydantoin plasma levels and a six-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 1h), whereas a relative latency was associated with

the phenobarbital effect. Simultaneous alterations in cortex and plasma

concentrations of several amino acids were detected, but there appeared

to be no correlation between these changes and the extent of depression

of leucine incorporation. Dose-related increases in cortex leucine,

isoleucine and valine levels occurred following treatment with either

drug. Cortex glycine concentrations were elevated only in response to the largest doses administered. Whereas plasma leucine, isoleucine and valine levels were also elevated, no change in plasma glycine concentrations were noted following any dose of either drug. The results indicate that diphenylhydantoin and phenobarbital inhibit the in vivo incorporation 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 following much lower doses in the rat. The relationship of the altered amino acid levels to the effects on protein metabolism remains uncertain.

INTRODUCTION

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 and phenobarbital on the in vivo incorporation 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 diphenylhydantoin and phenobarbital was of interest because the two drugs have extensive application in the

therapy of grand mal type seizures, and a similarity with respect to their effects on cerebral protein metabolism could have practical, as well as functional significance. Although a diphenylhydantoin-induced depression of cerebral protein synthesis has already been characterized in incubated tissue slice preparations,[9-11] in vivo experiments 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 phenobarbital on cerebral protein synthesis in vivo. In vivo techniques 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, diffusion problems in cortical slices, etc.). The results indicate that both drugs, in therapeutic concentrations, inhibit the in vivo incorporation 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, having free access to standard rat food

and water. The sodium salts of diphenylhydantoin (Sigma Chemical Co.)

and phenobarbital (Parke, Davis & Co.) were prepared fresh daily in

aqueous 10% gum arabic, the control solution.

Each experiment consisted of a control group, a diphenylhydantoin-

treated group and a phenobarbital-treated group. Animals received intra-

peritoneal 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 twelve hour intervals. Schedule III animals

received three injections at twelve hour intervals; diphenylhydantoin was

administered in 100, 50 and 50 mg/kg doses and phenobarbital was

administered in 50, 25 and 25 mg/kg doses. Plasma drug levels were

determined by gas liquid chromatography.[16]

One hour following a single dose (Schedule I) or two hours following

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, specific radioactivity 47.6 Ci/mmol) 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. Radio-

isotope purity was routinely evaluated by paper chromatography using

a butanol-acetic acid-water (4:1:1, by vol.) solvent system and What-

man number 41 paper. At various times following 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 percent and the c.p.m. were converted

to d.p.m. using a suitable program for the PDS/3 computer. Samples

were counted until the statistical uncertainty associated with the d.p.m.

was less than 2 percent.

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

Protein specific activity. Prior to decapitation, each animal was anesthetized lightly with ether and exanguinated 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°C 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, by vol.) and once with ether. Each wash and extraction was accomplished by gentle shaking for ten minutes in an Erlenmeyer flask containing 5 ml of the appropriate solvent. 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. The reagents used in the above method were modified, however, as described elsewhere.[19] Protein was collected (GF/C glass fiber discs, 10 mm diameter) and processed as described above for radioactivity determinations. Results were expressed as d.p.m. per milligram 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.[20] 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[21] 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.[22] Amino acid concentrations were expressed as micromoles per gram wet weight of cortex or per ml of plasma. The radioactivity in leucine and its metabolites was determined by collecting the column effluent at two minute intervals and analyzing for radioactivity as described above. Specific activities were expressed as d.p.m. per micromole amino acid.

Relative protein specific activity. Relative specific activities were derived by dividing the uncorrected values by the corresponding free leucine specific activities.[23]

Statistical analysis. Differences between control and experimental groups of animals were analyzed by one-way analysis of variance or the studentized range test.[24]

RESULTS

The plasma concentrations of diphenylhydantoin and phenobarbital
at various times following intraperitoneal injection are presented in
Table 1 for the specified treatment schedules. The plasma diphenyl-
hydantoin levels following a single 100 mg/kg injection (Schedule I)
resemble closely the blood levels reported by Lee and Bass[25] following
the same dosage. Substantial variability was observed in diphenyl-
hydantoin concentrations following 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.)
Phenobarbital plasma concentrations were much less variable, as were
the overt symptoms of toxicity. Although Schedule I phenobarbital
levels are clearly within a range compatible with the comatose state
in humans,[26] the majority of rats used in these experiments were
arousable upon stimulation. Schedule II levels, however, were consis-
tent with a uniform production of coma. No overt symptoms of toxicity
were associated with Schedule III plasma levels at the time of measure-
ment.

The therapeutic serum diphenylhydantoin 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.[27] Control of seizures with phenobarbital 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 a clear sensorium.[26] Thus, the diphenylhydantoin concentrations following Schedules I and III experiments were in a range clearly consistent with a therapeutic level in humans, whereas following Schedule II treatments the plasma levels approached the range of minimal toxicity. The phenobarbital plasma levels were in a therapeutic range only following Schedule III treatments; Schedules I and II levels would produce variably light or deep coma, respectively in humans.

The acute (Schedule I) effects of diphenylhydantoin and phenobarbital on the specific activity (dpm/mg protein) of total cerebral cortical protein, uncorrected for differences in the precursor leucine specific activity, are depicted in Fig. 1. Illustrated is the time-dependent change in protein specific activity following radioisotope injection. The very rapid incorporation during the initial 15 minutes, and the gradual tapering off thereafter, are characteristic for such measurements in brain.[28] Specific activities in the drug-treatment groups exceeded those in the control groups when radioisotope incorporation was conducted for periods between 15 and 120 minutes. The "chronic" (Schedule II) effects illustrated in Fig. 2, likewise uncorrected for

differences in the free leucine specific activity, demonstrate an approximate reversal of the specific activities in the drug-treatment groups relative to the control groups seen in Fig. 1. Concurrent measurements of the cortex TCA-soluble radioactivity (Figs. 3 and 4) 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 following drug treatment (Fig. 1) might be due to an elevated free leucine specific activity, the lower drug-associated specific activities observed following "chronic" administration (Fig. 2) are unlikely due to a decrease in specific activity of the precursor.

The relationship between the precursor (free leucine) 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 Schedules I and II plasma drug levels is shown in Table 2. Although the data for Schedule III experiments do not significantly differ from one another ($P>0.05$), a similar increase is suspected. 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 Figs. 3 and 4 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 unlabelled 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. 1 are a consequence of an elevated free

leucine specific activity, whereas the lower drug-associated specific

activities displayed in Fig. 2 are, in fact, overestimates of the actual

values.

Numerical values for the uncorrected protein specific activities

depicted in Figs. 1 and 2 are presented for the 5 and 15 minute incor-

poration periods in the upper half of Table 4. Also shown are the spe-

cific activities for Schedule III experiments where incorporation was con-

ducted for 7.5 and 15 minutes. The lower half of Table 4 contains data

derived by dividing the uncorrected values by the average of the cor-

responding leucine specific activities in Table 3.[23] These corrected

(relative) protein specific activities were consistently lower in the drug-

treatment groups than in the control groups. The maximum diphenyl-

hydantoin-induced depressions associated with the various treatment

schedules were quantitatively similar despite as much as a three-fold

difference in plasma drug levels (Tables 1 and 4). The inhibition by

phenobarbital also appeared to be fairly insensitive to dose, since an

approximate six-fold increase in plasma drug concentrations 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.

Patel and Balazs[29] have shown that an injected dose of radio-

labelled 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 metab-

olism. 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 experi-

mental group. The apparent depression in leucine metabolism seems

to correlate with the extent to which the incorporation of $L-[4,5-^3H]$

leucine into cerebral protein was depressed. For example, approximate-

ly 26% of the radioactivity remained in leucine in cerebral extracts from

animals receiving control injections (Schedule I, 5 minute incorporation);

an average 35% remained in leucine in animals where incorporation was

depressed approximately 19% (phenobarbital), and an average 44%

remained in the amino acid when incorporation was depressed approxi-

mately 38% (diphenylhydantoin).

By 15 minutes metabolites accounted for approximately 91% of the

total acid-soluble radioactivity in control groups (Table 5). This is in

close agreement with the data of Banker and Cotman[30] which indicated

a 94-95% metabolism in 16 minutes. The major radioactive metabolite

eluted with the taurine-urea fractions and, as pointed out by Banker

and Cotman,[30] 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 quantitites of these labelled 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[31] seem to bear this out: The radio-

activity in a protein hydrolysate obtained 15 minutes after the injection

of L-[U-^{14}C] leucine resided almost exclusively in leucine.

As already described, both diphenylhydantoin and phenobarbital

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 following Schedule II treat-

ments ($P<0.01$), but no change was apparent at lower plasma drug

levels. The alanine response was not uniform between the various

treatment schedules. A significant increase ($P<0.01$) in cerebral ala-

nine occurred following a single 100 mg/kg dose of diphenylhydantoin

(Schedule I), whereas following three such doses (Schedule II) cerebral alanine levels appeared to be lower than controls (P<0.05). Lower alanine levels (P<0.01) were also found following Schedule III diphenyl-hydantoin treatments. On the other hand, phenobarbital appeared to lower cerebral alanine levels regardless of the particular treatment schedule. Vary large doses of phenobarbital might be responsible for a small elevation in cortex phenylalanine (P<0.05, Schedule II). Like-wise, methionine levels appear to be elevated by high plasma pheno-barbital 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 following Schedule II diphenylhydan-toin (P<0.01) and phenobarbital (P<0.05) treatments; changes following 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 following Schedule II treatments. The alanine response was again not uniform between the various treat-ment schedules. Plasma alanine levels might have increased slightly following a single injection of diphenylhydantoin (Schedule I), but "chronic" administration (Schedule II) was associated with a significant decrease (P<0.05). Plasma alanine concentrations were lower following

phenobarbital treatment regardless of the particular treatment schedule.

High doses of diphenylhydantoin appear responsible for a small rise in

plasma phenylalanine levels ($P<0.05$, Schedule II).

The relative changes in the concentrations of the various amino

acids in the cortex and in the plasma following drug treatment were

approximated by the ratio: cerebral amino acid (μmoles gram^{-1})/plasma

amino acid (μmoles ml^{-1}). This ratio, however, does not necessarily

reflect the extent of transport, or uptake of the respective amino acid

into the brain (see Discussion). Schedule I diphenylhydantoin treatment

appears to be associated with increased cerebral concentrations of

leucine, isoleucine and glycine relative to the respective plasma con-

centrations; relative cerebral concentrations of alanine, tyrosine and

phenylalanine were reduced (Tables 2 and 7). The ratios following the

Schedule II diphenylhydantoin treatment were uniformly lower than for

the controls, except for the amino acids glycine and alanine. Finally,

the cortex/plasma amino acid ratios were increased (except for alanine)

following phenobarbital regardless of the treatment schedule.

If these ratios were presumed to measure the extent of cerebral

uptake of the various amino acids, the lower leucine ratio following

Schedule II diphenylhydantoin treatment would seem to be consistent

with the lower uptake of radioactive leucine observed in vitro by

Yanagihara and Hamberger.[10] Unlike the in vitro studies, however,

the present results indicate a net drug-related increase in cerebral

leucine, presumably due to a specific effect on amino acid transport

(see Discussion).

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% TCA. Several assump-
tions 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.[32]
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 possi-
ble at present to estimate the error inherent in the fourth assumption.
Compartmentalization of free leucine would hinder the equilibration of
the labelled and the unlabelled 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 strong evidence that diphenylhydantoin and phenobarbital are capable of inhibiting cerebral protein synthesis. Maximum depressions (in relative protein specific activities) of approximately 42% and 63% were observed for the two drugs, respectively (Schedule II, Table 4). The induction period for the diphenylhydantoin effect is relatively short, since an approximate 38% depression was observed in Schedule I only one hour after a single dose. This is consistent with the drug's rapid onset of anticonvulsant action (The time of peak effect is about 15 minutes in the rat.[33]), and is probably related to the facility with which it enters the central nervous system (see below).

The induction period for the phenobarbital effect is comparatively longer than that for diphenylhydantoin. An approximate 19% depression was observed one hour after a single dose (Schedule I), whereas a 42% depression occurred following multiple (Schedule III) dosage when, in fact, the plasma drug levels were one-fourth those following the single dose (Tables 1 and 4). The relative latency of the phenobarbital effect can likely be explained in terms of two physical properties: the dissociation constant and lipid solubility. Phenobarbital (pk_a 7.3) is only 44.3% unionized at pH 7.4, whereas diphenylhydantoin (pk_a 8.3) is 88.8% unionized at this pH. Furthermore, the lipid solubility (based on a chlorobutane/aqueous partitian coefficient) of phenobarbital is only 21% of that of diphenylhydantoin.[34] Since drugs enter the central nervous system at rates roughly proportional to (a) the concentration

of undissociated molecules at pH 7.4 and (b) the lipid-solubility of the undissociated molecules,[35] phenobarbital probably attains a steady state brain concentration much more slowly than diphenylhydantoin.

Following "chronic" diphenylhydantoin treatment similar to Schedule II, Yanagihara and Hamberger[10,11] observed an approximate 25% inhibition of leucine incorporation in the homogenate fraction in studies involving the in vitro incubation of cortical slices. These investigators failed to detect a significant inhibition in the homogenate fraction when leucine incorporation was evaluated acutely (in a treatment program similar to Schedule I). Studies involving "cell-enriched" fractions, however, revealed a prompt and sustained inhibition of incorporation in the neuronal fraction, whereas a significant inhibition was observed in the glial fraction only after multiple dosage.[11] Thus, the above studies revealed changes in the homogenate fraction which appeared to reflect glial rather than neuronal metabolism.

The results of the present investigation might reflect alterations of neuronal as well as glial metabolism. Several investigations have shown that brain protein synthesis in the rat is very active at parturition and declines continuously thereafter until it reaches a plateau at about 90 days.[36,37] This decline with age correlates approximately with (a) histological evidence for the proliferation of glial cells,[38] (b) with increased concentrations of the presumably glial specific S-100 protein,[39] and (c) with increased activities of the enzyme carbonic anhydrase, a glial marker.[40] Thus, measurements of protein synthesis in the

unfractionated cortex of the adult rat might be expected to involve substantial contributions from glial metabolism, since in the mature brain the number of glial cells greatly exceed the number of neurons.[38] Unlike the studies of Yanagihara and Hamberger, however, the present investigation was conducted with relatively immature rats, in which neurons constitute a greater proportion of the cellular population. Therefore, the prompt inhibition of leucine incorporation in the homogenate fraction described herein could be due primarily to changes in neuronal metabolism.

Yanagihara and Hamberger[10] have proposed that the diphenylhydantoin-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 following diphenylhydantoin (or phenobarbital) 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[31] 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 non-related protein precursors. In addition, leucine incorporation was itself 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 consistently to be elevated following the various treatment schedules (Table 6). Thus, the inhibition by diphenylhydantoin and phenobarbital 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 in vitro inhibition described by Yanagihara and Hamberger (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, as for 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[41] have observed that brain levels of most amino acids are not altered in response to elevated plasma concentrations. Therefore, unless the brain and the plasma levels are elevated independently, the mechanism probably involves a specific effect on amino acid transport.

If, for example, drug treatment induced the release of amino acids from the liver or muscle, plasma concentrations would undoubtedly increase. Brain levels would not be expected to change unless there was an accompanying alteration in blood-brain barrier permeability. Rapid exchange between plasma and brain free amino acid pools is

known to occur for many amino acids,[42] despite the fact that net uptake from the blood is usually impeded; and there is evidence that their entry and exit from the brain occur by specific mechanisms.[41,43] Since the transport of L-leucine, L-isoleucine and L-valine are presumably mediated by the same system (the L system proposed by Oxender and Christensen[43]), the spectrum of changes described presently is consistent with a specific transport effect. The elevated cerebral amino acid levels probably result from a stimulation of transport into the brain, although an inhibition of transport from cerebrospinal fluid to plasma might produce the same result. The present data are not sufficient to distinguish between the alternatives.

Although specific mechanisms have been demonstrated for the transport of non-metabolized amino acids from the cerebrospinal fluid to plasma,[44] the locus of this process in vivo has not been clarified; both the choroid plexus[45] and brain slices[46] have been shown to concentrate amino acids. Leucine is very rapidly metabolized in the brain (this paper and refs 30 and 31), and if a significant proportion of this amino acid is transported into the brain, its metabolism there may intervene with its further transport. The studies of Lajtha and Toth[41] did not account for this leucine "sink" effect. Thus, the inhibition of cerebral protein synthesis described herein might account for the elevated levels of this amino acid by interfering with its metabolism.

The elevated cerebral glycine levels following 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.[47] Glycine is apparently actively transported into nerve cells from the synaptic cleft (a process thought to be involved in the termination of its action at this site).[47] Since alanine transport is probably mediated by the same system,[43] the low cerebral alanine concentrations following Schedule II drug treatments probably reflect its reciprocal inhibition by glycine.

It is doubtful that functional significance can be attributed to the elevated glycine levels. Although there is strong evidence implicating glycine as an inhibitory transmitter in the spinal cord[48] and medulla,[49] a similar role in more rostral parts of the brain seems unlikely. Furthermore, cerebral glycine levels apparently do not change following therapeutically more reasonable doses of either drug (Schedules I and III, Table 6).

Van Gelder and co-workers[50,51] have correlated regions of epileptogenicity in the cortex with substantial increases in glycine concentration. Observations that seizure activity is apparently associated with an inhibition of protein synthesis in the affected tissue[52,53] have led these investigators to suggest that the accumulation of glycine in these tissues is due to a diminished re-utilization of this amino acid under these circumstances. The apparent refractoriness of glycine to metabolic degradation at the synaptic cleft[47] 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 diphenylhydantoin can elicit convulsive behavior in the rat; and the Schedule II dosage is similar to that to which clonic seizure activity has developed.[11] Therefore, the increase in cerebral glycine levels following 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 minute 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 minute incorporation period, 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 relative long half-life. The inhibition of this protein following large doses of either drug could result in the accumulation of glycine for the reason noted above. These observations might be relevant to the search for a "seizure defense protein."[54] In contrast, the anticonvulsant properties at lower doses might be related to the specific inhibition of a more rapidly metabolized protein (see below).

Another indirect mechanism of inhibition could involve a drug-induced decrease in available energy. Barbiturates are known to inhibit

the respiratory chain in mitochondria, possibly by disrupting the struc-
ture of FAD (see below).[55] Gaitonde and Richter[5] have demonstrated an
inhibition by pentobarbital of the in vivo incorporation of L-[^{35}S] methio-
nine into rat brain proteins; they suggested that the inhibition may be
related to the lower oxygen consumption of the brain under such condi-
tions. However, Shuster and Hannan[13] have correlated the pentobarbital
inhibition with the hypothermic response of rodents to this drug. Fur-
thermore, investigation has failed to detect an effect of either diphenyl-
hydantoin or phenobarbital on tissue respiration or glycolysis in incu-
bated brain slices.[56] Thus, a more relevant concern might be the
possibility of a drug-induced hypothermia. This is especially true for
the sedative and hypnotic agents, including phenobarbital.

Diphenylhydantoin and phenobarbital might conceivably alter
cerebral protein synthesis in a more direct way. Recent work has shown
that certain hydantoins[57] and barbiturates[58] form highly specific,
hydrogen-bonded dimers with adenine, or with the adenine moiety of
FAD and NAD.[55] The affinities of diphenylhydantoin and phenobarbital
for adenine are both greater than those of uracil and thymine for adenine.[59]
Thus, a preferential association of these drugs with the recently dis-
covered poly A-rich segments of a particular messenger RNA might
effectively interfere with the transcription of protein.

Direct evidence for such a phenomena, however, is lacking. Kemp
and Woodbury[60] have demonstrated that the microsomal fraction is the
major binding site for diphenylhydantoin in rat cerebral cortex.

Accumulation in this fraction was noted for periods up to 12 hours following a single intracisternal injection; and 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 following diphenylhydantoin treatment in the rat. Thus, diphenylhydantoin accumulates and appears to inhibit protein synthesis in those subcellular structures with which the majority of cellular RNA is normally associated.

The hydrogen-bonding affinity of phenobarbital[58] for adenine is substantially greater than that of diphenylhydantoin[57] (with adenine). Therefore, if the inhibition of protein synthesis by these drugs is a consequence of their hydrogen-bonding affinity for adenine in RNA, phenobarbital should presumably be the more potent inhibitor. This was not verified by the present results, since approximately equal effects were achieved only when phenobarbital plasma concentrations were three-fold greater than the diphenylhydantoin levels (Schedules III, Tables 1 and 4); but conclusions based upon these observations are complicated without a more complete knowledge of the dose-response curves. Furthermore, the actual concentration of phenobarbital at the site of action may, according to the principles outlined above for its penetration into the brain, be substantially lower than that of diphenyl-hydantoin. It is probable that the participation of such mechanisms in

the inhibition of protein synthesis would be more appropriately evaluated in a cell-free system.

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. Although the average half-life of the proteins of rat brain is about 14 days, there is considerable variability in this parameter; estimates of the half-lives of individual components have ranged from seconds to several months.[5,6] Since the present results are based upon observations of total acid-precipitable protein, information relating to specific effects on individual proteins is obviously lacking.

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. Especially interesting in this context are certain "brain specific" proteins with no known enzymatic activity, but for which evidence implicating their role in synaptic function is rapidly accruing. These are the soluble, acidic proteins whose abundance, high turnover rates and unusual physical properties have provoked much investigation.[1,61]

The physiological function of these acidic proteins remains uncertain. However, they appear to serve as integral components of transmitter release. They are known to occur in the nerve endings in association with the various transmitter substances in storage granules. Upon stimulation they are released together with the transmitter into the

synaptic cleft. Increased frequency of stimulation is presumably associated with an enhanced release and turnover of these soluble proteins.[1] Thus, an inhibition of protein turnover could conceivably interfere with transmitter release.

High concentrations of rapidly metabolized soluble protein have been detected in areas of the cerebellum known to contain substantial quantities of acetylcholine.[61] The reported ability of diphenylhydantoin and phenobarbital to inhibit the synthesis of free acetylcholine in brain slices[62] might be related to an inhibition of a soluble protein associated with its release. 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 diphenylhydantoin for protection against maximal electroshock is about 15 minutes in rats.[33] 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.[63] 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 minutes of somal inhibition.[64] An effect on local (synaptic) synthesis, therefore, may be more consistent with the latency of the anticonvulsant effect.

Although axons and nerve endings do not contain visible ribosomes, their capacity to synthesize protein has been demonstrated repeatedly.[65]

Austin and Morgan[66] have described a substantial non-mitochondrial

protein synthesis in synaptosomes; at least 75% of the total precursor

amino acid was found in soluble and non-mitochindrial membrane-

bound proteins. Since the prolonged inhibition by diphenylhydantoin

of protein synthesis in the synaptosomal fraction appears to correlate

with the duration of its anticonvulsant effect in rats,[10] further study

of the drug's actions on synaptic protein metabolism appears warranted.

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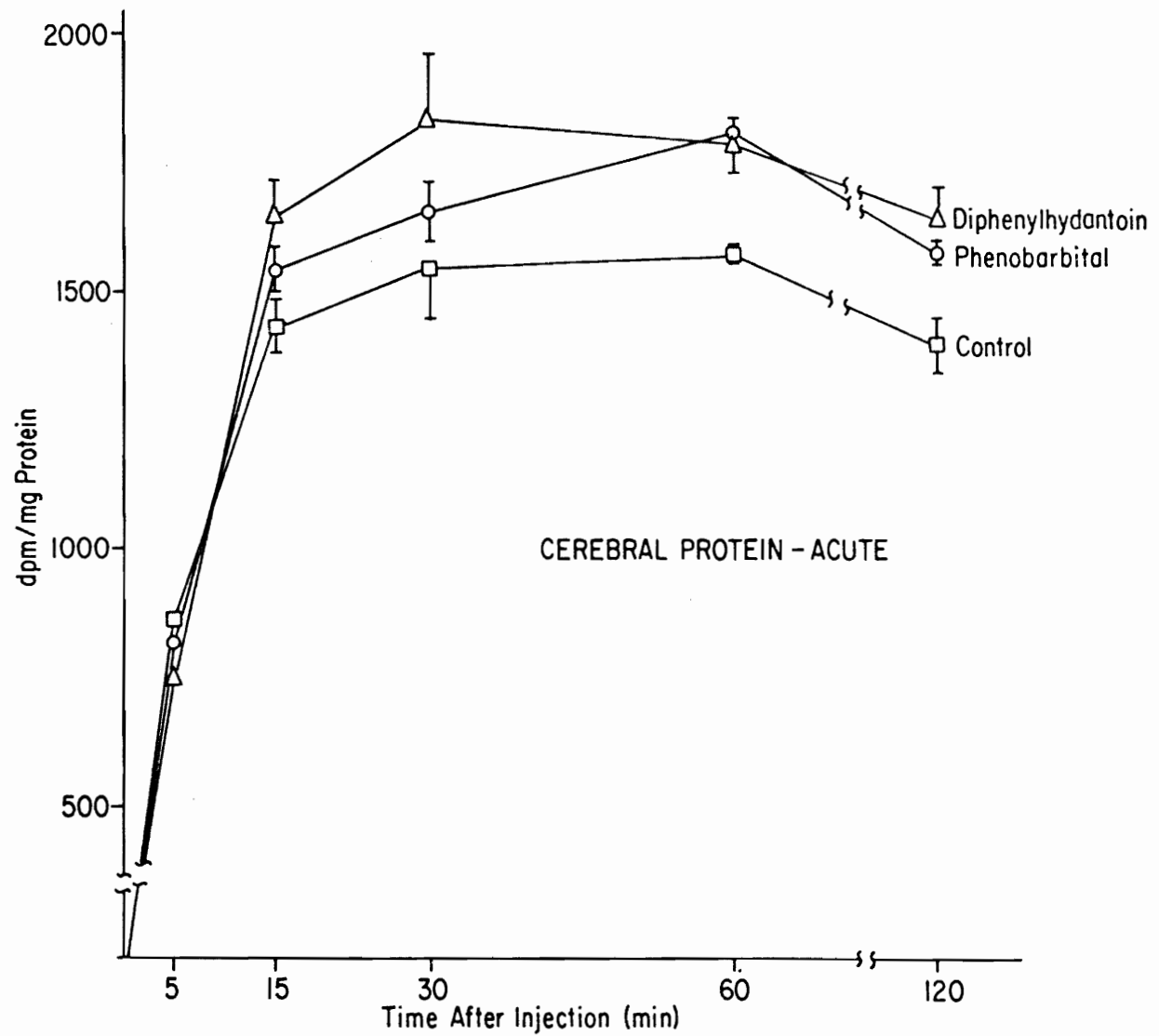66. L. AUSTIN and I.G. MORGAN, J. Neurochem. 14, 377 (1967).

Fig. 1. The acute (Schedule I) effects of diphenylhydantoin and pheno-
barbital on the specific activity (dpm/mg protein) of the trichloroacetic
acid-precipitable fraction of rat cerebral cortex measured at various
times following 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.
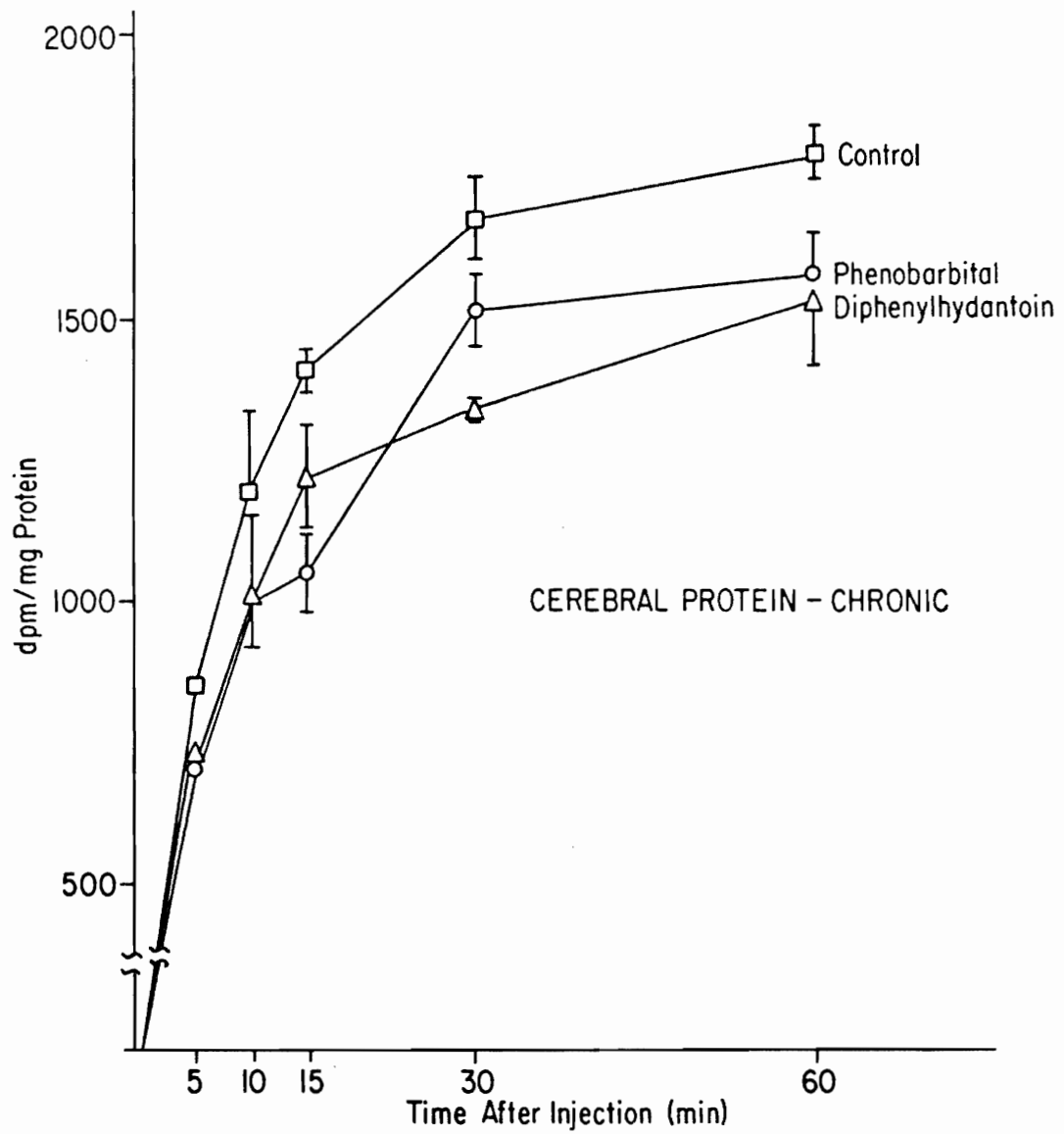The vertical bars represent the standard errors of the mean.

Fig. 2. The chronic (Schedule II) effects of diphenylhydantoin and phenobarbital on the specific activity (dpm/mg protein) of the trichloro-acetic acid-precipitable fraction of rat cerebral cortex measured at various times following 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.
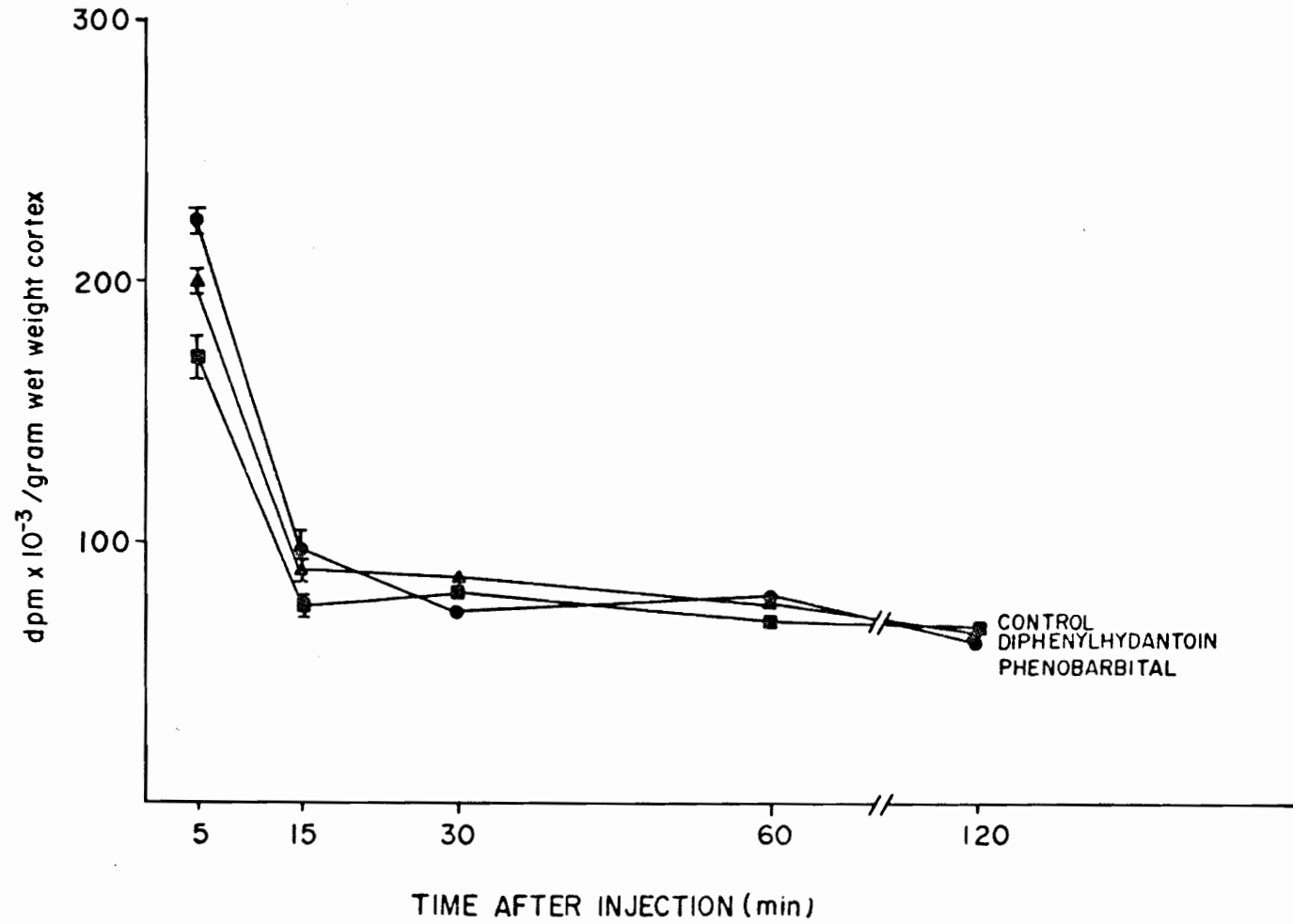
CEREBRAL PROTEIN — CHRONIC

Fig. 3. The acute (Schedule I) effects of diphenylhydantoin and pheno-

barbital on the radioactivity contained in the trichloroacetic acid-

soluble fraction (dpm/g wet wt.) of rat cerebral cortex measured at

various times following L-[4,5-^{3}H] leucine injection. Each point repre-

sents the mean of three to six determinations on separate animals. The

vertical bars represent the standard errors of the mean.

CORTEX TCA SOLUBLE – Acute High Dose

Fig. 4. The chronic (Schedule II) effects of diphenylhydantoin and phenobarbital on the radioactivity contained in the trichloroacetic acid-soluble fraction (dpm/g wet wt.) of rat cerebral cortex measured at various times following 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.

CORTEX TCA SOLUBLE — Chronic High Dose
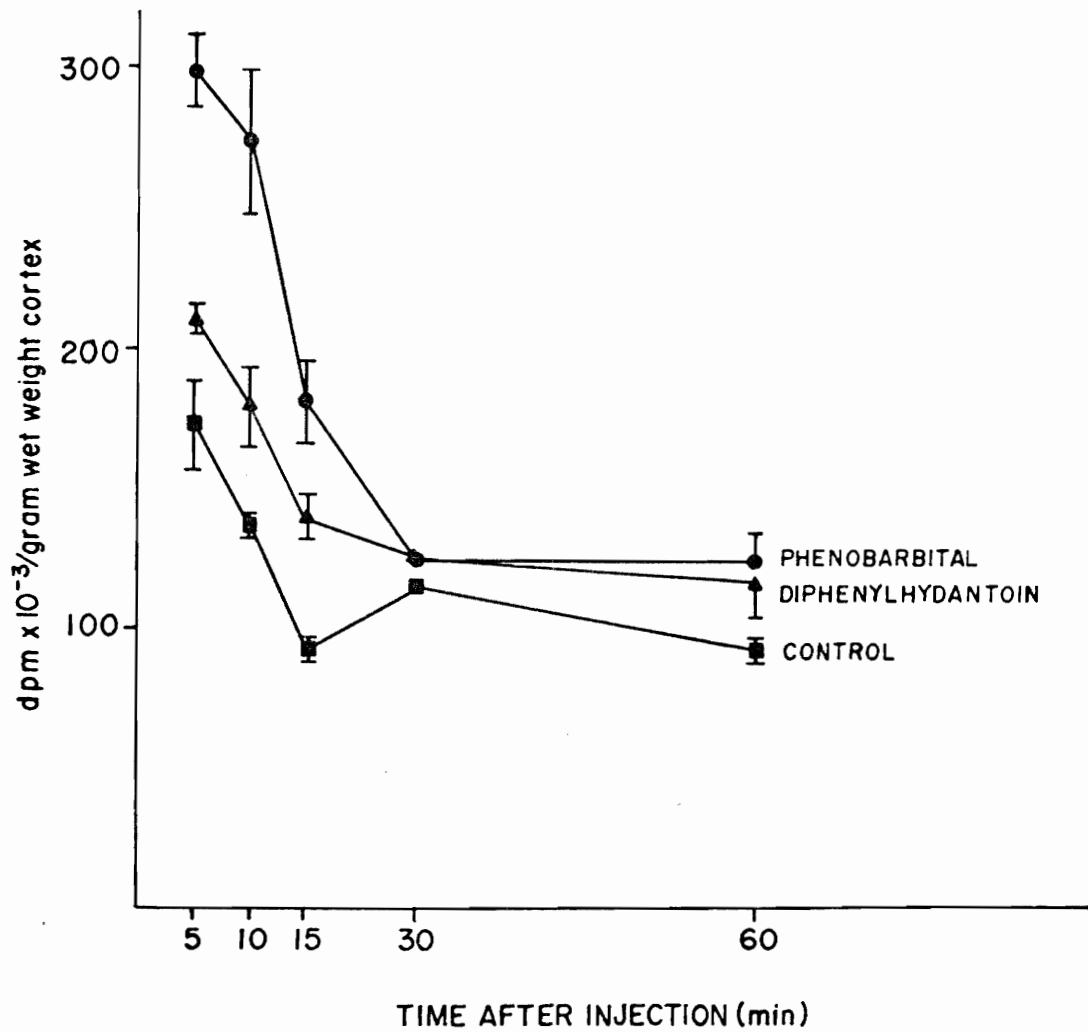
Table 1. Plasma Drug Levels Following Specified Treatment Schedules

Schedule[†]	Diphenylhydantoin (µg/ml)				Phenobarbital (µg/ml)			
	Hours Following Final Injection							
	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*	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.[20] Each value represents the mean $\pm$ S.E.M. of three to four determinations on separate animals, except where a single determination is noted (*).

†Schedule I: Single 100 mg/kg intraperitoneal injection; Schedule II: Three 100 mg/kg intraperitoneal injections at 12 hour intervals; Schedule III: Three intraperitoneal injections at 12 hour intervals; 100, 50 and 50 mg/kg (diphenyl-hydantoin) and 50, 25 and 25 mg/kg (phenobarbital).

Table 2. Effects of Diphenylhydantoin and Phenobarbital on

Free L-Leucine Levels in Rat Plasma and Cerebral Cortex

Schedule	A. Plasma Leucine (μ moles/ml)			B. Cerebral Leucine (μ moles/g)			B/A*		
	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 one hour following a single drug injection (Schedule I) or approximately two hours following 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 3-7 extracts involving 4-5 animals per extract.

*Cerebral leucine/plasma leucine.

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

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

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

Table 3. Effects of Diphenylhydantoin and Phenobarbital on L-Leucine
Specific Activity in Rat Plasma and Cerebral Cortex Precursor Pools

Schedule	Control		Diphenylhydantoin		Phenobarbital	
	Incorporation Period (min)					
	5(7.5)*	15	5(7.5)*	15	5(7.5)*	15
Plasma Specific Activity (dpm/μ mole leucine)						
I	211617	51886	265733	70642	144989	41314
II	187100	54259	215780	64734	269413	80824
	210129	65140	241845	70135	278707	83612
Cortex Specific Activity (dpm/μ mole leucine)						
I	158541	41129	241980	67022	204240	52397
	203678	51428	298364	91124	233069	75805
	225534	65163		101219	254844	79443
		69542				
II	173881	45209	243211	78461	319844	102246
	194794	60386	261537	85124	349571	122350
III	119219	60746	140794	85238	151446	84892
	125413	63911	154151	89789	168321	88878

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 minutes.

Table 4. Effects of Diphenylhydantoin and Phenobarbital on Incorporation
In Vivo of L-[4,5-^{3}H] Leucine into Rat Cerebral Protein

Schedule	Control		Diphenylhydantoin		Phenobarbital	
	Incorporation Period (min)					
	5(7.5)	15	5(7.5)	15	5(7.5)	15
	Specific Activity, Uncorrected (dpm/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 (dpm/mg protein)					
I	451 ± 32	2553 ± 100	278 ± 13† (38.4)	1927 ± 85† (24.5)	364 ± 19*‡ (19.3)	2254 ± 70*‡ (11.7)
II	469 ± 39	2657 ± 56	278 ± 35† (40.7)	1532 ± 119† (42.3)	204 ± 18† (56.5)	972 ± 44†§ (63.4)
III	3224 ± 209	6374 ± 238	1915 ± 118† (40.6)	3984 ± 219† (37.5)	1863 ± 74† (42.2)	4077 ± 165† (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 experiments were 7.5 and 15 minutes. 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 percent depression in that respective value relative to its control.

*P < 0.05 for comparison of treatment value with control value.
†P < 0.01 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 5. Effects of Diphenylhydantoin and Phenobarbital on
L-[4,5-^{3}H] Leucine Metabolism in Rat Cerebral Cortex

Schedule	Control		Diphenylhydantoin		Phenobarbital	
	Incorporation Period (min)					
	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 percent 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 minutes. Raw data are
presented for one to three determinations per experimental group.

Table 6. Effects of Diphenylhydantoin and Phenobarbital on Free Amino Acid

Concentrations in Rat Plasma and Cerebral Cortex

Schedule	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 3-7 extracts involving 4-5 animals per extract.

*P < 0.05 for comparison of treatment value with control value.

+P < 0.01 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 7. Effects of Diphenylhydantoin and Phenobarbital on
Amino Acid Uptake*Into Rat Cerebral Cortex

Schedule	Amino Acid	Cerebral amino acid (μ moles/g)/Plasma amino acid (μ moles/ml)		
		Control	Diphenylhydantoin	Phenobarbital
I	Glycine	2.22	2.39	2.54
	Alanine	2.24	2.15	2.16
	Valine	1.24	1.28	1.58
	Methionine	1.06	1.06	1.16
	Isoleucine	1.00	1.12	1.29
	Tyrosine	1.59	1.38	1.80
	Phenylalanine	1.27	1.20	1.77
II	Glycine	2.33	3.11	3.00
	Alanine	1.84	3.14	1.54
	Valine	1.41	1.08	1.74
	Methionine	1.14	0.88	1.48
	Isoleucine	1.07	0.82	1.35
	Tyrosine	1.42	1.29	1.85
	Phenylalanine	1.31	1.11	1.60

The ratios (cerebral amino acid/plasma amino acid) are constructed from the data in Table 6.

*These ratios may not necessarily reflect the extent of transport, or uptake of the respective amino acid into the brain (see Discussion).

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