THE PHARMACOLOGY OF PHENYTOIN IN THE FROG: SEIZURE MODIFICATION, DRUG DISPOSITION, AND EFFECTS ON CEREBELLAR PURKINJE CELLS AND CYCLIC NUCLEOTIDES IN THE CENTRAL NERVOUS SYSTEM

by

Steven W. Johnson

A THESIS

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APPROVED:

[redacted]

William K. Riker, M.D.
Professor in Charge of Thesis

[redacted]

Jules V. Hallum, Ph.D.
Chairman, Graduate Council

DEDICATION

To my mother, Ruth B. Johnson, and my father, William L. Johnson.

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GENERAL INTRODUCTION TO THE THESIS

This work was undertaken with the specific aim of evaluating two hypotheses concerning the mechanism(s) by which phenytoin exerts its anticonvulsant effect. The first hypothesis tested was based upon the work of Julien and Halpern (1971), who found that the firing rates of cerebellar Purkinje cells were significantly elevated in cats following phenytoin administration. Because Purkinje cells exert inhibitory influences on neurons concerned with motor function (Ito and Yoshida, 1964; Ito, Yoshida, and Obata, 1964; Tsukahara, Toyama, and Kosaka, 1964), it was suggested by Julien and Halpern (1971) that increased Purkinje cell activity might be a mechanism by which phenytoin produces its anticonvulsant action (Halpern and Julien, 1972). Julien and Halpern's results have been confirmed by some investigators (Shimizu, Manaka, Hori, and Sano, 1977; Fernandez-Guardiola, Calvo, and Condes-Lara, 1979) but refuted by others (Puro and Woodward, 1973; Pieri and Haefely, 1976; Latham and Paul, 1976 and 1980). The conflicting results could be attributable to differences between experimental animals, anesthetic paradigms, recording procedures, phenytoin doses and routes of administration, or times during which the activity of Purkinje cells was monitored.

The frog (Rana pipien) was selected as the experimental animal in which Julien and Halpern's (1971) hypothesis was tested, because preliminary studies in this species with anticonvulsant drug modification of pentylenetetrazole (Johnson and Riker, 1977) and electrically-induced

seizures (Johnson and Riker, 1980) produced results qualitatively similar to those obtained in mammals. Also, the frog possesses a relatively small cerebellum, which is histologically simpler than the mammalian counterpart yet complete with respect to the major cerebellar cell types (Larsell, 1923; Sotelo, 1976).

Besides our use of the frog, the approach employed in testing this hypothesis also differed from the studies cited above in that the first step was to establish complete time-effect and dose-effect relationships for the anticonvulsant action of phenytoin in the intact animal. Only then were the effects of phenytoin on Purkinje cell activity studied, using those doses and times after injection which were associated with anticonvulsant efficacy. Because the classic anticonvulsant effect of phenytoin is to modify the pattern of electrically-induced maximal seizures (Toman, Swinyard, and Goodman, 1946), this experimental model of epilepsy, initiated by a standard method of electroshock delivered via corneal electrodes (Woodbury and Davenport, 1952), was used as the seizure challenge in this and subsequent portions of this thesis.

The second hypothesis tested in this thesis was based upon the finding that phenytoin, in modifying the motor manifestations of electrically-induced maximal seizures, also antagonizes the seizure-associated increase in central nervous system (CNS) levels of adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) (Lust, Kupferberg, Yonekawa, Penry, Passonneau, and Wheaton, 1978; McCandless, Feussner, Lust, and Passonneau, 1979). Cyclic GMP has been shown to increase the excitability of central neurons (Stone, Taylor,

and Bloom, 1975; Hoffer, Seiger, Freedman, Olson, and Taylor, 1977; Freedman, Taylor, Seiger, Olson, and Hoffer, 1979), and elevated CNS levels of cGMP have also been shown to occur prior to the onset of impending seizures (Berti, Bernareggi, Folco, Fumagalli, and Paoletti, 1976; Ferrendelli and Kinscherf, 1977a) or of paroxysmal EEG activity (Ferrendelli, Blank, and Gross, 1980). Because of these observations, it was suggested that phenytoin may raise seizure threshold by preventing the elevation of cyclic nucleotide levels in the CNS (Ferrendelli and Kinscherf, 1977b; Ferrendelli, 1980). If this hypothesis is accepted, then one would expect a good degree of correlation between the dose-effect relationships for phenytoin anticonvulsant action and phenytoin reduction of seizure-associated levels of cGMP. However, the literature relating to this hypothesis lacks systematic dose-effect analysis. Many of the cited investigations have employed only a single dose level of phenytoin (25 mg/kg, i.p.) in mice to achieve a reduction in electroshock-induced elevation of cyclic nucleotide levels (Kupferberg, Lust, Yonekawa, Passonneau, and Penry, 1976; Lust, Kupferberg, Yonekawa, Penry, Passonneau, and Wheaton, 1978; McCandless, Feussner, Lust, and Passonneau, 1979), but in the present thesis work, this was found to be at least 2-3 times that needed to modify the pattern of electrically-induced maximal seizures in mice.

To test the hypothesis that phenytoin exerts its anticonvulsant action by modifying CNS cyclic nucleotide levels, anticonvulsant dose-effect relationships for phenytoin were first established in two species, frogs and mice (CF #1 strain). Subsequently, whole brain

levels of cAMP and cGMP were determined in both species before and at various times after electroshock. The final step was dose-effect analysis of phenytoin action on cyclic nucleotide levels in specific CNS tissues after electroshock. In addition to the foregoing procedures in frogs and mice subjected to electroshock, it was deemed important to compare electroshocked animals with those having spontaneous seizures. Therefore, the effects of phenytoin on CNS levels of cyclic nucleotides were also studied in quaking mice (qk/qk), a mutant strain with deficient myelinization and spontaneously occurring seizures (Sidman, Dickie, and Appel, 1964).

It is clear from the preceding paragraphs that systematic dose-effect analysis was a critically important part of evaluating the two hypotheses stated earlier. In addition, other aspects of phenytoin pharmacodynamics in the frog had to be characterized, such as the time course of action and the margin of safety between anticonvulsant and toxic doses. Also, since little is known about the disposition of phenytoin in frogs, it was necessary to define the pharmacokinetics and major metabolites of phenytoin, especially because it had previously been claimed that frogs were unable to metabolize lipid-soluble drugs (Brodie and Maickel, 1962). Therefore, a large portion of this thesis is devoted to the presentation of new information about the pharmacodynamics and the disposition of phenytoin in the frog.

This thesis is divided into four manuscripts. Manuscript I contains the results of the pharmacodynamic studies of phenytoin in the intact frog, and includes comparative studies with the CF #1 strain of mice.

Manuscript II describes the pharmacokinetics, tissue distribution, and metabolism of phenytoin in the frog. Manuscript III contains the results of testing the hypothesis that the anticonvulsant mechanism of phenytoin may involve regulation of cAMP or cGMP levels in the CNS, and Manuscript IV evaluates the hypothesis that phenytoin-induced augmentation of Purkinje cell activity is a mechanism of anticonvulsant action. Briefly, the results show that phenytoin is an effective and relatively selective anticonvulsant agent in the frog, with effects qualitatively similar to those of phenytoin in CF #1 mice. Elimination of phenytoin from frog lymph followed concentration-dependent kinetics similar to that found in rodents and higher mammals, including man, and the tissue distribution and major metabolites were also similar to those previously reported in many other animals. However, phenytoin did not influence CNS levels of cyclic nucleotides at doses effective in modifying maximal seizures in frogs, CF #1 mice, or quaking mice, nor did anticonvulsant doses of phenytoin influence spontaneous activity of Purkinje cells in frogs. Thus, the two hypotheses that were tested are not tenable as mechanisms for the anticonvulsant action of phenytoin in the frog.

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MANUSCRIPT I

A COMPARATIVE STUDY OF THE EFFECTS OF PHENYTOIN AND PHENOBARBITAL ON ELECTRICALLY-INDUCED MAXIMAL SEIZURES IN FROGS AND MICE

ABSTRACT

This study was undertaken to compare seizure patterns and anticonvulsant effects of phenytoin in frogs (Rana pipiens) and the CF #1 strain of mice. Maximal seizures were induced with electroshock via corneal electrodes, and phenytoin anticonvulsant effects were determined by measuring the duration of tonic hindlimb extension (THE) and prevention of THE. To control for any possible species difference that might be unique to phenytoin, the anticonvulsant effects of phenobarbital also were compared in frogs and mice. It was found that the dose-effect relationships for shortening THE duration and for prevention of THE by phenobarbital in frogs and mice were not significantly different in terms of slopes and potencies. With phenytoin, however, the dose-effect relationship for prevention of THE in mice was significantly steeper than that in frogs, whereas the slopes of the dose-effect relationships for shortening THE duration were not significantly different in these two species. Frogs decapitated 1.5 sec after electroshock exhibited THE durations equal to those in intact frogs, and phenytoin and phenobarbital efficacies in shortening THE duration were unchanged by decapitation. These results show that shortening of THE duration by both phenytoin and phenobarbital may reflect drug action in spinal or peripheral neural pathways. Also, it is suggested that phenytoin, but not phenobarbital, prevents THE by a selective action on cortical structures which are well developed in mice but are poorly developed or absent in frogs.

INTRODUCTION

Preliminary experience with pentylenetetrazole (Johnson and Riker, 1977) and electrically-induced maximal seizures (Johnson and Riker, 1980) in frogs suggested this animal was a suitable test object for the study of anticonvulsant drugs. In particular, we wished to evaluate current hypotheses that implicate the cerebellum and cyclic nucleotides in the mechanism of anticonvulsant action of phenytoin. A necessary prerequisite, therefore, was to establish dose-time-effect relationships for the anticonvulsant actions of phenytoin in the frog. During pursuit of this primary goal, several differences were discovered between the maximal seizure pattern in frogs and those reported in the literature for mammals. Consequently, we began a comparative study of frogs and the CF #1 strain of mice, the latter a well-established species in epilepsy research (Anticonvulsant Screening Program, 1976), and investigated the effects of phenytoin as well as phenobarbital on maximal seizures induced by electroshock through corneal electrodes. Because tonic hindlimb extension (THE) is the definitive criterion for the occurrence of an electrically-induced maximal seizure (Toman, Swinyard, and Goodman, 1946; Swinyard, 1949), the anticonvulsant effects of phenytoin and phenobarbital were assayed using prevention of THE as the endpoint. In addition to the use of this classical endpoint, a less widely used endpoint, duration of THE, was also employed.

The results of the present study suggest that electrically-induced maximal seizures may originate predominantly or exclusively, at a spinal cord level in frogs and that the duration of THE is regulated solely by

spinal cord pathways in frogs and perhaps also in mice. Phenytoin and phenobarbital were each capable, in a dose-related fashion, of preventing the occurrence of THE and of shortening the duration of THE when it occurred in frogs and mice. The decreased THE duration in both species is likely effected by drug action on spinal cord pathways. However, phenytoin, but not phenobarbital, prevents the occurrence of THE more selectively in mice than in frogs. Since frogs lack higher brain centers such as neocortex, the data thus suggest both supraspinal and spinal sites of phenytoin action in mice.

METHODS

ANIMALS AND DRUGS

Rana pipiens (northern variety) of both sexes weighing 10-100 gm were obtained from William Lemberger Assoc. (Germantown, Wisc.) and housed in plastic containers tipped at one end to allow for dry and wet areas with continuously flowing tap water. Frogs were kept at room temperature (23° C) in a room with artificial lighting and were force-fed raw hamburger every 2-3 weeks until sacrificed. During an experiment, frogs were placed in individual plastic boxes with enough tap water to keep the frog moist at all times. CF #1 mice of both sexes, weighing 20-40 gm, were obtained from Charles River (Wilmington, Mass.) and housed in wire cages in a room supplied with light 12 hrs/day. Mice were fed mouse chow obtained from Oregon State University Mills (Corvallis, Oreg.) and water *ad libidum*. Sodium phenytoin (Parke, Davis and Co.) and phenobarbital sodium (Mallinckrodt) were dissolved in 50 mM NaOH (pH 12.6) and 0.9% NaCl, respectively. Drugs or drug solvents were injected into the ventral lymph sac (v.l.s.) in frogs and subcutaneously in mice, with the volume of material injected being less than 0.5% of body weight whenever possible. Anticonvulsant effects were measured 3 hrs after phenytoin and 2 hrs after phenobarbital in both species, these being the times of peak effect in mice (Swinyard, Brown, and Goodman, 1952) as well as in frogs (*vide infra*) following the respective routes of drug administration. Control animals, injected with drug solvents, were also tested at these times.

ELECTRICALLY-INDUCED MAXIMAL SEIZURES

Maximal seizures, those in which tonic hindlimb extension (THE) occurs, were elicited by the electroshock technique of Woodbury and Davenport (1952). Corneal electrodes, coated with Redux Creme^{R} (Hewlett Packard) to facilitate contact with corneae, were used to deliver the electrical stimulus generated by a constant current (60 Hz) electroshock device (Wahlquist Instrument Co., Salt Lake City, Utah). Preliminary experiments showed that frogs required a relatively long stimulus duration (0.5 sec) in order to induce maximal seizures with reasonably low amperages (60-90 mA). In mice, however, seizure thresholds were not significantly different, with stimulus duration of 0.2 vs. 0.5 sec, although some deaths were seen with the longer stimulus duration. Therefore, all subsequent experiments were performed using stimulus durations of 0.2 sec in mice and 0.5 sec in frogs. No deaths occurred in either species which could be attributed to electrically-induced seizures using these stimulus durations.

ENDPOINTS FOR MEASURING ANTICONVULSANT EFFECTS

Anticonvulsant effects of phenytoin and phenobarbital were measured with two endpoints: a quantal endpoint, prevention of THE, and a graded endpoint, duration of THE, and an extension was not considered tonic in nature (THE) unless it was sustained for at least one second. The quantal endpoint was the same as that used by Swinyard *et al.* (Swinyard, Brown, and Goodman, 1952) except that we selected an electroshock amperage intended to produce THE in 95% of animals (convulsant amperage 95%, CA 95), while Swinyard *et al.* used amperages which were 5-6 times

that needed to produce maximal seizures. This alteration was made because it was thought that a better comparison of the effects of phenytoin and phenobarbital in frogs vs. mice could be made if amperages were equally efficacious in producing maximal seizures in each species. Preliminary studies showed approximate CA 95's to be 98 mA in frogs and 24 mA in mice, and these amperages were subsequently used in experiments which utilized the quantal endpoint (presence or absence of THE). At the completion of the experiments reported in this communication, we found 98 mA in frogs was closer to a CA 87 (40/46), while 24 mA in mice was closer to a CA 100 (28/28). Because individuals of some species are incapable of THE (Toman, Swinyard, and Goodman, 1946), frogs which did not show THE with 98 mA were electroshocked with 200 mA 1.5 hrs later, and all showed THE. Therefore, it is likely that all members of the species R. pipiens, as well as the CF #1 mice, are capable of exhibiting maximal seizures.

In experiments that utilized the graded endpoint, THE duration was timed with mice in a supine position, while frogs were timed while supported in a vertical position. The vertical position was chosen for frogs because cessation of tonic extension was more obvious in this position. Comparisons of vertical vs. supine positions, in frogs, showed no significant difference in the duration of THE. High amperages (300-425 mA) were frequently used to elicit THE in animals treated with phenytoin or phenobarbital because these animals were protected from THE when challenged with lower amperages. Preliminary studies† showed that THE, once established, was sustained for a constant duration regardless of

the current intensity used to elicit THE, which agrees with the results of studies using rabbits and rats (Toman, Swinyard, and Goodman, 1946).

DECAPITATION OF ANIMALS

In some experiments, animals were decapitated with a guillotine (Harvard Apparatus, Inc.) 1.5 sec after the onset of THE, and the total duration of THE was timed. Duration of THE was markedly reduced in decapitated mice, but frogs subjected to decapitation had THE durations which were not significantly different from those in intact frogs. We were thus able to perform experiments which measured the effect of phenytoin and phenobarbital on THE duration in decapitated vs. intact frogs.

SUPPRESSION OF POST-ROTATORY HEAD TURNING

The most sensitive measure of phenytoin-induced toxicity in frogs was found to be suppression of post-rotatory head turning (PRHT). PRHT, which is assumed to be a vestibular-dependent phenomenon, was induced by placing a frog in a plastic box on the seat of a swivel chair which was rotated clockwise at 1 Hz for 10 seconds. After this time, the rotation was suddenly stopped, and the behavior of the frog was noted. It was found that control frogs reproducibly turned their heads in the direction in which they had been rotated (clockwise) and occasionally displayed 2-3 counter clockwise saccades of the head. The dose-response relationship for the suppression of PRHT by phenytoin was determined by measurements 3 hrs after injection. Finally, the lethal dose-response relationship for phenytoin was established by monitoring the incidence of mortality in the 36-hour period following injection.

STATISTICAL ANALYSES OF DATA

Statistical analyses of dose-response curves were performed according to the method of Litchfield and Wilcoxon (1949), and differences were accepted as significant when 95% probability limits were exceeded. Student's t-test was used to evaluate the significance of differences between characteristics of maximal seizures in frogs and mice, and the "Up and Down" method (Dixon and Mood, 1948) was used to establish the time-effect relationship for the anticonvulsant effects of phenytoin and phenobarbital in frogs.

†Effects of current intensity on THE duration are reported in Appendix III.

RESULTS

PATTERNS OF ELECTRICALLY-INDUCED MAXIMAL SEIZURES

In CF #1 mice, the established species for anticonvulsant drug screening (Anticonvulsant Screening Project, 1976), the well-known (Toman, Swinyard, and Goodman, 1946; Woodbury and Davenport, 1952) pattern of the maximal seizure following corneal electroshock was confirmed. A latency period of 1-2 seconds after electroshock was followed by tonic flexion, lasting 2-4 seconds, and then, abruptly, tonic hindlimb extension (THE) began, lasting 10.3 ± 2.5 seconds (± S.D., n=16). Termination of THE was immediately followed by a brief (about 3 seconds) episode of clonus, the terminal motor event in the seizure. There ensued a period of post-ictal inactivity, and not until 130 ± 39 seconds (n=16) after electroshock did mice resume normal exploratory behavior. This classical pattern of the maximal seizure has also been described in many common laboratory mammals other than mice (Toman, Swinyard, and Goodman, 1946).

The maximal seizure pattern in frogs differed from that in mice in several important respects: (1) There was no latency between electroshock and seizure onset; (2) The initial seizure event was almost always THE, frequently accompanied by a shrill cry; (3) Tonic flexion was seen only occasionally, and then as hindlimb abduction; (4) The duration of THE (8.0 ± 1.7 seconds, n=31) was significantly ($p < 0.01$) shorter than that in mice; and (5) There was never a true terminal clonus, but occasionally some frogs displayed a few repetitive extensor thrusts as the terminal motor event. As with mice there was

post-ictal inactivity, and its duration (156 ± 59 seconds, n=12), measured as the time between electroshock and the resumption, by the frog, of a normal sitting posture, was not significantly ($p > 0.1$) different from that in mice.

Since the occurrence of THE is *sine qua non*, the definition of a maximal seizure, it is important to note that frogs and mice required different electroshock parameters to produce THE. Fig. 1 shows the incidence of THE as a function of current (mA) intensity in frogs and mice. Although slopes of these two dose-response curves are not significantly different, the current-response relationship in mice is significantly ($p < 0.05$) shifted to the left of that in frogs. Considering that stimulus durations were 0.2 seconds in mice and 0.5 seconds in frogs, it is evident that the spread between THE-inducing current intensities in these two species is even greater than depicted in Fig. 1.

PREVENTION OF TONIC HINDLIMB EXTENSION BY PHENYTOIN OR PHENOBARBITAL

The times of peak anticonvulsant action of phenytoin (3 hours) or phenobarbital (2 hours) following subcutaneous injection in mice have been established by Swinyard *et al.* (Swinyard, Brown, and Goodman, 1952). However, no comparable data existed for frogs given these drugs by injection into the ventral lymph sac. Consequently, the time-action curves were determined by measuring the drug-induced elevation of electroshock amperage required to produce THE in 50% of the frogs tested, i.e., the convulsant amperage 50% (CA 50). This was accomplished most efficiently by measuring the CA 50 in groups

of about 15 frogs, before and at various times after drug administration, by the "Up and Down" method of Dixon and Mood (1948), as modified by McCawley and Wayson (1974).†

In control frogs, the CA 50 did not vary significantly when they were electroshocked at frequent time intervals between 1 and 24 hours after injection of 50 mM NaOH. Peak elevation of the CA 50 in frogs given phenytoin, 20-40 mg/kg (12-16 frogs per dose), occurred between 2-4 hours, with a decay "half-time", from peak, of 10.5 hours. Thus, all subsequent testing of phenytoin effects on seizure parameters in frogs was done 3 hours after drug injection into the lymphatic circulation. By the same means, it was found that the peak time of phenobarbital anticonvulsant action in frogs was at 2 hours after injection into the ventral lymph sac.

The prevention of THE as a function of phenytoin dose in frogs and mice is illustrated in Fig. 2. It can be seen that the curve for prevention of THE by phenytoin in mice is significantly steeper in slope ($p < 0.05$) and shifted to the left of that obtained in frogs. In contrast, Fig. 3 reveals that the anticonvulsant action of phenobarbital, i.e., prevention of THE, is remarkably similar in frogs and mice. The slopes of the dose-response curves are not significantly different in frogs and mice, and phenobarbital is equipotent in these species (Fig. 3).

REDUCTION OF THE DURATION OF TONIC HINDLIMB EXTENSION BY PHENYTOIN AND PHENOBARBITAL

Although prevention of THE is the commonly used endpoint for measuring an anticonvulsant effect during a maximal seizure, it is

also known that anticonvulsant drugs can shorten the duration of THE (Bárány and Stein-Jensen, 1946; Toman, Loewe, and Goodman, 1946; Goodman, Toman, and Swinyard, 1948; Esplin and Freston, 1960). Therefore, the effects of phenytoin and phenobarbital on THE duration were also evaluated. Fig. 4 shows the dose-response relationships for shortening THE duration by phenytoin in frogs and mice. Whereas the slopes of the two curves are not different, phenytoin is significantly ($p < 0.05$) more potent in mice than it is in frogs. For the phenobarbital dose-response relationships, however, not only are the slopes similar, but there is no difference in potency of phenobarbital in frogs and mice (Fig. 5).

To distinguish between supraspinal and spinal (or peripheral) sites of drug action responsible for shortening THE duration, studies were conducted using animals which were decapitated 1.5 seconds after corneal electroshock had produced THE. Decapitation of 4 mice shortened THE duration about 50% compared to THE duration in intact mice, and, therefore, decapitation experiments were not pursued further with mice. However, decapitation of frogs 1.5 seconds after electroshock did not affect appreciably the duration of THE. Therefore, it was possible to investigate the effects of phenytoin and phenobarbital on THE duration in decapitated or "spinal" frogs.

As seen in Fig. 6, phenytoin and phenobarbital, in a dose-related fashion, shortened THE duration in decapitated frogs. In comparing these dose-response curves (Fig. 6) to those for phenytoin (Fig. 4) and phenobarbital (Fig. 5) in intact frogs, it was found that neither the

slopes of these curves nor the potencies of these drugs were altered by the decapitation.

One may notice none of the dose-response relationships for shortening THE duration (Figs. 4, 5, and 6) contain responses greater than 60-70%. In experiments involving decapitation of frogs, the low maximum response was due to a limitation imposed by the experimental protocol. Shortening of THE duration by 60% is nearly the maximum suppression possible because the time needed to decapitate the animal was about 40% of the total THE duration in control frogs. Relatively high doses of phenytoin or phenobarbital are capable of achieving a 90% shortening of control THE duration, but in experiments reported in this paper the doses used in intact frogs were limited to the doses used in decapitated frogs. In mice, a lack of data for responses above 60% was presumably due to the inability of the electroshock device to generate amperages large enough to elicit THE.

EFFECTS OF PHENYTOIN ON OTHER PHASES OF THE MAXIMAL SEIZURE

In addition to preventing and shortening the duration of THE, phenytoin also exacerbated the phases of tonic flexion and clonus in frogs and mice. These phenomena, which have also been observed in other laboratory animals (Toman, Swinyard, and Goodman, 1946), were less commonly seen in animals treated with phenobarbital. Phenytoin also affected the time necessary for frogs to recover from maximal electroshock. The time needed for frogs treated with 40 mg/kg phenytoin to recover from electroshock (65 ± 31 sec, n=6) was significantly shorter than the recovery time in untreated control

frogs ($p < 0.01$), a phenomenon also observed in rabbits, cats and rats (Toman, Swinyard, and Goodman, 1946). However, the recovery time for mice treated with 10 mg/kg phenytoin (113 ± 37 sec, n=8) was not significantly different from the recovery time for untreated control mice ($p > 0.3$). The lack of phenytoin effect on recovery time in mice may have been due to a definition of recovery in mice, resumption of exploratory activity, which was different from that in frogs, resumption of a normal sitting posture.

TOXIC EFFECTS OF PHENYTOIN IN FROGS

To determine the dose-selectivity of the anticonvulsant effect of phenytoin in frogs, toxic dose-effect relationships were studied. Several toxic effects of phenytoin are readily seen with a dose of 80 mg/kg and include ataxia, head drop, and loss of righting reflex.†† However, these toxicities were difficult to assess quantitatively. A less conspicuous but more sensitive measure of phenytoin-induced toxicity which was easily quantifiable was suppression of post-rotatory head turning (PRHT). PRHT, the production of which is described in METHODS, was seen in 94% of control frogs (34/36). Phenytoin was found to be a potent inhibitor of PRHT, with a TD 50 of 33.2 mg/kg (Fig. 7).

Because the slope of the dose-response curve for suppression of PRHT was significantly ($p < 0.05$) steeper than that of the curve for prevention of THE, comparing the TD and ED 50's via a protective index (P.I., TD 50/ED 50) would not have been meaningful. However, suppression of PRHT did not occur until doses greater than 20 mg/kg were administered (Fig. 7), and because 17 mg/kg was the ED 50 for prevention of THE, it

is clear that phenytoin is a relatively selective anticonvulsant drug in frogs. Using the rotorod to test for toxicity, a P.I. of 10.4 has been reported for phenytoin in mice (Raines, Niner, and Pace, 1973). Although this high P.I. in mice may be partially due to the use of the rotorod toxicity test, which is probably a less sensitive indicator of phenytoin-induced toxicity than suppression of PRHT in frogs, a P.I. of 10 could not have been obtained in frogs without exceeding the LD 50, which was found to be 147 mg/kg (Fig. 7). Therefore, it appears that phenytoin is a more selective anticonvulsant drug in mice than in frogs.

†Details of the "Up and Down" method can be found in Appendix I.

††More information on the righting reflex as an endpoint for phenytoin-induced toxicity is presented in Appendix IV.

Figure 1. Quantal current-response relationships for tonic hindlimb extension (THE) induced by corneal electroshock in frogs and mice. Current durations were 0.5 sec (frogs) and 0.2 sec (mice). At each data point, 10-11 animals were tested to determine the percentage responding with THE. CA 50's (and 95% confidence limits) were 67 mA (49-91) for frogs and 8.7 mA (7.8-9.7) for mice. The slopes of the two curves are not different, but the amperages eliciting THE in mice are significantly less than those required in frogs ($p < 0.05$).

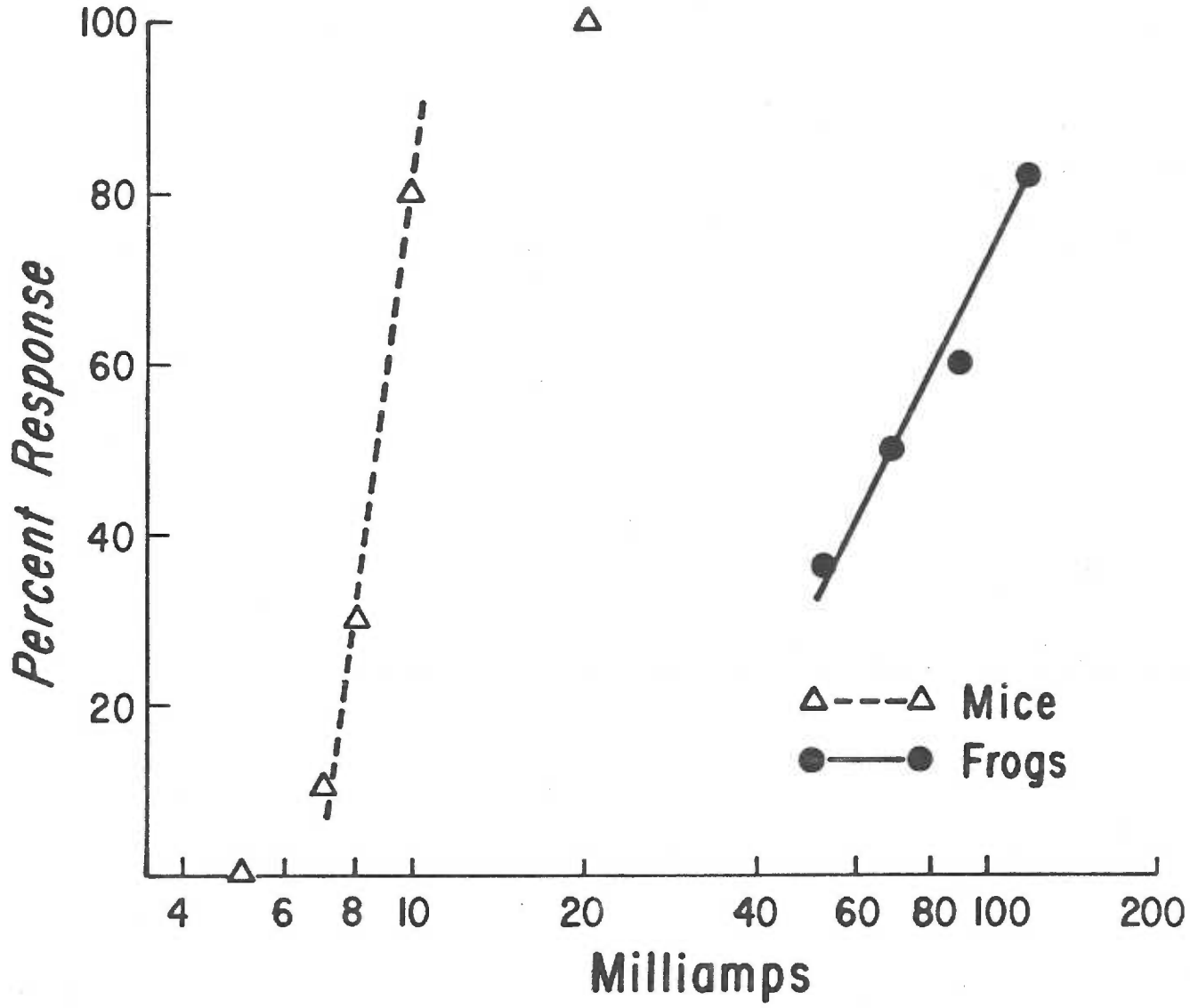

100
80
60
40
20
Percent Response
4
6
8
10
20
40
60
80
100
200
Milliamps
Mice
Frogs

Figure 2. Quantal dose-effect relationship for prevention, by phenytoin, of tonic hindlimb extension (THE) in frogs and mice. Each data point represents 12 animals. Electroshock parameters, 98 mA (0.5 sec duration) for frogs and 24 mA (0.2 sec) for mice, were selected to produce THE in 95% of control animals. Note the steeper slope of the dose-response curve for mice ($p < 0.05$). The ED 50's (and 95% confidence limits) were 17.1 mg/kg (10.4-28.0) for frogs and 7.2 mg/kg (6.7-7.8) for mice.

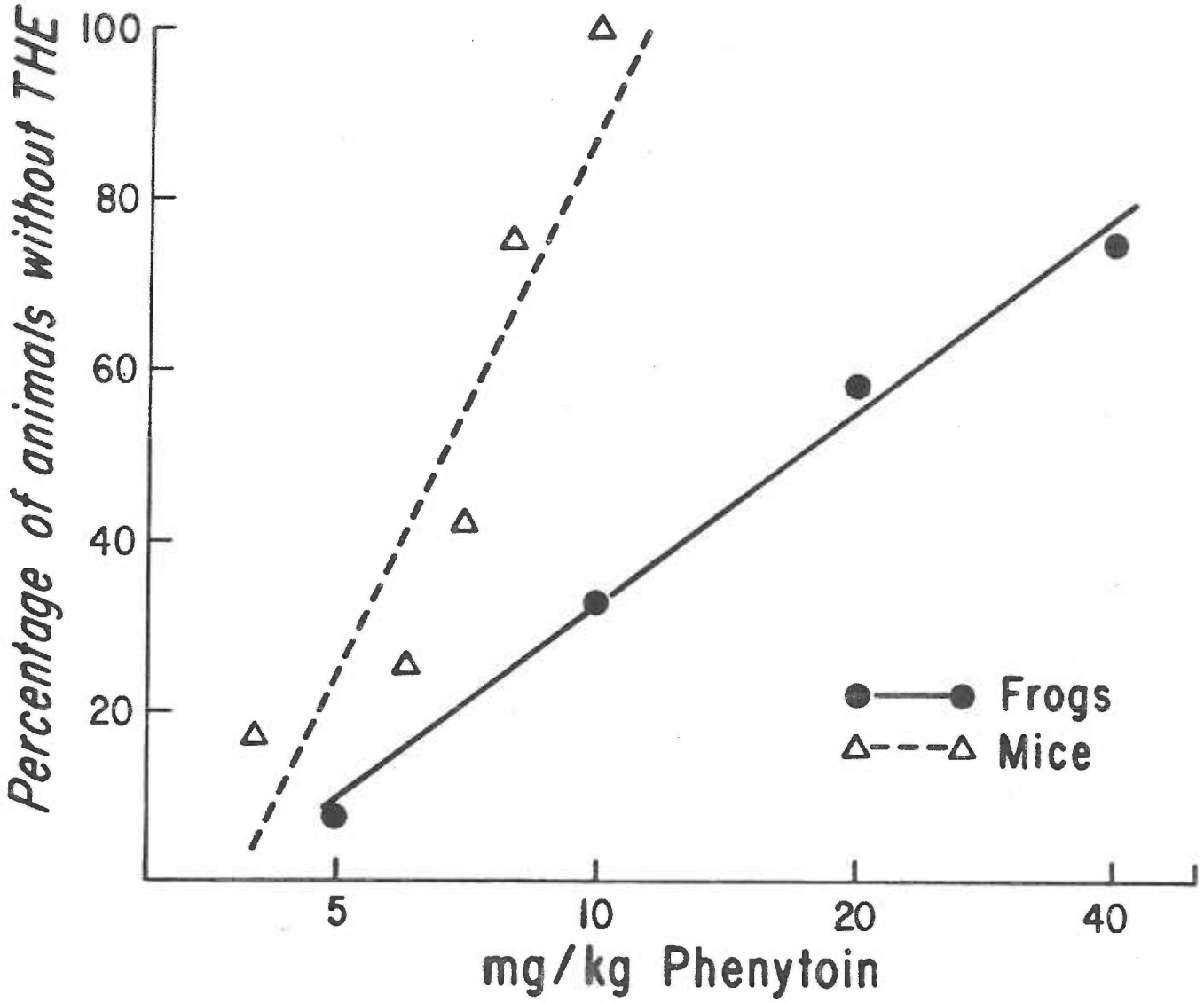

Percentage of animals without THE
100
80
60
40
20
5
10
20
40
mg/kg Phenytoin
Frogs
Mice

Figure 3. Quantal dose-effect relationship for prevention, by phenobarbital, of tonic hindlimb extension (THE) in frogs and mice. Each data point represents 9-10 animals. Electroshock parameters were the same as those described in Fig. 2. Note that the slopes and the potencies of phenobarbital in these two dose-response curves are virtually identical. The ED 50's (and 95% confidence limits) were 13.8 mg/kg (10.7-17.7) for frogs and 15.4 mg/kg (13.2-18.0) for mice.

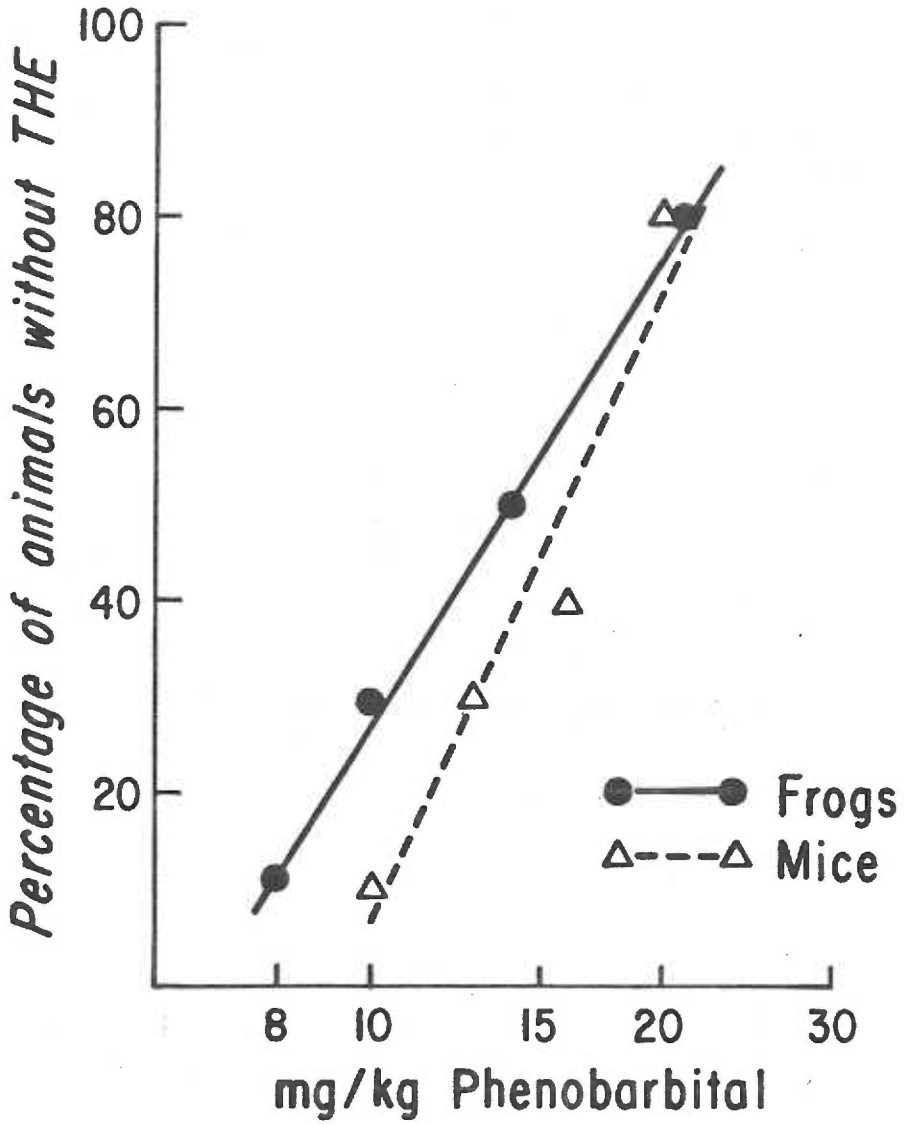

Percentage of animals without THE
100
80
60
40
20
8
10
15
20
30
mg/kg Phenobarbital
Frogs
Mice

Figure 4. Percentage reduction of the duration of tonic hindlimb extension (THE) by phenytoin in frogs and mice. 8-9 frogs and 3-10 mice per data point were electroshocked with 300-425 mA for durations of 0.5 and 0.2 sec, respectively. Control THE durations were 6.7 ± 1.5 sec (± S.D.) for frogs (n=8) and 10.3 ± 2.5 sec for mice (n=16). Error bars are standard deviations expressed as a percent of control THE duration. The slopes of the two curves are not different, but the potency of phenytoin is greater in mice than in frogs ($p < 0.05$). The ED 50's (and 95% confidence limits) were 26.5 mg/kg (13.3-52.8) for frogs and 10.7 mg/kg (7.8-14.6) for mice.

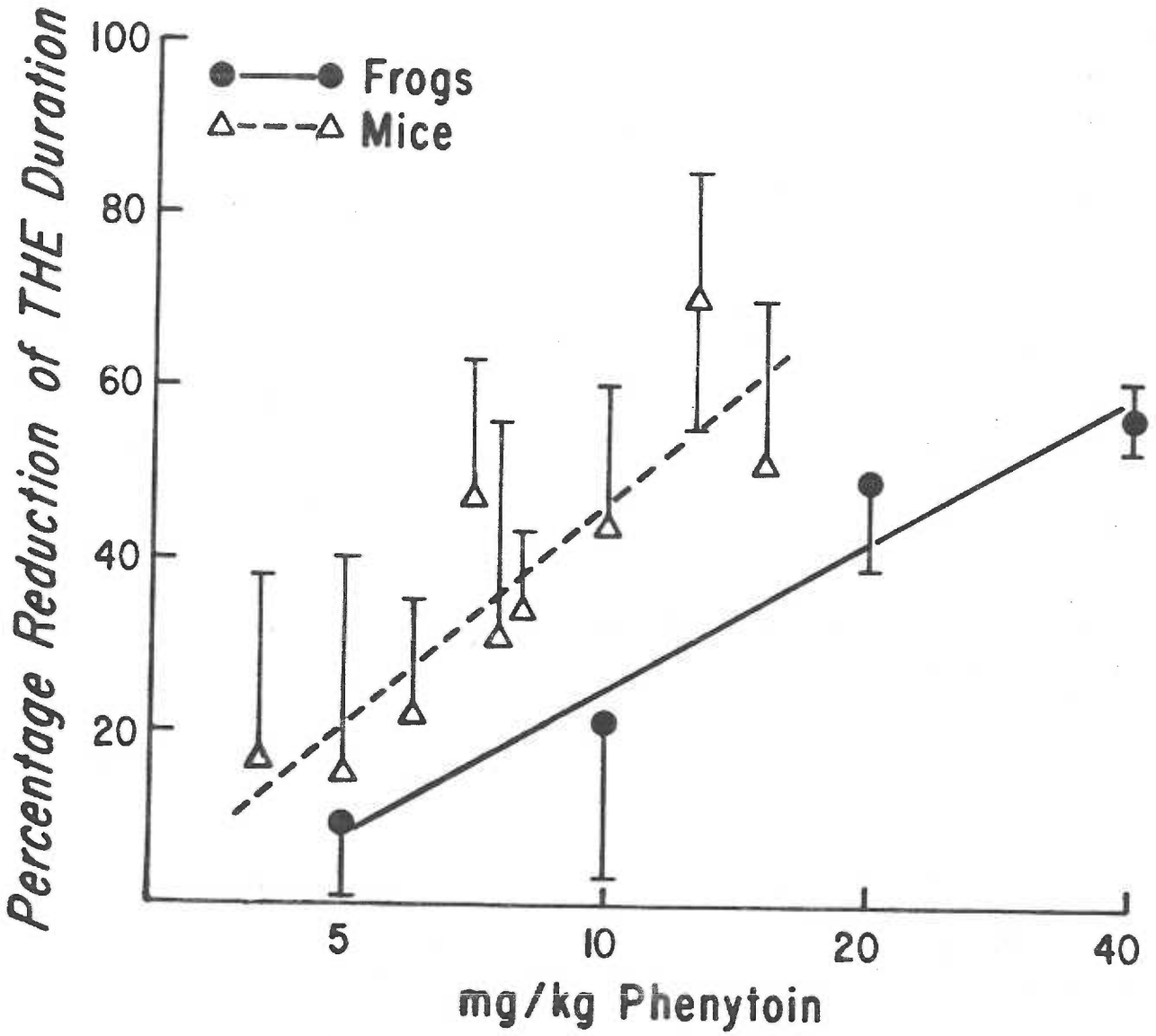

Percentage Reduction of THE Duration
100
80
60
40
20
Frogs
Mice
5
10
20
40
mg/kg Phenytoin

Figure 5. Percentage reduction of the duration of tonic hindlimb extension (THE) by phenobarbital in frogs and mice. Electroshock parameters were the same as those in Fig. 4. Data points represent the mean of 8-13 frogs and 5-10 mice. Control THE durations were 6.6 ± 0.9 sec (± S.D.) for frogs (n=10) and 11.9 ± 2.1 sec for mice (n=8). Error bars are standard deviations expressed as a percent of control THE duration. Neither the slopes of the two dose-response curves nor the potencies of phenobarbital were significantly different in frogs and mice. The ED 50's (and 95% confidence limits) were 38.0 mg/kg (19.0-76.1) for frogs and 19.6 mg/kg (12.1-31.8) for mice.

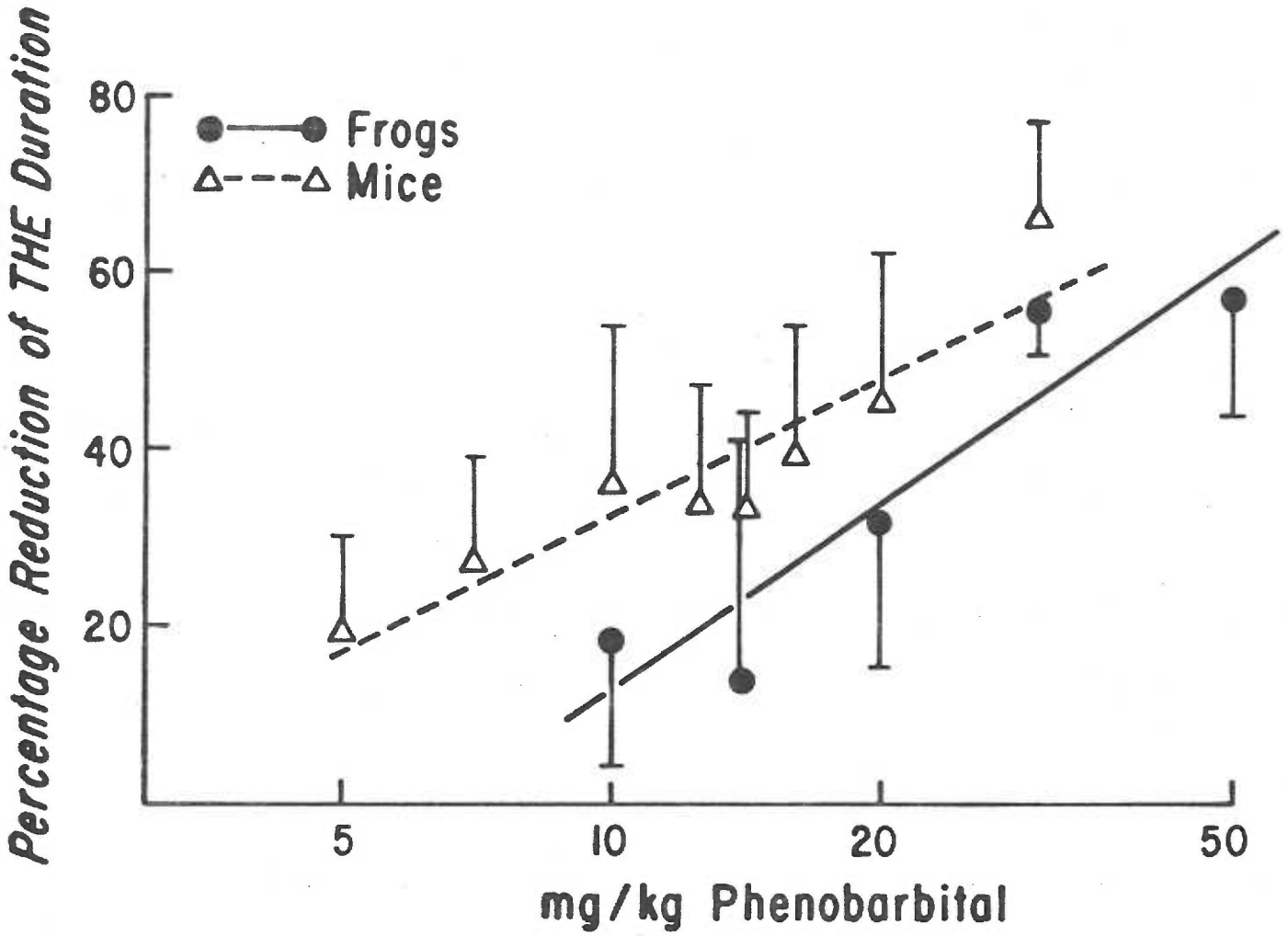

Percentage Reduction of THE Duration
80
60
40
20
Frogs
Mice
5
10
20
50
mg/kg Phenobarbital

Figure 6. Percentage reduction of duration of tonic hindlimb extension (THE) by phenytoin and phenobarbital in decapitated frogs. Electroshock parameters were the same as those in Fig. 4. Each data point represents the mean of 8-13 frogs which were decapitated 1.5 sec after electroshock. Control THE durations were 6.7 ± 1.5 sec (± S.D.) and 6.6 ± 0.9 sec for the phenytoin and phenobarbital groups, respectively. Error bars are standard deviations expressed as a percent of control THE duration. Drug potencies and slopes of the curves for decapitated frogs were not significantly different from those for intact frogs (see Figs. 4 and 5). ED 50's (and 95% confidence limits) in decapitated frogs were 22.6 mg/kg phenytoin (11.2-45.4) and 56.5 mg/kg phenobarbital (21.9-146).

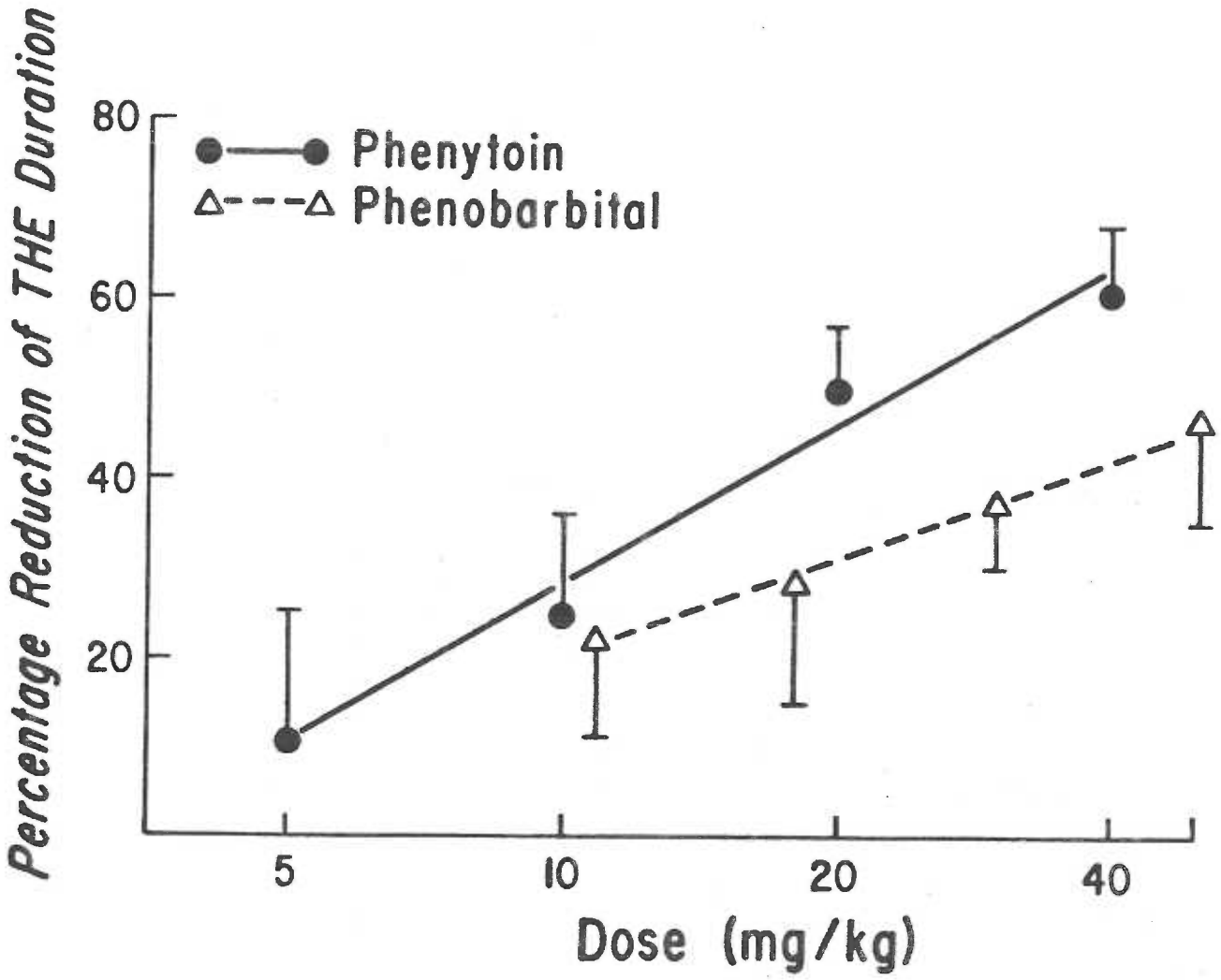
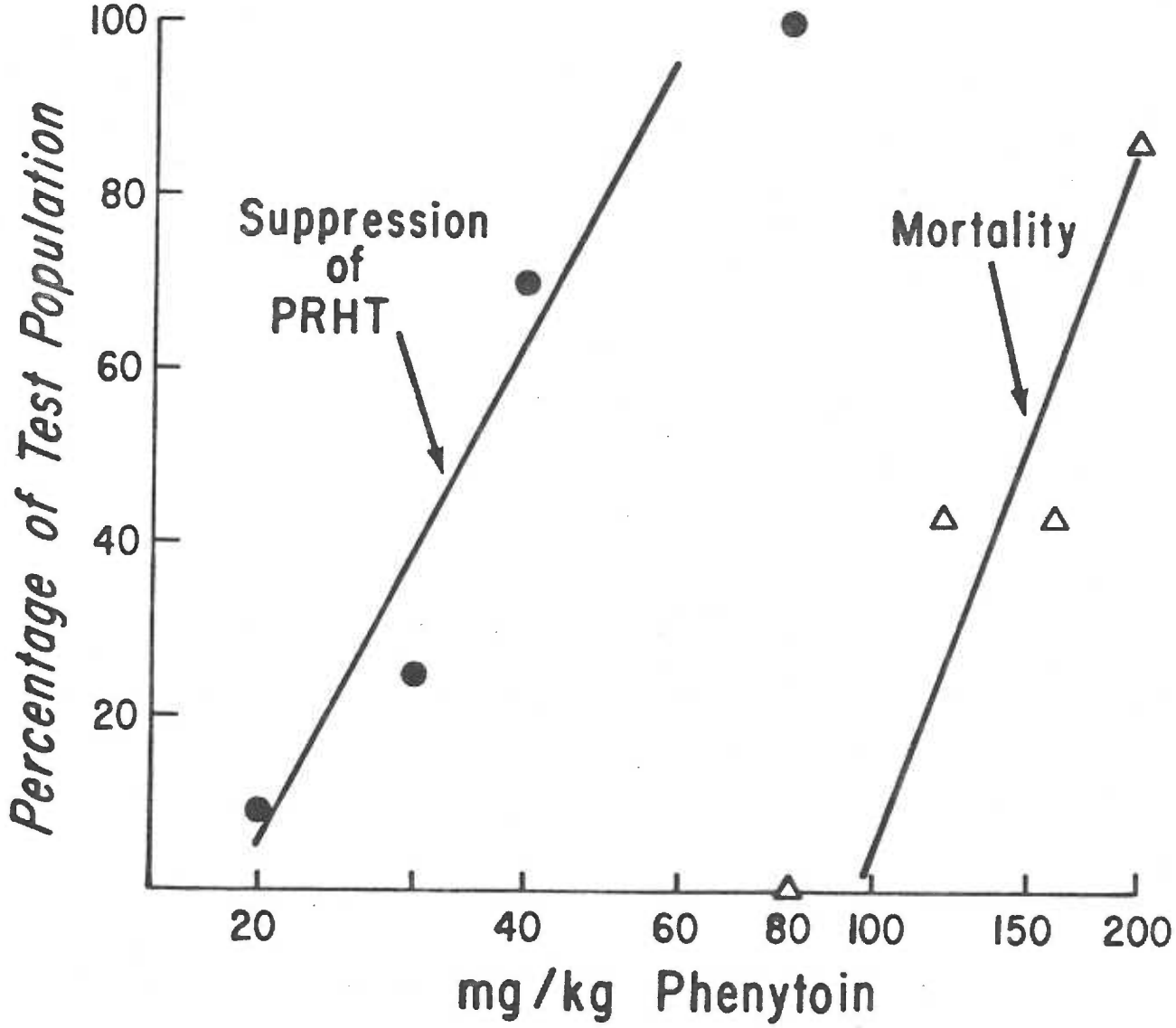

Percentage Reduction of THE Duration
80
60
40
20
Phenytoin
Phenobarbital
5
10
20
40
Dose (mg/kg)

Figure 7. Quantal dose-effect relationships for phenytoin-induced toxicities in frogs. Suppression of post-rotatory head turning (PRHT) was found to be the most sensitive measure of phenytoin-induced toxicity. To elicit PRHT, frogs were rotated clockwise at 1 Hz for 10 sec, after which rotation was stopped suddenly. 94% (34/36) of control frogs showed clockwise head turning following this procedure. Each data point in the PRHT-curve represents 10-12 frogs, and the TD 50 (and 95% confidence limits) was 33.2 mg/kg (27.3-40.4). The lethal dose-response curve for phenytoin was obtained by observing 7-12 frogs per dose level for a 36-hr period after injection. The LD 50 (and 95% confidence limits) was 147 mg/kg (125-172).


Percentage of Test Population
100
80
60
40
20
Suppression of PRHT
Mortality
20
40
60
80
100
150
200
mg/kg Phenytoin

DISCUSSION

In most animals in which the antigravity muscles are the extensors, maximal seizures are characterized by tonic extension preceded by tonic flexion. The discovery that dorsal rhizotomy abolishes tonic hindlimb extension (THE) during electrically-induced seizures in cats (Esplin and Laffan, 1957) led Esplin (1959) to propose that the initial tonic flexion activates Golgi tendon organs in flexor muscles, resulting in inhibition of flexor motor neurons and excitation of extensor motor neurons. Esplin (1959) also suggested that flexion precedes extension because the motor neurons which innervate the antigravity extensor muscles, more powerful than flexors, are under a greater degree of neuronal inhibition than are flexor motor neurons (see, also, Esplin and Freston, 1960). However, the present study found that frogs rarely displayed the flexion-extension sequence during maximal seizures. It is interesting to note that when tonic flexion did occur, it took the form of abduction of the hindlimbs. This feature, and the prevailing pattern of THE as the initial event in frog seizures, might be viewed as commensurate with the normal sitting posture in this species. In other words, the frog is an intermediate case between the antigravity flexor dominance of the sloth (Esplin and Freston, 1960) and the antigravity extensor dominance of mammalian bipeds and quadrupeds. Thus, neither the flexors nor the extensors, at least in the hindlimbs, are true antigravity muscles in the frog.

Besides the difference in seizure patterns, frogs and mice also differed in that the former required a larger magnitude and duration of

stimulating current to produce maximal seizures, which is consistent with the results of Servit (1959 and 1972), who showed that seizure threshold is inversely related to the evolutionary development of the test animal. However, the larger electroshock stimulus raises the likelihood of more distant spread of stimulating current, and therefore the possibility that the seizure focus in frogs might be a site distal from the stimulating electrodes, such as rostral spinal cord, while the focus in mice might be more proximal, such as neocortex. A spinal cord generator site in frogs is supported by the observations that there was no latency between electroshock and seizure onset, nor was there terminal clonus after THE. Both phenomena are also characteristic of maximal seizures in spinal rats, mice, rabbits and hamsters (Esplin and Freston, 1960). Furthermore, decapitation of frogs 1.5 sec after electroshock did not change the duration of THE from that observed in intact frogs. In sum, all these findings, as well as the large stimulation requirement, point strongly to a spinal cord origin for THE in the frog. Even if a hindbrain focus exists, it is at least evident that once THE is initiated in frogs it can be sustained solely by spinal cord pathways. In contrast, decapitation of mice markedly shortened THE duration. Although this may suggest that mouse brain is essential to sustain THE, it is equally likely that their high metabolic rate makes mice more susceptible to decapitation-induced ischemia in neural tissues necessary to sustain THE.

The finding that phenytoin and phenobarbital shorten THE duration in decapitated frogs agrees with the results of Esplin and Freston (1960), who showed that phenytoin and other anticonvulsant drugs were effective in preventing and shortening the duration of THE in spinal cats. We,

therefore, also agree with these authors that phenytoin and phenobarbital have sites of anticonvulsant action in spinal cord and/or peripheral neural pathways. However, unlike the work of Esplin and Freston (1960), the present study shows that phenytoin and phenobarbital are each equally potent in intact and "spinal" frogs, suggesting even more strongly that peripheral sites are important for the anticonvulsant effect in intact animals. Esplin and Freston (1960) found that modification of maximal seizures in spinal animals required anticonvulsant drug doses higher than those needed in intact animals. But this apparent potency difference is rendered dubious or invalid by the methodological approach these authors used. The seizures in their intact cats were generated by electroshock via corneal electrodes, whereas seizures in the spinal cats were initiated after cord section via electrodes in the spinal cord itself. Since direct electrical stimulation of the spinal cord cannot be compared to corneal electroshock, the present study rectified that deficiency, and was thus able to make a valid comparison of drug potencies for shortening THE duration in intact and "spinal" frogs by utilizing the same THE-inducing procedure, corneal electroshock, in both preparations. Thus, doses of phenytoin that achieved equal shortening (40%) of THE duration in intact and decapitated frogs were, respectively, 18 and 16 mg/kg; and for phenobarbital the respective doses were 25 and 35 mg/kg. Given this equipotency of phenytoin or phenobarbital in intact vs. decapitated frogs, as well as the fact that dose-effect slope values for drug-induced shortening of THE duration in mice were not significantly different from those in frogs, it is likely that phenytoin and phenobarbital also act on spinal/peripheral pathways

to shorten THE duration in mice. Indeed, Raines et al. (1976) showed in mice subjected to electrically-induced maximal seizures that THE could be completely prevented by administration of agents that selectively depress muscle spindle activity.

The γ-motor neuron loop is essential for normal muscle tone, and would also be necessary to sustain THE during a maximal seizure. Therefore, depression of the γ-motor neuron loop would likely shorten the duration of THE, and phenytoin has been shown to interfere with the functioning of at least 3 segments of this loop, namely muscle spindles, and both afferent and efferent limbs. Anticonvulsant doses of phenytoin (2.5-40 mg/kg) in the cat interfere with the functioning of muscle spindles, as evidenced by the suppression of spontaneous activity, prolongation of post-stretch silence period, and a diminution of stretch-evoked firing of Ia and II afferent fibers originating in the triceps surae (Anderson and Raines, 1974). Phenytoin has also been reported to inhibit stretch-induced discharges originating in frog muscle spindles (Kontani, Kudo, and Fukuda, 1976). Raines and Standaert (1967) showed that anticonvulsant doses of phenytoin (2-10 mg/kg) in the cat depress the dorsal root IV reversal potential, indicating a drug-induced depression of the afferent pathway, specifically the afferent terminals (Lloyd, 1952), of the γ-motor neuron loop. Anticonvulsant doses of phenytoin (30 mg/kg) in the cat also diminish the 2N spike amplitude recorded in the ventral root following tetanic stimulation of the dorsal root (Esplin, 1957). The efferent limb is also affected by phenytoin, as Raines and Standaert (1966) have shown that anticonvulsant doses (5-20 mg/kg) in the cat selectively reduce post-tetanic

potentiation of neuromuscular transmission in soleus muscle. Data cited above make it clear that phenytoin has potent effects on afferent and efferent limbs of the γ-motor neuron loop, as well as on muscle spindles. Thus, phenytoin action on any or all of these sites in the γ-motor neuron loop may be responsible for phenytoin-induced shortening of THE duration.

With respect to the quantal endpoint, prevention of THE, Fig. 3 reveals that there is no significant species difference for the anticonvulsant action of phenobarbital. Not only are the threshold doses in frogs (8 mg/kg) and mice (10 mg/kg) extremely close, but the extended dose-effect relationships are virtually parallel. The proposed anticonvulsant mechanism for phenobarbital is direct depression of neural structures, thereby elevating seizure threshold at the epileptogenic focus, with only limited ability to prevent spread of excitation from a focus (Morrell, Bradley, and Ptashne, 1959). Considering that the focus for electrically-induced seizures in mice is almost certainly neocortex, and in the frog is hindbrain or spinal cord, the dose-effect identities in Fig. 3 suggest that phenobarbital is not site-selective in its ability to depress seizure foci.

The virtually common dose-effect relationships for phenobarbital prevention of THE in frogs and mice are also distinguished by their relatively steep slopes (Fig. 3). In frogs, however, phenytoin prevention of THE has a dose-effect slope which is significantly less steep than that in mice, but it is especially noteworthy that phenytoin anticonvulsant action in both mouse and frog has a common threshold dose level of 4-5 mg/kg (Fig. 2). Clearly, the present comparison of

phenobarbital and phenytoin for prevention of THE in mouse and frog discloses only one striking species-related difference: the very low slope of the phenytoin dose-effect relationship in frog. Concerning this difference, the following interpretation may have important implications for the inclusion of the frog in future anticonvulsant drug testing.

Phenytoin's general mechanism of anticonvulsant action has recently been described by Rall and Schleifer (1980), as follows: "In various species, as revealed by a variety of stimulation-recording technics, the ability of phenytoin to reduce the duration of afterdischarge and to limit the spread of seizure activity is more prominent than its effect on threshold for stimulation. Moreover, elevation of threshold is relatively selective for the cerebral cortex and hippocampus." The ability of phenytoin to inhibit cortical spread of seizure activity is well known (Louis, Kutt, and McDowell, 1968), and phenytoin is superior to phenobarbital in this regard (Morrell, Bradley, and Ptashne, 1959). However, phenytoin is inferior to phenobarbital in raising the threshold of a focus (Morrell, Bradley, and Ptashne, 1959). Consequently, phenytoin limitation of seizure spread and its suppression of a focus are both site-selective actions in cerebral cortex and hippocampus. These structures are well-developed in the mouse, but in the frog, cerebral cortex is absent and hippocampus is very poorly differentiated (Kicliter and Ebbesson, 1976).

Recalling the respective cortical and non-cortical focal origins of electrically-induced THE in mouse and frog, the near identity of the

phenobarbital dose-effect curves for preventing THE thus exemplifies focus suppression (and probably depression of other spinal and peripheral pathways) that is neither site- nor species-selective. Conversely, phenytoin's exceptional site selectivity for cortical and hippocampal structures is well demonstrated by the wide disparity between the dose-effect slopes in the mouse with cortical, and the frog with non-cortical, seizures. Indeed, the common threshold anticonvulsant dose level in mouse and frog (Fig. 2) suggests that phenytoin actions and other, non-cortical, neural pathways proceed *pari passu* with the cortical actions, but the latter dominate as dose is increased.

If one viewed the frog not as a frog but as a model of a decorticate, intact animal, it could be speculated that the phenytoin dose-effect slope for prevention of THE in this animal might be a vectorial representation of anticonvulsant contributions from all non-cortical (including peripheral) sites of action. The difference between this vector and that in the mouse would thus be an expression of the relative contribution of cortical (and hippocampal) sites of action to the totality of phenytoin's anticonvulsant effect.

Since the frog is, effectively, an intact decorticate animal, its regular inclusion, as an adjunct to the mouse, in anticonvulsant drug testing might have value in assessing the relative importance of cortical vs. non-cortical sites of drug action. At the least, it would be worthwhile to evaluate this possibility by extending the kind of comparative study reported here to include other established and new anticonvulsant drugs.

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MANUSCRIPT II

METABOLISM, TISSUE DISTRIBUTION, AND ELIMINATION KINETICS OF PHENYTOIN IN THE FROG

ABSTRACT

In this laboratory, recent investigations of the anticonvulsant effects and mechanism(s) of action of phenytoin have made extensive use of the frog (Rana pipiens), and these pharmacodynamic and mechanistic studies required detailed knowledge of the disposition of phenytoin by this animal. Since information of this type is not available in the literature, the present study was undertaken. The elimination of ^{14}C-phenytoin from frog lymph, investigated using a technique in which phenytoin is selectively extracted into 1-chlorobutane, was found to proceed by concentration-dependent kinetics which were accurately described by an integrated form of the Michaelis-Menten equation. From the extrapolation of elimination curves to "zero" time, phenytoin was found to have an apparent volume of distribution (V_d) of 3.5 l/kg. This relatively large V_d was found by tissue distribution analysis to primarily reflect distribution of phenytoin away from lymph and plasma and into carcass. Bile had the highest concentration of phenytoin, followed by kidney, pancreas, and brain. Bile also had the highest concentration of phenytoin metabolites, which were extracted into acetone after the removal of parent drug. Gas chromatography/mass spectrometry was used to identify major metabolites as 4'-hydroxyphenylphenylhydantoin (p-HPPH) and the glucuronide conjugate of p-HPPH, and minor metabolites as 3'-hydroxyphenylphenylhydantoin, 3',4'-dihydroxyphenylphenylhydantoin (catechol of phenytoin), and an O-methyl catechol of phenytoin. It is concluded that the metabolism, tissue distribution, and elimination kinetics of phenytoin in the frog are remarkably similar to those in many mammals, including humans.

INTRODUCTION

The anticonvulsant and toxic dose-response relationships of phenytoin in the frog have recently been studied in our laboratory. However, interpretations of pharmacodynamic data and the utilization of such data in future experiments would be limited without some knowledge of the disposition of phenytoin by this animal. For example, a knowledge of the kinetics governing the elimination of phenytoin is necessary to plan therapeutically-efficient chronic dosing schedules. Also, the metabolic fate and tissue distribution of phenytoin in the frog would be important in considering possible sites and mechanisms of action for both anticonvulsant and toxic effects. Furthermore, because the ability of the frog to metabolize lipid-soluble drugs has been questioned (Brodie, Maickel, and Jondorf, 1958; Brodie and Maickel, 1962), we found it necessary to define the metabolic fate of phenytoin. The present study examines the elimination, distribution, and metabolism of phenytoin in the bullfrog (Rana catesbiana) and leopard frog (Rana pipiens). Our results demonstrate that the elimination of phenytoin by dose-dependent elimination kinetics, the tissue distribution of phenytoin, and the production of major and minor metabolites of phenytoin by the frog, were remarkably similar to those found in man and most other mammals. Preliminary results of this study have been published previously (Johnson and Riker, 1978 and 1979).

METHODS

ANIMALS AND DRUGS

Bullfrogs (Rana catesbiana) of both sexes (200-465 gm), obtained from Davis Farms (Clovis, Calif.), and leopard frogs (Rana pipiens) of both sexes (45-100 gm), obtained from William Lemberger Assoc. (Germantown, Wisc.), were housed separately in metal or plastic containers tipped at one end to allow for dry and wet areas with continuously flowing tap water. Frogs were kept at ambient room temperature (20-25° C) in a room supplied with artificial light 8-12 hrs/day. Although not fed during captivity, frogs were usually kept no longer than 2-3 weeks before sacrifice. During an experiment, frogs were placed individually in plastic boxes with enough tap water to keep the frog moist at all times.

Sodium phenytoin, obtained from Parke, Davis and Co., and ^{14}C-phenytoin, obtained from New England Nuclear Corp., were dissolved in 50 mM NaOH (pH 12.6) and injected into the ventral lymph sac. 200-1000 units/kg of sodium heparin, obtained from A. H. Robins Co., were administered intramuscularly to frogs used in studies on elimination kinetics, and injected into the ventral lymph sac of frogs used in tissue distribution studies. Whenever possible, the volume of material injected was less than 0.5% of body weight.

SOLVENTS AND EQUIPMENT

Solvents used were 1-chlorobutane (butyl chloride, BuCl), chloroform, dimethyl sulfoxide (DMSO), acetone (Burdick and Jackson Labs.), and diethyl ether (ether) (J. T. Baker). ^{14}C assays were

performed in a Beckman LS-330 liquid scintillation counter using 10 ml Aquasol^{R} (New England Nuclear) for aqueous samples, and 5 ml of a concentrated dioxane-based scintillation fluid for BuCl and ether samples. The dioxane-based scintillation fluid was made by dissolving 300 gm naphthalene (scintillation grade, Matheson, Coleman and Bell Co.), 15 gm PPO (2,5-diphenyloxazole, scintillation grade, Packard), and 0.10 gm POPOP (p-bis-(2-(5-phenoxyoxazolyl))-benzene, scintillation grade, Research Products International Corp.) in 1 liter of 1,4-dioxane (Fischer Scientific Co.). Efficiency was determined both by external standard ratio and by addition of ^{14}C-toluene standard (New England Nuclear) to individual samples. Gas chromatography was performed in a 5830A Hewlett-Packard instrument equipped with a flame ionization detector, and chemical ionization and electron impact mass spectra were obtained at 70 eV using a Finnigan model 4000 gas chromatograph/mass spectrometer equipped with an Incos data system.

ASSAY OF PHENYTOIN AND METABOLITES IN WHOLE BULLFROG

Sixteen bullfrogs were injected with 14 mg of ^{14}C-phenytoin (0.31 μc/mg) and placed in individual plastic boxes containing 500 ml tap water. At successive 10-hr intervals, 4 frogs were sacrificed by a sharp blow to the head and the volume of water in each box was measured. Each frog and enough tap water to make a total volume of 2.5 liters were added to a Waring Blendor and the contents were homogenized for 3 minutes. Another 4 frogs were used to establish drug levels in frog homogenate at "zero" time by adding 14 mg of ^{14}C-phenytoin to the homogenate after 2 minutes of homogenization. Frog

homogenate and box water were filtered through cheesecloth before extraction with solvents.

Assay of phenytoin and metabolites were performed by the sequential solvent extraction method of Gerber et al. (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971). Three ml of filtrate from frog homogenate or box water were mixed with 1 ml of 0.4 M phosphate buffer (pH 6.8) and extracted 3 times with BuCl (40 ml), once with ether (25 ml), and once with acetone (15 ml). After equilibration with the filtrate, BuCl and ether layers were washed with 4 ml of 0.1 M phosphate buffer (pH 6.8). Prior to the equilibration of acetone with a sample, 12 ml of 4 M phosphate buffer (pH 6.8) were added to facilitate the separation of acetone and aqueous layers. After equilibration with aqueous layers, volumes of ether and acetone increased and, therefore, were always measured before aliquots were assayed for ^{14}C. A 5-ml aliquot of the acetone layer was evaporated under N_2 and the residue was dissolved in 2 ml distilled H_2O prior to the assay of ^{14}C, while 15 ml of each BuCl and ether layer were assayed directly. Organic material not assayed for ^{14}C was discarded.

Gerber et al., using counter current distribution, showed that BuCl selectively extracted the parent compound (phenytoin), ether preferentially extracted more polar compounds such as hydroxylated phenytoin metabolites, while acetone extracted the most polar compounds such as conjugated phenytoin metabolites from rat tissues (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971). We also found that BuCl selectively extracts phenytoin from the box water from frogs

treated with phenytoin using counter current distribution, according to the method of Bush and Densen (1948), and gas chromatography.†

ELIMINATION OF PHENYTOIN FROM FROG LYMPH

Phenytoin concentrations were measured in lymph from 14 bullfrogs given 40, 4, or 0.4 mg/kg ^{14}C-phenytoin, and from 5 leopard frogs given 30 mg/kg ^{14}C-phenytoin. Each frog, whose weight did not vary by more than 10% within each group, was given 8-10 µc of ^{14}C-phenytoin. To prepare for the collection of lymph, the frog was immobilized in the extended position by wrapping wet cheesecloth around the hindlimbs. The frog was lowered feet first through the eye of a ring stand, the diameter of which was sufficiently small that the frog's hindlimbs and torso passed through, but not the upper limbs. Thus, the frog was suspended by the axillae in a vertical position. A small hole was made in the frog's skin over the tendinous calcaneous with a 27-gauge needle, and a micropipette, made from a glass capillary tube, was slipped through the hole into the posterior crural lymph sac. Lymph (150-400 µl) passed through the micropipette and was collected in a small vial. After this procedure, which lasted about 5 minutes, frogs were released from their constraints and returned to their boxes. Collected lymph was stored at -20° C until the concentration of phenytoin in lymph was determined by extracting phenytoin from 100 µl lymph into 10 ml of BuCl, 9 ml of which was assayed for ^{14}C.

PREPARATION OF SAMPLES FOR GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

A bullfrog, injected with 40 mg/kg ^{14}C-phenytoin (0.19 μc/mg), and a leopard frog, injected with 100 mg/kg ^{14}C-phenytoin (0.64 μc/mg), were placed in separate plastic boxes with 100 ml tap water. Every 12 hours the volume of box water was removed, measured, stored at -20° C, and replaced with 100 ml fresh tap water. After at least 50% of the total ^{14}C was recovered in the box water, the frogs were sacrificed by pithing brain and spinal cord. Bile, feces, and urinary bladder and contents were then collected and prepared for the extraction of phenytoin and metabolites into acetone as described in METHODS for tissue distribution (vide infra).

Box water was used as the primary source of metabolites of phenytoin. A 50-ml aliquot of box water was equilibrated with two volumes of acetone (200 ml) after the addition of 145 ml of 4 M phosphate buffer (pH 6.8). Acetone extractions were pooled and evaporated to a volume of about 20 ml in a rotor evaporator (Büchi Rotavapor, Brinkman Instrument Co.), transferred to 2 Reacti-vials^{R} (Pierce Chemical Co.), evaporated under N_2, and dessicated overnight. The dessicated residues were permethylated or perdeuteriomethylated with CH_3I or CD_3I (vide infra).

Some aliquots of box water were treated with HCl to hydrolyze conjugates of phenytoin metabolites prior to extraction into acetone. Fifty ml of concentrated HCl (J. T. Baker) were added to the aliquot of box water (50 ml) and the solution was heated at 100° C for 2 hours. The solution was allowed to cool, 10 M NaOH was added dropwise to

neutralize the solution (final pH 6.5-7.5), and distilled H_2O was added until a final volume of 50 ml was obtained. Phenytoin and metabolites were extracted into acetone as described above.

Other aliquots of box water were equilibrated with ether instead of acetone. Box water (50 ml) was mixed with 1.25 ml of 4 M phosphate buffer (pH 6.8) and equilibrated with 250 ml of ether. The ether was evaporated in a rotor evaporator and the residue was dissolved in acetone (20 ml), transferred to a Reacti-vial^R, and evaporated under N_2. The residue was dessicated overnight and derivatized with trimethylchlorosilane (vide infra).

Bile, feces, and urinary bladder and contents were not subjected to acid hydrolysis, and were equilibrated with acetone. Acetone from each sample was transferred to 2 Reacti-vials^R, evaporated under N_2, dessicated overnight, and permethylated or perdeuteriomethylated with CH_3I or CD_3I (vide infra).

Permethylation and perdeuteriomethylation of dessicated samples were performed according to the method of Thompson et al. (Thompson, Gerber, Seibert, and Desiderio, 1973). The residue was dissolved in 100 µl of anhydrous DMSO, and 25 µl of the sodium salt of DMSO (Leclercq and Desiderio, 1971) were added. Ten minutes later, 10 µl of CH_3I or CD_3I (Aldrich Chemical Co.) were added and the mixture was vortexed. After 10 minutes, the reaction was stopped by the addition of 1 ml of H_2O and the products were extracted into 1 ml of chloroform and washed twice with 1 ml of H_2O. Chloroform was evaporated under N_2 and the residue was dissolved in 50 µl of fresh chloroform.

Trimethylsilylation of the residue from the ether extraction of box water was performed according to the method of Gerber et al. (Gerber, Thompson, Smith, and Lynn, 1979) in an attempt to identify the trimethylsilane derivative of a dihydrodiol metabolite of phenytoin. The dessicated residue was dissolved in 200 µl of pyridine (silylation grade, Pierce Chemical Co.). Five µl of BSTFA with 1% TNCS ((Bis-(trimethylsilyl)-trifluoroacetamide) with 1% trimethylchlorosilane) (Pierce Chemical Co.) were added to the sample and the mixture was heated at 65° C for 10 minutes. A 1-10 µl sample was injected onto the gas chromatograph/mass spectrometer within 2 hours of the sample's preparation.

TISSUE DISTRIBUTION OF PHENYTOIN

Three female leopard frogs were injected with 30 mg/kg ^{14}C-phenytoin (4.44 µc/mg) and placed in individual plastic boxes to which 300 ml of tap water had been added. After 3 hours, frogs were injected with heparin, the volume of water in the box was measured, and lymph was collected from the frogs as described above. Immediately following the collection of lymph, the spinal cord was pithed and blood was collected by cardiac laceration and centrifuged. Samples of other tissues, weighing not more than 0.5 gm, were added to 2-3 ml of 0.1 M phosphate buffer (pH 6.8), and tissue plus buffer was weighed prior to homogenization with a Polytron homogenizer (Brinkman Instruments). One gm of homogenate was mixed with 3 ml of 0.1 M phosphate buffer (pH 6.8) and extracted twice with BuCl (40 ml). Each BuCl layer was washed with 4 ml of 0.1 phosphate buffer (pH 6.8) prior to the taking

of a 15-ml aliquot for assay of ^{14}C. Metabolites of phenytoin were extracted into 15 ml of acetone after the addition of 12 ml of 4 M phosphate buffer (pH 6.8). After equilibration, the volume of acetone was measured and a 5-ml aliquot was evaporated under N_2. The residue was dissolved in 2 ml of distilled H_2O and assayed for ^{14}C.

Table 1 lists all the tissues assayed for ^{14}C. In the cases of gall bladder, stomach, and large intestine, these tissues were rinsed with saline to separate contents which were also homogenized and assayed for ^{14}C. Muscle and skin were sampled from two sites: medial thigh and upper abdomen. The abdominal site was included specifically to determine whether phenytoin had been sequestered at the site of injection (abdominal lymph sac), while muscle and skin of the thigh served as control tissues. Lymph, plasma, erythrocytes, bile, and 1 gm box water were not homogenized as were other tissues, but were weighed, mixed with 3 ml of 0.1 M phosphate buffer (pH 6.8), and sequentially equilibrated with BuCl and acetone. After removal of all tissues listed in Table 1, including fat bodies which are intraperitoneal structures in the frog, the carcass was weighed and homogenized in distilled H_2O (total volume 200-300 ml) with a Waring Blendor. One gm of homogenized carcass was assayed for ^{14}C.

A separate experiment was performed to investigate the distribution of phenytoin between brain and plasma 8 hours after phenytoin injection. Brain and plasma from a female leopard frog were assayed for ^{14}C as previously described 8 hours after an injection of 40 mg/kg ^{14}C-phenytoin (1.97 μc/mg).

STATISTICS

Analysis of variance was performed using Student's t-test, and results of a two-tailed test were considered significant if 95% confidence limits were exceeded.

†Data on the phenytoin-selectivity of the 1-chlorobutane extraction are presented in Appendix VI.

RESULTS

ELIMINATION KINETICS OF PHENYTOIN

Disappearance of phenytoin from whole bullfrog (Fig. 1) appeared to follow first-order elimination kinetics with a $t_{1/2}$ of 25.6 hours and could largely be accounted for by the appearance of metabolites which were extracted into acetone and ether from frog homogenate and box water. However, the elimination of phenytoin from the bullfrog was also accompanied by the time-dependent accumulation of 3-10% of the total parent drug in box water. Because of the gradual and progressive appearance of phenytoin in box water, this probably was not due to leakage of drug from the site of injection, but to the excretion and/or secretion of parent drug by the frog. The majority of phenytoin metabolites in frog homogenate and box water were extracted into acetone as opposed to ether (Fig. 1).

The apparent first-order $t_{1/2}$ of phenytoin measured in bullfrog lymph was dose-dependent, as seen in Fig. 2. The $t_{1/2}$ of phenytoin in frogs given 40 mg/kg was 15.3 hours, which was significantly greater than the $t_{1/2}$ of 5.4 hours obtained in frogs given 4 mg/kg ($p < 0.05$). Despite this implication of non-first-order elimination kinetics, all elimination curves were apparently linear when the logarithm of drug concentration was plotted vs time (Fig. 2). By extrapolation of elimination curves in Fig. 2 to zero time, an average volume of distribution for phenytoin of 3.5 l/kg was found.

Concentration-dependent kinetics were observed for the elimination of phenytoin from lymph in leopard frogs given 30 mg/kg phenytoin, a

dose which is effective in antagonizing electrically-induced seizures (Johnson and Riker, 1980). Seen in Fig. 3, between 3-19 hours, phenytoin was eliminated at a constant rate (0.43 µg/ml/hr), which implies zero-order elimination kinetics. However, after the concentration of phenytoin in lymph declined to less than 3 µg/ml (about 20 hours after injection), phenytoin was eliminated with a constant $t_{\frac{1}{2}}$ (4.8 hours) which implies that at these concentrations the elimination followed first-order kinetics. These elimination data, plotted by the method of Lundquist and Wolthers (1958), are adequately described by the integrated form of the Michaelis-Menten equation, as seen in Fig. 4.†

METABOLISM OF PHENYTOIN

Box water, obtained from a phenytoin-treated leopard frog, was treated with acid to hydrolyze conjugates, and the gas chromatographic separation of the permethylated components of box water is shown in Fig. 5. The mass spectrum of peak "a" in the chromatogram was identified as that of the dimethyl derivative of phenytoin and was virtually identical to that of permethylated phenytoin published by Thompson et al. (Thompson, Gerber, Seibert, and Desiderio, 1973). Peaks "b" and "c" represented the trimethyl derivatives of 3'-hydroxyphenylphenylhydantoin (m-HPPH) and 4'-hydroxyphenylphenylhydantoin (p-HPPH), respectively, the identification of which were aided by comparing these mass spectra to those obtained from permethylated samples of synthetic m-HPPH and p-HPPH (Parke, Davis and Co.). Peak "d" was identified as the tetramethyl derivative of 3',4'-dihydroxyphenylphenylhydantoin

(phenytoin catechol), the structure of which was confirmed by comparing the mass spectrum to that obtained from a permethylated sample of synthetic phenytoin catechol (Parke, Davis and Co.). However, peak "d" from a perdeuteriomethylated sample of box water was shown to be composed of two metabolites: a tetradeuteriomethylated catechol of phenytoin and a trideuteriomethyl derivative of an O-methyl catechol of phenytoin. Thus, the frog made the catechol and the O-methyl catechol metabolites of phenytoin, and judging from relative abundances of ion fragments, the frog made approximately equal amounts of these two metabolites. Peaks "e" and "f" were identified as isomers of a trimethyl derivative of a bromohydroxyphenylphenylhydantoin (Br-HPPH), and the electron impact mass spectrum for peak "f" is shown in Fig. 6. The methane chemical ionization mass spectrum contained doublets at 389-391, 417-419, and 429-431, thus confirming that the doublet 388-390 seen in Fig. 6 is due to the molecular ion fragment. Natural isotopes of bromine are responsible for doublets. Because mass spectroscopy did not identify brominated impurities in our stock supply of phenytoin (Parke, Davis and Co.), and Br-HPPH was only identified in box water which had been subjected to acid hydrolysis, it is possible that Br-HPPH is an artifact of the acid treatment, although the mechanism for this remains obscure. In contrast, all other metabolites described in this study could be identified in bile, feces, urinary bladder and contents, and box water, which had not been subjected to acid hydrolysis, being instead extracted directly into acetone prior to permethylation.††

Peak "g", in the chromatogram showing the separation of components of box water not subjected to acid hydrolysis (Fig. 7), was identified as the permethylated derivative of the glucuronide conjugate of p-HPPH. The mass spectrum of peak "g" was virtually identical to that of the permethylated glucuronide of p-HPPH published by Thompson et al. (Thompson, Gerber, Seibert, and Desiderio, 1973).

Para-HPPH was the major phenytoin metabolite found in acid-treated box water and comprised about 84% of all metabolites as judged from the peak heights in the chromatogram in Fig. 5. Minor metabolites were m-HPPH (9%), catechol (1.5%), O-methyl catechol (1.5%), and the two isomers of Br-HPPH (4%). Because the major metabolite, p-HPPH, is very soluble in ether (Maynert, 1960; Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971), one would expect large amounts of ^{14}C in the extractions with ether of box water and homogenates of frogs treated with ^{14}C-phenytoin. However, as seen in Fig. 1, most radiolabelled metabolites appeared in the extractions with acetone. Because the glucuronide of p-HPPH was present in significant amounts in box water and is much more soluble in acetone than in ether (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971), it is likely that the majority of ^{14}C in acetone was due to the glucuronide conjugate of p-HPPH.

Metabolites of phenytoin obtained from box water and tissues from the leopard frog given 100 mg/kg ^{14}C-phenytoin were identical to those from the bullfrog given 40 mg/kg ^{14}C-phenytoin. The dihydrodiol metabolite of phenytoin, which we attempted to isolate as the trimethylsilane derivative, was not found in the ether extraction of box water from either species.

TISSUE DISTRIBUTION OF PHENYTOIN

Table 1 gives the tissue distribution of phenytoin and metabolites of phenytoin in leopard frogs 3 hours after injection of 30 mg/kg. Several samples had tissue/plasma ratios (T/P) greater than 3, with that for bile being the highest (4.71), followed by kidney (4.11), pancreas (3.78), and brain (3.18, a weighted average for the 3 areas of brain sampled). Of the tissues of the nervous system which were sampled, cerebellum had the highest (3.83) and peripheral nerve the lowest (2.15) T/P, but this difference was not statistically significant ($p > 0.3$). The carcass had the greatest total amount of phenytoin (52.2% of the total radiolabel injected), followed by box water (7.8%), egg sac (7.1%), liver (2.8%), and small intestine (2.7%). Phenytoin levels in upper abdominal muscle and skin were not significantly different from those in muscle and skin of the thigh, indicating that sequestration of drug at the site of injection (ventral lymph sac) was not a significant factor governing the distribution of phenytoin.

Carcass, with a T/P of 2.69, seemed to concentrate phenytoin more than did muscle and skin. Bone, another major constituent of carcass, was not sampled in the tissue distribution experiment, but was sampled with plasma in a later experiment and the two samples were analyzed for phenytoin. Femoral bone and plasma, taken from a female leopard frog 48 hours after an injection of 100 mg/kg ^{14}C-phenytoin (0.71 μc/mg), were assayed for phenytoin as described in METHODS and the T/P for bone was found to be 1.74.

Lymph was one of the few tissues to have a T/P less than unity, the other tissue being stomach contents. Because phenytoin is highly

bound to plasma proteins in most species (Woodbury and Swinyard, 1972), the unequal distribution of phenytoin between lymph and plasma may have been due to a difference in protein content in these fluids. Concentrations of protein were measured in blood and lymph from 3 leopard frogs by the method of Lowry et al. (Lowry, Rosebrough, Farr, and Randall, 1951) and found to be 1.6 ± 0.9 gm% in lymph (± S.D.), 3.3 ± 0.6 in plasma, and 34.1 ± 5.6 gm% in erythrocytes.

Table 1 also provides concentrations of phenytoin metabolites in various tissues. Bile had the highest concentration of metabolites (1740 nmoles/gm) followed by kidney (94 nmoles/gm), fat bodies (79), egg sac (72), liver (72), and lung (58 nmoles/gm). The egg sac had the greatest total amount of metabolites (9.3% of the total ^{14}C injected), followed by carcass (9.2%), and bile (1.7%). Tissues with the lowest concentrations of metabolites were brain and contents of large intestine, with concentrations of 6.0 (a weighted average) and 1.5 nmoles/gm, respectively.

T/P values in brain 8 hours after phenytoin injection were 3.74 in cerebrum, 3.35 in optic lobes, and 4.29 in cerebellum. Since these values fall within the standard deviations for values obtained in these tissues at 3 hours, the distribution of phenytoin between brain and plasma is apparently complete 3 hours after injection.

†The derivation and use of the integrated form of the Michaelis-Menten equation are presented in Appendix V. Results of experiments which employed the integrated form of this equation in planning chronic dosing-schedules of phenytoin are given in Appendix II.

††Mass spectra of phenytoin and metabolites not presented in Manuscript II are presented in Appendix VII.

Figure 1. Phenytoin and metabolites of phenytoin extracted from bullfrog homogenate and box water. Each bullfrog was given 14 mg of ^{14}C-phenytoin (0.31 μc/mg) which resulted in doses of 35-47 mg/kg phenytoin. Each data point represents the mean of 3-4 frogs, and error bars are standard errors expressed as a percent of the total amount of phenytoin injected. ^{14}C extracted into 1-chlorobutane (BuCl) is parent drug, ether extracted the more polar metabolites, and acetone extracted the most polar metabolites. Phenytoin was eliminated from whole bullfrog with an apparent $t_{\frac{1}{2}}$ of 25.6 hrs as determined by linear regression ($r=0.9950$). Because phenytoin was added directly to frog homogenates at "zero" time, no box water data was obtained at this time. However, the amount of phenytoin in box water steadily increased over time, and the amount of phenytoin at 40 hrs was significantly greater than that present at 10 hrs ($p < 0.01$). Recovery of ^{14}C from box water plus frog homogenate at each time was 96.7 ± 7.8% (± S.D.).

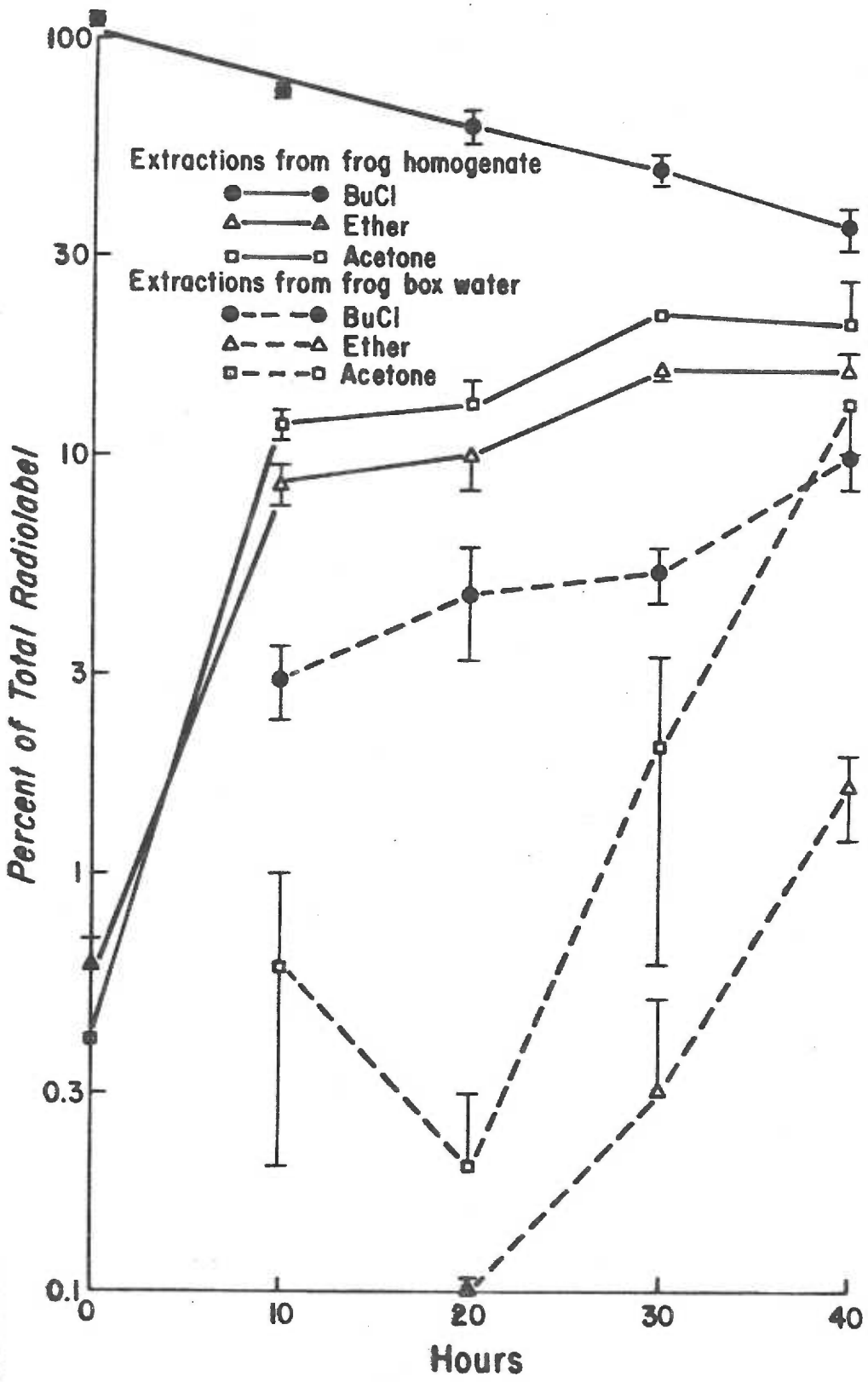
Extractions from frog homogenate
BuCl
Ether
Acetone
Extractions from frog box water
BuCl
Ether
Acetone
Percent of Total Radiolabel
100
30
10
3
1
0.3
0.1
0
10
20
30
40
Hours

Figure 2. Elimination of phenytoin from the lymph of bullfrogs. Lymph was collected from the posterior crural lymph sac at the indicated times following injection of ^{14}C-phenytoin into the ventral lymph sac. Data points represent the average of 4-5 frogs, and error bars refer to standard errors. After the exclusion of phenytoin concentrations at 1 and 2 hrs, $t_{\frac{1}{2}}$'s of phenytoin in individual frogs were determined by linear regression, and regression coefficients ranged from 0.9858-0.9992. Regression coefficients and $t_{\frac{1}{2}}$'s given in this figure are average values. The $t_{\frac{1}{2}}$ of phenytoin in frogs given 40 mg/kg was significantly greater than the $t_{\frac{1}{2}}$ in frogs given 4 mg/kg ($p < 0.05$), while the $t_{\frac{1}{2}}$'s in frogs given 4 or 0.4 mg/kg were not significantly different ($p > 0.15$). By extrapolation of elimination curves to zero time, an average volume of distribution for phenytoin of 3.5 l/kg is obtained.

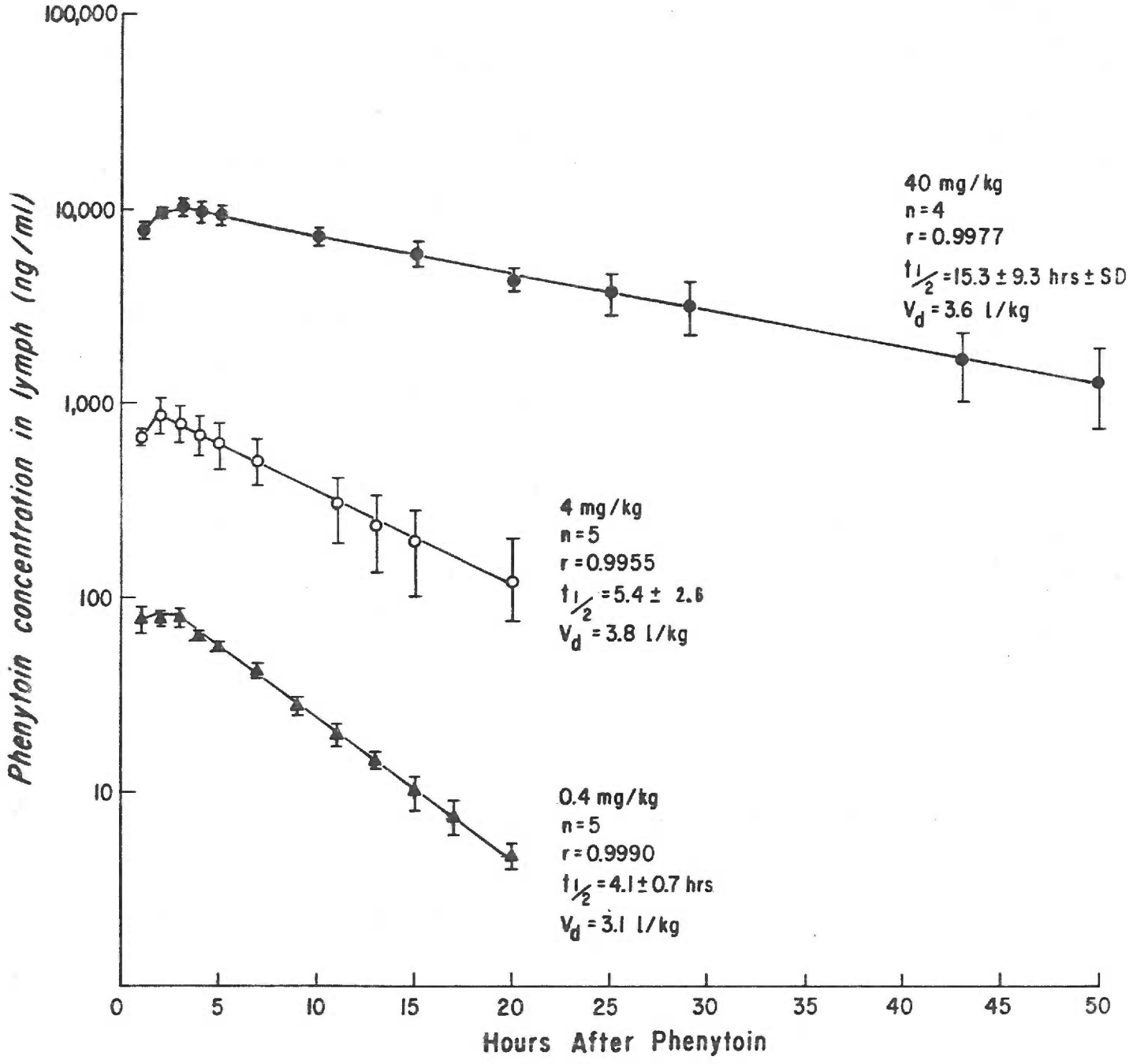
100,000
10,000
1,000
100
10
Phenytoin concentration in lymph (ng/ml)
40 mg/kg
n=4
r=0.9977
t1/2=15.3±9.3 hrs±SD
Vd=3.6 l/kg
4 mg/kg
n=5
r=0.9955
t1/2=5.4± 2.6
Vd=3.8 l/kg
0.4 mg/kg
n=5
r=0.9990
t1/2=4.1±0.7 hrs
Vd=3.1 l/kg
0
5
10
15
20
25
30
35
40
45
50
Hours After Phenytoin

Figure 3. Elimination of phenytoin from lymph of leopard frogs injected with 30 mg/kg phenytoin (3.63 μc/mg). Note the arithmetic plot. Each data point represents the mean of 5 frogs and error bars are standard errors. As determined by linear regression, phenytoin was eliminated at a constant rate of 0.43 μg/ml/hr between 3-19 hrs (r=0.9969), while between 23-43 hrs, the elimination of phenytoin followed first-order kinetics with a $t_{\frac{1}{2}}$ of 4.8 hrs (r=0.9948).

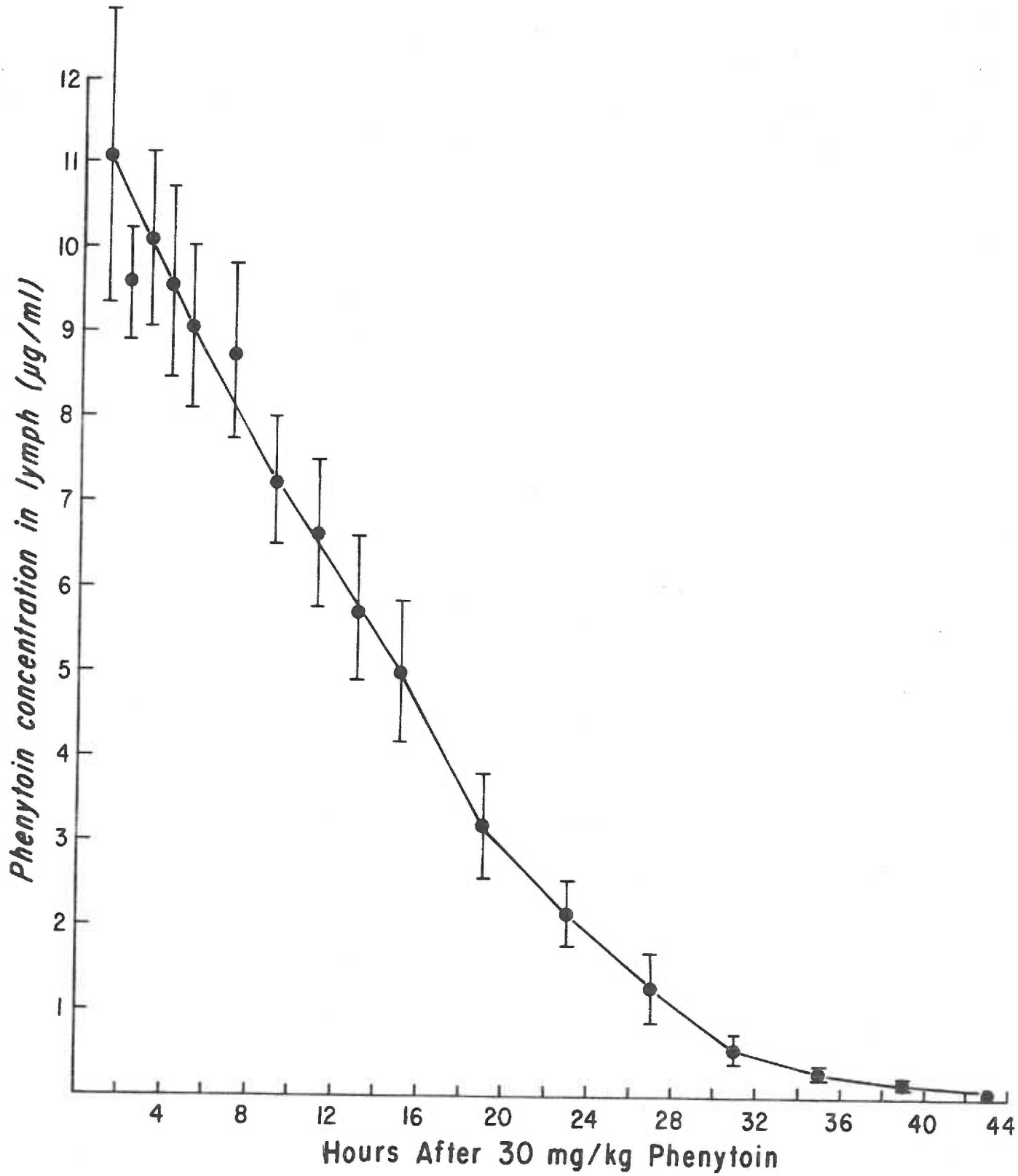
Phenytoin concentration in lymph (µg/ml)
12
11
10
9
8
7
6
5
4
3
2
1
4
8
12
16
20
24
28
32
36
40
44
Hours After 30 mg/kg Phenytoin

Figure 4. Elimination of phenytoin from lymph fitted to a form of the integrated Michaelis-Menten equation and plotted according to the method of Lundquist and Wolthers (1958). Data used in this graph were obtained from Fig. 3 with the deletion of one datum (phenytoin concentration at 2 hrs). This plot was based upon the equation:

$$\frac{s_1 - s_2}{t_2 - t_1} = -K_m \left(\frac{\ln \frac{s_1}{s_2}}{t_2 - t_1} \right) + V_{max}$$

where s_1 and s_2 represent concentrations of substrate (phenytoin) at times t_1 and t_2, respectively. $(s_1-s_2)/(t_2-t_1)$ was plotted as a function of $(t_2-t_1)^{-1}\ln(s_1/s_2)$ resulting in a line with slope $-K_m$, the negative value of the Michaelis-Menten constant, and an ordinate intercept of V_{max}, the maximum rate of the elimination of substrate (phenytoin). Coordinates for axes were derived at each experimental time interval, and s_2 and s_1 represented the higher and lower concentrations in each time interval, respectively.

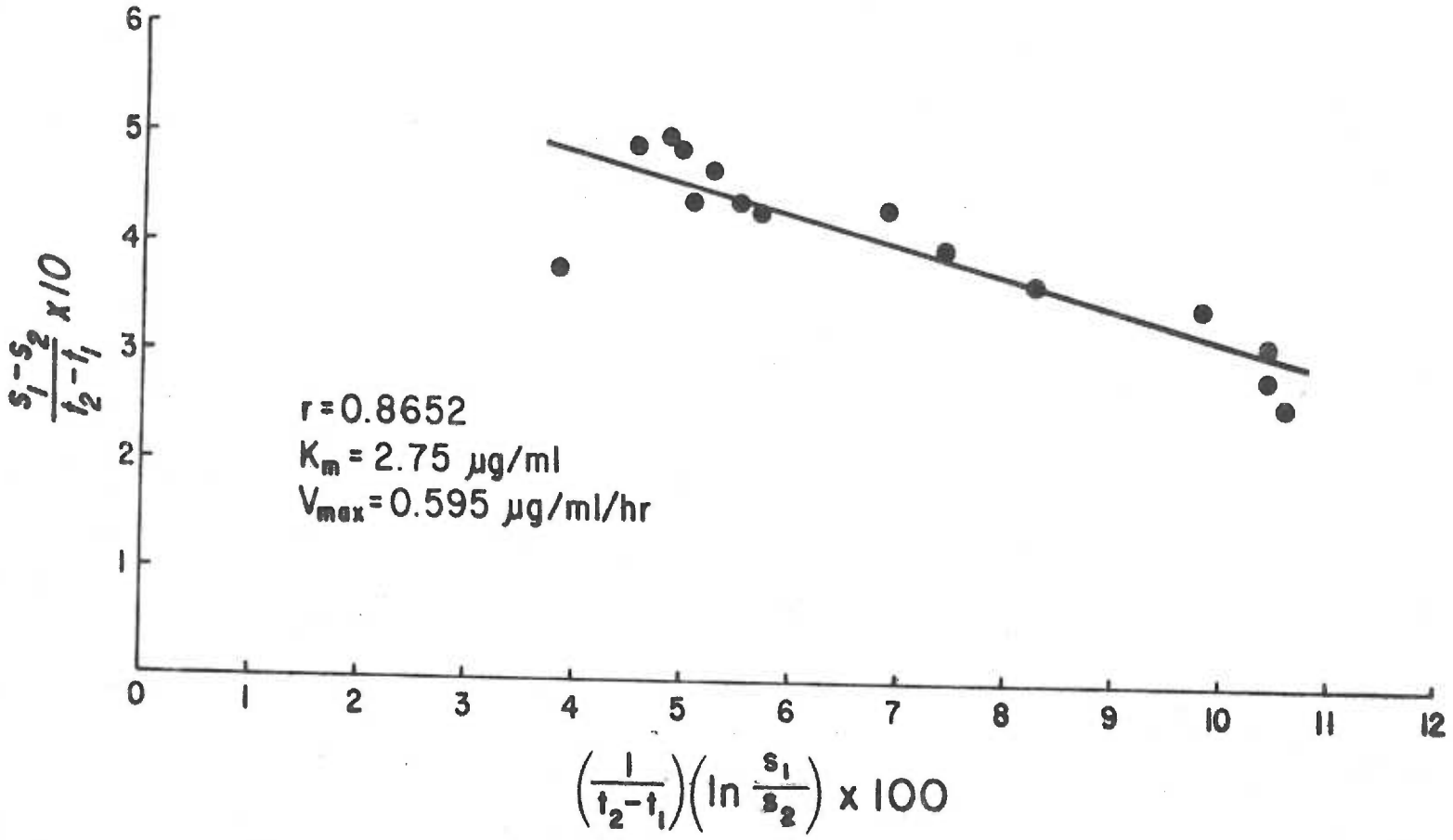
$\frac{S_1-S_2}{t_2-t_1}$ x10
r = 0.8652
K_m = 2.75 µg/ml
V_{max} = 0.595 µg/ml/hr
$\left(\frac{1}{t_2-t_1}\right)\left(\ln \frac{S_1}{S_2}\right)$ x 100

Figure 5. Gas chromatographic separation of permethylated components of box water from a leopard frog injected with 100 mg/kg phenytoin. Box water was treated with HCl to hydrolyze conjugates of phenytoin prior to permethylation. Numbers above peaks in the chromatogram refer to time after the injection of 1 µl of sample onto a 5830A Hewlett-Packard gas chromatograph equipped with a 3% OV-17 column (Pierce, Rockford, Ill.). Peak "a" was identified by mass spectroscopy as the permethylated parent compound, while peaks "b" through "d" were permethylated m-HPPH, p-HPPH, and phenytoin catechol, respectively, and peaks "e" and "f" were permethylated isomers of a brominated, hydroxylated phenytoin. Injection temperature was 260° C, flame ionization detector temperature was 400°, and the rate of carrier gas flow was 44 ml/min. Oven temperature was programed to hold at 200° for 1 min, increase at a rate of 10°/min, and hold at 320° for 5 min.

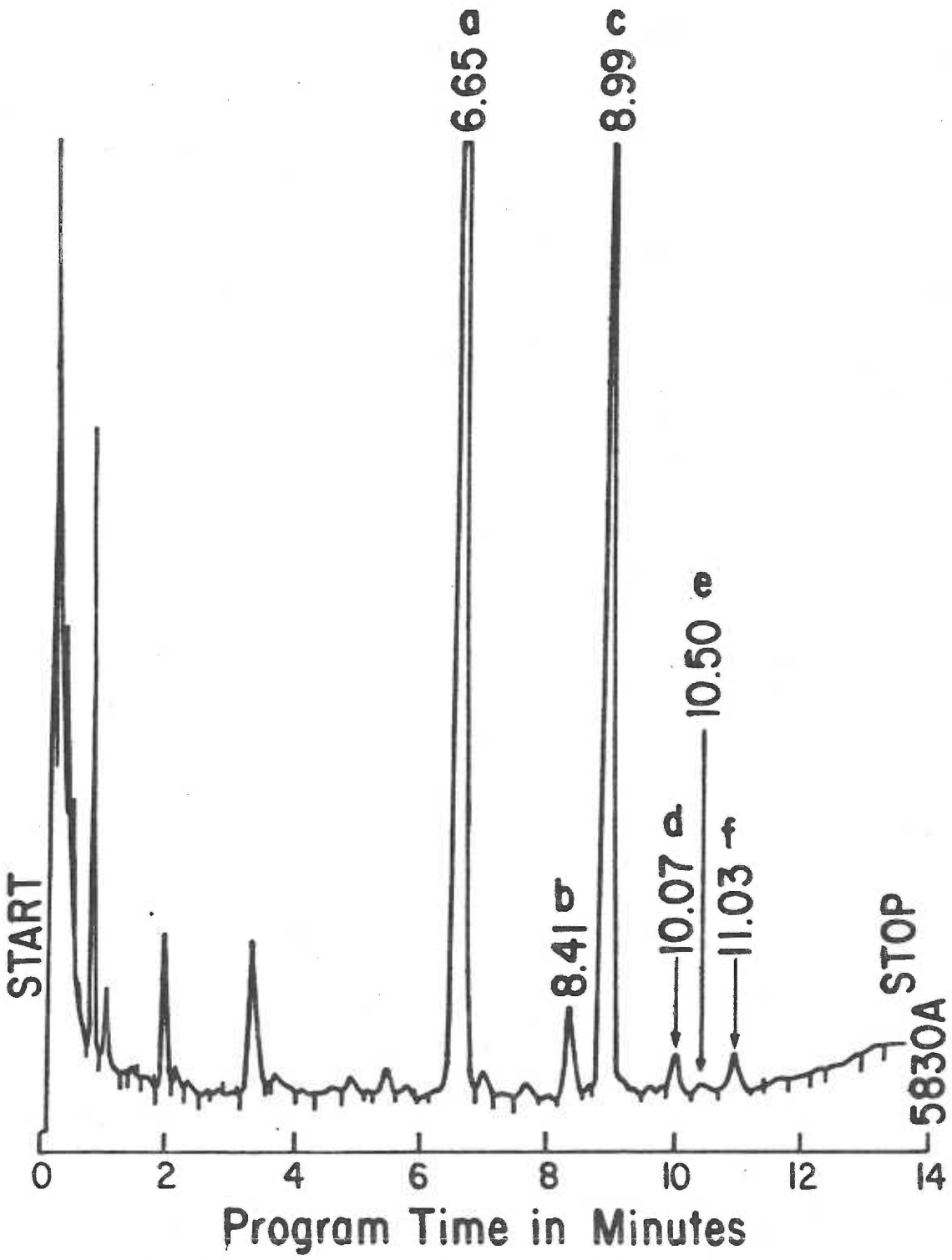
6.65 a
8.99 c
10.50 e
10.07 d
11.03 f
8.41 b
START
STOP
5830A
0
2
4
6
8
10
12
14
Program Time in Minutes

Figure 6. Mass spectrum of peak "f" in the chromatogram shown in Fig. 5. The spectrum is that of a trimethylated derivative of a bromohydroxyphenylphenylhydantoin and doublets in this spectrum (388-390, 359-361, 330-332, 302-304, and 226-228) are due to natural isotopes of bromine. Parentheses beside the molecular ion and fragment ions indicate the number of methyl groups added during derivatization as determined by perdeuteriomethylation. Experimental conditions were the same as those described in Fig. 5.

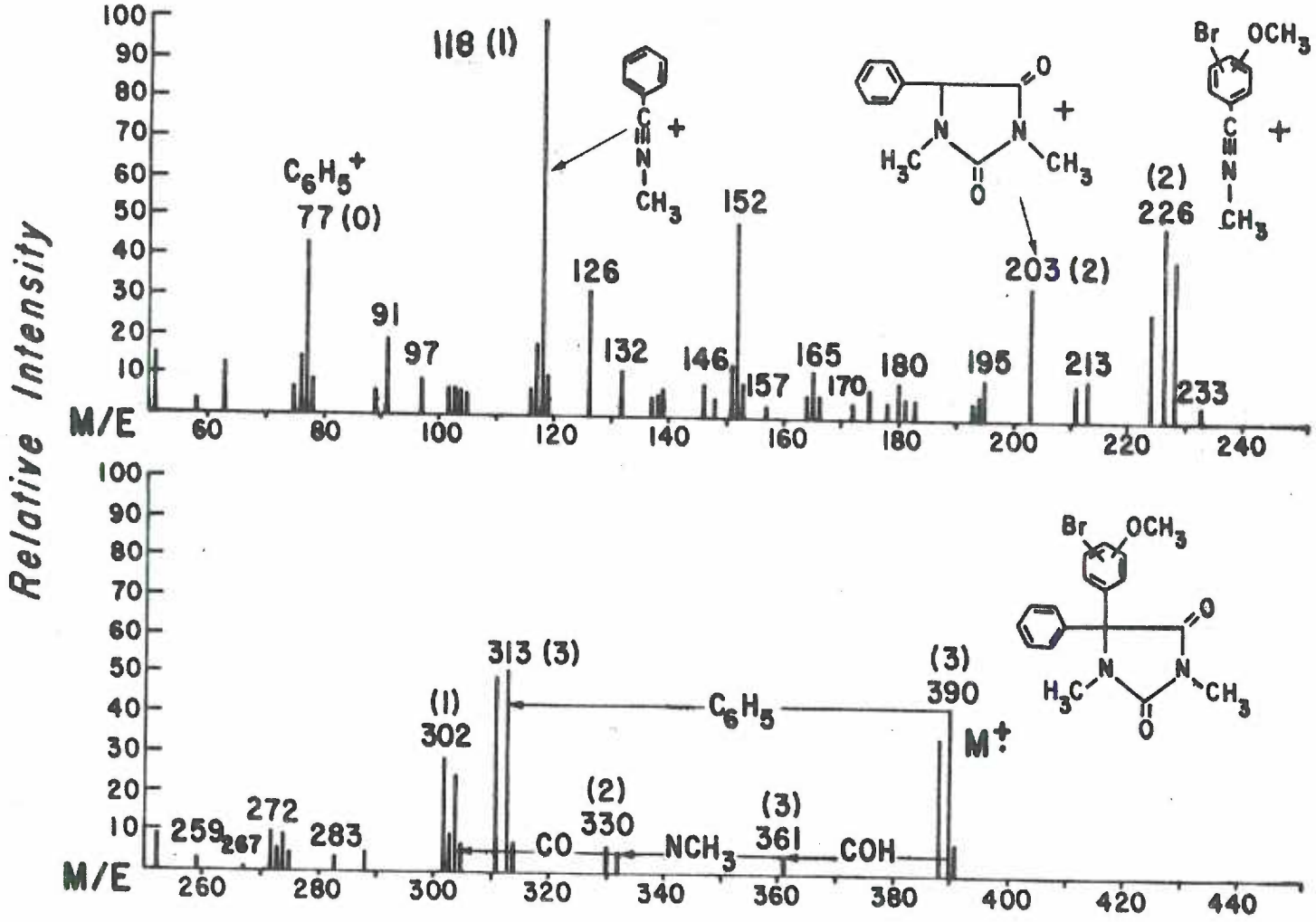
Relative Intensity
M/E
118 (1)
$C_6H_5^+$
77 (0)
91
97
126
132
146
152
157
165
170
180
195
203 (2)
213
(2)
226
233
Br
OCH_3
CH_3
313 (3)
(1)
302
(3)
390
C_6H_5
M^+
(2)
330
(3)
361
CO
NCH_3
COH
259
267
272
283

Figure 7. Gas chromatographic separation of permethylated components of box water from a leopard frog injected with 100 mg/kg phenytoin. Components of box water were extracted into acetone without being subjected to acid hydrolysis, and peak "g" in this chromatogram was identified by mass spectroscopy as the permethylated derivative of the glucuronide conjugate of p-HPPH. Except for the use of a 1½% OV-17 column, the experimental conditions were the same as those explained in Fig. 5.

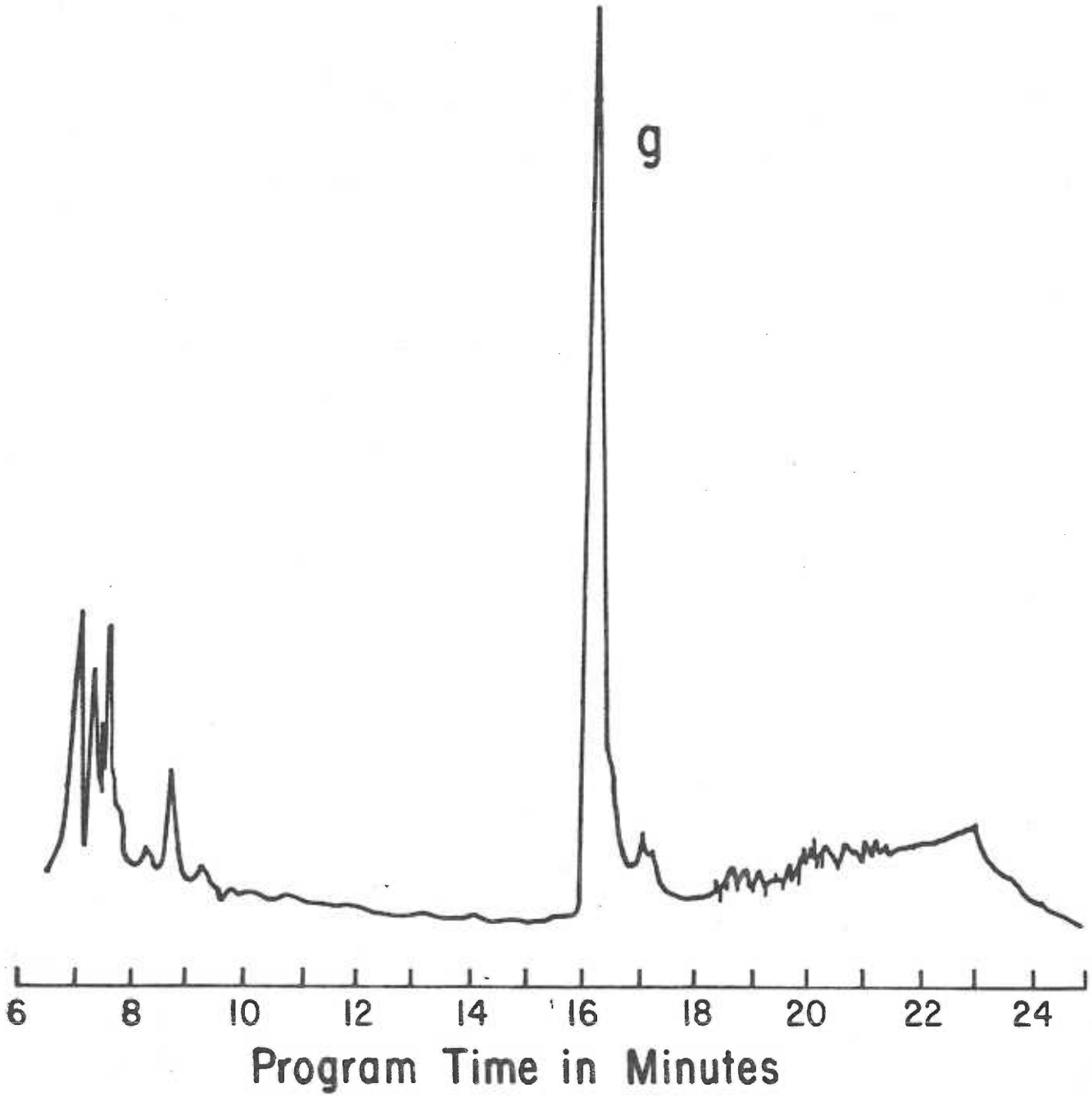
g
6
8
10
12
14
16
18
20
22
24
Program Time in Minutes

Table 1. Tissue distribution of phenytoin and metabolites of phenytoin in female leopard frogs 3 hrs after receiving 30 mg/kg phenytoin (4.44 μc/mg). Each entry is the mean ± S.E. from 3 frogs with the exception of entries for the cerebellum (n=2) and data in parentheses (n=1). An average of 6964 ± 402 nmoles (± S.E.) of phenytoin was injected into each frog and 76.8% of the total ^{14}C was extracted into 1-chlorobutane (representing parent drug), while 26.6% was extracted into acetone (metabolites of phenytoin). The average recovery of ^{14}C was 103.4%.

TISSUE	PHENYTOIN		METABOLITES	
	Nanomoles per gram Tissue	Tissue/Plasma Ratio	Nanomoles per gram Tissue	Tissue Weight (gm)
Bile	138.3 ± 39.2	4.71 ± 2.15	1740 ± 1110	0.067 ± 0.024
Brain: Cerebellum	136.8 ± 29.8	3.83 ± 0.95	15.5 ± 11.9	0.009 ± 0.001
Cerebrum	109.8 ± 17.7	3.78 ± 1.61	7.5 ± 2.8	0.047 ± 0.008
Optic Lobes	82.8 ± 13.7	2.59 ± 0.58	3.6 ± 1.4	0.058 ± 0.009
Carcass	80.3 ± 4.7	2.69 ± 0.67	14.1 ± 5.8	45.3 ± 7.33
Egg Sac	55.1 ± 9.2	1.80 ± 0.65	72.0 ± 44.9	9.00 ± 0.91
Erythrocytes	52.5 ± 14.3	1.52 ± 0.01	19.2 ± 8.9	0.24 ± 0.07
Fat Bodies	93.4 ± 18.8	2.86 ± 0.62	79.2 ± 43.0	0.065 ± 0.048
Gall Bladder	(158.7)	(2.75)	(49.4)	(0.014)
Heart	87.4 ± 7.3	2.75 ± 0.53	40.2 ± 16.1	0.185 ± 0.008
Kidney	124.3 ± 10.7	4.11 ± 1.26	93.5 ± 27.0	0.225 ± 0.026
Large Intestine	63.9 ± 17.3	1.95 ± 0.63	11.0 ± 5.3	0.17 ± 0.06
Large Int. Cont.	(98.9)	(1.71)	(1.5)	(0.20)
Liver	89.7 ± 22.5	2.61 ± 0.39	71.6 ± 10.7	2.17 ± 0.31
Lung	70.5 ± 20.0	2.06 ± 0.29	57.7 ± 21.2	0.53 ± 0.14
Lymph	28.5 ± 5.0	0.90 ± 0.10	8.0 ± 1.6	0.20 ± 0.01
Muscle, Abdominal	48.6 ± 34.6	1.06 ± 0.59	10.1 ± 6.1	0.57 ± 0.10
Muscle, Thigh	57.4 ± 22.4	1.56 ± 0.15	36.1 ± 16.4	0.39 ± 0.04
Nerve, Peripheral	72.3 ± 19.8	2.15 ± 0.64	18.0 ± 14.6	0.056 ± 0.012
Pancreas	116.8 ± 12.7	3.78 ± 1.01	34.8 ± 23.2	0.042 ± 0.008
Plasma	35.4 ± 11.3	1.00	10.9 ± 1.7	0.49 ± 0.12
Skin, Abdominal	83.5 ± 73.4	1.61 ± 1.41	8.5 ± 2.1	0.30 ± 0.05
Skin, Thigh	46.8 ± 35.3	1.00 ± 0.62	10.2 ± 4.3	0.20 ± 0.02
Small Intestine	45.8 ± 15.0	1.30 ± 0.18	23.9 ± 9.4	4.15 ± 0.28
Spleen	49.9 ± 39.1	1.05 ± 0.70	44.4 ± 19.3	0.043 ± 0.008
Stomach	35.3 ± 11.7	0.99 ± 0.05	18.0 ± 6.8	0.91 ± 0.03
Stomach Cont.	(37.4)	(0.65)	(4.2)	(0.20)
Water, Frog Box	3.2 ± 0.8	0.10 ± 0.03	0.4 ± 0.1	169 ± 59

DISCUSSION

Dose or concentration-dependent elimination of phenytoin, first discovered in dogs (Dayton et al., 1967) and subsequently in mice (Gerber and Arnold, 1969), rats (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971), and man (Arnold and Gerber, 1970), has, as a result of this study, also been shown in bullfrogs and leopard frogs. Also, the integrated form of the Michaelis-Menten equation, which has been used to describe accurately the concentration-dependent elimination of phenytoin from rats and man (Gerber and Wagner, 1972), was shown in this communication to fit accurately the data for the elimination of phenytoin from leopard frog lymph. Moreover, the Michaelis-Menten constant V_{max} for the elimination of phenytoin from the frog, 0.60 µg/ml/hr, was similar to values for V_{max} reported for adult man, which ranged from 0.25-0.68 (Gerber and Wagner, 1972; Eadie, Tyrer, Bochner, and Hooper, 1976; Garrettson and Jusko, 1975). Although the K_m for the elimination of phenytoin from frog lymph, 2.8 µg/ml, was somewhat smaller than values of K_m reported for the elimination of PHT from human plasma or whole blood, 3.6-14.4 µg/ml (Gerber and Wagner, 1971; Garrettson and Jusko, 1975; Eadie, Tyrer, Bochner, and Hooper, 1976), this difference can be attributed partially to the finding that the concentration of phenytoin in lymph is less than in plasma or whole blood.

Two mechanisms have been postulated to account for the non-first-order elimination kinetics of phenytoin: inhibition of enzymes(s) by metabolic products (Ashley and Levy, 1972) and saturation of enzymes(s)

by the substrate (Dayton, Cucinell, Weiss, and Perel, 1967; Eadie, Tyrer, Bochner, and Hooper, 1976; Albert, Hallmark, Sakmar, Weidler, and Wagner, 1974). However, regardless of the mechanism responsible for the concentration-dependent elimination of phenytoin, interpretations of these data in terms of enzyme kinetics are limited because V_{max} and K_m are composite constants, encompassing all mechanisms of elimination and/or metabolism of phenytoin. But while theoretical interpretations are limited, a knowledge of the values for V_{max} and K_m can be of significant practical importance. For example, Michaelis-Menten constants determined in man have been utilized in planning more effective chronic dosing schedules for therapeutically administered phenytoin (Chiba, Ishizaki, Miura, and Minagawa, 1980), and we have utilized values of V_{max} and K_m in planning dosing schedules for the chronic administration of phenytoin to frogs.

When the concentration of phenytoin is below the K_m, elimination proceeds by first-order kinetics, and under these circumstances, the average $t_{1/2}$ in bullfrogs and leopard frogs is 4.8 ± 0.7 hrs (± S.D.). This first-order $t_{1/2}$ compares favorably with that of 5 hrs in mice (Gerber and Arnold, 1969), but is much longer than the $t_{1/2}$ of 0.6 hrs in rats (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971). In contrast, the first-order $t_{1/2}$ for phenytoin in man is much longer than that in frogs, being between 15-24 hrs (Glazko, Chang, Baukema, Dill, Goulet, and Buchanan, 1969; Arnold and Gerber, 1970; Dill, Baukema, Chang, and Glazko, 1971), calculated subsequent to the acceptance of an average K_m of 5.3 μg/ml in man (Jusko, 1976).

Despite the elimination of phenytoin at concentrations well above the K_m of 2.8 µg/ml in frogs, Fig. 2 shows the curve describing the elimination of 40 mg/kg phenytoin to be apparently linear as plotted semi-logarithmically. The apparent linearity of this curve, implying a first-order process, is deceiving because the "half-lives" for elimination differ with the dose administered. In comparing elimination curves for 40 mg/kg phenytoin measured in lymph (Fig. 2) and whole bullfrog (Fig. 1), another apparent discrepancy may be noted, in that the "half-life" for phenytoin in whole bullfrog (2[illegible].6 hrs) is longer than the "half-life" in lymph (15.5 ± 9.3 hrs, ± S.D.). This difference in "half-lives" is probably not significant and may be due to a large variation in values of V_{max} for individual frogs which would cause large variations in the rates by which frogs eliminate phenytoin at concentrations above the K_m. The variability in "half-lives" obtained during a dose-dependent process may have been accentuated in the whole bullfrog experiment (Fig. 1) because, in an effort to administer the same amount of ^{14}C to each animal, frogs were given doses which ranged from 35-47 mg/kg phenytoin.

The average volume of distribution (V_d) for phenytoin in bullfrogs was apparently not a function of the order of the kinetics by which elimination proceeded, because all 3 elimination curves in Fig. 2 gave approximately the same V_d for phenytoin, 3.5 l/kg. However, the V_d for phenytoin in bullfrog is considerably larger than V_d's in mammals, such as the rat with a V_d of 1.0-1.1 l/kg (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971; Noach, Woodbury, and Goodman, 1958), dog, with a V_d of 0.5-1.5 l/kg (Dayton, Cucinell, Weiss, and Perel, 1967),

and man, with a V_d of 0.7-0.8 l/kg (Glazko, Chang, Baukema, Dill, Goulet, and Buchanan, 1969; Garrettson and Jusko, 1975). Because phenytoin was less concentrated in lymph than in plasma or whole blood, which were used to measure phenytoin concentration in mammals, this contributed in part to the large V_d in frogs. But probably of greater importance are the facts that a high percentage of phenytoin is normally bound to plasma proteins (Woodbury and Swinyard, 1972) and in the frog, concentrations of protein in lymph (1.6 gm%) and plasma (3.3 gm%) are relatively low compared to 6-8 gm% normally found in human plasma. Therefore, the large V_d in the frog may be due to a distribution of phenytoin away from the protein-poor media of lymph and plasma and into protein-rich tissues such as carcass, which comprised 68% of total body weight and had a tissue/plasma ratio (T/P) for phenytoin of 2.69, and egg sac, which in female frogs can comprise 14% of body weight with a T/P of 1.80. Although protein binding studies were not performed, a low retention of phenytoin by frog plasma is suggested by the fact that the T/P for phenytoin in frog erythrocytes (1.5) is much higher than the values, less than unity, obtained in man and rat (Borondy, Dill, Chang, Buchanan, and Glazko, 1973), and an inverse relationship between percent of phenytoin bound to plasma proteins and the T/P for phenytoin in erythrocytes has been reported (Borondy, Dill, Chang, Buchanan, and Glazko, 1973).

Relative concentrations of phenytoin and metabolites in tissues probably reflect the chemical composition and/or biological function of these tissues. Phenytoin concentrations were especially high in the CNS, with an average T/P of 3.18, and were highest in cerebellum

(T/P = 3.83). Although the high lipid/water partition coefficient for phenytoin might be a factor governing the accumulation of phenytoin in the nervous system, Firemark et al. suggested the binding of phenytoin to non-lipid constituents might be the most important factor (Firemark, Barlow, and Roth, 1963). High concentrations of phenytoin in cerebellum relative to other CNS structures have been reported in rats (Westmoreland and Bass, 1971) and cats (Kokenge, Kutt, and McDowell, 1965) following chronically administered phenytoin, and phenytoin has recently been shown to concentrate preferentially in a Purkinje cell-enriched fraction of rat cerebellum (Savolainen, Iivanainen, Elovaara, and Tannisto, 1980). It has been suggested that the accumulation of phenytoin in the cerebellum may cause the cerebellar-related toxicities of phenytoin (Kokenge, Kutt, and McDowell, 1965). However, other investigators have found no preferential accumulation of phenytoin in cerebella from acutely treated rats (Leppik and Sherwin, 1979) and cats (Firemark, Barlow, and Roth, 1963) or in chronically treated dogs and cats (Nakamura, Masuda, Nakatsuji, and Hiroka, 1966), and the high T/P for phenytoin in frog cerebellum (Table 1) was not significantly different from T/P's in other sampled areas of the CNS.

The accumulation of phenytoin in the frog liver (T/P = 2.61) and the extremely high concentration of phenytoin metabolites in bile (1740 nmoles/gm vs 11 nmoles/gm in plasma) suggests phenytoin is taken up by the liver where it is metabolized and secreted into bile. This pattern was first observed by Noach et al. (1958) in the rat, and the predominant role of the mammalian liver in the metabolism of phenytoin

has since become well established (Kutt and Verebely, 1970; Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971; Chang and Glazko, 1972).

The concentration of phenytoin in frog kidney (T/P = 4.11) was 2-5 times higher than levels reported in kidneys from rats (Dill, Kazenko, Wolf, and Glazko, 1956; Noach, Woodbury, and Goodman, 1958) and rabbits (Hine and Kozelka, 1941). This may reflect the fact that the adrenal gland, which is not readily separable from the kidney in the frog as it is in mammals, was assayed with the kidney. Adrenal glands in dogs and cats accumulate high concentrations of phenytoin, and this has been suggested as a factor in the development of hirsutism, an adverse effect of phenytoin in man (Nakamura, Masuda, Nakatsuji, and Hiroka, 1966). Accumulation with subsequent secretion of phenytoin metabolites by the kidney into urine probably accounts for the high concentration of metabolites in the frog kidney (94 nmoles/gm).

Pancreatic accumulation of phenytoin which was significant in the frog (T/P = 3.78) has also been reported in pancreases from dogs and cats (Nakamura, Masuda, Nakatsuji, and Hiroka, 1966) and may be due to the trapping of phenytoin, a weak acid, in the alkaline medium of the pancreas. Conversely, the low T/P for phenytoin in stomach contents (T/P = 0.65) may be due to the low solubility of phenytoin in an acidic, aqueous medium.

Para-HPPH and the glucuronide of p-HPPH, found to be the major metabolites of phenytoin in bullfrogs and leopard frogs, are also the major metabolites in man (Butler, 1957; Maynert, 1960) and in many other mammals (Chang and Glazko, 1972). Meta-HPPH, a minor metabolite in frogs, was shown by Atkinson et al. to be also a minor metabolite in

man, but the major metabolite in the dog (Atkinson, MacGee, Strong, Garteiz, and Gaffney, 1970). Other minor metabolites in the frog, the catechol and O-methyl catechol of phenytoin, were first identified in 1972, the catechol being identified in the urine from rats (Chang, Okerholm, and Glazko, 1972b) and man (Borgå, Garle, and Gutová, 1972), and the O-methyl catechol being identified in rat urine (Chang, Okerholm, and Glazko, 1972a). Another minor metabolite, the dihydrodiol of phenytoin, has been identified in the urine from rats, monkeys (Chang, Savory, and Glazko, 1970), and man (Horning, Stratton, Wilson, Horning, and Hill, 1971), but was not identified as a metabolite made by the frog. However, we do not conclude the frog does not make this metabolite, as the dihydrodiol is a likely intermediate in the formation of the catechol of phenytoin (Glazko, 1973) which was produced by the frog.

The two isomers of the brominated metabolite of HPPH (Br-HPPH) found in box water from frogs have never been reported. Indeed, bromination of xenobiotics by vertebrates is extremely rare with the bromination of phthalein dyes (Burger and Loo, 1959; Loo, Burger, and Adamson, 1963) and fluoresceins (Adamson and Burger, 1966) by the pregnant uterus of the spiny dogfish (Squalus acanthias) the only cases which appear in the literature. Because Br-HPPH could only be isolated from samples which had been subjected to hydrolysis with HCl (containing trace elements including Br_2), we suggest Br-HPPH may have been produced artifactually.

The ability of frogs to metabolize drugs and other xenobiotics has by no means been taken for granted by researchers, and several

metabolic processes known to occur in man have not been demonstrated in frogs. For example, intact frogs or preparations of frog liver (Rana pipiens, R. catesbiana, R. esculenta, R. temporaria, or R. ridibunda) failed to metabolize p-nitrobenzoic acid to p-aminobenzoic acid indicating a lack of nitro-reductase activity (Adamson, Dixon, Francis, and Rall, 1965), did not acetylate sulfonamides (Failey, Anderson, Henderson, and Chen, 1943), and failed to hydroxylate hexobarbital, hydrolyze p-ethoxyacetanilide, or demethylate aminopyrine and N-methylaniline (Brodie, Maickel, and Jondorf, 1958). Also, it was reported that frogs were unable to metabolize common drugs such as phenacetin, acetanilide, chlorpromazine (Brodie and Maickel, 1962), and amphetamine (Gaudette, Maickel, and Brodie, 1958). These and other data prompted Brodie and Maickel (1962) to conclude that frogs were unable to oxidize lipid-soluble drugs. However, there exists a large body of data which indicate that frogs are indeed capable of oxidizing xenobiotics. Frogs have been reported to hydroxylate benzene (Tschernikow, Gadaskin, and Gurewitsch, 1930), chlorobenzene (Gessner and Smith, 1960), biphenyl (Creaven, Parke, and Williams, 1965a), coumarin (Creaven, Parke, and Williams, 1965b) and Δ^9-tetrahydrocannabinol (Karler, Cely, and Turkanis, 1974), and to desulfurate parathion to paraoxon (Potter and O'Brien, 1964). Other biotransformations of xenobiotics by frogs have been reported, including azo-reduction (Adamson, Dixon, Francis, and Rall, 1965), O-dealkylation (Creaven, Davies, and Williams, 1967), N-demethylation (Gutman and Kidron, 1971), sulfuration (Smith, 1964), ester hydrolysis (Wayson, Downes, Lynn, and Gerber, 1976b), and the conjugation of

aromatic alcohols with sulfate and glucuronic acid (Maickel, Jondorf, and Brodie, 1958; DeMeio, 1945). Although several investigators found that frogs metabolized lipid-soluble xenobiotics at rates slower than in mammals (Creaven, Davies, and Williams, 1967; Gutman and Kidron, 1971; Wayson, Downes, Lynn, and Gerber, 1976a and 1976b), the conclusion of Brodie and Maickel (1962) was clearly based upon insufficient information. That the frog is capable of oxidizing and otherwise biotransforming xenobiotics is clear from the literature cited and from our own results with phenytoin.

In summary, the results of this study show that the elimination of phenytoin by frogs is a concentration or dose-dependent process similar to that found in man and many other mammals. Also, the distribution of phenytoin among tissues was similar to that found in mammals, although a relatively large volume of distribution for phenytoin was found in the frog, and was probably a result of low concentrations of protein in lymph and plasma. Major metabolites of phenytoin made by the frog, p-HPPH and the glucuronide of p-HPPH, as well as minor metabolites (m-HPPH, catechol of phenytoin, and O-methyl catechol of phenytoin) were the same as those made by man and many other mammals. Therefore, it is concluded that the disposition of phenytoin by frogs is not significantly different than that reported for man. It is suggested that this study may be useful in providing background information upon which future studies of the disposition of other drugs in the frog may be based.

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MANUSCRIPT III

RELATIONSHIPS BETWEEN THE ANTAGONISM OF ELECTRICALLY-INDUCED MAXIMAL SEIZURES BY PHENYTOIN AND CNS LEVELS OF ADENOSINE 3',5'-MONOPHOSPHATE AND GUANOSINE 3',5'-MONOPHOSPHATE IN FROGS AND MICE

ABSTRACT

Phenytoin antagonized the electroshock-induced increase in levels of adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) in cerebrum and cerebellum, respectively, from CF #1 mice. However, the effective dose range of phenytoin for significant reduction of the elevated levels of cAMP and cGMP was 2-5 times higher than that for prevention of tonic hindlimb extension (THE) in 95% of mice. The effective dose range of phenytoin for preventing tonic flexion and clonus was nearer to that for alteration of cyclic nucleotide levels, but these endpoints have less relevance to anticonvulsant efficacy than does the classic endpoint, prevention of THE. Also, the greatest reduction in cyclic nucleotide levels occurred at a dose (100 mg/kg) which produced toxic signs in mice. Quaking mice (qk/qk), a mutant strain which exhibits spontaneous seizures, did not have abnormal levels of cAMP or cGMP in cerebrum or cerebellum, and a dose of phenytoin (15 mg/kg) which abolished all seizure activity did not alter levels of these cyclic nucleotides. In frogs, the electroshock-associated increase in levels of cAMP and cGMP in the CNS was not altered by phenytoin even when the doses administered were up to twice the ED 95 for prevention of THE. Since these data from mice and frogs show that the anticonvulsant effect of phenytoin is dissociated, by dose, from effects on CNS cyclic nucleotide levels, it is doubtful that the alteration of cyclic nucleotide levels is a mechanism by which phenytoin exerts its anticonvulsant effect.

INTRODUCTION

The discovery that electrically and chemically-induced seizures were associated with large increases in CNS levels of adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) (Sattin, 1971; Lust, Passonneau, and Goldberg, 1972; Mao, Guidotti, and Costa, 1974; Lust, Goldberg, and Passonneau, 1976; Berti, Bernareggi, Folco, Fumagelli, and Paoletti, 1976; Folbergrova, 1975) prompted speculation about the relationships between cyclic nucleotides and seizures. Because many anticonvulsant drugs antagonize the rise in levels of cAMP and cGMP associated with seizures (Folbergrova, 1975; Lust, Kupferberg, Yonekawa, Penry, Passonneau, and Wheaton, 1978; McCandless, Fuessner, Lust, and Passonneau, 1979; Folbergrova, 1980), it has been suggested that the regulation of cyclic nucleotide levels may be a mechanism by which anticonvulsant drugs exert their effect (Ferrendelli and Kinschert, 1977b; McCandless, Fuessner, Lust, and Passonneau, 1979; Ferrendelli, 1980a; Ferrendelli, 1980b). However, most studies attempting to correlate anticonvulsant effects with changes in levels of cyclic nucleotides employed only a single dose of an anticonvulsant drug; one which successfully antagonized manifestations of the seizure, but was in excess of the minimum dose needed to produce maximal anticonvulsant effects. Thus, without demonstrable concordance of the dose-effect relationships for control of seizures and for alteration of cyclic nucleotide levels in the CNS, the viability of the proposed mechanism for anticonvulsant action has not been convincingly demonstrated. To investigate the proposed mechanistic

link between the control of seizures and regulation of cyclic nucleotide levels, we first established the effective dose range of phenytoin for the prevention of electroshock-induced tonic hindlimb extension (THE), the classic endpoint used to assay anticonvulsant efficacy, in frogs and mice. We then examined the effects of a broad range of phenytoin doses on levels of cAMP and cGMP in the central nervous systems (CNS) of these animals, before and after electroshock. Quaking mice (qk/qk), a myelin-deficient mutant strain which seize spontaneously (Sidman, Dickie, and Appel, 1964), were also used in this study, and the effects of phenytoin on seizure activity and CNS levels of cAMP and cGMP were examined. Our results reveal no correlation between doses of phenytoin effective in preventing THE and doses needed to alter levels of cyclic nucleotides.

METHODS

ANIMALS AND DRUGS

Rana pipiens (northern variety) of both sexes (20-40 gm) were obtained from William Lemberger Assoc. (Germantown, Wisc.) and housed in plastic containers tipped at one end to allow for dry and wet areas with continuously flowing tap water. During an experiment, frogs were placed in individual plastic boxes with enough tap water so the frog was exposed to water at all times. Mice of both sexes from the CF #1 strain (20-40 gm) were obtained from Charles River (Wilmington, Mass.), while female quaking mice (17-20 gm) were obtained from Jackson Labs (Bar Harbor, Me.). Mice were fed mouse chow obtained from O.S.U. Mills (Corvallis, Oreg.) and water ad libidum and housed in wire cages in a room supplied with 12 hours light and 12 hours dark. Sodium phenytoin, obtained from Parke, Davis and Co., was dissolved in an aqueous NaOH solution (pH 12.6) and administered subcutaneously to mice and injected into the ventral lymph sac of frogs. Whenever possible, the volume of material injected was less than 0.5% of body weight.

ELECTRICALLY-INDUCED MAXIMAL SEIZURES

Electrically-induced maximal seizures (maximal electroshock seizures, MES), defined as seizures in which tonic hindlimb extension (THE) is present, were elicited by the technique of Woodbury and Davenport (1952). Current (60 Hz), generated by a Wahlquist electroshock device (Wahlquist Instrument Co., Salt Lake City, Utah), was delivered by corneal electrodes coated with Redux Creme^{R} (Hewlett Packard) to facilitate contact with cornea. Electroshock parameters,

designed to produce THE in 95% of control animals, were 24 mA for a duration of 0.2 sec in mice, and 122 mA for a duration of 0.5 sec in frogs. All electroshock testing was done 3 hrs after the injection of phenytoin or phenytoin-solvent, this being the time of peak anticonvulsant effect for phenytoin with the respective routes of administration in frogs (Johnson and Riker, 1979) and mice (Swinyard, Brown and Goodman, 1952).

ANALYSIS OF CYCLIC NUCLEOTIDES

At appropriate times during an experiment, animals were sacrificed by immersion in liquid nitrogen and stored at -70° C. While still frozen, specific regions of the CNS were dissected in a room maintained at 4° C. Samples were added to 2 ml of ice cold 6% TCA, homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y.) and centrifuged at 1000 x g for 20 min. The supernatant was decanted and the pellet was dissolved in 1 N NaOH for the determination of protein concentration using the method of Lowry et al. (Lowry, Rosebrough, Farr, and Randall, 1951). TCA was removed by extracting it into 3 volumes of water-saturated ether (4 ml per volume) after the addition of 0.1 ml of 1 N HCl. The supernatant was freeze-dried in a Lab-Con-Co lyophilizer and the residue was dissolved in TRIS buffer (50 mM TRIS with 4 mM EDTA, pH 7.5).

Concentrations of adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) in reconstituted supernatants were determined using analytical kits supplied by Amersham Corp. (Arlington Heights, Ill.). Cyclic AMP was assayed by a competitive protein

binding assay developed by Gilman (1970), while cGMP was assayed by RIA as developed by Steiner et al. (Steiner, Parker, and Kipnis, 1972). 95-100% of exogenous cAMP and cGMP added to 40 mg BSA (Sigma Chemical Co., St. Louis, Mo.) was recovered following homogenization in TCA, centrifugation, ether washing, drying, and reconstitution in TRIS buffer. Percent recovery of exogenous cyclic nucleotides was used to adjust the levels of endogenous cAMP and cGMP recovered from tissue samples.†

†Detailed descriptions of assays of cyclic nucleotides and protein are provided in Appendix VIII.

RESULTS

FROGS

Cyclic GMP levels in whole brains from control frogs (Fig. 1) reached their peak elevation, 70% above control, 10 sec after electroshock. At this time the frogs exhibited tonic hindlimb extension (THE) or had just finished THE. The maximal increase in cAMP levels was only 18% above control levels and occurred 30 sec after electroshock, a time at which the frogs displayed post-ictal depression. Figure 1 also shows that phenytoin (40 mg/kg) did not significantly alter levels of cAMP or cGMP in whole brain before or after electroshock, compared to control levels. It is important to stress that 40 mg/kg of phenytoin is an effective anticonvulsant dose, preventing the occurrence of THE in 88% of intact frogs (Fig. 2).

To exclude the possibility that whole brain assay might have obscured significant phenytoin effects on cyclic nucleotide levels in discrete regions of the CNS, cAMP and cGMP were assayed in pooled samples of olfactory bulbs, cerebrum, optic lobes, cerebellum/medulla, and spinal cord obtained from 13 groups of frogs (8 frogs/group) treated with 5, 10, 20, 40, 80, or 160 mg/kg phenytoin, or phenytoin-solvent. Basal levels of cAMP, which were highest in olfactory bulb (7.0 pmoles/mg protein) and lowest in spinal cord (2.8 pmoles/mg), and basal levels of cGMP, which were highest in olfactory bulb (280 fmoles/mg) and lowest in cerebrum (200 fmoles/mg), were not altered by threshold anticonvulsant to lethal doses of phenytoin (5-160 mg/kg). Furthermore, 10 sec after electroshock, elevated levels

of cyclic nucleotides in these regions were not affected by anti-convulsant doses of phenytoin (5-80 mg/kg). Of the five CNS areas sampled, electroshock produced the highest level of cGMP in the cerebrum (360 fmoles/mg, an 83% increase over control level), while the level in olfactory bulb actually fell 18% from the control level. Levels of cAMP in the five areas of CNS were not significantly altered 10 sec after electroshock.

CF #1 MICE

Maximally elevated levels of cGMP (630% above basal level) and cAMP (35% over basal level) in the whole murine brain were reached 30 sec after electroshock (Fig. 3), a time at which intact mice exhibited post-ictal depression. Figure 3 also shows that 10 mg/kg phenytoin did not significantly alter whole brain levels of cGMP or cAMP before or after electroshock despite the finding that this dose of phenytoin prevents the occurrence of THE in 90% of intact mice (Fig. 4).

To determine whether phenytoin produced dose-related alterations in cyclic nucleotide levels in cerebrum or cerebellum, these brain regions were assayed for cGMP and cAMP before, and 30 sec after, electroshock in mice treated with phenytoin (4 to 100 mg/kg), or with phenytoin-solvent. Figure 5 shows that electroshock produced a 314% increase in cerebellar levels of cGMP, and although phenytoin caused a dose-related decrease in these elevated levels, significant reductions were achieved only by relatively high doses (25-100 mg/kg). These doses of phenytoin, as seen in Fig. 4, are not only greater than the anticonvulsant ED 90 (prevention of THE), but are even much higher than

the doses needed to abolish tonic hindlimb flexion. The phenytoin dose levels that prevent clonus, 60 to 100 mg/kg, and those that produce toxicities, such as the lethargy, difficulty in righting, and wide-based gait observed in mice given 100 mg/kg, seem to best correspond to those doses needed to reduce the electroshock-induced elevation in cerebellar levels of cGMP.

Cerebellar cAMP levels, which increased 40% after electroshock, were not affected by phenytoin (Fig. 5). Cerebrum, which consisted of cerebral hemispheres, hypothalamus, thalamus, basal ganglia, and hippocampus, contained an electroshock-influenced level of cGMP which was 66% greater than the control level, but phenytoin failed to reduce this elevated level (Fig. 6). In contrast, electroshock-influenced levels of cAMP in cerebrum, which were elevated 69%, were decreased by phenytoin in a dose-dependent fashion, as seen in Fig. 6. However, significantly altered levels of cAMP occurred only at the two highest doses of phenytoin (60 and 100 mg/kg), which, as previously described, are relatively high and toxic effects are produced with the highest dose (100 mg/kg).

QUAKING MICE

Maximal seizures (THE) in quaking mice, which occurred spontaneously every 60-80 min, differed from those elicited in the CF #1 strain by electroshock in that post-ictal depression was absent. Other seizure activities, such as tonic flexion, whole body clonus, and facial clonus were more frequently observed, and a rocking movement which involved the whole body was apparent whenever the mouse attempted

movement. Because handling often produced a seizure in quaking mice, we held the mice for a period of time until no seizure activity was apparent before sacrificing the mice in liquid nitrogen. Thus, cyclic nucleotide levels in the CNS from quaking mice represent basal, or more appropriately, inter-ictal levels.

Inter-ictal levels of cAMP and cGMP in cerebellum or cerebrum from quaking mice were not significantly different from those found in the CF #1 mice. Although phenytoin (15 mg/kg) abolished spontaneously occurring and handling-induced seizures, while not modifying the intention-dependent rocking movement, cerebellar and cerebral levels of cGMP and cAMP were not significantly different from those found in untreated quaking mice.

Figure 1. Cyclic nucleotide levels in whole brains from frogs treated with phenytoin or phenytoin-solvent. Each data point represents the mean ± S.E. of 5-6 frogs. Frogs were sacrificed before electroshock ("0" time), during or just after tonic hindlimb extension (5-10 sec), during post-ictal depression (30 sec), or after the frog assumed a normal sitting posture, i.e., recovery from post-ictal depression (180 sec). There were no statistically significant differences between levels of cyclic nucleotides in brains from control or phenytoin-treated frogs.

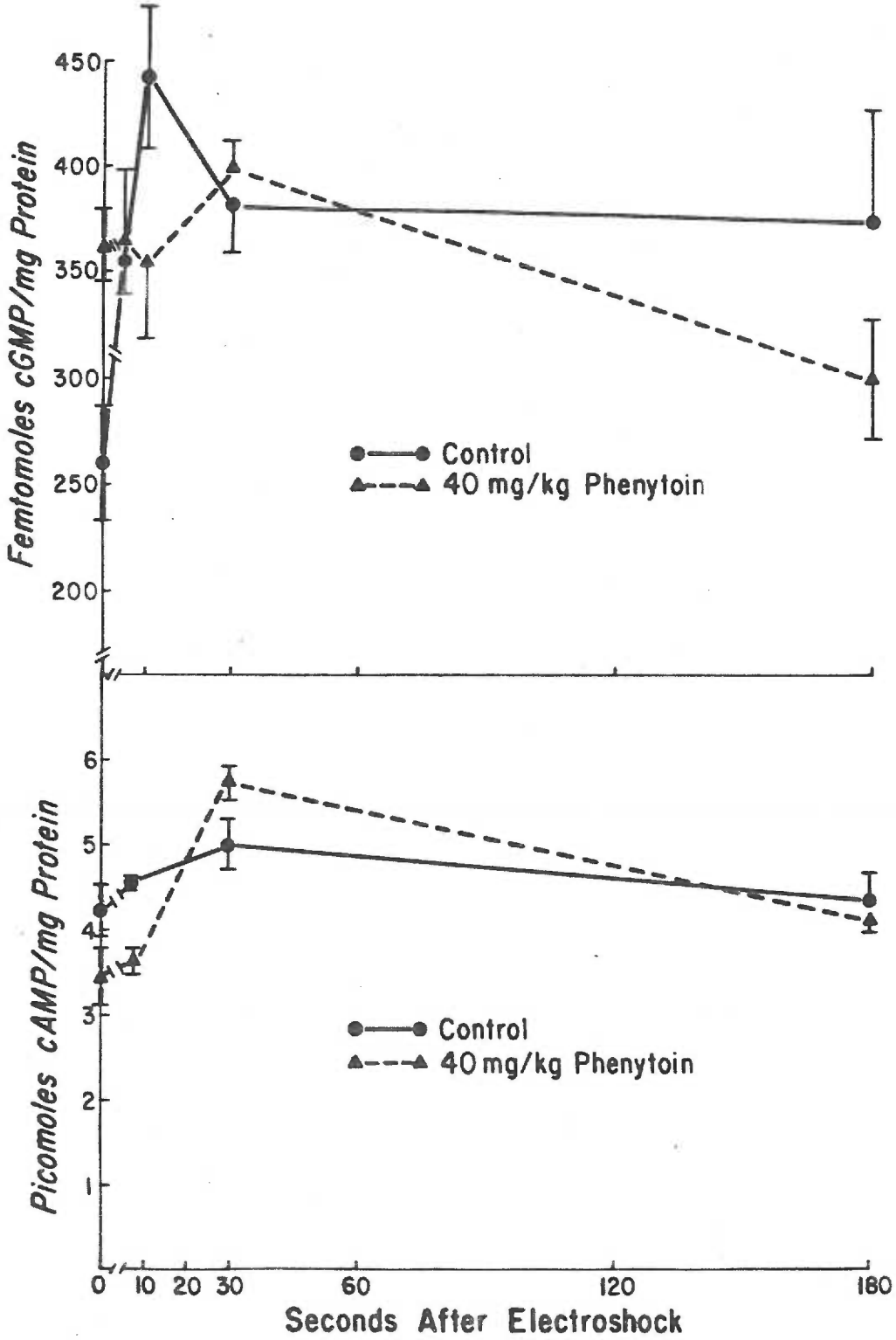

Femtomoles cGMP/mg Protein
450
400
350
300
250
200
Control
40 mg/kg Phenytoin
Picomoles cAMP/mg Protein
6
5
4
3
2
1
Control
40 mg/kg Phenytoin
0
10
20
30
60
120
180
Seconds After Electroshock

Figure 2. Quantal dose-response relationship showing protection against electroshock-induced tonic hindlimb extension (THE) by phenytoin in frogs. Each data point represents 8 frogs taken from the same population as frogs used in the analyses of cyclic nucleotides (Fig. 1). The ED 50 (and 95% confidence limits) was 15.6 mg/kg (9.4-25.8 mg/kg) as determined by the method of Litchfield and Wilcoxon (1949).

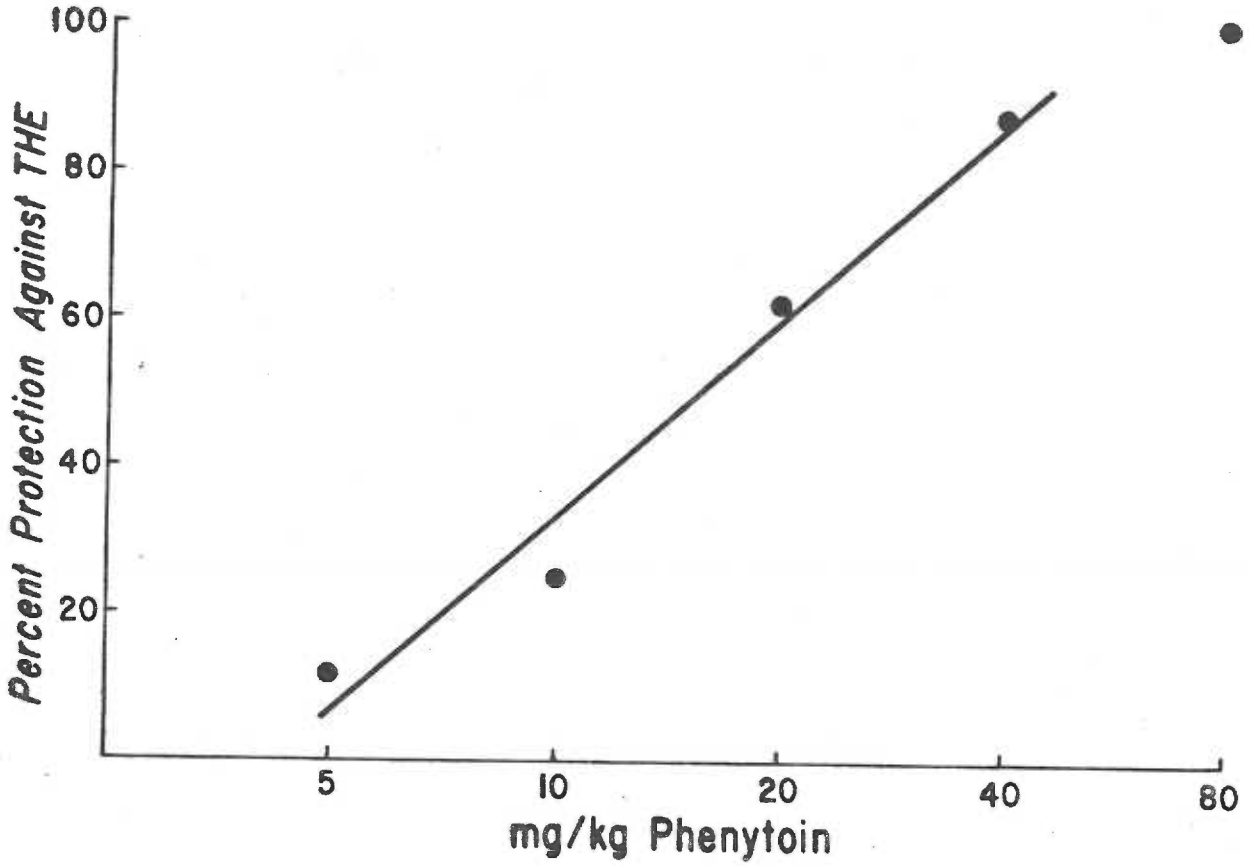

Percent Protection Against THE
100
80
60
40
20
5
10
20
40
80
mg/kg Phenytoin

Figure 3. Cyclic nucleotide levels in whole brains from mice treated with phenytoin or phenytoin-solvent. Each data point represents the mean ± S.E. of 5 mice. Mice were sacrificed before electroshock ("0" time), during or just after tonic hindlimb extension (10 sec), during post-ictal depression (30 sec), or after mice resumed normal exploratory behavior, i.e., recovery from post-ictal depression (130 sec). There were no statistically significant differences between levels of cyclic nucleotides in brains from control or phenytoin-treated mice.

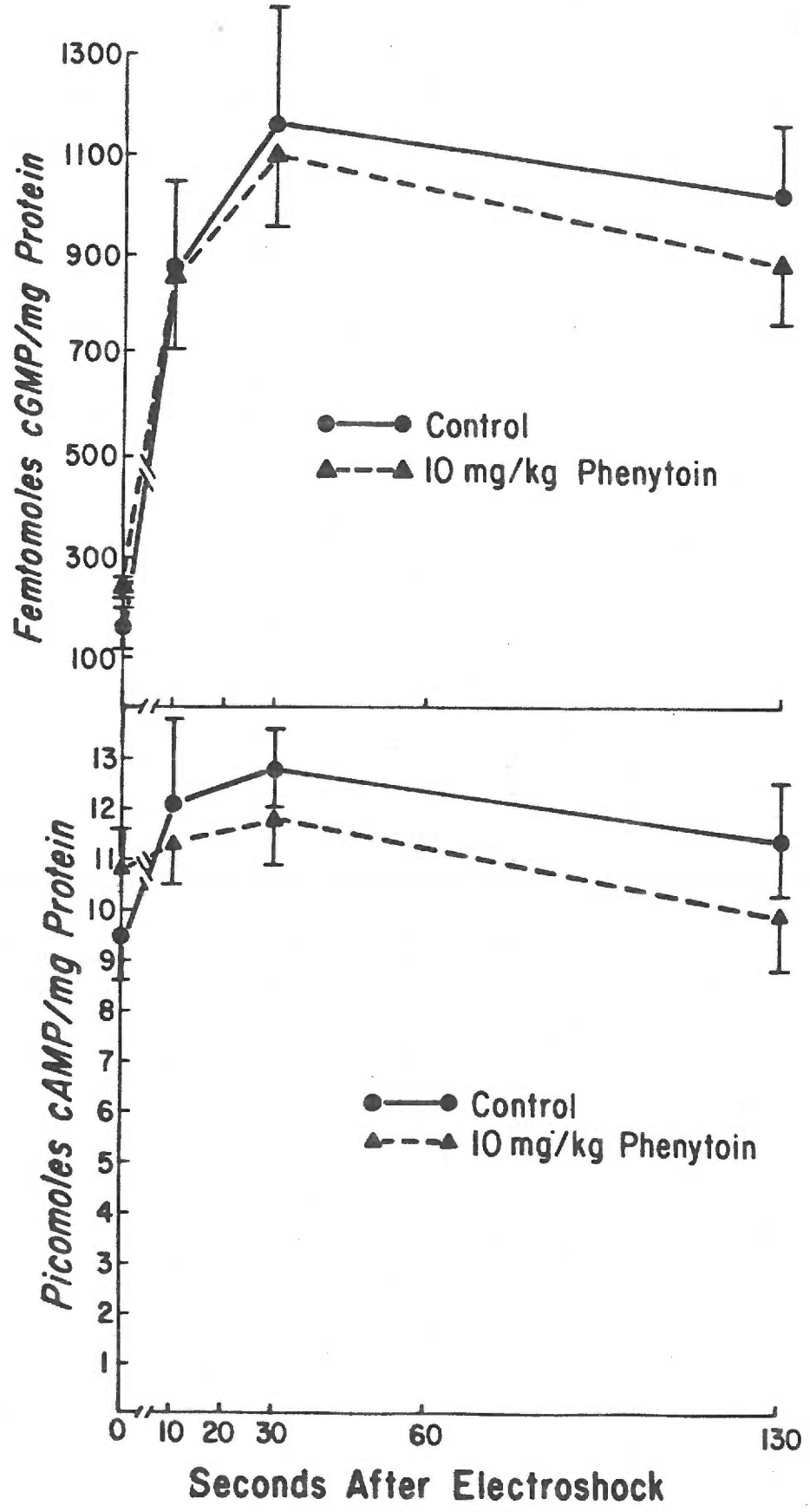

Femtomoles cGMP/mg Protein
1300
1100
900
700
500
300
100
Control
10 mg/kg Phenytoin
Picomoles cAMP/mg Protein
13
12
11
10
9
8
7
6
5
4
3
2
1
Control
10 mg/kg Phenytoin
0
10
20
30
60
130
Seconds After Electroshock

Figure 4. Quantal dose-response relationships showing protection against electroshock-induced tonic hindlimb extension (THE) and tonic hindlimb flexion (THF) by phenytoin in mice. Each data point represents 10 mice taken from the same population as mice used in the analyses of cyclic nucleotides (Fig. 3). ED 50's (and 95% confidence limits) were 6.0 mg/kg (5.0-7.2 mg/kg) for the prevention of THE, and 16.0 mg/kg (10.9-23.5 mg/kg) for prevention of THF as determined by the method of Litchfield and Wilcoxon (1949).

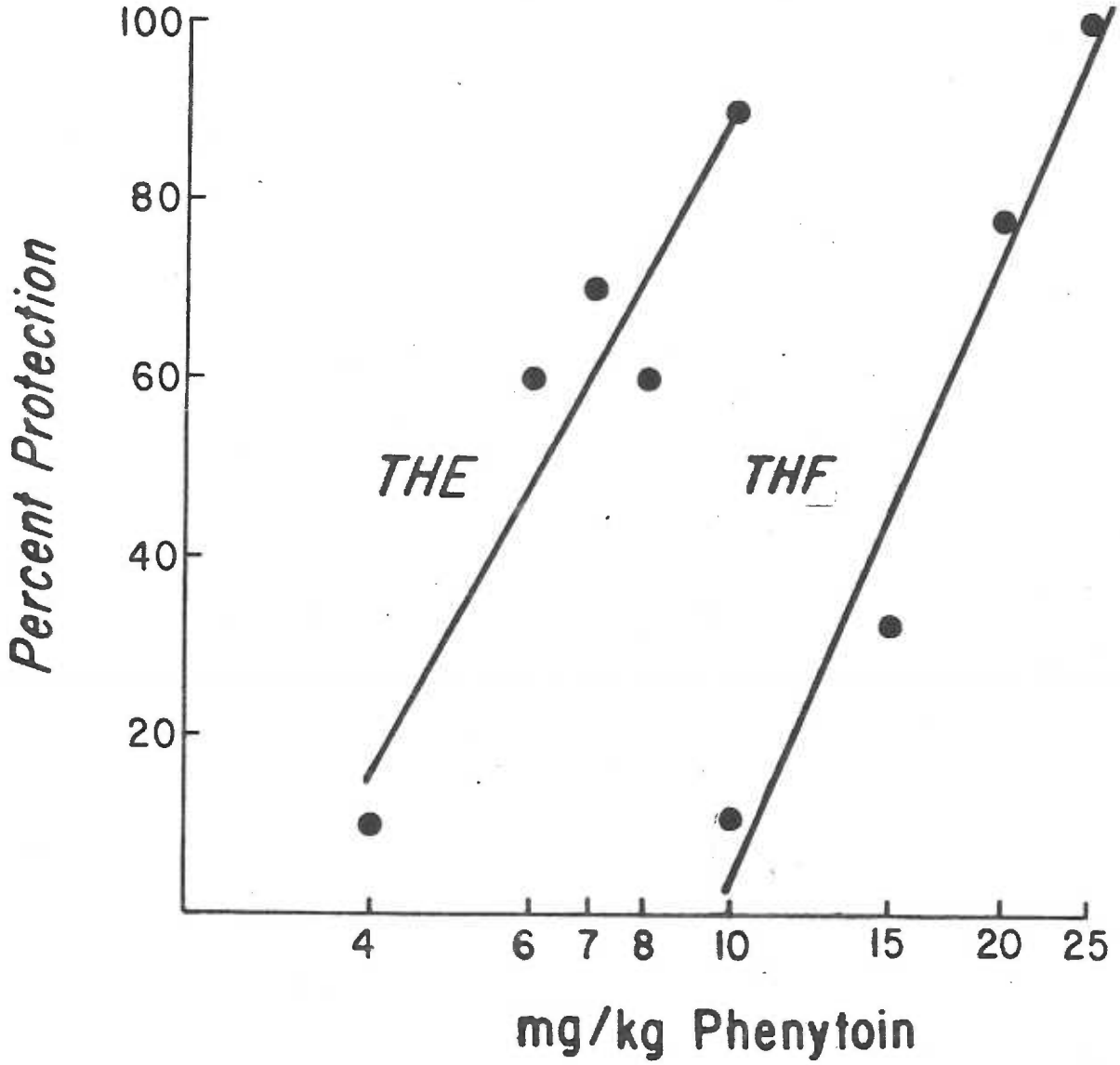

Percent Protection
100
80
60
40
20
THE
THF
4
6
7
8
10
15
20
25
mg/kg Phenytoin

Figure 5. Cyclic nucleotide levels in cerebella from mice treated with phenytoin or phenytoin-solvent. Each data point represents the mean ± S.E. of 5 mice. Mice were sacrificed before maximal electroshock seizure (MES) (pre-MES control) or 30 sec after electroshock, the time at which levels of cAMP and cGMP are maximally elevated. Student's t-test was used to test whether cyclic nucleotide levels in phenytoin-treated mice were significantly different from those in electroshocked control mice (post-MES control). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

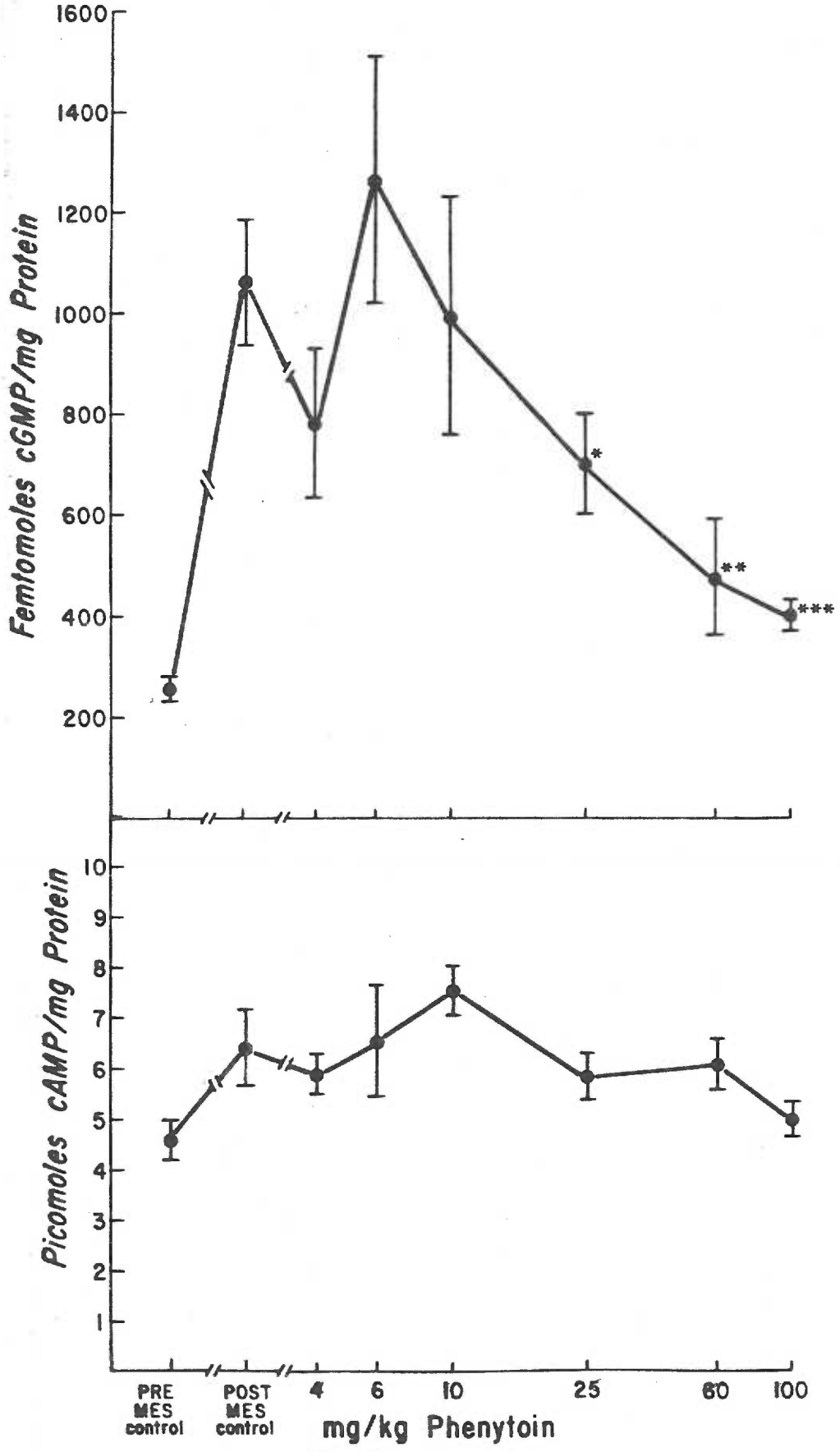
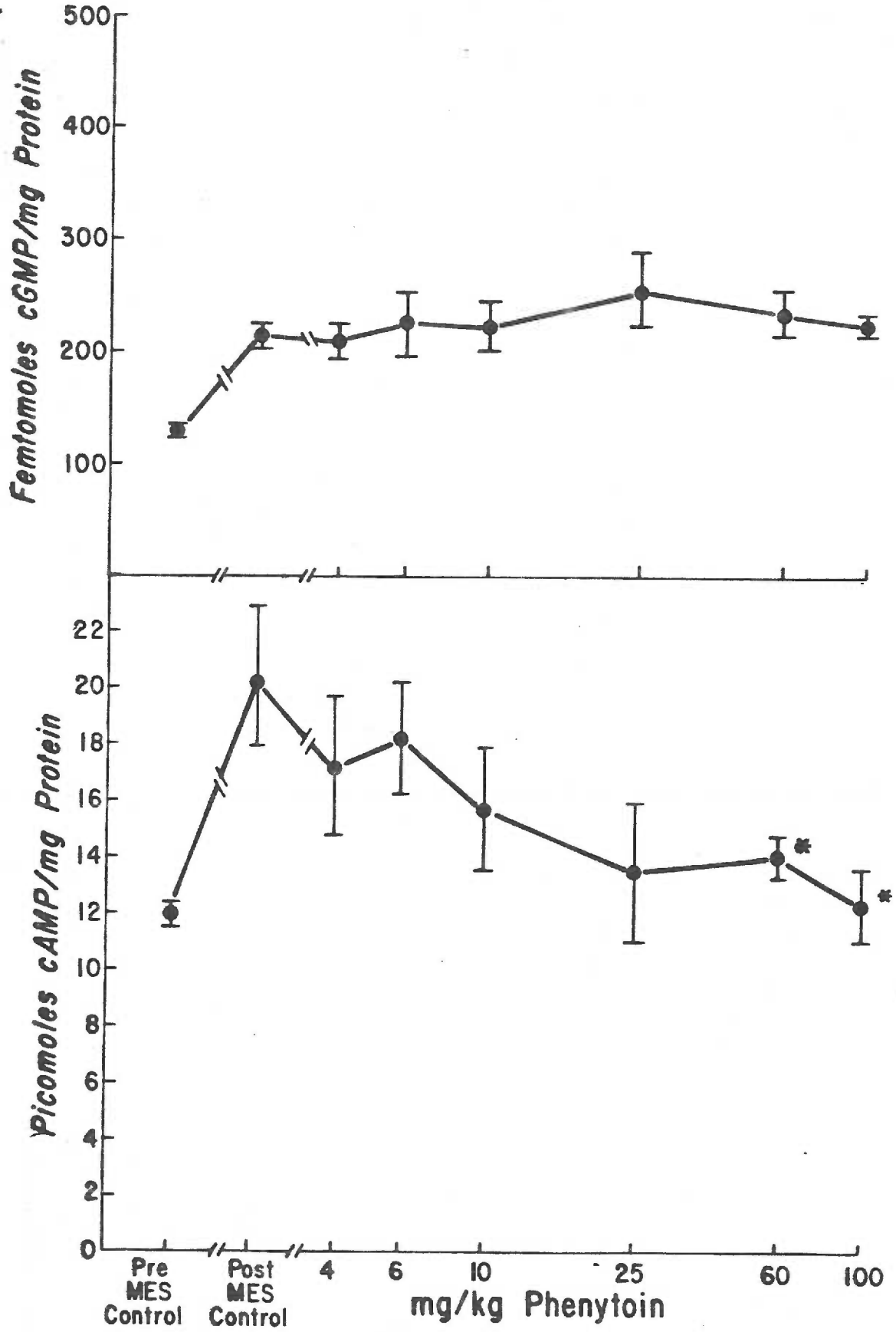

Femtomoles cGMP/mg Protein
1600
1400
1200
1000
800
600
400
200
*
**
***
Picomoles cAMP/mg Protein
10
9
8
7
6
5
4
3
2
1
PRE MES control
POST MES control
4
6
10
25
60
100
mg/kg Phenytoin

Figure 6. Cyclic nucleotide levels in cerebral hemispheres from mice treated with phenytoin or phenytoin-solvent. Each data point represents the mean ± S.E. of 5 mice. Experimental conditions and the statistical treatment of data were the same as described in Fig. 5


Femtomoles cGMP/mg Protein
500
400
300
200
100
Picomoles cAMP/mg Protein
22
20
18
16
14
12
10
8
6
4
2
0
*
*
Pre MES Control
Post MES Control
4
6
10
25
60
100
mg/kg Phenytoin

DISCUSSION

Electrically induced seizures have been reported to increase CNS levels of cAMP and cGMP in mice (Lust, Goldberg, and Passonneau, 1976; Lust, Kupferberg, Yonekawa, Penry, Passonneau, and Wheaton, 1978). Our study confirms these results in mice and also provides evidence that the same phenomenon can be elicited in frogs. Peak CNS levels of cAMP and cGMP in whole brains from frogs and mice were found 30 sec after electroshock (during post-ictal depression) except for cGMP in frog brain, which reached maximal levels 10 sec after electroshock (during or just after tonic hindlimb extension (THE)). Quantitatively, electroshock-induced increases in cyclic nucleotide levels were much greater in murine than in frog brain. Basal levels of cyclic nucleotides were also somewhat higher in murine than in frog brain, with the greatest difference being the cAMP level in murine cerebrum, which was approximately twice as high as in the frog.

Regional differences and temporal changes in cyclic nucleotide levels after electroshock are undoubtedly due to a complex interplay of biochemical and neurophysiological events. The spread of the seizure-inducing stimulus along preferred neuronal pathways, the relative abilities of neurons to accommodate to the stimulus, and differences in enzyme kinetics and the relative availability of biochemical substrates may be important factors which contribute to these regional and temporal differences. Because the quantitative importance of each of these (or other) factors is largely unknown, it would be premature to speculate on the significance of regional and temporal

differences in cyclic nucleotide levels.

Anoxia, ischemia, and massive neuronal depolarization are possible mechanisms responsible for the rise in CNS levels of cAMP during seizures. The contribution of anoxia to seizure-modified levels of cAMP was discovered by Sattin (1971) who showed that mice convulsing in an atmosphere of 100% O_2 have an increase in forebrain levels of cAMP smaller than that in mice convulsing in air. Others have shown that exposure of mice to an atmosphere of 100% N_2, or decapitation with subsequent exposure to air for 1 min, raised CNS levels of cAMP while cGMP levels were lowered (Steiner, Ferrendelli, and Kipnis, 1972; Ferrendelli and Kinscherf, 1977a). Although anoxia and ischemia may be important in mediating increased levels of cAMP, possibly via the release of endogenous adenosine (Sattin, 1971; Stefanovich, 1979; Paul, Pank, and Ditzion, 1970), these cannot be the only mechanisms because we found that phenytoin, at doses which abolished the anoxia-producing tonic phase of maximal seizures, did not lower the elevated levels of cAMP in murine cerebellum and in the whole frog brain. More likely, massive neuronal depolarization plays a larger role in mediating the electroshock-induced rise in cAMP levels. Membrane depolarizing agents such as potassium (64 mM)(Ferrendelli, 1976), glutamate (10 mM), and veratridine (50 μM)(Ferrendelli, Kinscherf, and Chang, 1975) produce elevated levels of cAMP in incubated slices of murine cerebellum. Also, pretreatment of mice with reserpine, 6-hydroxydopamine, or propranolol has been shown to antagonize pentylenetetrazol-induced rise in CNS levels of cAMP while not preventing the seizure or the seizure-associated increase in cGMP levels (Gross and Ferrendelli,

1979). These results suggest that a beta-adrenergic agonist such as norepinephrine may mediate the seizure-associated rise in cAMP levels (Gross and Ferrendelli, 1979).

It thus seems likely that electroshock-induced elevations of cAMP levels are a result of, rather than a cause of, seizures. Therefore, in order for phenytoin to prevent the seizure-induced rise in cAMP levels, it may be necessary to block the biochemical response to CNS stimulation, which is presumably mediated by neuronal depolarization (Ferrendelli, 1976). In our study, phenytoin blocked the peripheral motor manifestations of maximal seizures in frogs and mice at doses which did not lower the elevated cAMP levels. Indeed, only at nearly toxic doses (60-100 mg/kg) in mice was a significant lowering of cerebral cAMP levels seen, and even these doses did not reduce the elevated levels in murine cerebellum. These results suggest the peripheral motor manifestations of a seizure may be blocked by phenytoin at doses which do not alter the biochemical response to CNS stimulation, and that interference with the biochemical response, a consequence of neuronal depolarization, occurs only at doses of phenytoin which approach the toxic range.

It is interesting to note that although the elevation of cAMP levels is believed to be the consequence, not the cause, of seizures, it may also play a role in the termination of seizures. Microiontophoretic application of cAMP onto pyramidal tract neurons (Stone, Taylor, and Bloom, 1975), Purkinje cells (Siggins, Hoffer, and Bloom, 1969), or superfusion of cAMP onto hippocampal transplants (Freedman, Taylor, Seiger, Olson, and Hoffer, 1979) inhibited the firing of these

cells. However, since phenytoin does not raise basal levels of cAMP in cerebellum or cerebral cortex (Lust, Kupferberg, Yonekawa, Penry, Passonneau, and Wheaton, 1978; McCandless, Feussner, Lust, and Passonneau, 1979), it is doubtful that phenytoin exerts its anticonvulsant effect by this mechanism.

Unlike cAMP, seizure-induced elevation of cGMP levels cannot be attributed to anoxia or ischemia, because these conditions lower rather than raise CNS levels of cGMP (Ferrendelli and Kinscherf, 1977a; Lust, Kupferberg, Yonekawa, Penry, Passonneau, and Wheaton, 1978). More likely, the elevated CNS levels of cGMP during a seizure are, again, results of increased neurotransmission and depolarization of neuronal membranes. Oxotremorine, a centrally active agent which possesses marked peripheral cholinergic activity, produced elevated CNS levels of cGMP and coarse body tremors in mice, both of which were antagonized by atropine (Ferrendelli, Steiner, McDougal, and Kipnis, 1970). Thus, part of the seizure-associated rise in cGMP levels might be due to the activation of central cholinergic pathways. Depolarization of neuronal membranes as a mechanism producing elevated cGMP levels is supported by the findings that agents capable of depolarizing membranes, such as ouabain, veratridine, glutamate, or high concentrations of potassium, produce elevated levels of cGMP in superfused murine cerebellar slices (Ferrendelli, Kinscherf, and Chang, 1973; Ferrendelli, Chang, and Kinscherf, 1974). Because potassium (121 mM)-induced elevation of cerebellar cGMP levels is dependent upon Ca^{++}, is not inhibited by 0.1 mM concentrations of atropine, phentolamine, or propranolol, but is inhibited by drugs with significant local anesthetic properties such

as procaine (10 mM) and chlorpromazine (0.5 mM) (Ferrendelli, 1976), it has been suggested that seizure-induced elevations of cGMP levels may be due to the depolarization of neuronal membranes causing an influx of Ca^{++} into intracellular spaces with the subsequent activation of a Ca^{++}-dependent guanylate cyclase (Ferrendelli, 1980a; Ferrendelli, 1980b; Ferrendelli and Kinscherf, 1977b). According to this proposed mechanism, the seizure-associated elevation of cGMP levels in the CNS is largely a product of the seizure.

However, there is also evidence that cGMP can increase neuronal excitability. Microiontophoretic application of cGMP onto pyramidal tract neurons (Stone, Taylor, and Bloom, 1975) and hippocampal transplants (Hoffer, Seiger, Freedman, Olson, and Taylor, 1977) increased the firing rates of these neurons. Also, elevated levels of cGMP have been found in rat cerebellum (Berti, Bernareggi, Folco, Fumagalli, and Paoletti, 1976) and murine striatum (Ferrendelli and Kinscherf, 1977a) prior to the onset of pentylenetetrazol-induced seizures. More recently, it has been shown that subconvulsant doses of pentylenetetrazol administered to paralyzed, ventilated guinea pigs produced significantly elevated levels of cGMP in cerebral cortex, cerebellum and hippocampus, but did not produce abnormal EEG activity (Ferrendelli, Blank, and Gross, 1980). Thus, it is possible that elevated levels of cGMP may precede seizure activity, and based upon the excitatory nature of directly applied cGMP, may be instrumental in triggering pentylenetetrazol-induced seizures. Also, elevated levels of cGMP could contribute to the perpetuation of seizure activity, although this issue has not been addressed experimentally.

Our results with quaking mice, however, showed that a state of increased susceptibility to seizures is not necessarily associated with elevated CNS levels of cGMP. Quaking mice, which displayed spontaneous maximal seizures every 60-80 min and minor seizures much more frequently, did not have elevated cerebellar or cerebral levels of cGMP. Therefore, cGMP is probably not involved in the initiation and/or propagation of seizures in these mice. Because there are many different types of seizures and probably many different mechanisms which trigger seizures, it cannot be concluded that cGMP has no epileptogenic role in all types of experimental or spontaneous seizures.

In summary, we and others (Lust, Kupferberg, Yonekawa, Penry, Passonneau, and Wheaton, 1978) have shown that phenytoin is capable of inhibiting the electroshock-associated rise in cGMP levels in cerebellum but not in cerebrum from mice. Because cGMP has been shown to increase neuronal excitability by microiontophoretic application, and levels of cGMP in the CNS have been shown to increase prior to seizure onset, it has been hypothesized that the reduction of cGMP levels by phenytoin, presumably by blocking Na^{+} and/or Ca^{++} channels in neuronal membranes (Ferrendelli and Kinscherf, 1977b; Ferrendelli and Kinscherf, 1978), is a mechanism by which the drug exerts an anticonvulsant effect. However, the results of the present study would lead to rejection of this hypothesis. The electroshock-induced increase in cerebellar cGMP was significantly reduced only by doses of phenytoin twice the ED 95 for prevention of THE. Indeed, the most marked reduction by phenytoin of cGMP levels occurred at 100 mg/kg, a dose we found to be toxic (see RESULTS) and which has been shown by the roto-rod test to be

approximately a TD 50 (Raines, Niner, and Pace, 1973). Also, the electroshock-associated increase in CNS levels of cGMP in frogs was not altered by phenytoin even at doses up to 2 times the ED 95 for prevention of THE, a dose which has been shown in our lab to inhibit substantially the righting reflex in frogs. Furthermore, seizure activity in quaking mice was abolished with a dose of phenytoin (15 mg/kg) which did not influence cerebellar or cerebral levels of cGMP. These results indicate that the dose function for phenytoin antagonism of the seizure-induced elevation of cGMP levels is significantly to the right of the dose function for prevention of THE, the accepted correlative endpoint for anticonvulsant efficacy. Therefore, it is unlikely that the regulation of cyclic nucleotide levels is a mechanism by which phenytoin exerts its anticonvulsant effect.

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MANUSCRIPT IV

A DOSE-RESPONSE ANALYSIS OF PHENYTOIN ON ELECTRICALLY-INDUCED SEIZURES AND SPONTANEOUS ACTIVITY OF CEREBELLAR PURKINJE CELLS IN THE FROG

ABSTRACT

The hypothesis that phenytoin exerts its anticonvulsant effect by increasing the spontaneous firing rate of cerebellar Purkinje cells was tested in frogs (Rana pipiens). To provide the functional corollaries essential for the interpretation of phenytoin effects on Purkinje cell firing, time-effect and dose-effect relationships were first established for the anticonvulsant effect of phenytoin in intact frogs. Maximal seizures were induced by corneal electroshock, and phenytoin, injected into the ventral lymph sac (v.l.s.), was found to be most effective in preventing tonic hindlimb extension (THE) 2-4 hrs after injection. At time of peak effect (3 hrs), 20-40 mg/kg phenytoin protected 60-80% of frogs against THE. With this functional data base, experiments were then undertaken to test the effect of phenytoin on Purkinje cell firing rates. Prior to surgery, phenytoin was injected (v.l.s.) and 30-45 min later the frog was anesthetized with tricaine. While the frog was anesthetized, the cranium was opened and the dura mater overlying the cerebellum was removed. After surgery, the frog was curarized and a sufficient amount of time was allowed for the frog to recover from anesthesia before recording Purkinje cell activity. Single unit extracellular recordings from Purkinje cells were made with NaCl-filled glass micropipettes at the expected time (2-6 hrs) of phenytoin anticonvulsant effect. Effective anticonvulsant doses (20-40 mg/kg) of phenytoin produced no alteration in the spontaneous firing rates of cerebellar Purkinje cells compared to the rates in solvent-injected

controls. Consequently, the hypothesis that the anticonvulsant effect of phenytoin is mediated by an action on Purkinje cell firing rates is not supported by the results of this study.

INTRODUCTION

There has been a long-standing interest in the role of the cerebellum in epileptogenesis and seizure control. Electrical stimulation of the cerebellum has been shown to inhibit experimentally produced seizures or paroxysmal EEG activity in cats (Cooke and Snider, 1955; Reimer, Grimm, and Dow, 1967; Hutton, Frost, and Foster, 1972) and rats (Dow, Fernandez-Guardiola, and Manni, 1962), while cerebellar ablation facilitated paroxysmal EEG activity in cats (Julien and Halpern, 1972) and rats (Dow, Fernandez-Guardiola, and Manni, 1962). Although others have found cerebellar stimulation of little value in suppressing experimentally produced seizures in rhesus monkeys (Lockard, Ojemann, Congdon, and DuCharme, 1979) and paroxysmal EEG activity in cats (Testa, Pellegrini, and Giaretta, 1979), Cooper et al. (1976, 1978) reported that cerebellar stimulation effectively reduced the frequency of seizures in epileptic patients who were otherwise refractory to anticonvulsant therapy.

In cases where cerebellar stimulation suppressed seizure activity, the hypothesis has been that electrical stimulation of cerebellar cortex increases the firing of Purkinje cells which exert inhibitory influences on nuclei concerned with the control of muscle tone and movement, such as the red nucleus (Tsukahara, Toyama, and Kosaka, 1964), Deiters nucleus (Ito and Yoshida, 1964), and deep cerebellar nuclei (Ito, Yoshida, and Obata, 1964). At present, the antiepileptic efficacy of cerebellar stimulation is by no means conclusively established. In those instances in which it appeared efficacious, the mechanism may be

other than originally hypothesized owing to the recent discovery that cerebellar stimulation in the cat causes inhibition rather than excitation of Purkinje cells (Dauth, Dell, and Gilman, 1978). Also, chronic cerebellar stimulation, which caused marked depletion of Purkinje cells in monkeys (Dauth, Defendini, Gilman, Tennyson, and Kremzner, 1977; Tennyson, Kremzner, Dauth, Defendini, and Gilman, 1977), was an effective antiepileptic therapy in some patients in whom the population of Purkinje cells was already severely depleted (Rajjoub, Wood, and Van Buren, 1976; Urich, Watkins, Amin, and Cooper, 1978). There is now evidence to suggest that therapeutic benefits from cerebellar stimulation are not due to stimulation of Purkinje cells, but to stimulation of the ascending reticular formation and non-specific thalamic nuclei (Bantli, Bloedel, and Tolbert, 1976) with subsequent suppression of activity in the thalamo-cortical pathway (Johnson, Charlton, Englander, Brickley, Nowack, and Hanna, 1979). Nevertheless, the potential role of Purkinje cells in seizure disorders is supported by the finding that "nervous" mice, which have an autosomal recessive mutation which results in selective degeneration of Purkinje cells (Sidman and Green, 1970; Landis, 1973), also have a reduced seizure threshold.

The augmentation of Purkinje cell activity by antiepileptic drugs was first proposed as a mechanism of action by Julien and Halpern (1971) who found that anticonvulsant doses of phenytoin increased the firing rate of Purkinje cells in unanesthetized, curarized cats. Although some investigators have confirmed these results in cats (Shimizu, Manaka, Hori, and Sano, 1977; Fernandez-Guardiola, Calvo, and Condes-Lara, 1979),

others have failed to demonstrate phenytoin-induced augmentation of Purkinje cell activity in cats (Latham and Paul, 1976) and rats (Pieri and Haefely, 1976; Puro and Woodward, 1973; Latham and Paul, 1980). These conflicting results may have arisen from differences in experimental animals, anesthetic protocols, microelectrode recording techniques, and/or phenytoin doses and routes of administration. As a further test of the Julien and Halpern (1971) hypothesis, we investigated the effects of phenytoin on Purkinje cell activity in the frog, an animal which possesses a relatively small and histologically simple cerebellum (Larsell, 1923; Sotelo, 1976). The present communication describes the effects of phenytoin on the spontaneous activity of cerebellar Purkinje cells in the unanesthetized, curarized frog. The phenytoin doses employed were those demonstrated to span the effective range for modification of maximal electrically-induced seizures in the frog.

METHODS

ANIMALS AND DRUGS

Rana pipiens (northern variety) of both sexes (20-100 gm) were obtained from William Lemberger Assoc. (Germantown, Wisc.) and housed in plastic containers tipped at one end to allow for dry and wet areas with continuously flowing tap water. Phenytoin, obtained from Parke, Davis and Co., was dissolved in 50 mM NaOH (pH 12.6), while tricaine methanesulfonate (Ayerst Laboratories, Inc.), d-tubocurarine (Calbiochem), and lidocaine (Astra Pharmaceutical Products, Inc.) were dissolved in 0.9% NaCl. Drug solutions were injected into the ventral lymph sac, with the exception of lidocaine which was topically applied, at a volume less than 0.5% of body weight.

ELECTRICALLY-INDUCED MAXIMAL SEIZURES

Maximal seizures, produced with electroshock via corneal electrodes using the technique of Woodbury and Davenport (1952), were used to test the anticonvulsant efficacy of phenytoin, the endpoint for which was prevention of tonic hindlimb extension (THE). To establish time-effect curves, CA 50's (convulsant amperage 50%; amperage needed to produce THE in 50% of a population) were measured according to the "Up and Down" method of Dixon and Mood (1948), as modified by McCawley and Wayson (1974). Using this method, 12-16 frogs per treatment (or control) group were electroshocked before and at various time intervals, up to 24-36 hrs, after phenytoin injection. Thus, with the electroshock of frogs at various intervals after injection, each group served as its own

control over time, and it is noteworthy (Fig. 1) that repeated electroshock in the solvent-injected control frogs did not result in any significant variation in the CA 50 throughout the experiment.† Subsequently, a phenytoin dose-effect curve was constructed by administering electroshock (98 mA for a duration of 0.5 sec) at the time of peak phenytoin effect (3 hrs). Electroshock parameters were shown previously to produce THE in approximately 90% of control frogs. Statistical analysis of the phenytoin dose-effect curve was performed by the method of Litchfield and Wilcoxon (1949).

PURKINJE CELL RECORDING

To insure that recordings of Purkinje cell activity were made at times of effective phenytoin anticonvulsant activity (2-6 hrs), the following procedure was employed: Thirty to forty-five min after an injection of phenytoin or phenytoin-solvent into the ventral lymph sac, frogs were anesthetized with tricaine (120 mg/kg). A rectangular piece of skin, extending between the tympani and the eyes, was removed to expose the skull, and toenail clippers (Revlon) were used to remove the cranium overlying the cerebellum and optic lobes. The dura mater covering the cerebellum was removed with fine tweezers and iris scissors, being careful not to damage the relatively large dorsal cerebellar vein. After dissection, frogs were curarized (8 mg/kg), and their heads were stabilized by clamping the mandible onto a lucite plate. Respiration in the frog, which is normally accomplished by cutaneous as well as pulmonary gas exchange (Krogh, 1904; Krogh, 1968), was facilitated by keeping

the skin moist at all times and by inflating the lungs 5-6 times every 5 minutes.

Spontaneous activity from single Purkinje cells was recorded extracellularly with glass microelectrodes filled with 4 M NaCl. To penetrate the pia more easily, and to reduce impedance to 2-8 MΩ , electrodes were beveled using the jet stream microbeveling method of Ogden, Citron, and Pierantoni (1978). Potentials from Purkinje cells, identified by their characteristic climbing fiber responses (Eccles, Ito, and Szentagothai, 1967), were amplified by a capacity-neutralized, high impedance preamplifier (Pico-metric amplifier, Instrumentation Laboratory, Inc., Boston, Mass.), displayed on a Tektronix RM-565 dual beam oscilloscope (Portland, Oreg.), and recorded on magnetic tape. Average firing rates were determined by analyzing up to 5 min of recorded spontaneous activity for each cell and counting a maximum of 1000 action potentials with a Beckman 6240 Eput^{R} and timer (Richmond, Calif.).

Because general anesthesia is known to depress the firing rates of Purkinje cells (Nacimiento, 1969; Murphy and Sabah, 1970), we analyzed data only from those frogs in which a sufficient amount of time had passed for recovery from the tricaine-induced anesthesia. Duration of anesthesia after 120 mg/kg tricaine in intact frogs, measured as the time needed for the frog to resume a normal sitting position, was found to be 64 ± 17 min (± S.D., n=5). Therefore, no data were recorded unless at least 75 min had passed since the frog was injected with tricaine. Since phenytoin administration preceded the tricaine injection by 30-45 min (see above), the earliest Purkinje cell recordings

were, therefore, approximately 2 hrs after phenytoin injection. In a few experiments, dissections were performed on curarized frogs after the skin overlying the dorsal cranium had been locally anesthetized with 1% lidocaine. Spontaneous firing rates of Purkinje cells in these frogs were not different from those recorded in frogs which had been subjected to tricaine-induced anesthesia.

†Detailed description of the "Up and Down" method and its use in obtaining this dose-time-effect relationship for phenytoin is provided in Appendix I.

RESULTS

DOSE-TIME-EFFECT RELATIONSHIPS FOR THE ANTICONVULSANT ACTION OF PHENYTOIN

Fig. 1 illustrates dose-time-effect characteristics for phenytoin elevation of the convulsant amperage 50% (CA 50) in frogs. Phenytoin doses from 20 to 40 mg/kg produced proportionately greater elevations of the CA 50 above that of controls, with the peak anticonvulsant effect occurring 2-4 hrs after injection. Significant anticonvulsant effects from 20 mg/kg lasted at least 8 hrs, while those from 40 mg/kg lasted at least 26 hrs. The average "half-time" for the decay of phenytoin protection, from peak, was approximately 10.5 hrs.

The dose-effect relationship for phenytoin prevention of THE is shown in Fig. 2. In this experiment, all frogs were electroshocked 3 hrs after drug administration (time of peak effect) with an amperage known to produce tonic hindlimb extension (THE) in about 90% of untreated animals. Fig. 2 shows that the threshold protective dose of phenytoin is near 5 mg/kg and that at 40 mg/kg nearly 80% of frogs fail to develop THE. The ED 50 of phenytoin was found to be 17.1 mg/kg.

EFFECT OF ANTICONVULSANT DOSES OF PHENYTOIN ON PURKINJE CELL ACTIVITY

Spontaneous firing rates of Purkinje cells from 3 control frogs averaged 4.6 ± 1.7 spikes/sec (± S.D., n=8), which is in agreement with the results of others (Matthews, Phillips, and Rushworth, 1958; Nacimiento, 1969). Phenytoin, at the doses of 20-40 mg/kg which prevented THE in 60-80% of frogs (Fig. 2), did not significantly affect the spontaneous firing rate of 27 Purkinje cells, as seen in Fig. 3. Data shown in

Fig. 3 were collected from 10 frogs 2-6 hrs after phenytoin injection (4.4 ± 1.3 hrs), a time period during which these doses were exerting anticonvulsant effects (Figs. 1 and 2).

Figure 1. Dose-time-effect relationship for anticonvulsant action of phenytoin in frogs. Each data point is the average CA 50 (convulsant amperage 50%), measured in milliamperes, of 12-16 frogs. Error bars are 95% confidence limits. CA 50's at "zero" hrs for the phenytoin-treated groups were determined 12 hrs before phenytoin injection. Control frogs were injected with phenytoin-solvent (50 mM NaOH, pH 12.6) at "zero" hrs.

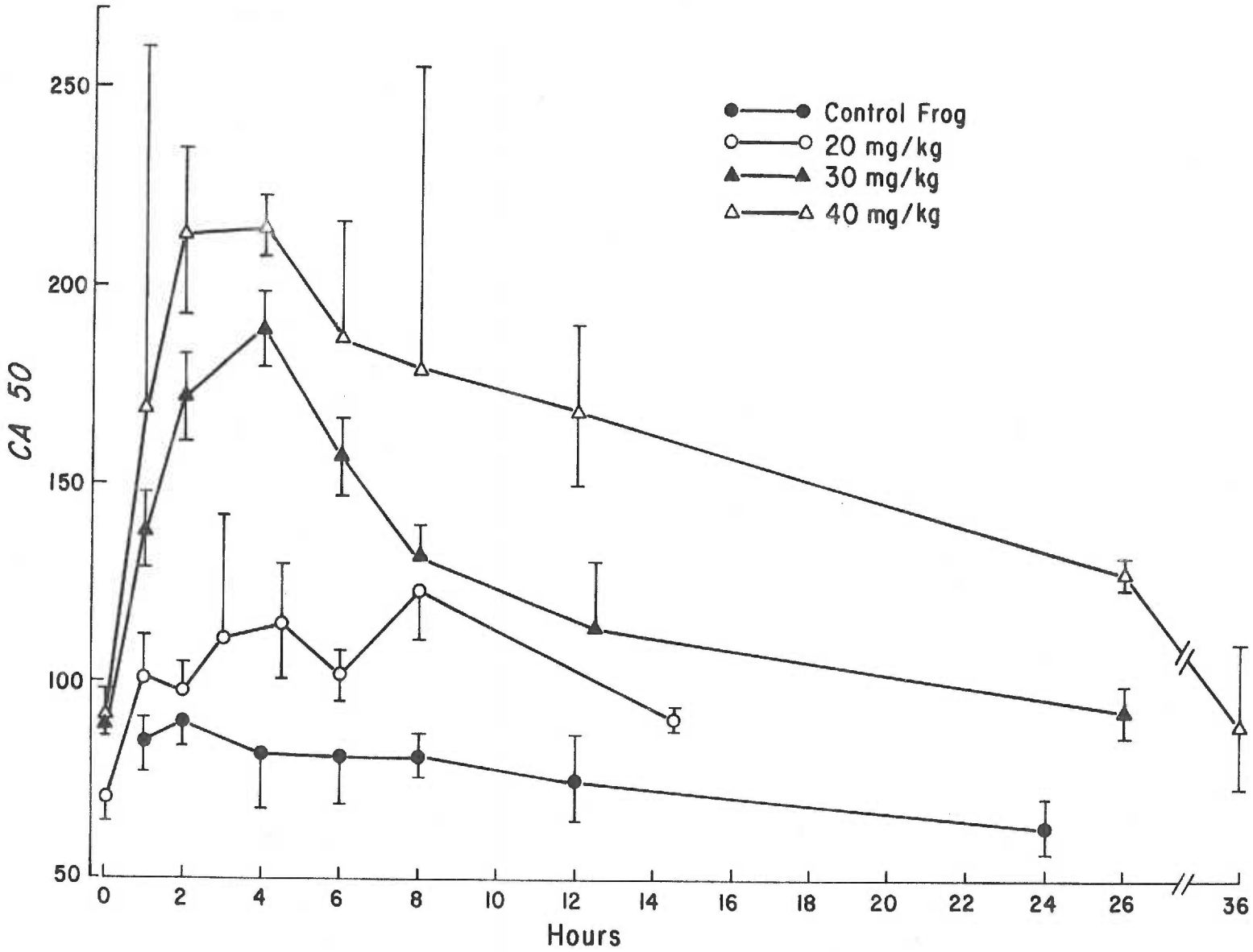

Control Frog
20 mg/kg
30 mg/kg
40 mg/kg
CA 50
250
200
150
100
50
0
2
4
6
8
10
12
14
16
18
20
22
24
26
36
Hours

Figure 2. Quantal dose-effect relationship for percentage of frogs protected against electrically-induced tonic hindlimb extension (THE) by phenytoin at the time of peak anticonvulsant effect (3 hrs). Each data point represents the quantal responses of 12 frogs which were electroshocked with an amperage which produces THE in about 90% of control frogs. The ED 50 (and 95% confidence limits) was 17.1 (10.4-28.0) mg/kg.

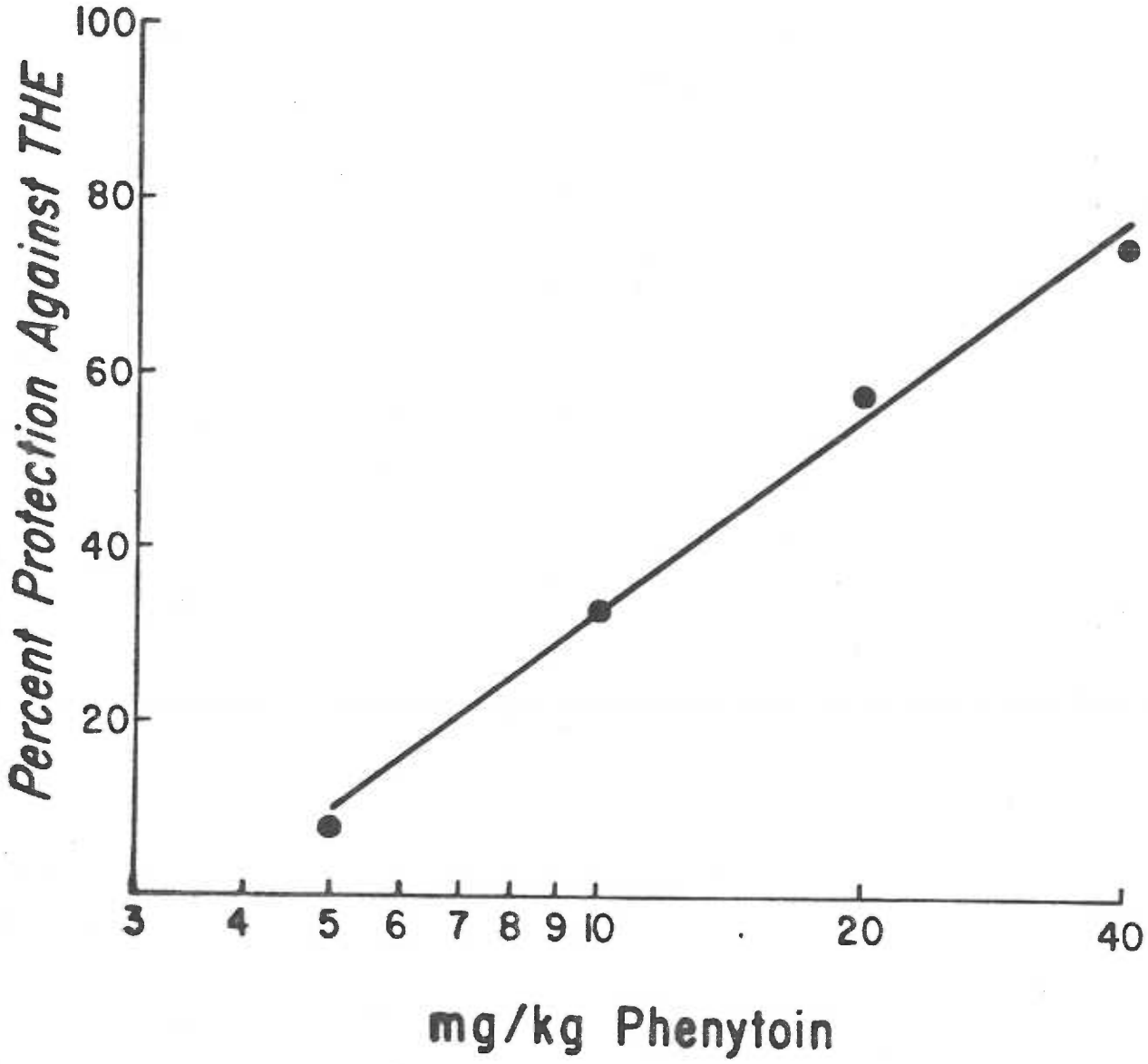
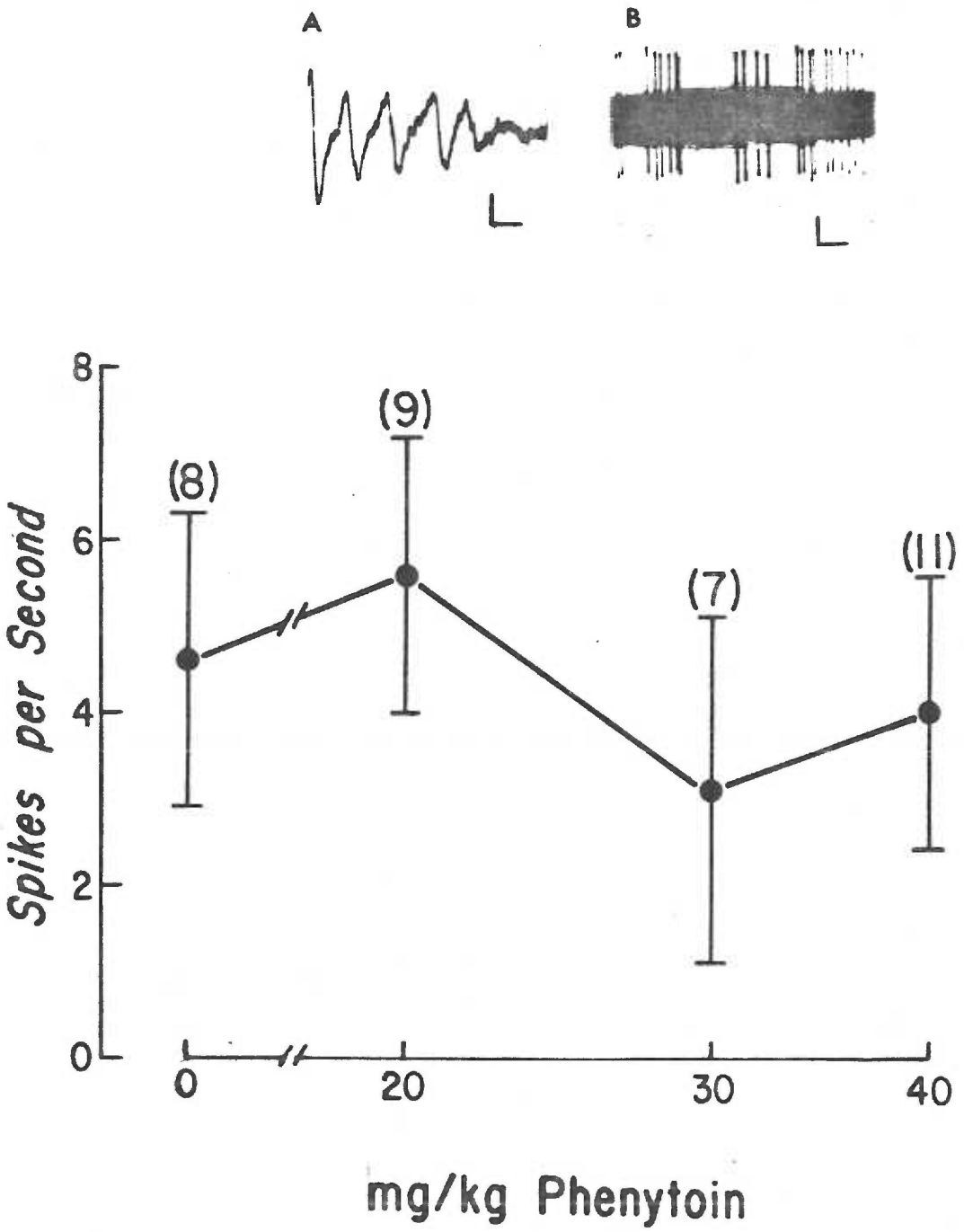

Percent Protection Against THE
100
80
60
40
20
3
4
5
6
7
8
9
10
20
40
mg/kg Phenytoin

Figure 3. Spontaneous firing rates of cerebellar Purkinje cells in frogs treated with phenytoin or phenytoin-solvent. Each data point represents the mean ± S.D. of the number of cells in parentheses. Data were collected from 13 unanesthetized, curarized frogs at an average time of 4.4 ± 1.3 hrs (± S.D.) after injection of phenytoin or phenytoin-solvent. Inset "A" displays an internally-triggered oscillographic sweep containing an action potential with the characteristic waveform of the climbing fiber response. Inset "B" shows a Purkinje cell discharging at the fairly typical rate of about 6 spikes/sec. Signals in both insets were filtered below 100 Hz and above 3000 Hz. Vertical calibration bars represent 1 mV, while horizontal bars represent 2 and 500 msec in insets "A" and "B", respectively.


A
B
8
6
4
2
0
Spikes per Second
(8)
(9)
(7)
(11)
0
20
30
40
mg/kg Phenytoin

DISCUSSION

The results of this study, which show that anticonvulsant doses of phenytoin do not augment spontaneous activity of cerebellar Purkinje cells in the frog, encourage the rejection of the hypothesis that the Purkinje cell is an important site of action for the anticonvulsant effect of phenytoin. Our results are in accord with those of some investigators who used rats and cats as experimental animals (Puro and Woodward, 1973; Latham and Paul, 1976 and 1980; Pieri and Haefely, 1976), but disagree with those using unanesthetized cats as experimental animals (Julien and Halpern, 1971; Halpern and Julien, 1972; Fernandez-Guardiola, Calvo, and Condes-Lara, 1979). An examination of the different experimental conditions prevailing in these studies might shed some light on why conflicting reports have appeared in the literature.

Julien and Halpern (1971), who first reported phenytoin-induced augmentation of Purkinje cell activity, used microelectrodes filled with 2.7 M KCl to record extracellularly (Halpern and Julien, 1972). Leakage of K^+ from electrodes could have artifactually produced the time-dependent acceleration of Purkinje cell activity. However, this mechanism cannot be used to explain the results of Fernandez-Guardiola et al. (1979), who confirmed the work of Julien and Halpern (1971) using tungsten microelectrodes.

Variations in cerebellar concentrations of phenytoin at the times of experiments, resulting from different routes of drug administration and different times at which Purkinje cell activity was monitored, could have contributed to conflicting results cited in the literature.

According to the original report by Julien and Halpern (1971), a single 10 mg/kg dose of phenytoin administered i.v. to cats caused Purkinje cell activity to increase from 24 Hz to a maximum rate of over 200 Hz 90 min after injection. Thereafter, Purkinje cell firing rates declined slowly over time, but were still significantly elevated (90 Hz) 4-5 hrs after injection. Although the route of administration was not reported, Shimizu et al. confirmed the results of Julien and Halpern in cats and found that the peak increase in Purkinje cell activity, occurring 90-120 min after injection of 10-40 mg/kg phenytoin, was preceded by a decrease in activity (Shimizu, Manaka, Hori, and Sano, 1977). Fernandez-Guardiola et al., recording Purkinje cell activity from cats given a constant i.v. infusion of phenytoin, found increased activity with cumulative doses greater than 20 mg/kg, but decreased Purkinje cell activity with doses less than 20 mg/kg (Fernandez-Guardiola, Calvo, and Condes-Lara, 1979). Thus, it appears from these reports that reduced Purkinje cell activity may precede the increase in activity, possibly due to a dose-time-dependent process, and that the i.v. route might be necessary to produce accelerated Purkinje cell activity. However, other investigators using cats and rats (Latham and Paul, 1976 and 1980; Pieri and Haefely, 1976) also administered anticonvulsant doses of phenytoin i.v. and monitored Purkinje cell activity for at least 1.5-3 hrs after injection without observing an increase in activity. Also, Puro and Woodward (1973) gave rats 15 or 150 mg/kg phenytoin i.p. and monitored Purkinje cell activity over a period of several hours to several days without observing an increase in firing rates. Furthermore, our study has shown that no

change in Purkinje cell activity occurred during times in which phenytoin is an effective anticonvulsant drug in frogs. Therefore, it appears that the route of administration and the times during which Purkinje cell activity have been monitored should not have contributed to the lack of concordance in the literature.

The choice of laboratory animal and the use of anesthesia are the remaining factors that may contribute to the conflicting reports about phenytoin augmentation of Purkinje cell activity. It seems significant that the only reports of phenytoin-induced acceleration of Purkinje cell activity were obtained using cats (Julien and Halpern, 1971; Halpern and Julien, 1972; Shimizu, Manaka, Hori, and Sano, 1977; Fernandez-Guardiola, Calvo, and Condes-Lara, 1979). Moreover, these positive results were obtained using unanesthetized, curarized animals, and the only reported case in which phenytoin failed to augment Purkinje cell activity in cats was when barbiturate anesthesia was maintained during the experiment (Latham and Paul, 1976), a condition which has been shown to interfere with neuronal transmission in the feline cerebellar cortex (Gordon, Rubia, and Strata, 1972; Gordon, Rubia, and Strata, 1973). On the other hand, phenytoin-induced augmentation of Purkinje cell activity was not observed by us in the curarized frog, and has not been observed in the rat, whether curarized (Pieri and Haefely, 1976) or when anesthetized with urethane (Latham and Paul, 1976 and 1980) or halothane (Puro and Woodward, 1973). These observations lead us to suggest that phenytoin-induced acceleration of Purkinje cell activity may be a species specific phenomenon, and may be suppressed by anesthesia.

In summary, our data show that phenytoin, at anticonvulsant doses and at times during which phenytoin exerts an anticonvulsant effect, does not alter Purkinje cell activity in frogs. Therefore, the Purkinje cell is not a demonstrable, nor necessary, site of anticonvulsant action of phenytoin in frogs. In reviewing the literature, this may also be true for rats. However, it is possible that the Purkinje cell may be an important site of action for phenytoin in cats. Furthermore, since a single i.p. dose of phenytoin (20 mg/kg) is known to protect cats from electrically-induced maximal seizures for an average of 7 days (Toman, Swinyard, and Goodman, 1946), one would expect increased Purkinje cell activity to persist for many days if this is, indeed, an anticonvulsant mechanism of phenytoin in cats. We suggest that a time-action study, correlating the anticonvulsant effect in the intact animal with the effect on Purkinje cell activity in the curarized animal, would answer the question of whether the Purkinje cell is an important site of action of phenytoin in cats.

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APPENDIX I

THE "UP AND DOWN" METHOD FOR DETERMINING THE CONVULSANT AMPERAGE 50%

THE "UP AND DOWN" METHOD FOR DETERMINING THE CONVULSANT AMPERAGE 50%

There are 3 variables in measuring the effect of a drug on electrically-induced seizures: the dose of the drug, the intensity of the electroshock stimulus, and the response of the test animal. The relationships between these variables can be established by keeping one constant, treating the second as the independent variable, and observing the third as the dependent variable. Therefore, there are 3 combinations of these variables with which drug action can be investigated. Although the majority of anticonvulsant dose-response curves presented in this thesis were obtained by delivering a constant current, varying the dose, and observing the response (see Manuscript I), a second method was also used in which the response was held constant, the dose was varied, and the amperage needed to maintain the constant response was observed. This second method employed the "Up and Down" method of Dixon and Mood (1948) to find the amperage needed to produce tonic hindlimb extension (THE) in 50% of a population, i.e, the convulsant amperage 50% (CA 50).

In the "Up and Down" method, amperages near the CA 50 are used so animals are not "wasted" in defining extremes of the dose-response curve. Animals are sequentially tested with a series of predetermined amperages spaced at equal logarithmic intervals about the previously approximated CA 50. The first animal is tested at one of the selected amperages, and a positive (THE) or negative (no THE) response forces use of the next lower or higher amperage, respectively, in the subsequent test animal.

Similarly, the response of this second animal determines whether the third animal is tested at lower or higher amperage, and this pattern is continued until all test animals, usually 12-16, have been electroshocked. As a result, very close to half the animals either display THE or do not, and the data are therefore centered around the CA 50.

Because the optimal interval between test amperages is equal to the standard deviation (Dixon and Mood, 1948), preliminary estimates of the CA 50 and standard deviation were made with a few animals to derive the series of test amperages spaced at equal logarithmic intervals. To determine the preliminary mean and standard deviation, 4-5 control animals were electroshocked at 4 different amperages and the CA 5, CA 50, and CA 95 were estimated by plotting percent response (percent with THE) vs. amperage on log-probit graph paper. Because the 95 and 5% responses are separated by approximately 4 standard deviations, the logarithmic interval representing one standard deviation was calculated by the formula $\ln(CA\ 95) - \ln(CA\ 5)/4$. Preliminary CA 50's (and ln standard deviations) were 89.3 mA (0.13456) for frogs and 15.0 mA (0.2231) for mice. As described in METHODS in Manuscript I, all frogs were electroshocked for a duration of 0.5 sec while mice were given a stimulus lasting 0.2 sec.

Test amperages spaced at equal logarithmic intervals were calculated by the formula $\exp(\ln(x) \pm dn)$ where "x" is the preliminary CA 50, "d" is the logarithmic interval, and "n" represents the integers 0, 1, 2, 3, ... etc. As illustrated by data obtained from frogs, the series of test amperages were calculated from the formula $\exp(\ln(89.3) \pm 0.13456n)$ and are listed in the first column in Fig. 1. Fig. 1 also

demonstrates the method by which the CA 50 was established in control frogs. Each "X" and "O" in Fig. 1 represents a frog which did or did not show THE, respectively, in response to electroshock with the indicated amperage. As one can see, the data in the figure are centered around the CA 50 with approximately half the frogs showing THE and half without.

The CA 50 was calculated by the method of Dixon and Mood (1948), as modified by McCawley and Wayson (1974). Theoretically, the median amperage which produced THE should be identical to the median amperage which failed to produce THE, i.e., these two CA 50's should be identical. However, in practice this is usually not the case. Therefore, CA 50's for responders and non-responders were averaged to obtain the best estimate of the CA 50. To calculate the CA 50 for control frogs, data from Fig. 1 were organized into two tables, one for the "responder" group and one for the "non-responder" group, as shown in Table 1. A CA 50 was calculated for each group by the equation:

$$m = \ln(Y_o) + d\left(\frac{\Sigma in}{\Sigma n} + \frac{1}{2}\right)$$

where "Y_o" is the lowest amperage at which an event occurred (either THE or no THE), "d" is the natural logarithmic interval (0.13456), and "m" is the exponent of the CA 50 and will be "m_x" for frogs showing THE and "m_o" for frogs without THE. The overall CA 50 was obtained by averaging m_x and m_o, i.e., CA 50 = $\exp(m_x + m_o/2)$. The CA 50 for control frogs was 75 mA.

A logarithmic function of the standard deviation, "s", was also calculated for both responders and non-responders (s_x and s_o, respectively) by the equation:

$$s = 1.62\ d\left[0.029 + \left(\frac{\Sigma n \Sigma i^2 n - (\Sigma in)^2}{(\Sigma n)^2}\right)\right]$$

The value of "s", taken as the average of s_x and s_o, was found to be 0.3458 which, after transformation into the same units (amperage) as the CA 50 by the formula $(CA\ 50)e^{\pm s}$, yielded a standard deviation of 26 mA. If "s" is significantly lower than the value of "d", the testing interval logarithm, errors in calculating the standard deviation may result. In this case, a correction can be made using data provided in the paper by Dixon and Mood (1948), which also provides methods for calculating the standard error and 95% confidence limits.

To assess the accuracy of the "Up and Down" method in our hands, control CA 50's from mice and frogs, determined by the "Up and Down" method, were compared to those determined by the method of Litchfield and Wilcoxon (1949). Fig. 2 shows these two methods gave CA 50's which were in close agreement.

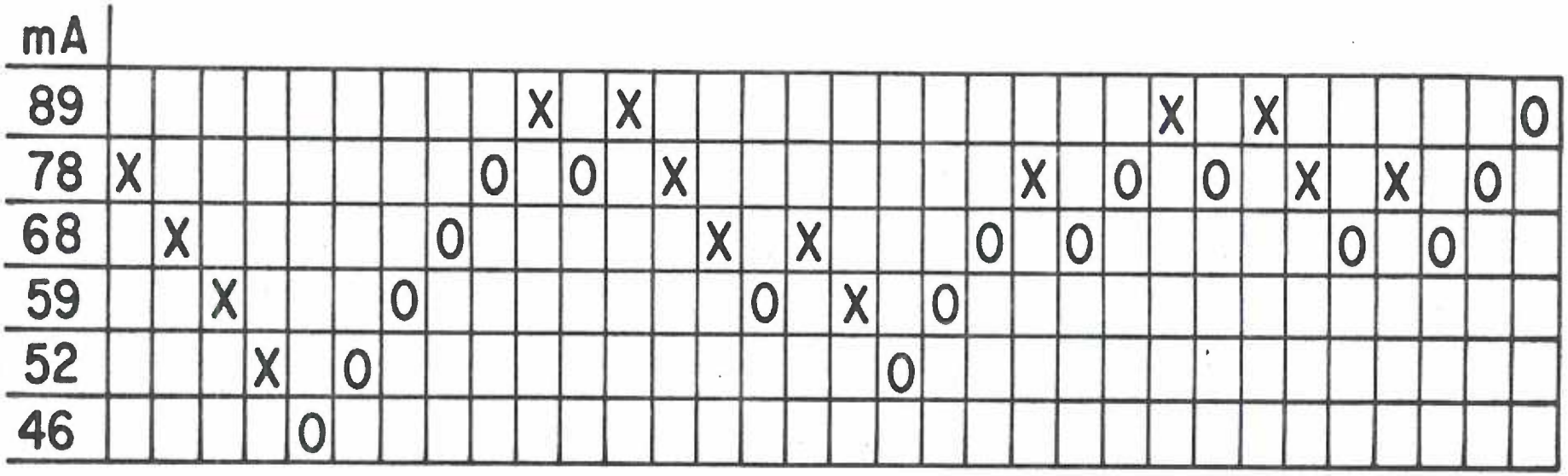
Because the "Up and Down" method requires fewer animals than does the method of Litchfield and Wilcoxon (1949), the former was used to characterize the time-action relationship for the anticonvulsant effect of phenytoin in frogs. Electroshock seizures were produced as described in METHODS, Manuscript I. Four groups of frogs (12-16/group) were injected with 20, 30, or 40 mg/kg phenytoin or phenytoin-solvent and CA 50's were determined in each group at various times after injection.

Fig. 3 shows that the CA 50 did not change upon repeated electroshock in frogs injected with phenytoin-solvent, while peak anticonvulsant effect of phenytoin occurred between 2 and 4 hours and lasted, in the case of 40 mg/kg, at least 26 hours after injection. Fig. 4 shows that the anticonvulsant effect decayed in apparent exponential fashion with an apparent "half-time" of about 10.5 hours.

In addition to the dose-effect analysis described in Manuscript I, performed by the method of Litchfield and Wilcoxon (1949), the "Up and Down" method was also employed to compare phenytoin dose-effect relationships in frogs and mice. Fig. 5 gives the results of this study, performed at the time of peak effect of phenytoin (3 hrs), and it can be seen that the slope of the dose-response curve for elevation of the CA 50 by phenytoin is much steeper in mice than in frogs. This result was in agreement with that obtained by the Litchfield and Wilcoxon method (Fig. 2, Manuscript I) and suggests that in mice phenytoin may have sites and/or mechanism(s) of action different from those in frogs.

It can be seen that the "Up and Down" method basically provides the same information on CA 50 as does the method of Litchfield and Wilcoxon (1949). When both economy and efficiency are desirable, as in the characterization of a time-action curve, the "Up and Down" method can be advantageous in that animals are not "wasted" on defining the extremes of a dose-response curve.

Figure 1. "Up and Down" method for the determination of convulsant amperage 50% (CA 50) in untreated control frogs. From left to right, each of 32 frogs was electroshocked in sequence with the indicated amperage, and the presence or absence of tonic hindlimb extension (THE) is indicated by an "X" or an "O", respectively. Amperage, beginning at 78 mA, was adjusted to the next lower level if the frog showed THE, and was raised to the next higher level if the frog failed to show THE. Thus, data were centered around the CA 50 which was calculated to be 75 ± 26 mA (± S.D.).



X = Tonic Hindlimb Extension (*THE*)

O = *THE* Absent

Table 1. Organization of data for the analysis of convulsant amperage 50% by the "Up and Down" method. Data presented in this table were taken from Fig. 1.

RESPONDERS (THE)

mA	i	n	in	i^2n
89	4	4	16	64
78	3	5	15	45
68	2	3	6	12
59	1	2	2	2
52	0	1	0	0

$\Sigma n = 15 \quad \Sigma in = 39 \quad \Sigma i^2n = 123$

NON-RESPONDERS (no THE)

mA	i	n	in	i^2n
89	5	1	5	25
78	4	5	20	80
68	3	5	15	45
59	2	3	6	12
52	1	2	2	2
46	0	1	0	0

$\Sigma n = 17 \quad \Sigma in = 48 \quad \Sigma i^2n = 164$

i = sequence number

n = number of events occurring at that mA

Figure 2. Amperage-response relationships in frogs and mice. Amperages were applied for a duration of 0.5 sec in frogs and 0.2 sec in mice. Percent-response curves were determined with 10-11 animals per amperage. CA 50's (and 95% confidence limits) of these curves as determined by the method of Litchfield and Wilcoxon (1949) were 67 mA (49-91) in frogs and 8.7 mA (7.8-9.7) in mice. CA 50's determined in 32 frogs and 13 mice by the "Up and Down" method of Dixon and Mood (1948) are represented by the open triangles, while error bars refer to 95% confidence limits. CA 50's (and 95% confidence limits) as determined by the "Up and Down" method were 75 mA (67-84) in frogs and 8.8 mA (8.3-9.4) in mice.

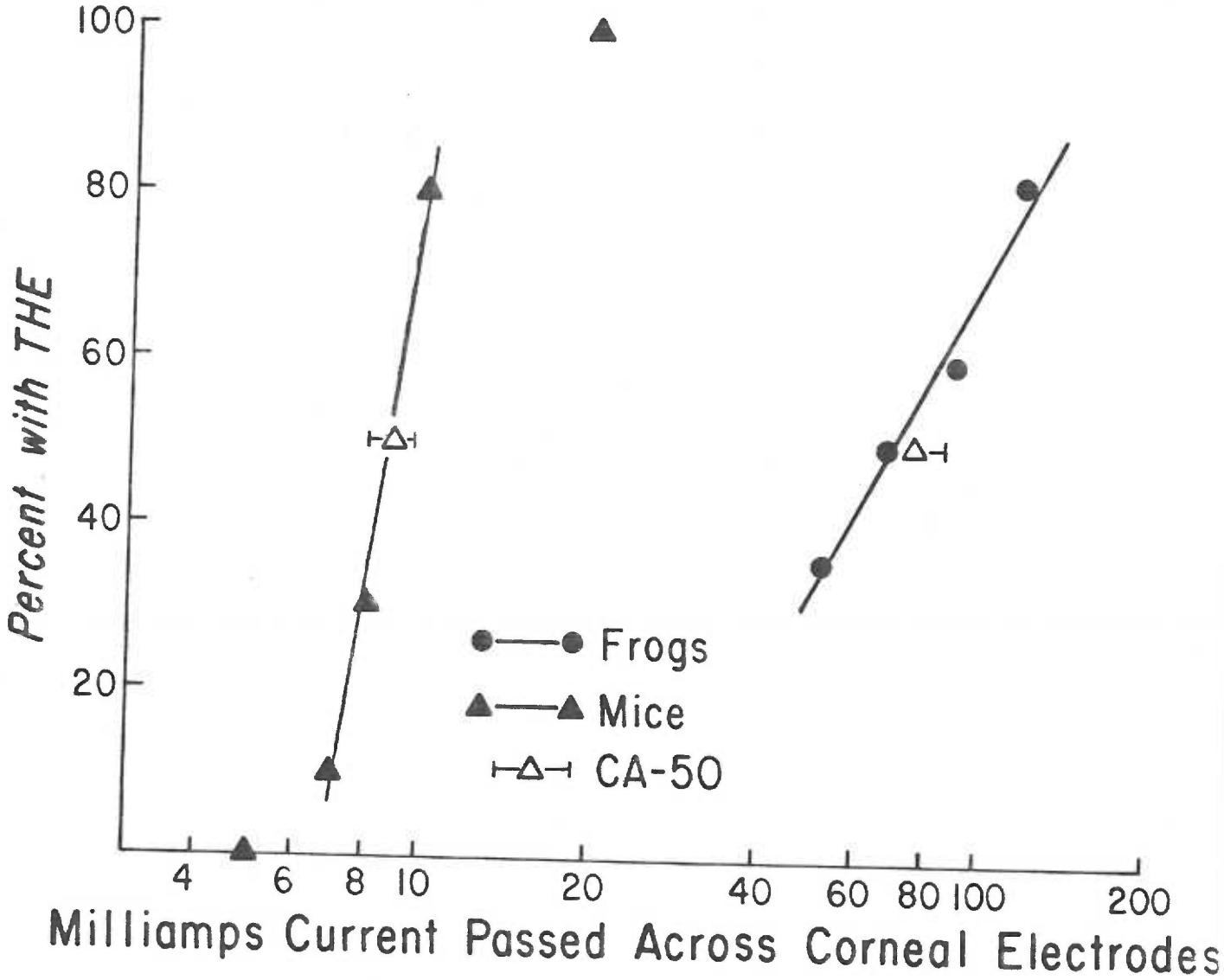

100
80
60
40
20
Percent with THE
Frogs
Mice
CA-50
4
6
8
10
20
40
60
80
100
200
Milliamps Current Passed Across Corneal Electrodes

Figure 3. Dose-time-effect relationship for anticonvulsant action of phenytoin in frogs. Each data point is the average CA 50 (convulsant amperage 50%), measured in milliamperes, of 12-16 frogs. Error bars are 95% confidence limits. CA 50's at "zero" hrs for the phenytoin-treated groups were determined 12 hrs before phenytoin injection. Control frogs were injected with phenytoin-solvent (50 mM NaOH, pH 12.6) at "zero" hrs.

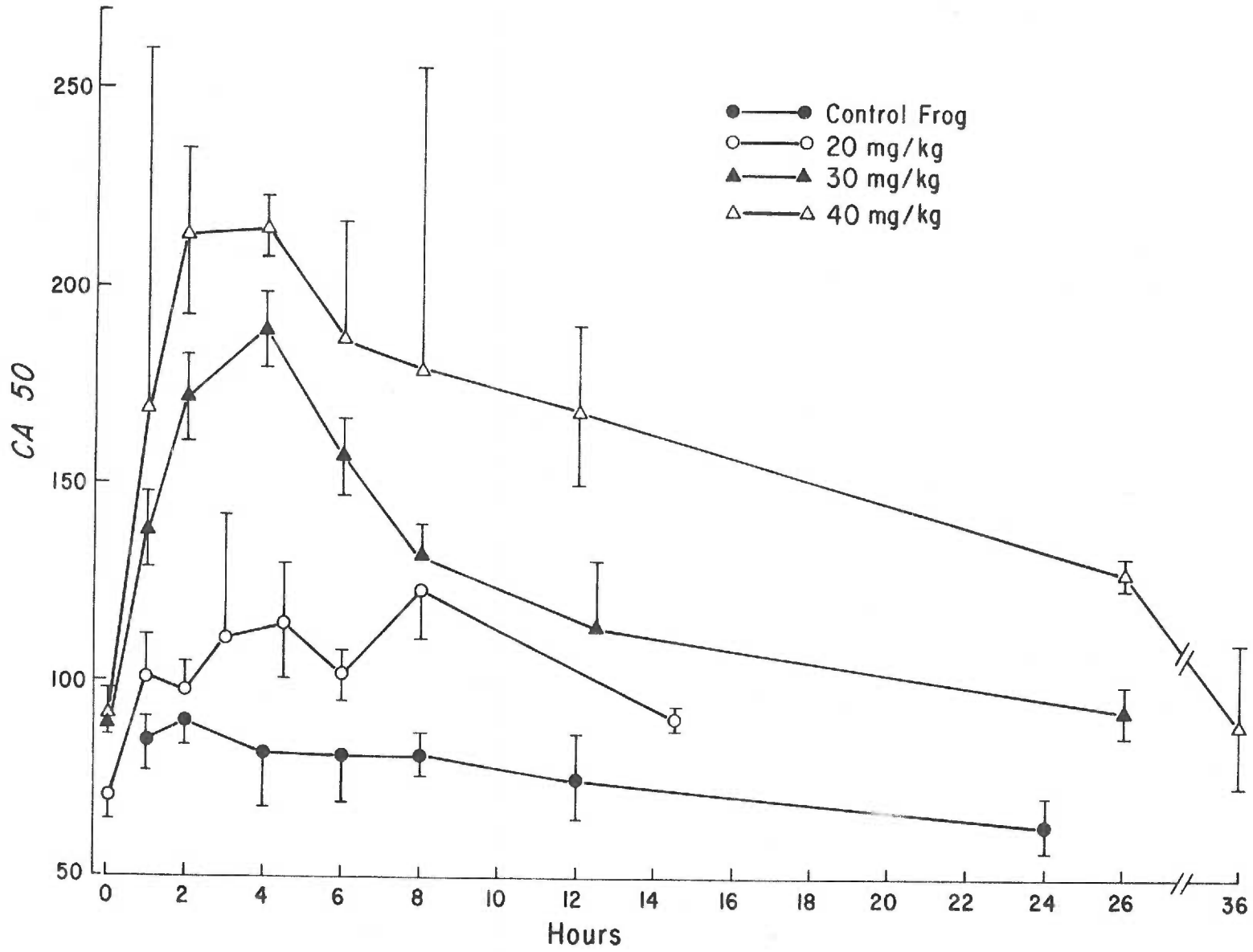

Control Frog
20 mg/kg
30 mg/kg
40 mg/kg
CA 50
250
200
150
100
50
0
2
4
6
8
10
12
14
16
18
20
22
24
26
36
Hours

Figure 4. Semilogarithmic plot of the percent increase in control CA 50 as a function of time. Data presented in this graph were taken from Fig. 3 after CA 50's prior to peak effect (3 hrs) had been omitted. CA 50's in phenytoin-treated frogs were compared to a control CA-50 (75 mA, n=32) obtained in frogs derived from the same population. The average "half-time" for the disappearance of the anticonvulsant effect was approximately 10.5 hrs.

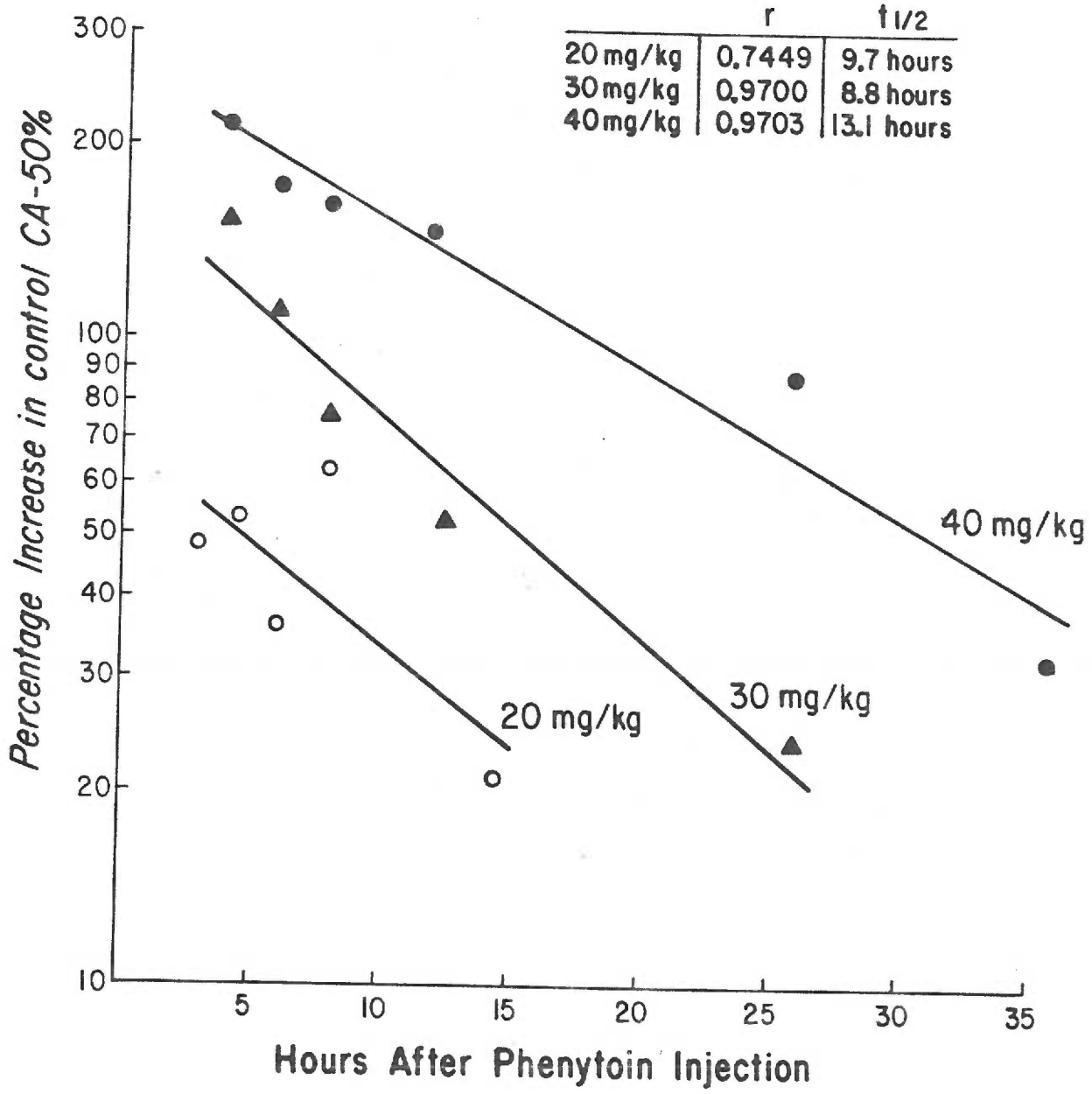
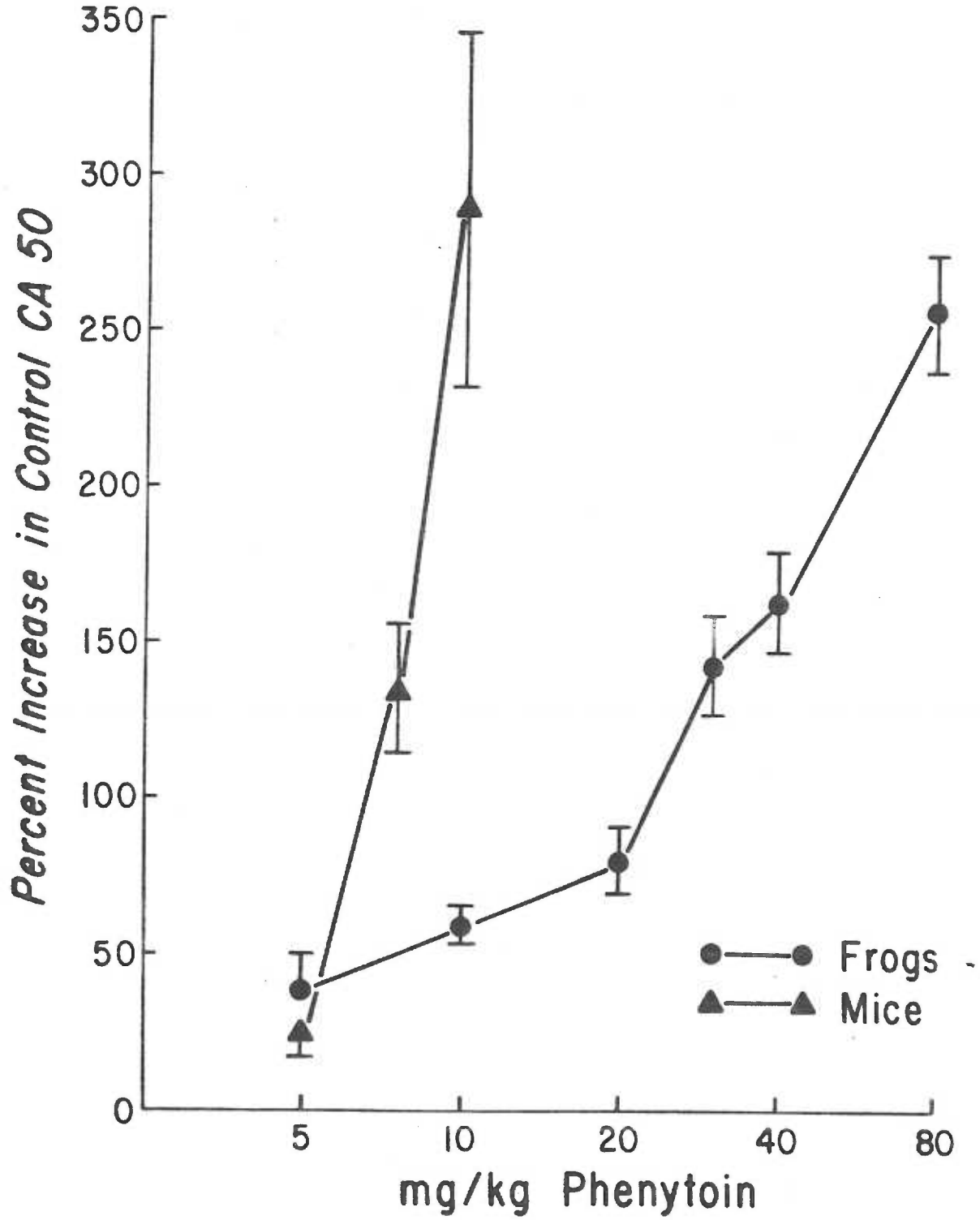

r
t1/2
20 mg/kg
0.7449
9.7 hours
30 mg/kg
0.9700
8.8 hours
40 mg/kg
0.9703
13.1 hours
300
200
100
90
80
70
60
50
40
30
20
10
Percentage Increase in control CA-50%
40 mg/kg
30 mg/kg
20 mg/kg
5
10
15
20
25
30
35
Hours After Phenytoin Injection

Figure 5. Effect of phenytoin on convulsant amperage 50% (CA 50), measured in milliamperes, in frogs and mice. Each data point represents the CA 50 as determined in 12-14 animals, while error bars are standard errors expressed as a percent of the control CA 50. CA 50's in all groups of animals were significantly elevated compared to the respective control CA 50's ($p < 0.05$ to $p < 0.001$, Student's t-test). The CA 50 for mice treated with 12.5 mg/kg (not shown in this figure) was estimated to be 209 mA, a 1380% increase in control CA 50, while current needed to determine a CA 50 in mice treated with 15 mg/kg was beyond the capacity of the electroshock device. Therefore, mice treated with this dose were electroshocked with 425 mA, which was found to be a CA 21 (3/14).


Percent Increase in Control CA 50
350
300
250
200
150
100
50
0
5
10
20
40
80
mg/kg Phenytoin
Frogs
Mice

APPENDIX II

ANTICONVULSANT EFFECTS OF ACUTE AND CHRONIC PHENYTOIN TREATMENT IN *RANA PIPIENS*

INTRODUCTION

Manuscript I of this thesis showed that an appropriate single, acute dose of phenytoin is an effective anticonvulsant treatment in frogs. However, phenytoin is usually administered chronically to patients for the treatment of grand mal epilepsy, and there is no convincing evidence that either tolerance or hypersensitivity develops over prolonged periods of treatment. Therefore, in order to prepare for anticipated studies comparing the effects of acutely vs. chronically administered phenytoin on frog cerebellar Purkinje cell activity, it was important to establish whether or not the anticonvulsant efficacy demonstrable in acutely treated frogs was significantly altered under conditions of chronic treatment. In this study, phenytoin was administered acutely and chronically to frogs, and the anticonvulsant efficacies of these treatments were compared. A preliminary report of these experiments has been published (Johnson and Riker, 1980).

METHODS

Rana pipiens (8-26 gm) were obtained from William Lemberger Assoc. (Germantown, Wisc.) and housed as explained in Manuscript I. The effects of chronically administered phenytoin were tested twice, once with winter frogs and once with summer frogs. On the first day of chronic treatment, loading doses of 10 or 30 mg/kg were injected into the ventral lymph sac of frogs. Thereafter, daily maintenance doses of 9.8 and 26.7 mg/kg were administered to frogs given loading doses of 10 or 30 mg/kg, respectively. These maintenance doses were calculated on the basis of Michaelis-Menten elimination kinetics as described in Appendix V. On the last day of chronic treatment, a convulsant amperage 50% (CA 50), determined by the method of Dixon and Mood (1948), was obtained in each group of frogs 3 hrs after the last dose of phenytoin was injected. Winter frogs were treated for a total of 13 days, while summer frogs were treated for 22 days. For the same length of time, control frogs were given daily injections of phenytoin-solvent (50 mM NaOH) and CA 50's were determined after the chronic treatment.

Immediately after electroshock, frogs were injected with 50 units of sodium heparin (A. H. Robins, Richmond, Va.) (ventral lymph sac) and 4 min later were sacrificed by a sharp blow to the head. Blood was collected by cardiac laceration, centrifuged, and the plasma was stored at -20° C until phenytoin concentrations in plasma could be determined.

Frogs acutely treated with 10-40 mg/kg phenytoin were used for the purpose of comparing CA 50's and plasma phenytoin concentrations with those of the chronically treated frogs. Winter and summer frogs acutely

treated with phenytoin were subjected to electroshock, sacrifice, and blood collection precisely in the way described for the chronically treated frogs.

Plasma phenytoin concentrations were determined by high pressure liquid chromatography (HPLC). 100 µl plasma were transferred to a 4 ml Reacti-vial^{R} (Pierce Chemical Co., Rockford, Ill.) and 50 µl of 4 M phosphate buffer (pH 6.8) and 3 ml of chloroform (Burdick and Jackson Labs., Muskegon, Mich.) containing 600 ng/ml hexobarbital (Riker Labs), internal standard, were added. The mixture was vortexed, centrifuged, the aqueous layer aspirated, and the organic layer was evaporated under a gentle stream of N_2. After dissolving the residue in 300 µl of 30% acetonitrile (Burdick and Jackson Labs) and 70% H_2O (HPLC grade, J. T. Baker, Phillipsburg, N.J.), 100-200 µl were injected onto a 1084 Hewlett Packard liquid chromatograph equipped with a Waters µ-Bondapak phenyl column and a 79875 Hewlett Packard variable wavelength spectrophotometer. The chromatographic separation of hexobarbital and phenytoin in frog plasma by HPLC is shown in Fig. 6.

An unknown concentration of phenytoin in frog plasma was determined by calculating the ratio of the area under the chromatographic peak for phenytoin to that for hexobarbital, the internal standard. Once the area ratio was known, the concentration of phenytoin in the sample was calculated using a standard curve (Fig. 7). The standard curve was made by adding known amounts of phenytoin dissolved in methanol (20 µg/ml) to Reacti-vials^{R}, evaporating the methanol under N_2, and performing the analysis of phenytoin by HPLC as described

above. Phenytoin standards were made in plasma from frogs which had received an injection of phenytoin-solvent.

RESULTS

The results of these experiments, given in Table 2, show that chronically administered phenytoin is an effective anticonvulsant treatment in frogs. CA 50's of winter and summer frogs treated chronically (13-22 days) with 10 or 30 mg/kg phenytoin were significantly increased compared to CA 50's of chronically treated (solvent) control frogs (Table 2). In contrast, the CA 50 values in chronically treated control frogs were not significantly different from those obtained in untreated control frogs.

Plasma levels of phenytoin in frogs given 10 mg/kg acutely or chronically were not different (Table 2). Although summer frogs treated chronically with 30 mg/kg phenytoin had plasma levels which were also not different from levels in acutely treated frogs, winter frogs treated chronically with 30 mg/kg had plasma levels which were significantly lower than those in the corresponding acutely treated frogs ($p < 0.001$, Student's t-test).

Fig. 8 shows that chronically treated frogs displayed no consistent sign of either diminished sensitivity (tolerance) or hypersensitivity to the anticonvulsant effect of phenytoin. In the experiment using winter frogs, plasma concentrations of phenytoin appeared to be more potent following chronic treatment than following acute treatment, while in summer frogs the reverse appeared to be true. However, these apparent differences in potency are not significant. Fig. 8 also shows there is no significant seasonal difference in the potency of acutely administered phenytoin.

Figure 6. High pressure liquid chromatographic (HPLC) separation of hexobarbital (internal standard) and phenytoin in frog plasma. Experimental conditions were isocratic. Solvent, flowing at a rate of 2 ml/min at 23° C, consisted of 30% acetonitrile and 70% H_2O, and absorbance was measured at 195 nm. Numbers in this figure refer to program time in minutes.

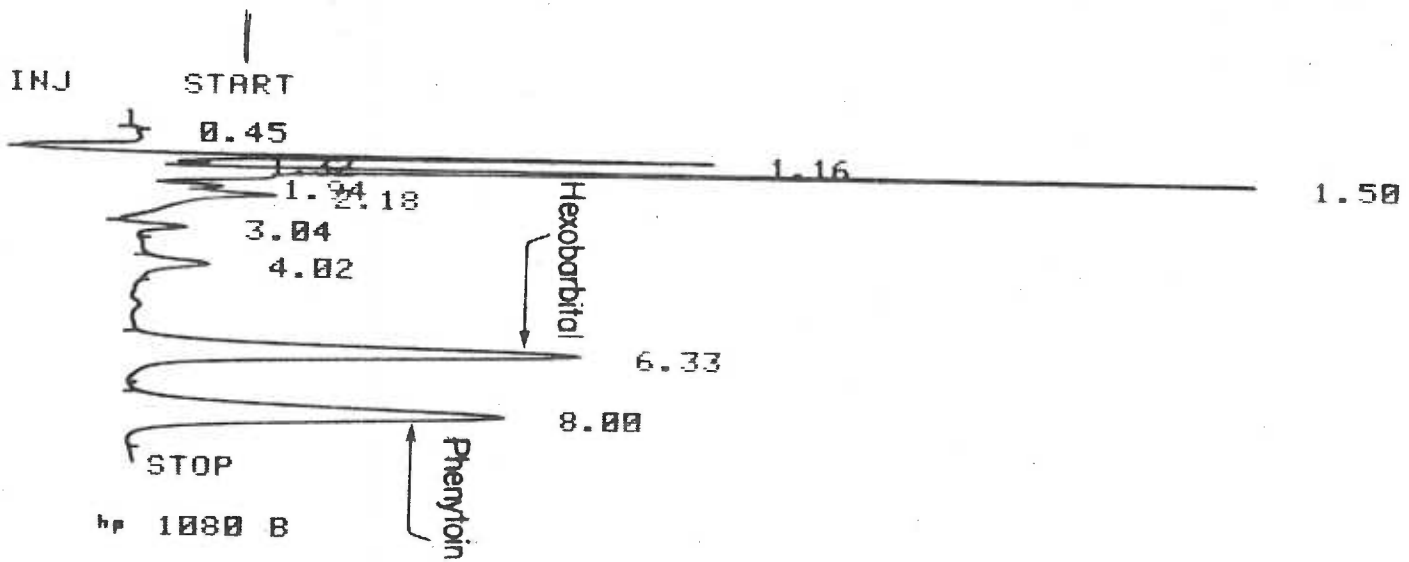

INJ
START
0.45
1.16
1.50
1.94
2.18
3.04
4.02
Hexobarbital
6.33
8.00
STOP
Phenytoin
hp 1080 B

Figure 7. Standard curve for the analysis of phenytoin concentration in frog plasma. Area ratio refers to the area under the liquid chromatographic peak for phenytoin divided by that for the internal standard hexobarbital.

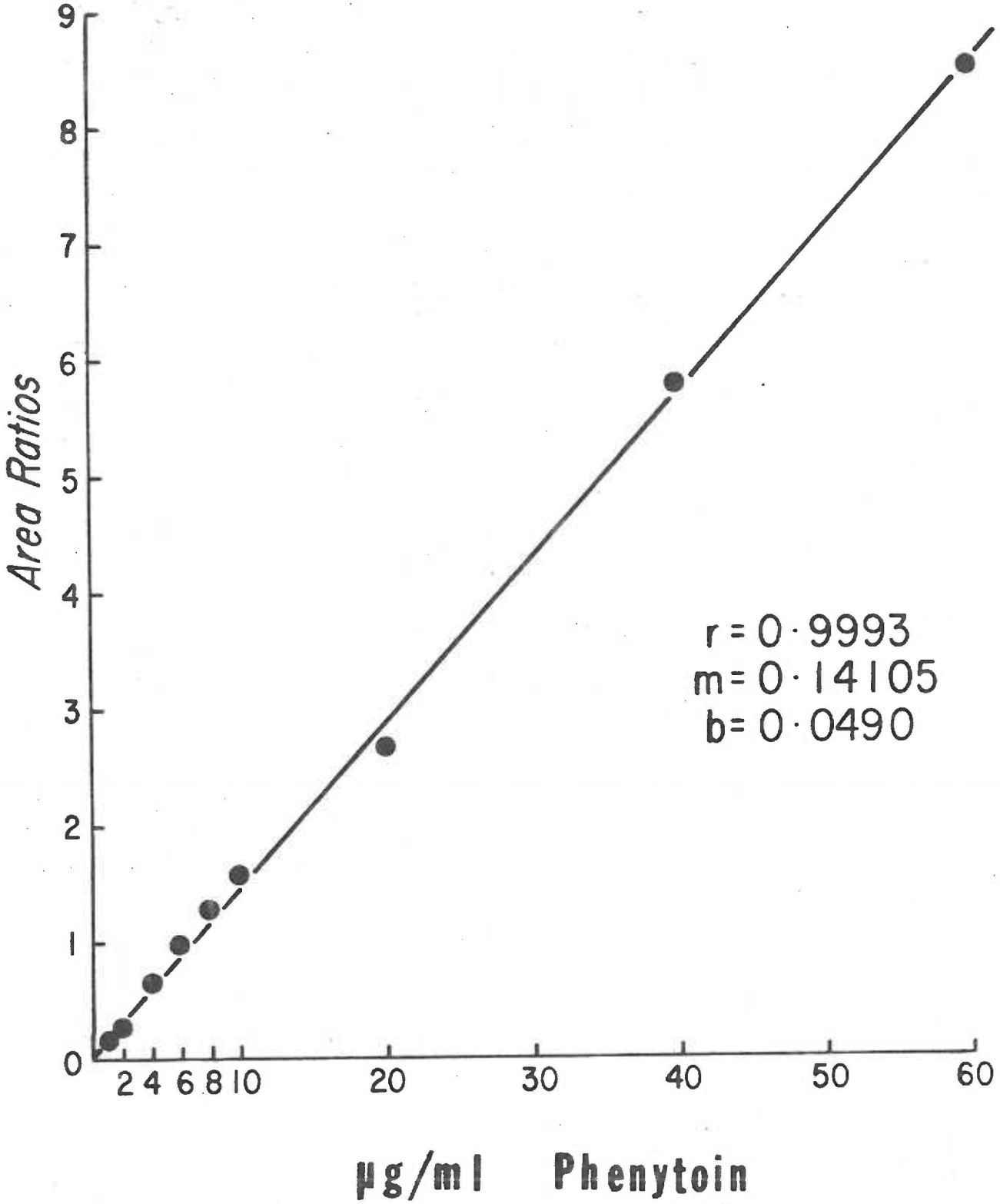

Area Ratios
0
1
2
3
4
5
6
7
8
9
2 4 6 8 10
20
30
40
50
60
r = 0·9993
m = 0·14105
b = 0·0490
µg/ml Phenytoin

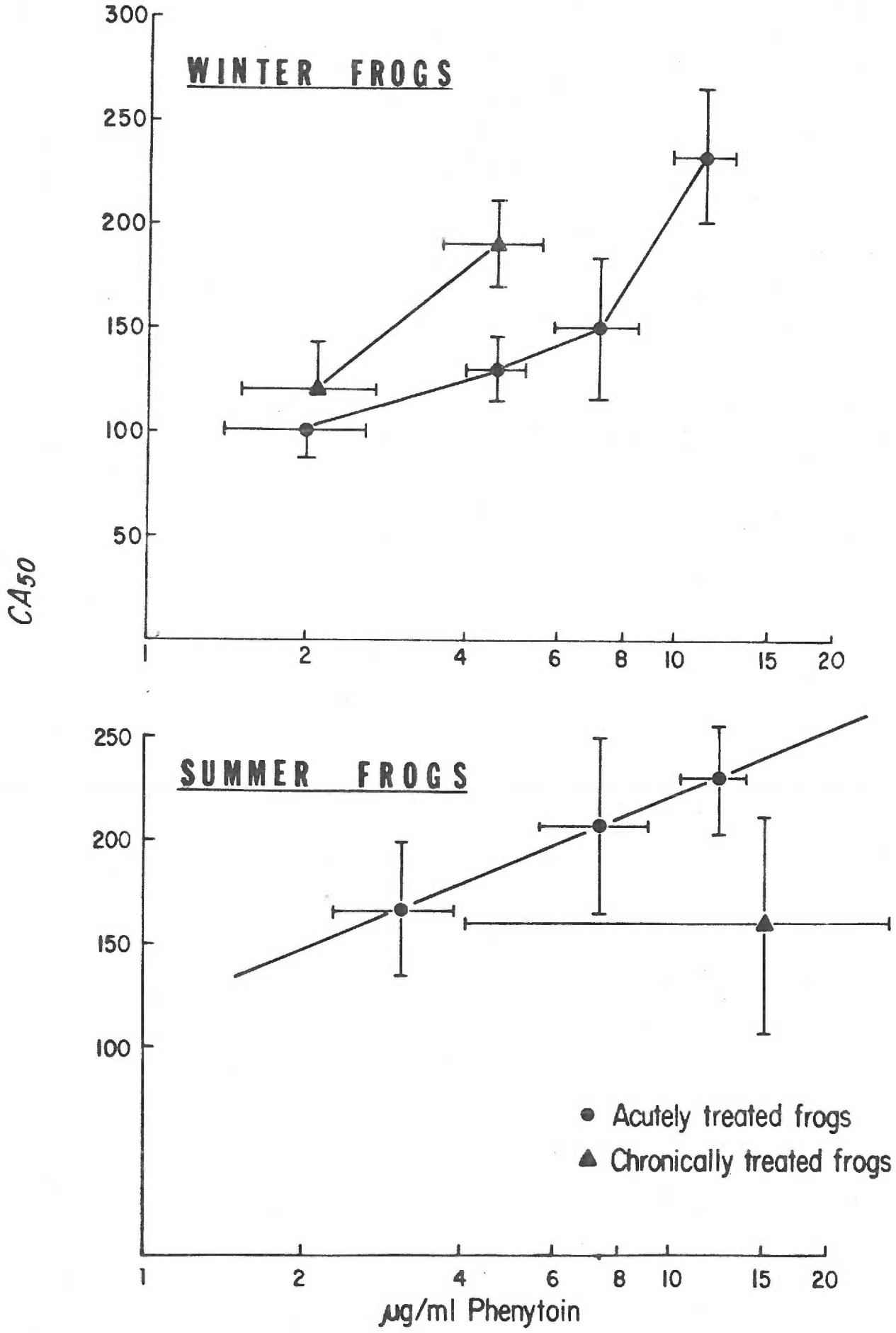
Table 2. Effects of acutely and chronically administered phenytoin on convulsant amperage 50% in winter and summer frogs.

	Acute Untreated Control*		Treated			µg/ml Plasma	
Winter Frogs	CA50 ± SD	N	CA50 ± SD	N	p**	[PHT] ± SD	N
Chronic Control	64 ± 20	8	81 ± 50	8	———	0	8
Chronic 10 mg/kg	74 ± 21	20	119 ± 24	18	< 0.01	2.1 ± 0.6	18
Chronic 30 mg/kg	74 ± 17	20	191 ± 22	16	< 0.001	4.6 ± 1.0	16
Acute 10 mg/kg	89 ± 8	11	101 ± 14	11	< 0.05	2.0 ± 0.6	11
Acute 20 mg/kg	86 ± 11	11	130 ± 16	11	< 0.001	4.6 ± 0.6	11
Acute 30 mg/kg	73 ± 16	11	149 ± 34	11	< 0.001	7.2 ± 1.3	11
Acute 40 mg/kg	72 ± 12	11	232 ± 32	11	< 0.001	11.4 ± 1.6	11
Summer Frogs							
Chronic Control	58 ± 32	16	63 ± 12	16	———	0	16
Chronic 30 mg/kg	———	—	159 ± 52	16	< 0.001	15.2 ± 11.1	16
Acute 20 mg/kg	87 ± 22	16	166 ± 32	16	< 0.001	3.1 ± 0.8	16
Acute 30 mg/kg	60 ± 9	12	206 ± 42	16	< 0.001	7.4 ± 1.7	13
Acute 40 mg/kg	57 ± 12	16	229 ± 26	16	< 0.001	12.4 ± 1.8	16

* Acute, untreated control CA 50's are the CA 50's determined 12 hrs before the beginning of chronic or acute treatment with phenytoin (PHT) or PHT-solvent.

** CA 50's of chronically treated frogs were compared to the CA 50 obtained in control frogs which had been chronically treated with NaOH ("chronically treated control frogs"). CA 50's of acutely treated frogs were compared to the CA 50's obtained in the same frogs 12 hrs before the injection of phenytoin ("acute, untreated controls").

Figure 8. Convulsant amperage 50% (CA 50), measured in milliamperes, as a function of plasma phenytoin concentration in winter and summer frogs treated acutely and chronically with phenytoin. Each data point represents the CA 50 as determined in 11-18 frogs, while error bars represent standard deviations. Chronically treated winter frogs were given loading doses of 10 or 30 mg/kg phenytoin and daily maintenance doses of 9.8 or 26.7 mg/kg, respectively, for 21 days. Acutely treated winter frogs were given 10, 20, 30, or 40 mg/kg phenytoin. Chronically treated summer frogs were given a loading dose of 30 mg/kg and a daily maintenance dose of 26.7 mg/kg for 12 days. Acutely treated winter frogs were given 20, 30, or 40 mg/kg phenytoin.


WINTER FROGS
SUMMER FROGS
300
250
200
150
100
50
250
200
150
100
1
2
4
6
8
10
15
20
CA50
Acutely treated frogs
Chronically treated frogs
µg/ml Phenytoin

DISCUSSION

Phenytoin, chronically administered to frogs, was shown to have unaltered efficacy as an anticonvulsant when compared to acute administration of a single dose. Thus, for the length of drug administration investigated (13-22 days), tolerance to the anticonvulsant effect of phenytoin does not develop in frogs. In mice, however, tolerance develops when phenytoin is administered for 2 weeks in drinking water (Frey and Kampmann, 1965) and may be partially due to induction of microsomal enzymes in the liver (Gerber and Arnold, 1968) with subsequent enhancement of the rate of drug elimination (Gerber and Arnold, 1969). Because tolerance to the anticonvulsant effect of phenytoin is not observed in humans (Buchthal and Lennox-Buchthal, 1972), the frog, in this respect, is perhaps a closer human comparator than is the mouse.

From data presented in RESULTS, it cannot be determined whether or not frogs treated chronically eliminated the drug at a rate different from that in acutely treated frogs. Although chronically treated winter frogs given 30 mg/kg (and daily maintenance doses of 26.7 mg/kg) had plasma levels which were lower than expected based upon levels in the corresponding acutely treated group, thus suggesting that phenytoin is eliminated more rapidly from chronically treated frogs, this difference in plasma levels may actually have been due to insufficient maintenance doses for this particular group of chronically treated frogs.

As a poikilotherm, the frog is liable to exhibit physiological changes according to the season and ambient temperature; potentially

important variables in any bioassay procedure in frogs. In the early 20th century the frog was commonly used to standardize samples of cardiac glycosides, the endpoint being arrest of the heart in systole. Cushny (1918) remarked upon the variability of this endpoint according to the season, as did Clark (1937) with respect to bioassays of ouabain and strophanthin. More recently, Karler and Turkanis (1976) reported that winter frogs, as opposed to frogs obtained during other seasons, are not protected against electrically-induced maximal seizures by relatively large doses of phenytoin or phenobarbital. In our study, we found no significant differences between the potency of phenytoin, given either acutely or chronically, when tested in summer and winter frogs. The storage of our frogs in a room supplied with artificial light at about 23° C in groups of 20-40, and for periods of 2-3 weeks prior to use, may have minimized seasonal variations that might otherwise be present in frogs recently taken from a natural habitat.

In summary, the results of this study show that phenytoin administered chronically to frogs is an effective anticonvulsant therapy in which tolerance, based upon plasma levels of phenytoin, is not a significant factor. Also, in our laboratory, no seasonal variation in response to phenytoin was observed in frogs. Therefore, it is concluded that the frog is a suitable test animal, in summer or winter, for studying anticonvulsant effects of chronically or acutely administered phenytoin.

APPENDIX III

EFFECT OF AMPERAGE ON DURATION OF TONIC HINDLIMB EXTENSION

EFFECT OF AMPERAGE ON DURATION OF TONIC HINDLIMB EXTENSION

Although prevention of tonic hindlimb extension (THE) following maximal electroshock is the classic endpoint for measuring the anticonvulsant effect of phenytoin, this drug also has the capacity, known for many years, to shorten the duration of THE (Bárány and Stein-Jensen, 1946; Toman, Loewe, and Goodman, 1947; Goodman, Toman, and Swinyard, 1948; Esplin and Freston, 1960). However, in order to utilize THE duration as a measure of phenytoin anticonvulsant effect, the electroshock amperage must be greater than maximal to overcome the protective effect of phenytoin, i.e., to elicit THE. The relationship between electroshock amperage and THE duration was therefore explored in frogs and mice to determine whether or not THE duration was independent of stimulus strength.

Maximal seizures were elicited in frogs and mice with corneal electroshock and durations of THE were timed, as explained in METHODS, Manuscript I. Frogs were injected with phenytoin (20 mg/kg) or phenytoin-solvent (50 mM NaOH) and electroshocked at varying amperages 3 hours later. Fig. 9 shows that the duration of THE in either control frogs or frogs treated with phenytoin is independent of electroshock amperage over a wide range. This is also the case in mice, in which THE duration after electroshock with 24 mA (11.9 ± 2.1 seconds, ± S.D., n=8) was not significantly different from that elicited with 350 mA (12.3 ± 1.4 seconds, n=10, $p > 0.6$). These results are in agreement

with those of Toman et al. who found that THE duration was independent of corneal electroshock amperage in rabbits and rats (Toman, Swinyard, and Goodman, 1946).

Because THE duration was not dependent on electroshock amperage, it was possible to conduct dose-response analyses for the shortening of THE duration by phenytoin. Fig. 10 shows that THE duration in frogs was progressively shortened by increasing doses of phenytoin, reaching a peak reduction of about 90%. Because extensions with a duration of less than 1 second were not considered tonic in nature (THE), a 90% reduction in THE duration is virtually the limit of the measurement technique.

In Manuscript I, it was shown that the dose-range of phenytoin or phenobarbital which shortened THE duration during maximal seizures in intact frogs was not significantly different from the dose-range which shortened THE duration in "spinal" frogs, i.e., frogs which had been decapitated 1.5 sec after corneal electroshock. It is suggested that THE duration is shortened by anticonvulsant drugs which interfere with the functioning of spinal cord pathways and, therefore, THE duration may be a useful adjunct to the use of prevention of THE as an endpoint for the testing of anticonvulsant drugs.

Figure 9. Duration of tonic hindlimb extension (THE) in frogs as a function of amperage. Each data point represents the mean of 5-8 frogs, and error bars represent standard deviations. As determined by linear regression, slopes (and correlation coefficients) were -0.3551 (0.2508) in frogs treated with NaOH (control frogs) and 0.5023 (0.4444) in frogs treated with 20 mg/kg phenytoin. Neither slope was significantly different from zero ($p > 0.5$, Student's t-test).

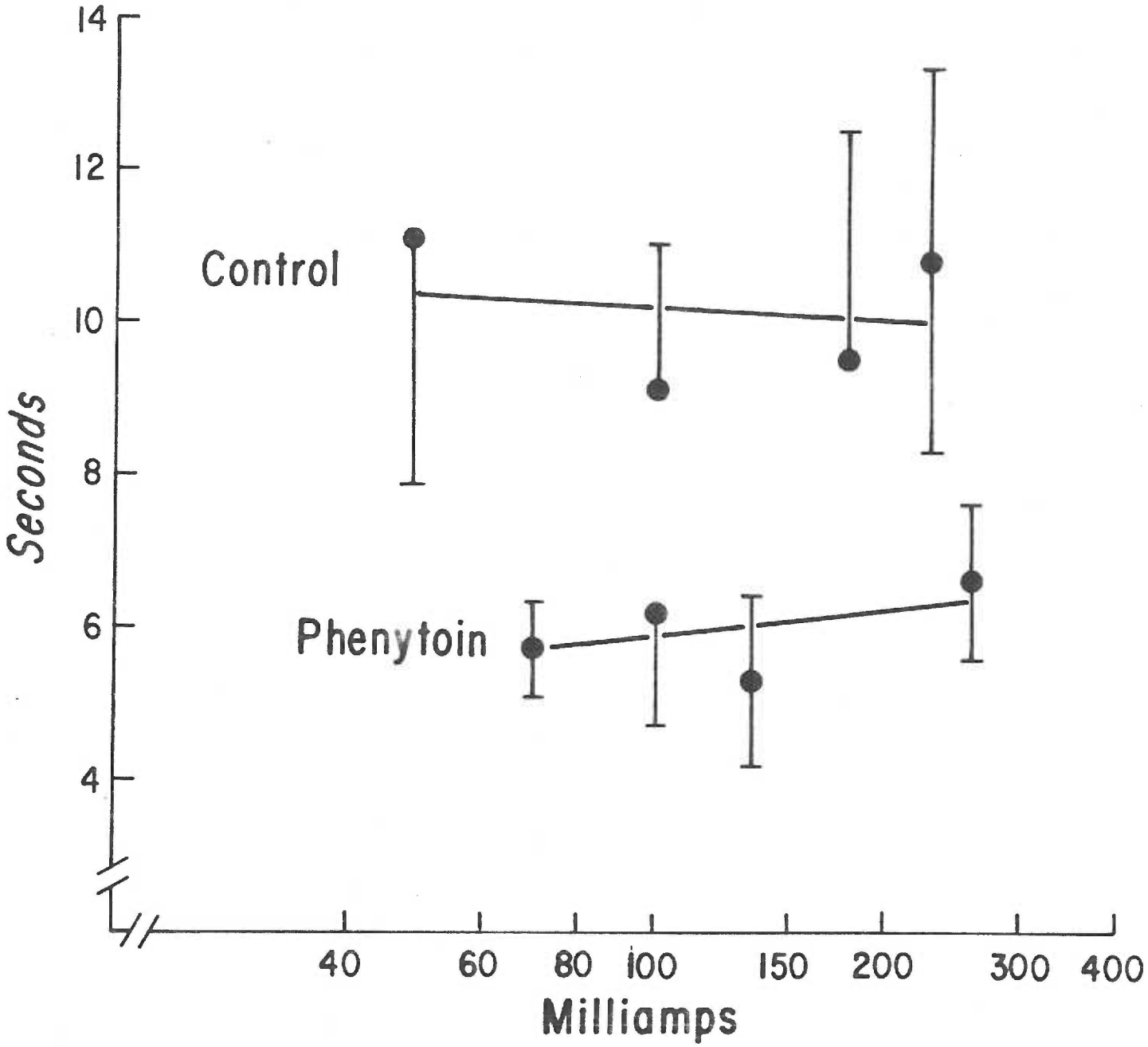
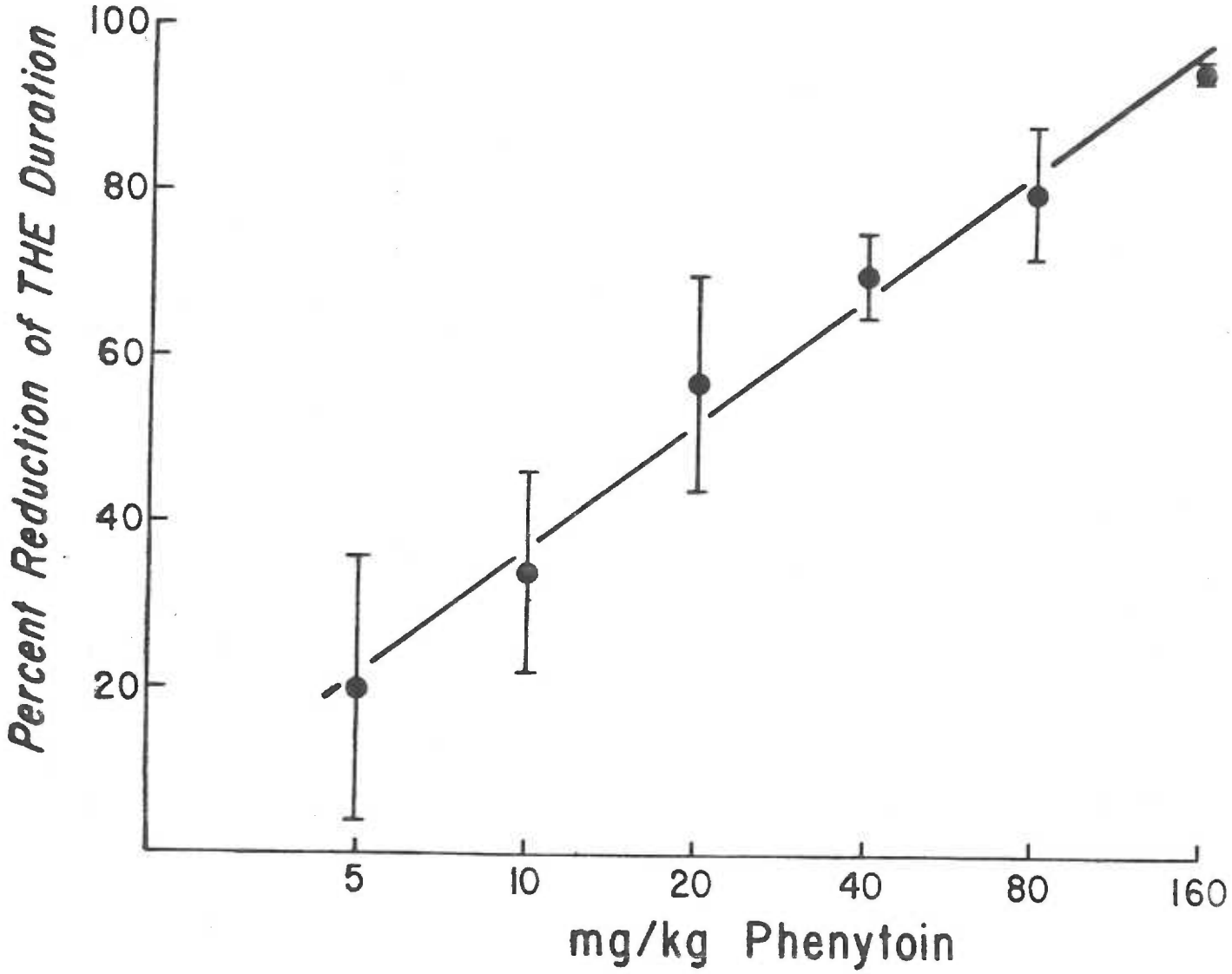

14
12
10
8
6
4
Seconds
Control
Phenytoin
40
60
80
100
150
200
300
400
Milliamps

Figure 10. Percentage reduction of the duration of tonic hindlimb extension (THE) by phenytoin in frogs. Each data point represents 11-12 frogs, while error bars are standard deviations expressed as a percent of control THE duration. Results shown in this figure were obtained in three experiments, each separated by at least one week, using the same population of frogs. All frogs were electroshocked with 400-425 mA, and control THE durations were 8.5 ± 1.5 seconds (± S.D.)(for 10 and 80 mg/kg), 7.4 ± 1.7 seconds (for 5 and 20 mg/kg), and 9.8 ± 1.9 seconds (for 40 and 160 mg/kg). According to the method of Litchfield and Wilcoxon (1949), the ED 50 (and 95% confidence limits) was 17.2 mg/kg (9.5-31.2).


Percent Reduction of THE Duration
100
80
60
40
20
5
10
20
40
80
160
mg/kg Phenytoin

APPENDIX IV

PHENYTOIN-INDUCED TOXICITY: LOSS OF RIGHTING REFLEX

PHENYTOIN-INDUCED TOXICITY: LOSS OF RIGHTING REFLEX

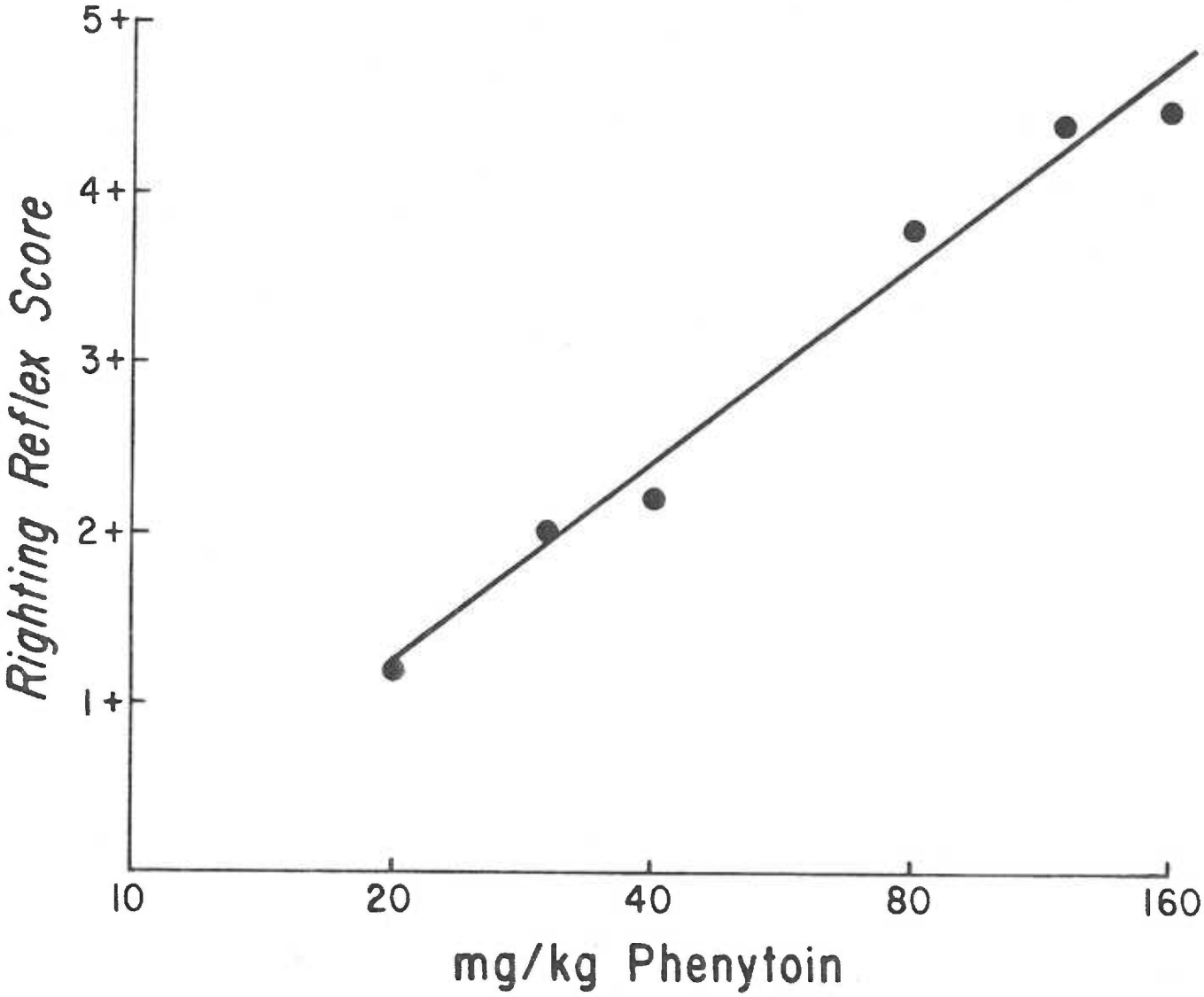
One of the most easily recognized toxicities of phenytoin in frogs was loss of righting reflex. However, depression of righting reflex was found not to be a simple, precise function of phenytoin dose. Therefore, a non-parametric scoring scale was devised and used to measure the impairment of this reflex by phenytoin in frogs.

A 1+ to 5+ scale was used to score the impairment in righting reflex. Normal frogs, when placed on their dorsal aspects, righted in less than 1 sec and were scored 1+. Frogs which righted in less than 5 sec but greater than 1 sec were scored 2+. Righting which occurred in less than 15 sec but greater than 5 sec was scored 3+. Righting which took place in less than 30 but greater than 15 sec was scored 4+. Frogs which righted only after 30 sec or more were scored 5+. As opposed to control frogs, frogs treated with phenytoin displayed decreased ability to right as the number of attempts increased. Therefore, the ability of frogs to right was tested at least three times and scores from each frog were averaged.

Three hours after injection of phenytoin into the ventral lymph sac there was a dose-dependent depression of the righting reflex, as seen in Fig. 11. Despite the use of a non-parametric scale, this dose-response curve is adequately described by linear regression ($r=0.9907$) and a TD 50, arbitrarily defined as a score of 3+, was found to be 57 mg/kg. This arbitrary TD 50 is higher than the TD 50 of 33.2 mg/kg determined by post-rotatory head turning (PRHT) (Manuscript I). Also, minimal signs of righting reflex depression are not seen until doses

greater than 20 mg/kg are used; doses which cause substantial suppression of PRHT. Therefore, depression of righting reflex is not as sensitive a measure of phenytoin-induced toxicity as suppression of PRHT and, because of this, suppression of PRHT instead of depression of righting reflex was used as the endpoint for phenytoin-induced toxicity (see RESULTS, Manuscript I).

Figure 11. Depression of the righting reflex by phenytoin in frogs. Each data point represents the average score of 8-14 frogs examined 3 hrs after phenytoin injection (ventral lymph sac). The righting reflex scale is a 1+ to 5+ system in which higher numbers indicate increasing impairment of the reflex. 1+ is the score for an unimpaired righting reflex. Although based upon a nonparametric scale, this dose-response was adequately described by linear regression ($r=0.9907$). A toxic dose 50% (TD 50), arbitrarily defined as a score of 3+ (frog righted between 5 and 15 sec), was 57 mg/kg. The two highest doses, 120 and 160 mg/kg, were near the LD 50 (see Manuscript I), and several frogs died from these doses of phenytoin before righting reflex could be tested.


5+
4+
3+
2+
1+
Righting Reflex Score
10
20
40
80
160
mg/kg Phenytoin

APPENDIX V

THE INTEGRATED MICHAELIS-MENTEN EQUATION: ITS DERIVATION AND USE

THE INTEGRATED MICHAELIS-MENTEN EQUATION: ITS DERIVATION AND USE

The Michaelis-Menten equation, originally used to describe the kinetics of the fermentation of sugars (Michaelis and Menten, 1913), has been found to describe accurately the non-first-order or concentration-dependent elimination kinetics of several commonly used drugs, such as ethanol (Lundquist and Wolthers, 1958), salicylate (Levy, Tsuchiya, and Amsel, 1972), and phenytoin (Gerber and Wagner, 1972). Based upon data presented in Manuscript I of this thesis, the integrated Michaelis-Menten equation also adequately describes the kinetics for the elimination of phenytoin from frog lymph, and these data were used to plan maintenance dosing schedules for frogs chronically treated with phenytoin (Appendix II). In this appendix, the integrated form of the Michaelis-Menten equation is derived and the method for planning maintenance dosing schedules is explained.

The unintegrated form of the Michaelis-Menten equation is given below:

$$V = \frac{V_{max} \cdot s}{K_m + s}$$

where V is the initial velocity of the reaction, i.e., the rate of appearance of product (dp/dt), V_{max} is the maximum velocity of the reaction, K_m is the Michaelis-Menten constant, and s is the concentration of substrate. Because in our studies we were more interested in measuring the disappearance of substrate (phenytoin) rather than the

appearance of product, dp/dt was replaced with -ds/dt and the equation was rewritten as:

$$-\frac{ds}{dt} = \frac{V_{max} \cdot s}{K_m + s}$$

To prepare for integration, the variables are separated:

$$-\left(\frac{K_m + s}{s}\right) ds = V_{max}\, dt$$

and integration between the boundaries "a" and "b" yields the equation:

$$-K_m \left(\ln \frac{s_b}{s_a}\right) - (s_b - s_a) = V_{max} (t_b - t_a)$$

After dividing by $(t_b - t_a)$ and rearranging, the form of the integrated Michaelis-Menten equation used in this thesis is obtained:

$$\frac{s_a - s_b}{t_b - t_a} = -\frac{K_m \left(\ln \frac{s_a}{s_b}\right)}{(t_b - t_a)} + V_{max} \quad \text{(Equation 1)}$$

The plotting of $(s_a - s_b)/(t_b - t_a)$ vs $(t_b - t_a)^{-1} \ln(s_a/s_b)$ should yield a straight line with slope = $-K_m$ and a "y" intercept of V_{max}. To fit empirical data to this equation, one may either choose a fixed value for s_a (e.g., s_1, the concentration of substrate at 1 hr) and subtract all subsequent values of s_b from s_a, or one can define s_a and s_b as the

higher and lower substrate concentrations, respectively, in each time interval. This second method was favored by Lundquist and Wolthers (1958) and was the method used in this thesis to plot the elimination of phenytoin from frog lymph. This plot can be seen in Fig. 4, Manuscript II.

The integrated Michaelis-Menten equation was used to calculate maintenance doses for frogs chronically treated with phenytoin (Appendix II). In this experiment, it was decided that maintenance doses would be given every 24 hours. Therefore, the maintenance dose, which is that fraction of the loading dose which is eliminated in 24 hrs, can be calculated by the equation:

$$\text{Maintenance Dose} = \text{Loading Dose} \left(\frac{S_0 - S_{24}}{S_0} \right) \quad \text{(Equation 2)}$$

where S_0 is the substrate (phenytoin) concentration extrapolated to "zero" time and S_{24} is the substrate concentration at 24 hrs. It is in the calculation of S_0 and S_{24} that the Michaelis-Menten equation is employed. However, because Michaelis-Menten kinetics are concentration-dependent, a knowledge of one point on the elimination curve is essential for calculating S_0 and S_{24}. This information is provided in Fig. 12, which shows the relationship between dose of phenytoin and plasma concentration of phenytoin measured 3 hrs after injection, a time at which an equilibrium is established between the concentration of drug in tissues and that in plasma (see tissue distribution study, Manuscript II). The use of the Michaelis-Menten equation and Fig. 12 in calculating maintenance doses is illustrated by two examples given below.

To calculate the 24-hr maintenance dose following a loading dose of 30 mg/kg, the expected plasma concentration of phenytoin at 3 hrs is estimated from Fig. 12. From this figure, one finds s_3 = 7.84 µg/ml. This value for s_3 is then used to calculate s_0 and s_{24}. First, s_0 is calculated by substituting into Equation 1 7.84 µg/ml and 3 hrs for s_b and t_b, and s_0 and 0 hrs for s_a and t_a, respectively. After entering the values of K_m = 2.75 µg/ml and V_{max} = 0.595 µg/ml·hr, which were determined using data presented in Fig. 4, Manuscript II, the equation is rearranged to give:

$$s_0 = -2.75 \ln\left(\frac{s_0}{7.84}\right) + 9.625$$

An approximate solution for s_0 is 9.19 µg/ml. Second, s_3 is used to calculate s_{24} in an analogous manner. Substituting 7.84 µg/ml and 3 hrs for s_a and t_a, and s_{24} and 24 hrs for s_b and t_b, respectively, this equation, after rearrangement, is obtained:

$$s_{24} = -2.75 \ln\left(\frac{s_{24}}{7.84}\right) - 4.655$$

An approximate solution for s_{24} is 1.00 µg/ml. The maintenance dose can now be calculated by substituting the values for s_0 and s_{24} into Equation 2:

$$(30 \text{ mg/kg}) \left(\frac{9{\cdot}19 - 1{\cdot}00}{9{\cdot}19}\right) = 26.7 \text{ mg/kg}$$

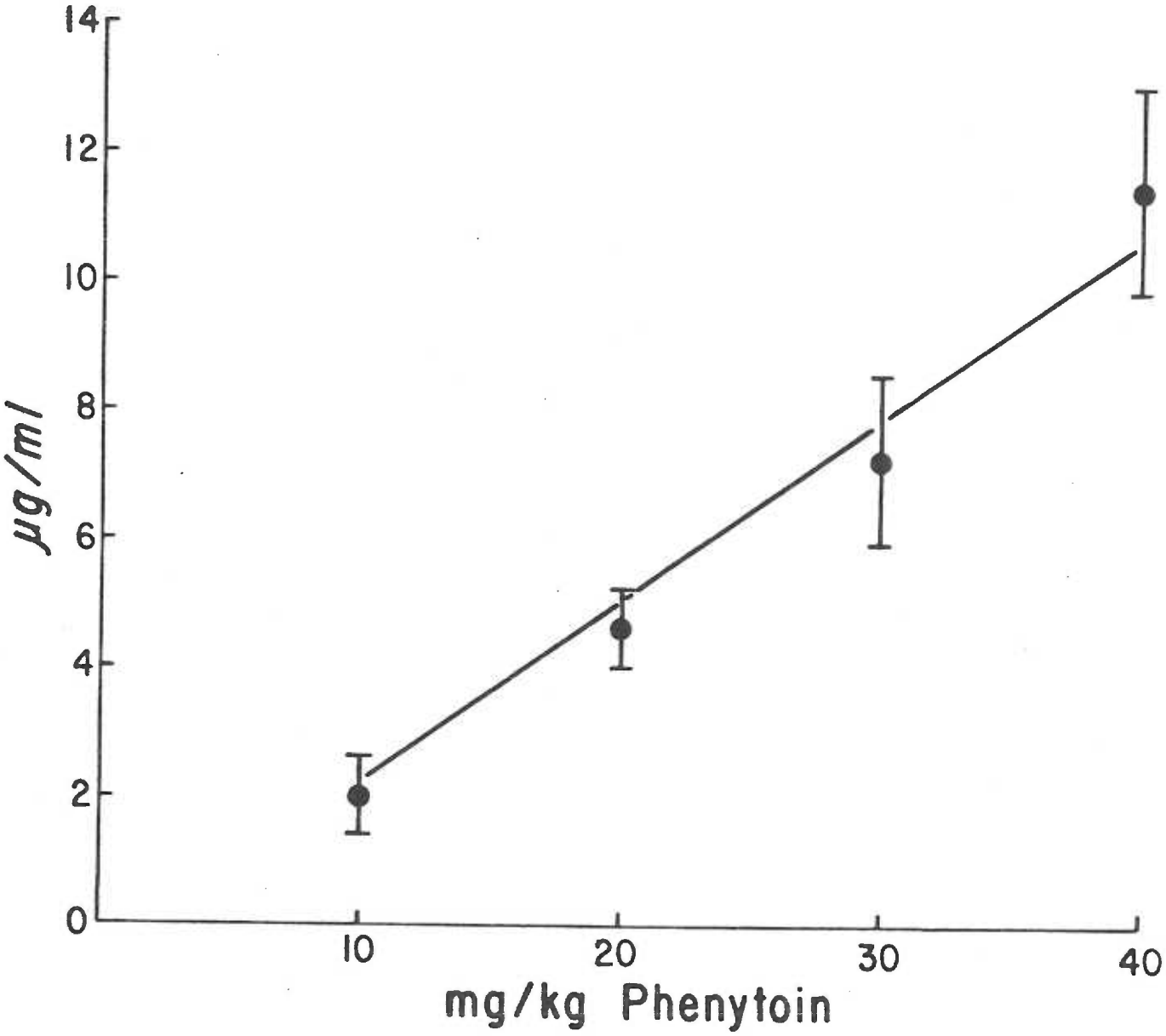
Therefore, the 24-hr maintenance dose, following a loading dose of 30 mg/kg, should be 26.7 mg/kg.

Phenytoin was also administered chronically to frogs following a loading dose of 10 mg/kg (Appendix II), and the 24-hr maintenance dose was calculated in the same way as that for 30 mg/kg. From Fig. 12, an injection of 10 mg/kg phenytoin is expected to produce a plasma concentration of 2.24 µg/ml 3 hrs after injection (s_3). Substituting into Equation 1 2.24 µg/ml and 3 hrs for s_b and t_b, and s_0 and 0 hrs for s_a and t_a, respectively, gives an approximate solution of 3.11 µg/ml for s_0. Substituting into Equation 1 2.24 µg/ml and 3 hrs for s_a and t_a, and s_{24} and 24 hrs for s_b and t_b, respectively, gives an approximate solution of 0.053 µg/ml for s_{24}. Therefore, by substituting these values of s_0 and s_{24} into Equation 2, the 24-hr maintenance dose following a loading dose of 10 mg/kg is calculated to be 9.8 mg/kg.

A possible source of error in these calculations stems from the fact that Michaelis-Menten constants were derived from data obtained from lymph while data from Fig. 12 were obtained from plasma. Because lymph contains only 90% of the phenytoin levels found in plasma (tissue distribution study, Manuscript II), the K_m would have been higher if the elimination curve had been measured in plasma, although the V_{max} probably would not have been altered. Although underestimating the value for K_m would lead to overestimation of the required maintenance doses, the magnitude of this error is probably insignificant when one considers the variations in K_m from frog to frog. From the results of experiments which used these 24-hr maintenance dosing schedules

(Appendix II), it can be seen that plasma levels of phenytoin in chronically treated frogs were approximately what one would predict on the basis of levels in frogs acutely treated with phenytoin.

Figure 12. Relationship between dose of phenytoin and concentration of phenytoin in frog plasma. Each data point represents the mean of 11 frogs, and error bars are standard deviations. Phenytoin was injected into the ventral lymph sac (v.l.s.) and 3 hrs later, 50 units of heparin (A. H. Robins, Richmond, Va.) was injected (v.l.s.), blood was collected, and plasma was prepared and analyzed for phenytoin by liquid chromatography as explained in Appendix II. Data in this figure were adequately described by linear regression ($r=0.9902$) with the equation $y = 0.28x - 0.56$.


14
12
10
8
6
4
2
0
µg/ml
10
20
30
40
mg/kg Phenytoin

APPENDIX VI

SPECIFICITY OF THE EXTRACTION OF PHENYTOIN INTO 1-CHLOROBUTANE

INTRODUCTION

Gerber et al. used counter current distribution (CCD) to show that 1-chlorobutane (BuCl) selectively extracted phenytoin from rat tissues (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971). In the present study, CCD and gas chromatography were used to show that BuCl selectively extracts phenytoin from box water gathered from frogs treated with phenytoin.

METHODS

Aliquots of box water used in this study were obtained from a bullfrog (Rana catesbiana) injected with 40 mg/kg ^{14}C-phenytoin (0.19 μc/mg) and a leopard frog (Rana pipien) injected with 100 mg/kg ^{14}C-phenytoin (0.64 μc/mg). These aliquots were taken from the same box water used to identify metabolites of phenytoin (see METHODS, Manuscript II).

Box water from the bullfrog was used in CCD. A 50-ml aliquot was mixed with 1.25 ml of 4 M phosphate buffer (pH 6.8) and equilibrated with 3 volumes of BuCl (500 ml per volume). The volumes of BuCl were pooled, evaporated in a rotor evaporator (Büchi Rotavapor, Brinkman Instrument Co.), and the residue was dissolved in acetone (20 ml). After being transferred to a conical centrifuge tube, the acetone, which contained about 20,000 dpm (0.19 μmoles), was evaporated under N_2. This centrifuge tube was used as "tube #1" in the counter current procedure.

Four-tube CCD was carried out between BuCl (5 ml) and 0.1 M phosphate buffer (pH 6.8) (35 ml) according to the method of Bush and Densen (1948). BuCl was added to tube #1, and after equilibration with aqueous buffer in tube #1, was quantitatively transferred to tube #2. Fresh BuCl was then added to tube #1. Buffer and BuCl were equilibrated and BuCl from tubes 1 and 2 were transferred to tubes 2 and 3, respectively. Fresh BuCl was again added to tube #1. This procedure was repeated until 4 BuCl layers had been equilibrated with each aqueous layer. Four ml of BuCl and 2 ml of buffer were assayed for ^{14}C as described in METHODS, Manuscript II.

Box water from the leopard frog was used as substrate for the gas chromatographic study. A 50-ml aliquot was mixed with 1.25 ml of 4 M phosphate buffer (pH 6.8) and equilibrated with BuCl (250 ml). BuCl was evaporated in a rotor evaporator and the residue was dissolved in acetone (20 ml). The acetone, which contained about 150,000 dpm (0.42 μmoles), was transferred to a Reacti-vial^{R}, evaporated under N_2, and permethylated with CH_3I as described in METHODS, Manuscript II. A 50-ml aliquot of box water from an untreated leopard frog was used as a control sample and was prepared for gas chromatography in the same way as the sample which contained ^{14}C. Also, 1 mg of sodium phenytoin (Parke, Davis and Co.) was prepared for gas chromatography (see METHODS, Manuscript I) for the purpose of comparing the retention time of phenytoin to retention times of unknown components of box water.

RESULTS

The results of CCD are plotted by the method of Bush and Densen (1948) in Fig. 13. CCD demonstrated that BuCl extracted only one radiolabelled compound in any significant amount from box water. The compound extracted into BuCl from the phosphate-buffered aqueous layer had a partition coefficient (C_o) of 1.86, which is essentially the same as the C_o of 1.9 of phenytoin obtained by Gerber et al. using similar experimental conditions (Gerber, Weller, Lynn, Rangno, Sweetman, and Bush, 1971).

As seen in Fig. 14, the chromatogram of the sample of box water which contained phenytoin and metabolites had one component which was absent in the chromatogram of the control sample. This component, which eluted from the column 6.65 min after the sample was injected, elutes at virtually the same time as does permethylated phenytoin under the same experimental conditions as seen in Fig. 15.

DISCUSSION

CCD showed that BuCl extracts a single ^{14}C-labelled compound from box water which contains ^{14}C-labelled phenytoin and metabolites. This compound has virtually the same partition coefficient as does phenytoin. Moreover, gas chromatography showed that BuCl extracted a compound from box water which elutes from the column at the same time as does phenytoin. It is concluded that phenytoin, in aqueous media buffered at pH 6.8, is selectively extracted into BuCl. Contamination of BuCl with metabolites of phenytoin does not occur appreciably.

Figure 13. Counter current distribution (CCD) of ^{14}C extracted into 1-chlorobutane (BuCl) from box water obtained from a bullfrog injected with ^{14}C-phenytoin. The 4-tube CCD was performed between BuCl (5 ml) and 0.1 M phosphate buffer (pH 6.8) (35 ml), and the results shown in this figure were plotted according to the method of Bush and Densen (1948). Numbers 1 to 4 are BuCl fractions, while numbers 5 to 8 are the aqueous fractions, left in the tubes, and numbered in reverse order: the first aqueous fraction is number 8, the second aqueous fraction is number 7, etc. The theoretical curve was based upon p = 0.21 and q = 0.79, where p and q are the fractions of ^{14}C in the upper and lower layers, respectively. The partition coefficient, calculated from the equation $C_o = (p/q)(V_y/V_x)$ where V_x and V_y are the volumes in the upper and lower layers, respectively, was found to be 1.86.

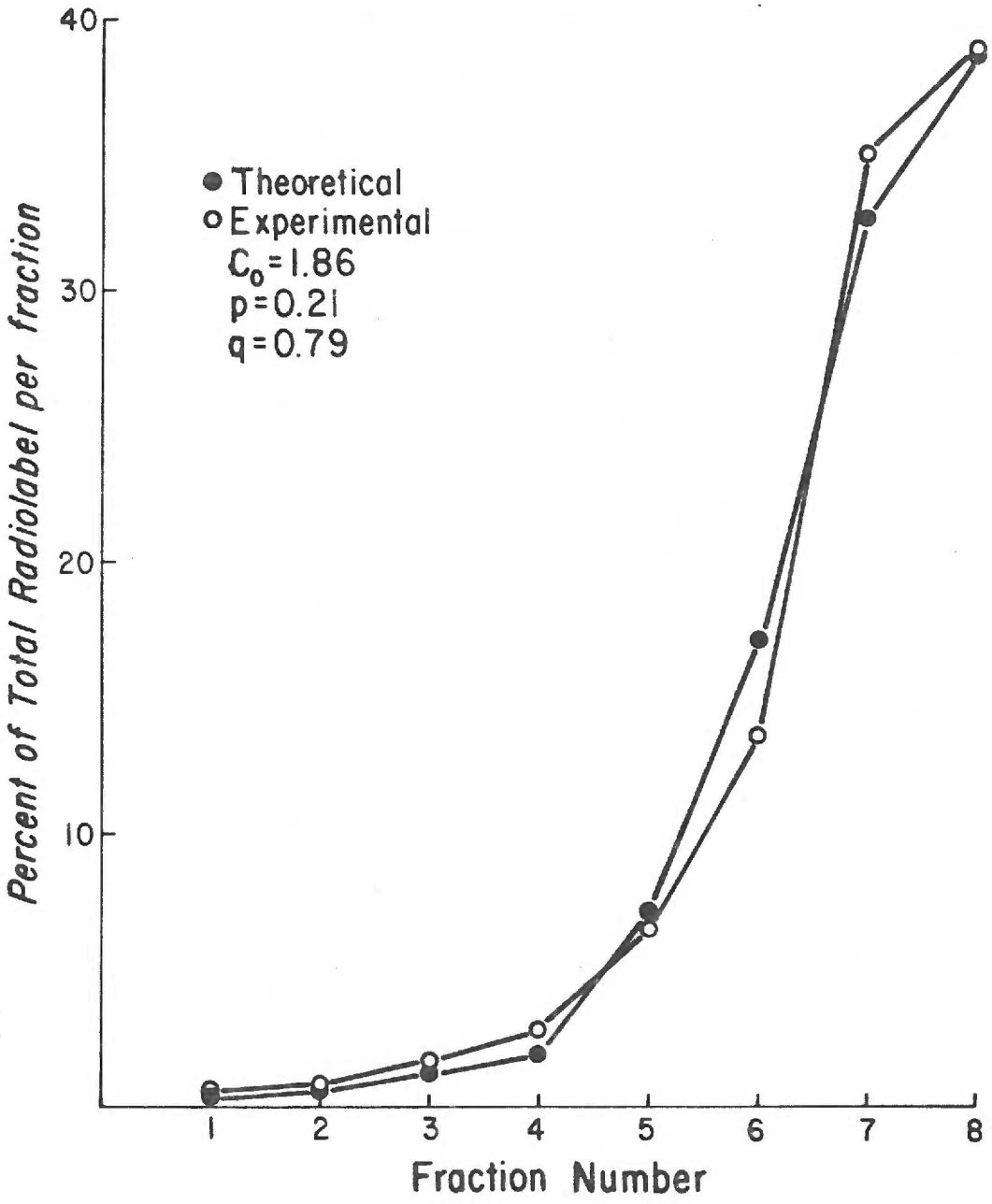
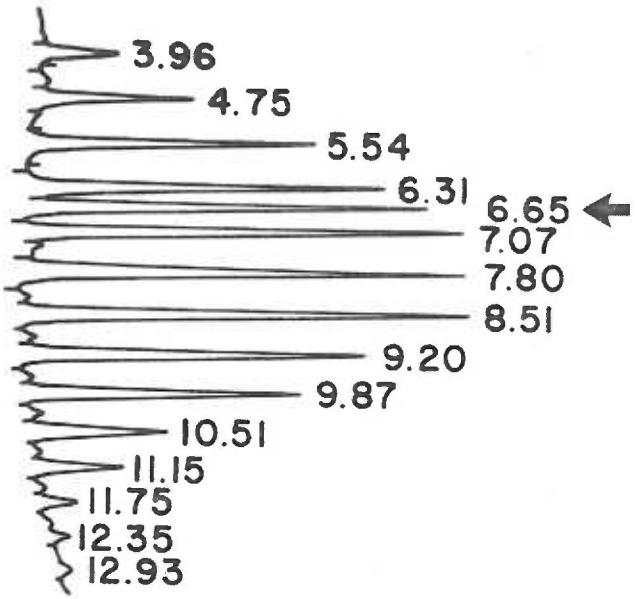

40
30
20
10
Percent of Total Radiolabel per fraction
● Theoretical
○ Experimental
C_o = 1.86
p = 0.21
q = 0.79
1
2
3
4
5
6
7
8
Fraction Number

Figure 14. Gas chromatograms of samples derived from 1-chlorobutane extracts of box water. Numbers refer to program time in minutes. Trace "a" is the chromatogram taken of box water from a leopard frog treated with ^{14}C-phenytoin, while trace "b" is the chromatogram taken of box water from an untreated leopard frog. The arrow in trace "a" points to a component which is not present in trace "b". These chromatograms were obtained from a 5830A Hewlett-Packard gas chromatograph equipped with a 3% OV-17 column (Pierce Chemical Co., Rockford, Ill.). Injection temperature was 260° C, flame ionization temperature was 400°, and the carrier gas (N_2) was flowing at a rate of 44 ml/min. Oven temperature was programed to hold at 200° for 1 min, increase at a rate of 10°/min, and hold at 320° for 5 min.

a


3.96
4.75
5.54
6.31
6.65
7.07
7.80
8.51
9.20
9.87
10.51
11.15
11.75
12.35
12.93

b

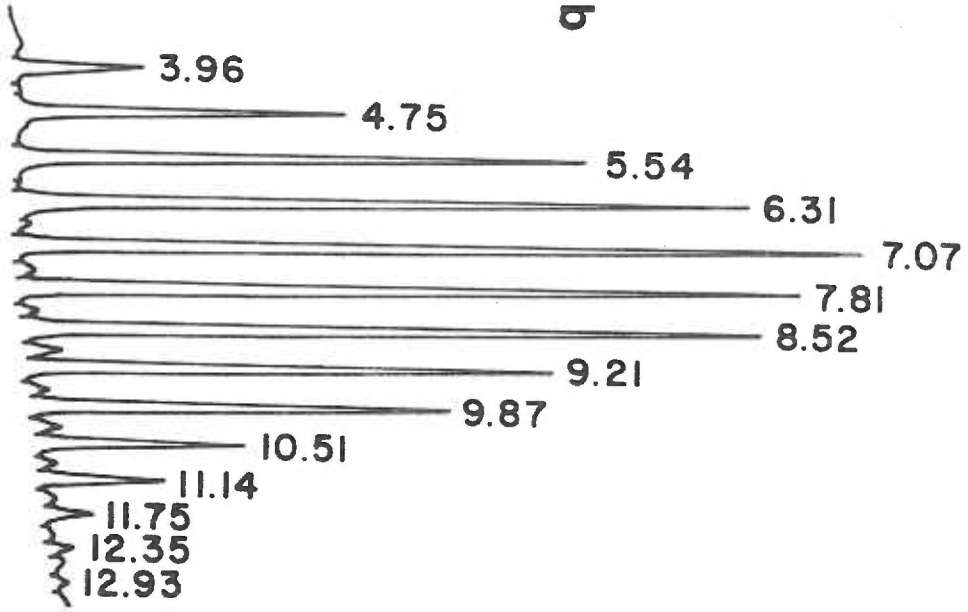
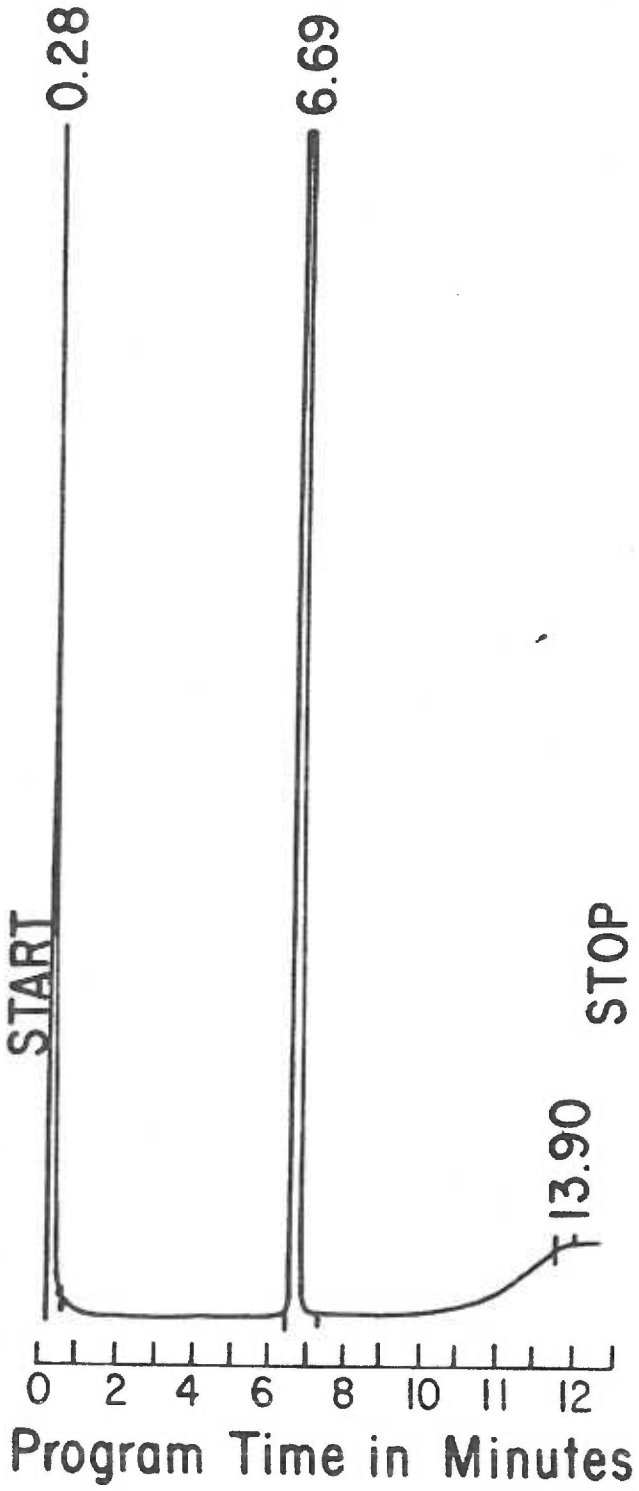

3.96
4.75
5.54
6.31
7.07
7.81
8.52
9.21
9.87
10.51
11.14
11.75
12.35
12.93

Figure 15. Gas chromatogram of permethylated phenytoin. Phenytoin is seen in this figure to elute from the column 6.69 min after injection. Experimental conditions were the same as explained in Fig. 14.


0.28
6.69
START
STOP
13.90
0 2 4 6 8 10 11 12
Program Time in Minutes

APPENDIX VII

MASS SPECTRA OF PHENYTOIN METABOLITES

MASS SPECTRA OF PHENYTOIN METABOLITES

This appendix contains the mass spectra of phenytoin and phenytoin metabolites which were not presented in Manuscript II. Electron impact mass spectra were obtained at 70 eV using a Finnigan model 4000 gas chromatograph/mass spectrometer (GC/MS) equipped with an Incos data system. Data presented were derived from box water gathered from a leopard frog (Rana pipien) injected with 100 mg/kg ^{14}C-phenytoin (0.64 μc/mg). Samples were prepared for GC/MS as explained in METHODS, Manuscript II. Although these data were obtained from a leopard frog, the same phenytoin metabolites were also identified in box water gathered from a bullfrog (Rana catesbiana) injected with 40 mg/kg ^{14}C-phenytoin (0.19 μc/mg).

Figure 16. Mass spectrum of N,N'-dimethyldiphenylhydantoin (permethylated phenytoin). Parentheses indicate the number of methyl groups added, as determined by perdeuteriomethylation. This mass spectrum corresponds to that of peak "a" in the gas chromatogram shown in Fig. 5, Manuscript II. The gas chromatograph/mass spectrometer was fitted with a 3% OV-17 column (Pierce Chemical Co., Rockford, Ill.). Oven temperature was programed to hold at 200° C for 1 min, increase at a rate of 10°/min, and hold at 320° for 5 min.

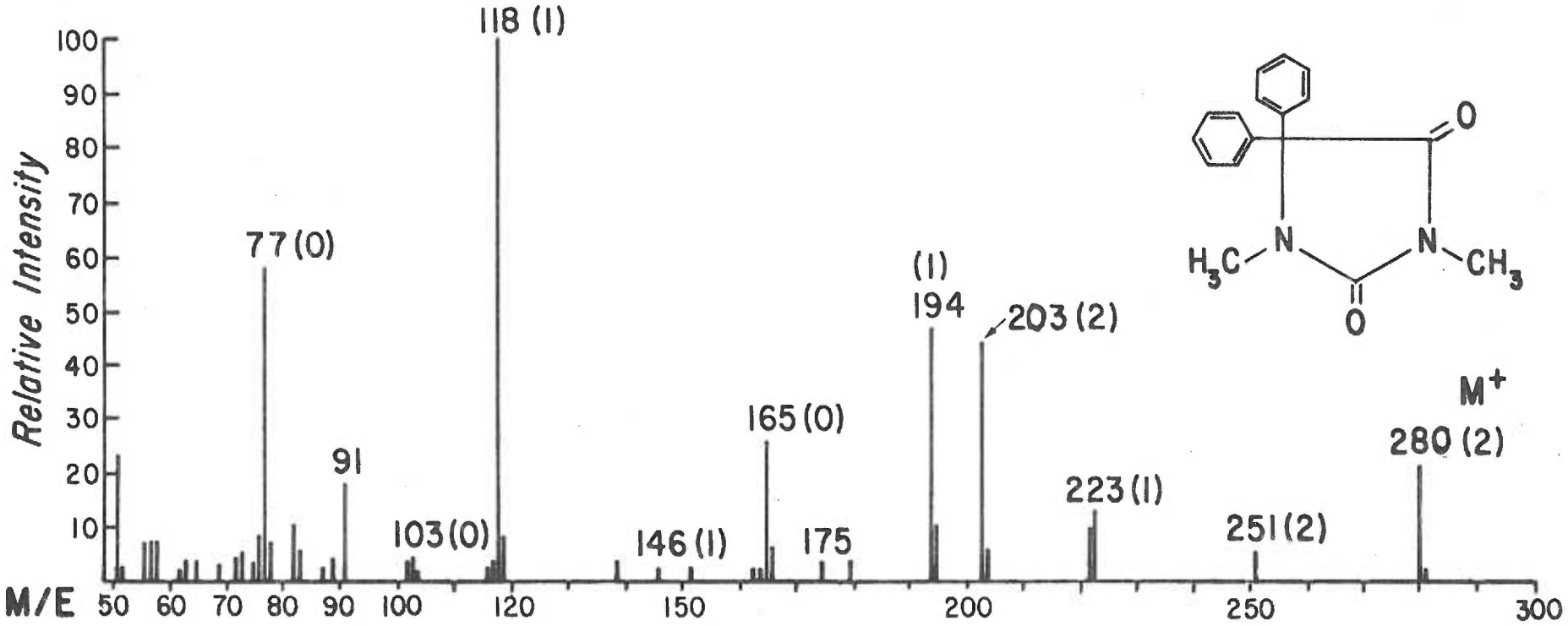

Relative Intensity
100
90
80
70
60
50
40
30
20
10
M/E
50 60 70 80 90 100 120 150 200 250 300
118 (1)
77 (0)
91
103 (0)
146 (1)
165 (0)
175
(1)
194
203 (2)
223 (1)
251 (2)
M+
280 (2)
O
O
N
N
H3C
CH3

Figure 17. Mass spectrum of permethylated 3'-hydroxyphenyl-phenylhydantoin (m-HPPH). Parentheses indicate the number of methyl groups added. This mass spectrum corresponds to that of peak "b" in the gas chromatogram shown in Fig. 5, Manuscript II. Experimental conditions were the same as explained in Fig. 16.

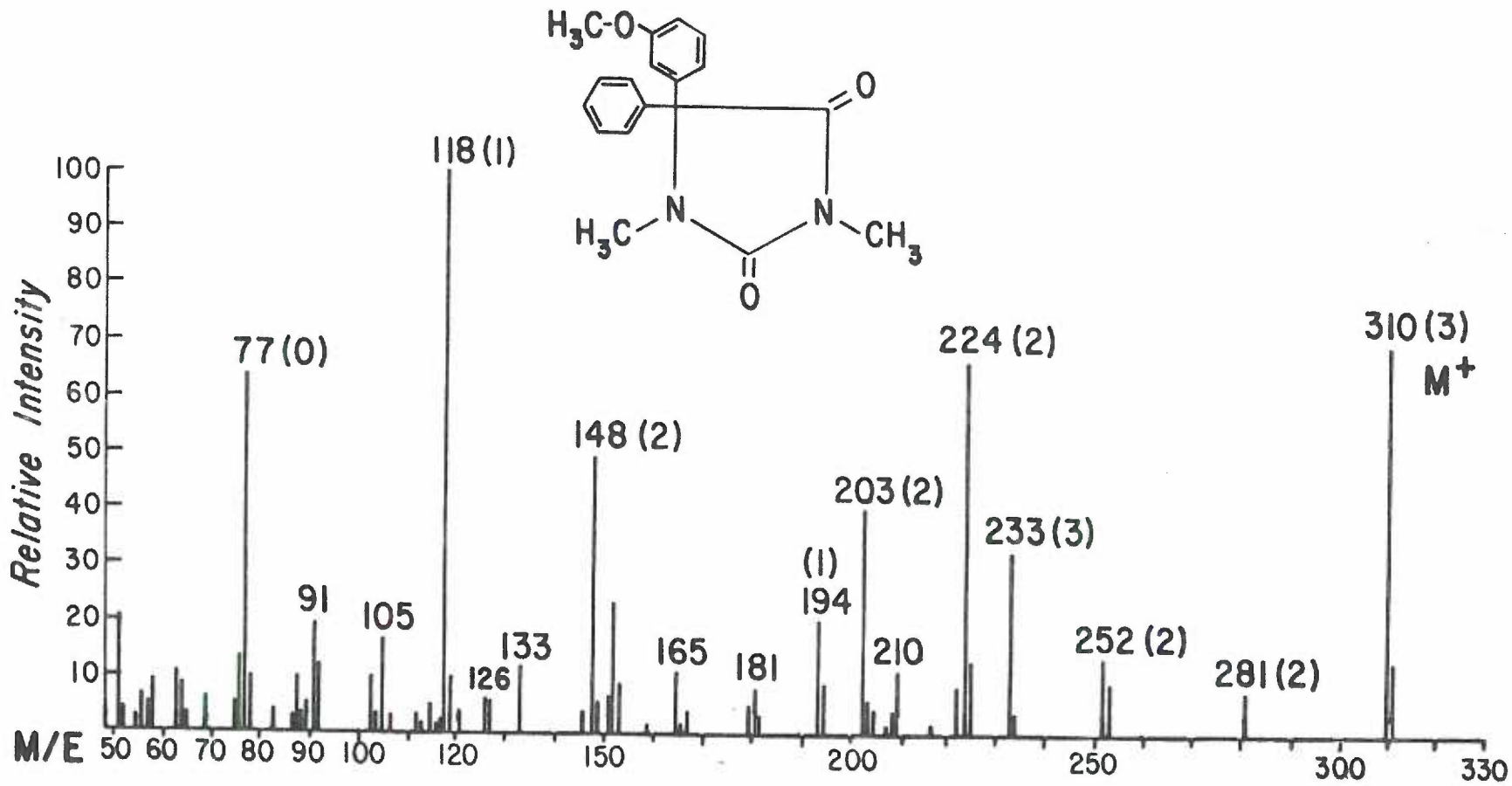

H3C-O
O
N
N
H3C
CH3
O
Relative Intensity
100
90
80
70
60
50
40
30
20
10
M/E
50 60 70 80 90 100 120 150 200 250 300 330
77 (0)
118 (1)
91
105
126
133
148 (2)
165
181
(1)
194
203 (2)
210
224 (2)
233 (3)
252 (2)
281 (2)
310 (3)
M+

Figure 18. Mass spectrum of permethylated 4'hydroxyphenylphenylhydantoin (p-HPPH). Parentheses indicate the number of methyl groups added. This mass spectrum corresponds to that of peak "c" in the gas chromatogram shown in Fig. 5, Manuscript II. The para isomer is distinguished from the meta isomer by the relative abundance of ion fragments at 148 and 233 m/e, and the relative lack of abundance of fragments at 118 and 203 m/e. Experimental conditions were the same as explained in Fig. 16.

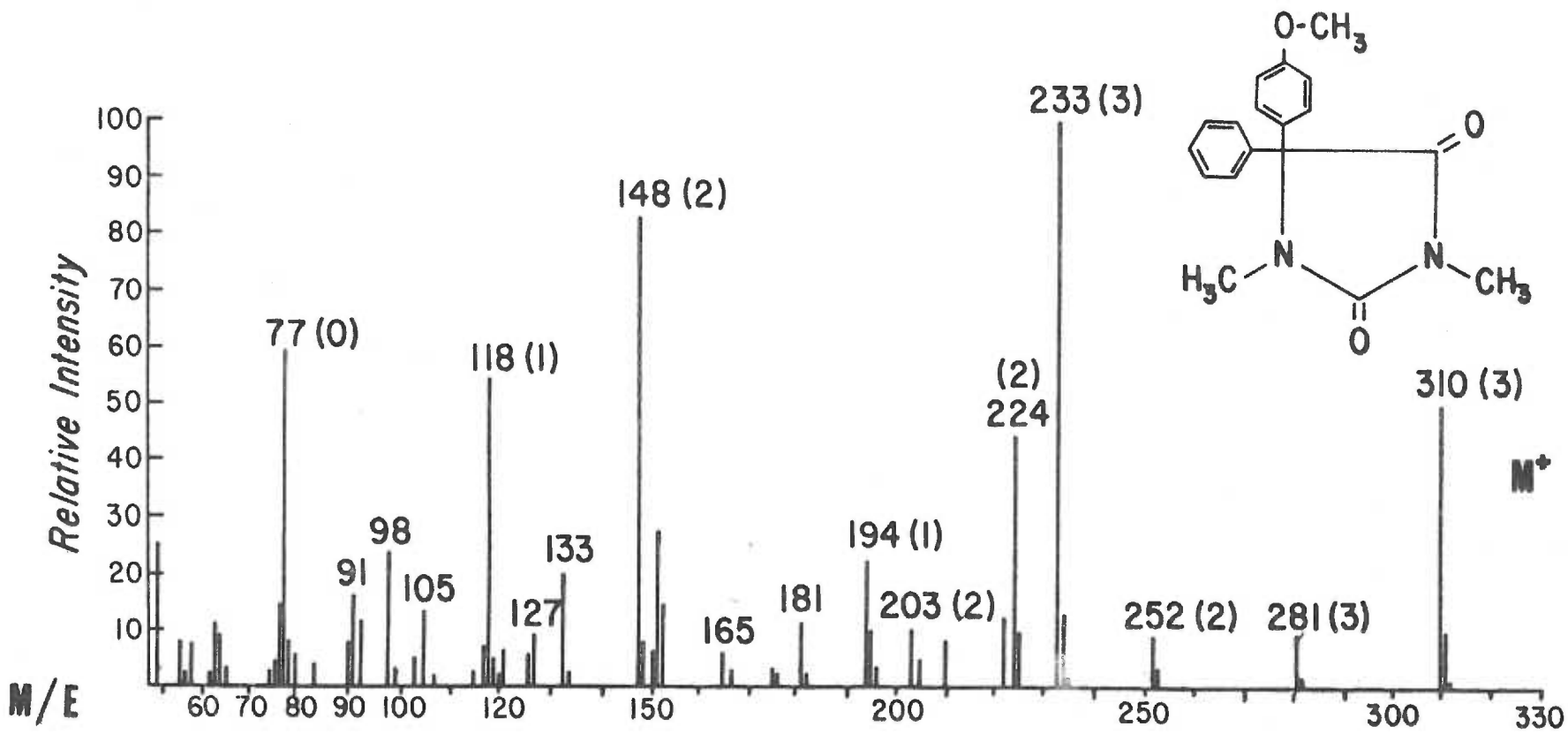

Relative Intensity
100
90
80
70
60
50
40
30
20
10
M/E
60 70 80 90 100 120 150 200 250 300 330
77 (0)
91
98
105
118 (1)
127
133
148 (2)
165
181
194 (1)
203 (2)
(2)
224
233 (3)
252 (2)
281 (3)
310 (3)
M+
O-CH3
O
H3C
N
N
CH3
O

Figure 19. Mass spectrum of permethylated 3',4'-dihydroxyphenyl-phenylhydantoin (permethylated catechol metabolite of phenytoin). Parentheses indicate the number of methyl groups added. This mass spectrum corresponds to that of peak "d" in the gas chromatogram shown in Fig. 5, Manuscript II. The gas chromatograph/mass spectrometer was fitted with a 1½% OV-17 column (Pierce Chemical Co., Rockford, Ill.). Oven temperature was programed to hold at 150° C for 1 min, increase at a rate of 10°/min, and hold at 300° for 5 min.

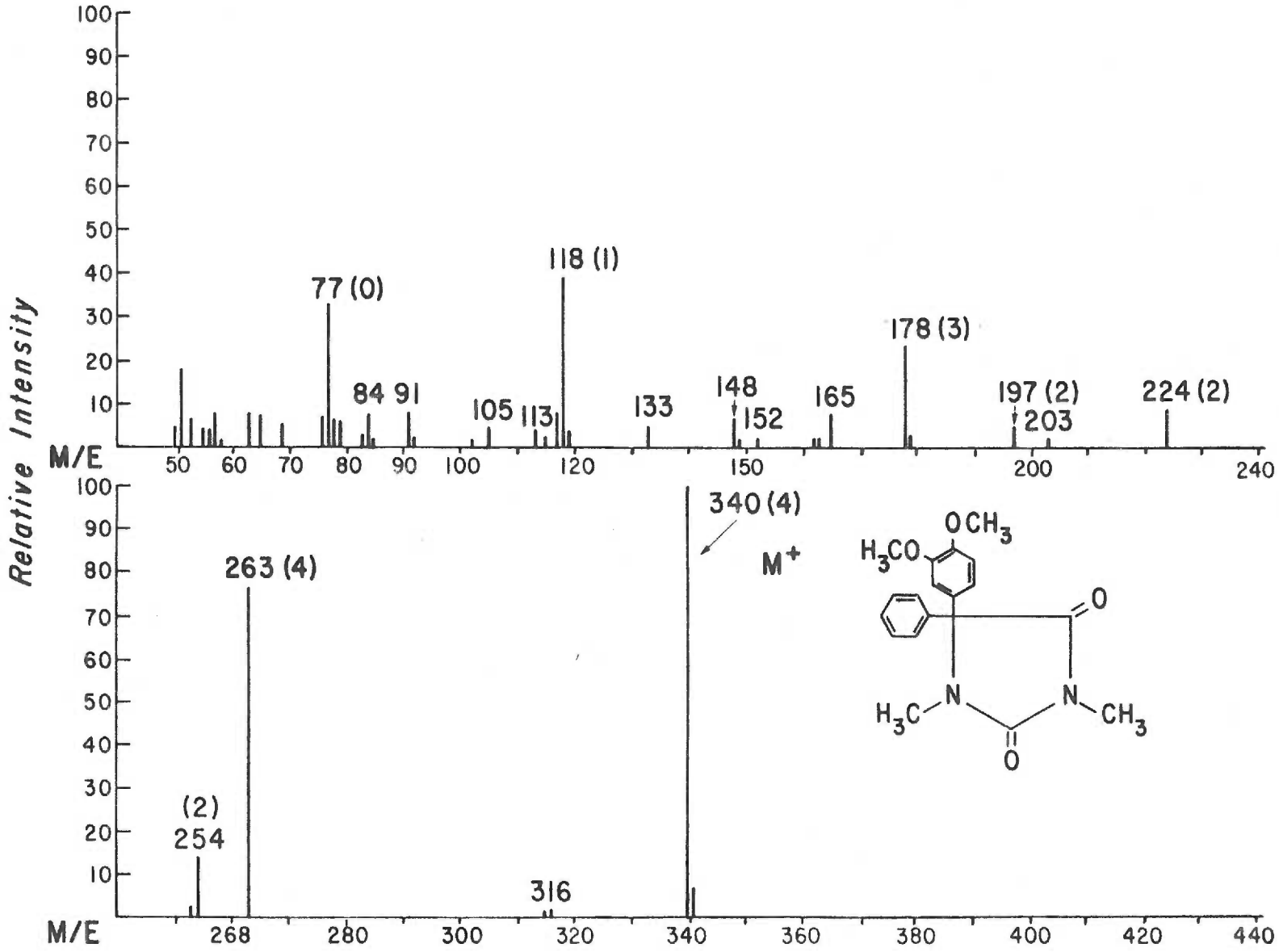

Relative Intensity
M/E
77 (0)
118 (1)
84 91
105 113
133
148
152
165
178 (3)
197 (2)
203
224 (2)
263 (4)
340 (4)
M+
(2)
254
316
OCH3
H3CO
O
N
H3C
CH3
O

Figure 20. Mass spectrum of peak "d" (Fig. 5, Manuscript II) in the chromatogram of a perdeuteriomethylated sample of box water subjected to acid hydrolysis. This spectrum shows the existence of an O-methyl catechol metabolite of phenytoin. Doublets seen in the spectrum (349-352, 272-275, and 184-187) resulted from the tetradeuteriomethylation of the catechol metabolite of phenytoin and the trideuteriomethylation of an O-methyl catechol metabolite of phenytoin. Judging from the relative abundance of ion fragments in doublets, the catechol and O-methyl catechol metabolites of phenytoin were equally plentiful in box water. Parentheses indicate the number of deuteriomethyl groups added to the O-methyl catechol metabolite. Experimental conditions were the same as explained in Fig. 19.

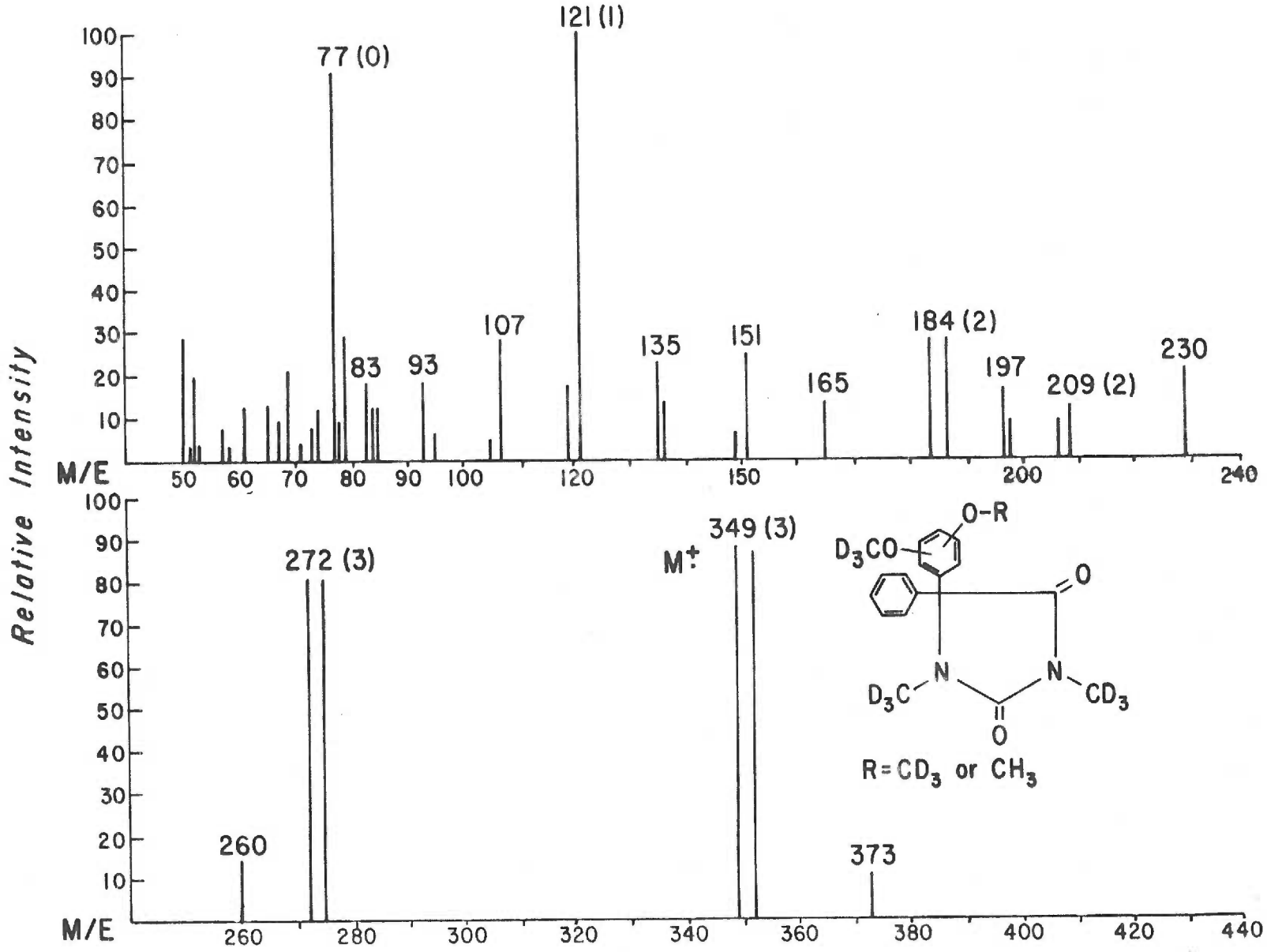
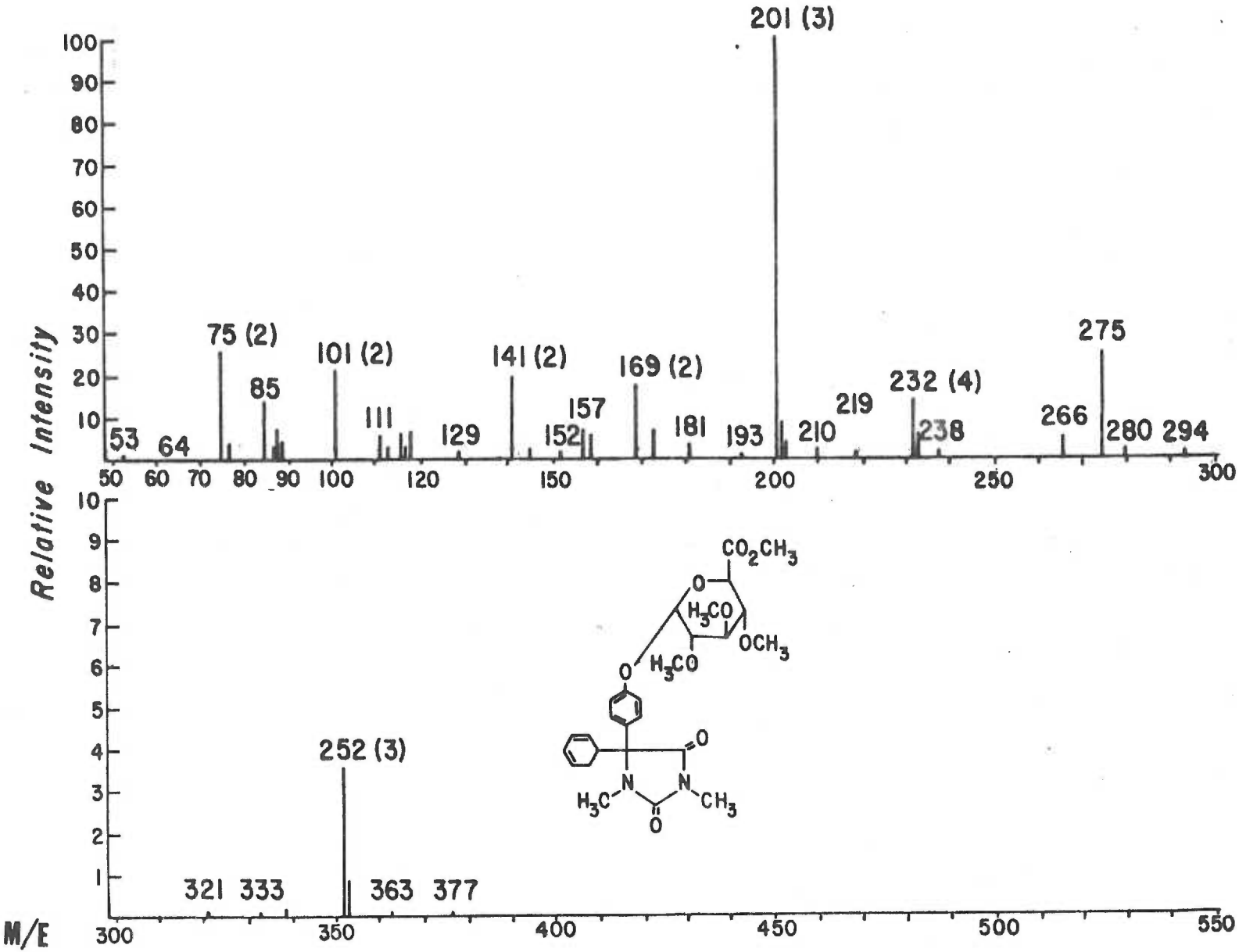

Relative Intensity
M/E
77 (0)
121 (1)
83
93
107
135
151
165
184 (2)
197
209 (2)
230
272 (3)
260
M+
349 (3)
373
O-R
D3CO
O
N
N
D3C
CD3
O
R = CD3 or CH3

Figure 21. Mass spectrum of the glucuronide conjugate of 4'-hydroxyphenylphenylhydantoin (p-HPPH). This spectrum is that of peak "g" in the gas chromatogram shown in Fig. 7, Manuscript II. Numbers in parentheses are the number of methyl groups added. The molecular ion (528 m/e) is absent in this spectrum. The gas chromatograph/mass spectrometer was fitted with a 1½% OV-17 column (Pierce Chemical Co., Rockford, Ill.). Oven temperature was programed to hold at 200° C for 1 min, increase at a rate of 10°/min, and hold at 320° for 5 min.


Relative Intensity
M/E
201 (3)
75 (2)
85
101 (2)
111
129
141 (2)
152
157
169 (2)
181
193
210
219
232 (4)
238
266
275
280
294
53
64
252 (3)
321
333
363
377
CO2CH3
H3CO
OCH3
H3CO
O
N
CH3
H3C

APPENDIX VIII

ANALYSES OF cAMP AND cGMP LEVELS IN THE CNS FROM FROGS AND QUAKING MICE

METHODS

Concentrations of adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) were determined, using analytical kits supplied by Amersham Corp. (Arlington Heights, Ill.). Cyclic AMP was assayed by a competitive protein binding assay developed by Gilman (1970), while cGMP was assayed by RIA, as developed by Steiner, Parker, and Kipnis (1972). Methods used in these assays were those recommended by Amersham, with the exceptions that the incubation volume used in the assay of cAMP was increased from 0.2 to 0.9 ml and the incubation volume for the cGMP assay was increased from 0.2 to 1.0 ml. These larger incubation volumes were used to facilitate the ease of decanting supernatants and to allow larger volumes of samples to be assayed. Based upon the comparison of standard curves, no loss of sensitivity or reproducibility was encountered in using the larger incubation volumes.

MATERIALS

Cyclic AMP standard (320 pmoles/ml), 8-^{3}H-cAMP (28 μc/nmole, 18 pmoles/ml, dextrose-coated charcoal, and the CAMP binding protein purified from bovine muscle, were obtained from Amersham Corporation. Cyclic GMP standard (80 pmoles/ml), 8-^{3}H-cGMP (20 μc/nmole, 8 pmoles/ml), and rabbit antiserum raised against cGMP conjugated with bovine serum albumin were also obtained from Amersham Corp. Ammonium sulfate and EDTA were obtained from J. T. Baker (Phillipsburg, N.J.), TCA was

obtained from Fisher Scientific Co. (Fairlawn, N.J.), and the enzyme research grade of TRIS was obtained from General Biochemicals (Chagrin Falls, Ohio).

ANALYSES OF CYCLIC NUCLEOTIDES

As described in METHODS, Manuscript III, tissue samples were homogenized in TCA, washed with ether, centrifuged, and the supernatant was lyophilized. Residues from supernatants were dissolved in TRIS buffer (50 mM TRIS with 4 mM EDTA, pH 7.5), with a final volume of at least 3.5 ml. The pH of reconstituted supernatants was measured with a Corning Digital 110 pH meter (Medfield, Mass.) and the pH was adjusted to pH 7.5, as needed, with measured volumes of either NaOH or HCl. A reconstituted supernatant from a given tissue sample was the substrate for the analyses of both cAMP and cGMP.

For the analysis of cAMP, reconstituted supernatant (0.1-0.75 ml) or cAMP standard (0.8-16 pmoles) was added to a small test tube, and ice-cold TRIS buffer was added until a final volume of 0.75 ml was reached. Fifty µl of the solution of 8-^{3}H-cAMP and 100 µl of the solution containing binding protein were added to the sample, and the mixture was vortexed. After an overnight incubation at 4° C, 100 µl of the solution containing dextrose-coated charcoal were added and the mixture was vortexed and centrifuged at 1000 x g for 30 min. The supernatant was decanted, mixed with 5 ml of Aquasol^{R} (New England Nuclear, Boston, Mass.), and ^{3}H was assayed in a Beckman LS-330 or LS-3133-P liquid scintillation counter.

For the analysis of cGMP, reconstituted supernatant (0.1-0.9 ml) or cGMP standard (0.4-8 pmoles) was added to a small test tube, and ice-cold TRIS buffer was added until a final volume of 0.9 ml was reached. Fifty µl of the solution containing 8-^{3}H-cGMP and 50 µl of the solution of rabbit antiserum were added to the sample, and the mixture was vortexed. After an overnight incubation at 4° C, one ml of a saturated solution of ammonium sulfate was added. The mixture was vortexed, centrifuged at 1000 x g for 30 min, and the supernatant was decanted and discarded. 1.1 ml of distilled H_2O was added to the pellet, and the solution was vortexed and assayed for ^{3}H as described for the analysis of cAMP.

RECOVERY OF CYCLIC NUCLEOTIDES

Standard curves were constructed with samples which were not subjected to TCA, homogenization, or lyophilization. Therefore, controls were used to measure the percent of the cyclic nucleotides which were lost as a result of these procedures. Standard amounts of cAMP and cGMP (supplied by Amersham Corp.) were added to a vial which contained 0.4 ml of a 10% aqueous solution of BSA (Sigma Chemical Co., St. Louis, Mo.). The sample was homogenized in TCA, washed with ether, centrifuged, and the supernatant was lyophilized, as were tissue samples. Analyses of cAMP and cGMP were performed, and recovery of cyclic nucleotides was usually between 95 and 100% of the expected level. These recovery data were used to adjust the levels of cyclic nucleotides recovered from tissue samples.

LOWRY PROTEIN ASSAY

The protein content of tissue samples was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951). The following reagents were prepared: (A) 2 gm of Na_2CO_3 were dissolved in 100 ml of distilled water; (B_1) 1 gm of $CuSO_4 \cdot 5H_2O$ was dissolved in 100 ml of distilled water; (B_2) 2 gm of $KNaC_4H_4O_4 \cdot 4H_2O$ were dissolved in 100 ml of distilled water; (C) 50 ml of reagent A were mixed with 0.5 ml of reagent B_1 and 0.5 ml of reagent B_2.

After centrifugation, each pellet from an homogenized tissue sample was mixed with 1 ml of 1 N NaOH and heated at 80° C for 90 min. Samples were allowed to cool to room temperature (about 23° C) and 9 ml of distilled water were added to each sample. Redissolved tissue samples were brought to a final volume of 20 ml by the addition of 0.1 N NaOH. Aliquots of samples containing 10-100 µg of protein were transferred to test tubes, and 0.1 N NaOH was added to bring each volume to 2 ml. Two ml of reagent C, which was prepared on the day the protein assay was carried out, were then added to each tube, and solutions were vortexed. After 10 min, 0.2 ml of 1 N folin phenol reagent was added to each tube and the solutions were vortexed. The samples were allowed to stand for 30 min, and then the resulting color in the solutions was quantified spectrophotometrically at a wavelength of 750 nm with a Cary-15 Varian spectrophotometer (Monrovia, Calif.), using 1 N HCl as the reference.

Standard curves were prepared, using a stock solution of BSA dissolved in 0.1 N NaOH (1 mg/ml). Standard concentrations of BSA were prepared from the stock solution and varied from 5-50 µg/ml.

RESULTS

FROGS

As seen in Fig. 22, resting levels of cGMP were highest in olfactory bulbs and were lowest in cerebrum, while levels of cAMP were highest in olfactory bulbs and were lowest in spinal cord. Also seen in Fig. 22, phenytoin, at doses of 5-160 mg/kg, did not alter resting levels of cAMP or cGMP in any of the 5 regions of the frog CNS which were sampled.

The influence of electroshock on cyclic nucleotide levels in specific regions of the CNS is seen in Fig. 23. The largest electroshock-induced increase in cGMP levels was found in the cerebrum, while the level of cGMP in olfactory bulbs actually decreased subsequent to electroshock. Electroshock did not alter levels of cAMP in any region of the CNS at the time the frogs were sacrificed, which is in agreement with data obtained using whole brain (see RESULTS, Manuscript III). Also seen in Fig. 23, phenytoin (5-80 mg/kg) did not produce dose-related changes in levels of cAMP or cGMP in any region of the CNS which was sampled. This is despite the fact that the dose-range of phenytoin employed was effective in modifying the characteristics of electroshock-induced seizures in frogs (see RESULTS of Manuscripts I and III).

QUAKING MICE

Levels of cAMP and cGMP in brains of quaking mice are listed in Table 3. In comparing cyclic nucleotide levels in quaking mice to

those in CF #1 mice (Manuscript III), the level of cAMP in cerebellum was marginally higher and the level of cGMP in cerebrum was somewhat lower in quaking mice ($p < 0.05$, Student's t-test), but levels of cAMP in cerebrum and cGMP in cerebellum were not different in these two strains of mice ($p > 0.3$). There were no significant differences in levels of cAMP and cGMP in cerebella and cerebral hemispheres from control vs. phenytoin-treated quaking mice ($p > 0.1$). This is despite the fact that quaking mice treated with phenytoin (15 mg/kg, s.c.) ceased to display seizure activity, while quaking mice treated with phenytoin-solvent (NaOH) continued to spontaneously seize.

Figure 22. Resting levels of cyclic nucleotides in specific regions of the CNS from frogs with and without phenytoin treatment. Each data point is the pooled average of 8 frogs. Frogs were injected with phenytoin or phenytoin-solvent (50 mM NaOH) and sacrificed 3 hrs later, the time of peak anticonvulsant effect (Appendix I).

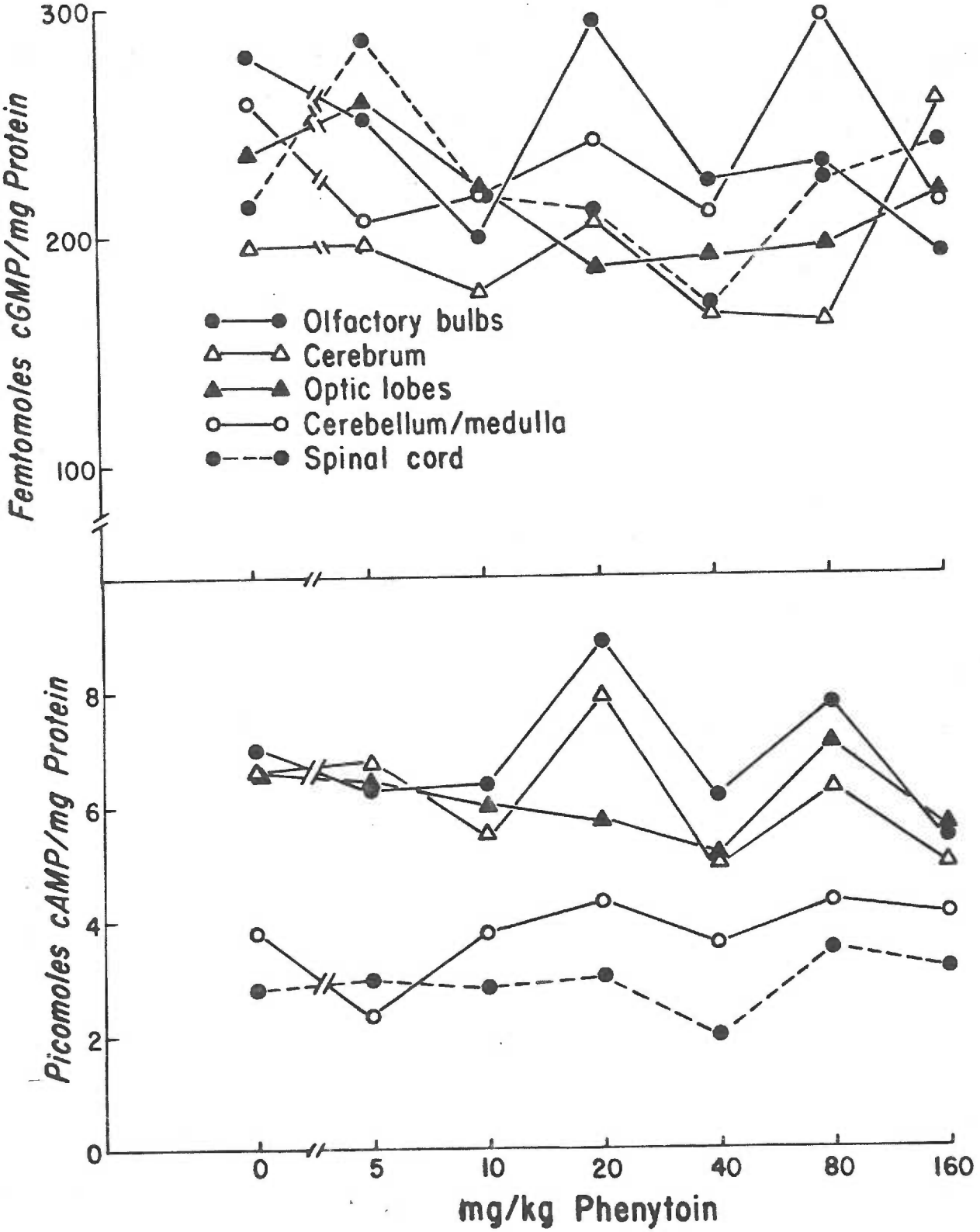

300
200
100
Femtomoles cGMP/mg Protein
Olfactory bulbs
Cerebrum
Optic lobes
Cerebellum/medulla
Spinal cord
8
6
4
2
0
Picomoles cAMP/mg Protein
0
5
10
20
40
80
160
mg/kg Phenytoin

Figure 23. Levels of cyclic nucleotides in specific regions of the CNS from frogs with and without phenytoin treatment, and before and after corneal electroshock. Each data point is the pooled average of 8 frogs. Three hrs after injection, frogs were subjected to electroshock (122 mA, 0.5 sec duration) and sacrificed 10 sec later, a time at which the intact frog displayed tonic hindlimb extension (THE) or had just finished THE.

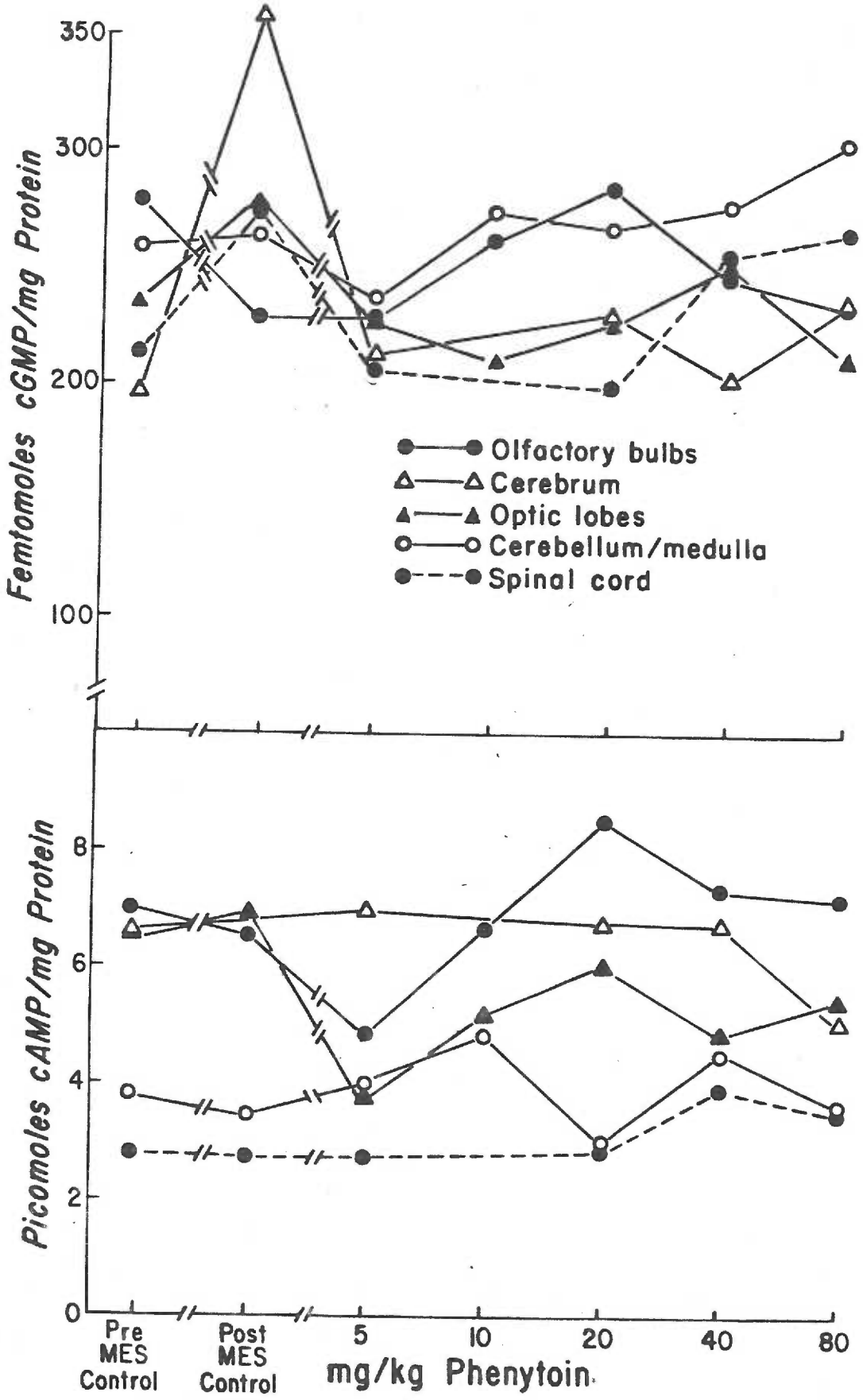

350
300
200
100
Femtomoles cGMP/mg Protein
Olfactory bulbs
Cerebrum
Optic lobes
Cerebellum/medulla
Spinal cord
8
6
4
2
0
Picomoles cAMP/mg Protein
Pre MES Control
Post MES Control
5
10
20
40
80
mg/kg Phenytoin

Table 3. Levels of cyclic nucleotides (mean ± S.D.) in cerebella and cerebral hemispheres from quaking mice. Units for cAMP levels are pmoles/mg protein, while those for cGMP levels are fmoles/mg protein. Three hrs after injection, mice treated with NaOH continued to exhibit spontaneous seizures, while mice treated with phenytoin (15 mg/kg, s.c.) exhibited no seizure activity. Control mice were sacrificed during inter-ictal periods.

	cAMP (pmoles/mg)	n	cGMP (fmoles/mg)	n
Cerebellum				
Control	6.32 ± 0.51	3	215 ± 52	3
Phenytoin-treated	5.52 ± 0.43	3	222 ± 26	4
Cerebrum				
Control	11.43 ± 2.66	3	192 ± 44	4
Phenytoin-treated	(9.54, 12.27)	2	246 ± 122	4

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GENERAL CONCLUSION TO THE THESIS

Two hypotheses concerning mechanism(s) of anticonvulsant action of phenytoin were evaluated in this thesis by experimental studies in the frog. The first hypothesis states that the anticonvulsant effect is a result of phenytoin-induced increase in cerebellar Purkinje cell activity, and the second hypothesis states that phenytoin is anticonvulsant because it regulates CNS levels of guanosine 3',5'-monophosphate (cGMP) and/or adenosine 3',5'-monophosphate (cAMP). Acceptance of these hypotheses as a basis for further investigation would have required dose-related changes in these parameters within the phenytoin dose-range covering threshold to maximum anticonvulsant effects. Because this necessary condition was not met (indeed, neither parameter was modified at all within the phenytoin anticonvulsant dose-range), the two hypotheses are not tenable as mechanisms responsible for phenytoin's anticonvulsant effect, at least in the frog. Dose-time-effect analyses and correlations equivalent to those performed in this thesis have not been done for phenytoin in mammals, hence the acceptability of the hypotheses in this class has not yet been adequately tested.

The manuscripts comprising this thesis also disclose that in the process of testing these hypotheses, a corollary framework of basic data was developed concerning electrically-induced seizures, and the pharmacodynamics, pharmacokinetics, and metabolism of phenytoin in the frog. Previously, very little was known about any of these areas of anticonvulsant pharmacology in the frog, but the present thesis work provides new and useful information on these topics. For example, the elimination

of phenytoin from frogs was found to proceed via concentration-dependent kinetics, which is also the case in many mammals, including man. Also, major and minor phenytoin metabolites produced by the frog were the same as those produced by humans. Thus, in these aspects, the frog closely follows the mammalian paradigm, and an earlier claim that the frog has limited or no ability to metabolize lipid soluble drugs has been refuted by the present study.

Much new information has also been presented on the modification of electrically-induced maximal seizures by anticonvulsant drugs in frogs. Although phenytoin and phenobarbital have long been known to shorten the duration of tonic hindlimb extension (THE) in mammals, this is a new observation in frogs. Furthermore, this parameter of anticonvulsant drug action has been relatively ignored in recent years. The present evidence obtained with phenytoin and phenobarbital in post-electroshock decapitated frogs shows that these drugs act on spinal cord pathways to shorten THE duration. Therefore, this previously neglected endpoint, THE duration, may provide a valuable measure of drug action on spinal cord and/or peripheral sites which most likely involve elements of the γ-motor neuron loop.

Lastly, the comparative data derived by contemporaneous study of frog and mouse have established the facts that these animals hardly differ with respect to the anticonvulsant effects of phenobarbital or to the shortening of THE duration by phenytoin. The most exceptional difference between frog and mouse was in the slopes of the respective phenytoin dose-effect relationships for prevention of THE. In view of

phenytoin's site-selectivity for cerebral cortex and hippocampus, together with the fact that the former structure is absent in the frog and the latter is primordial, the dose-effect slope difference is understandable. The neuroanatomical and phenytoin slope differences between frog and mouse thus prompted the speculation that inclusion of both animals in anticonvulsant drug testing might be a useful means of evaluating neocortical vs non-cortical sites of drug action.

In summary, the results reported in this thesis provide a new fund of basic information for further studies on the pharmacodynamics and disposition of anticonvulsant drugs and other xenobiotics in the frog. Along with rejection of the hypotheses concerning Purkinje cell activity and CNS levels of cyclic nucleotides, the thesis data further suggest that future studies of anticonvulsant drug mechanisms give more attention to the role of spinal cord pathways, in particular the γ-motor neuron loop.