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Blum, Milton Robert

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PHARMACOKINETIC STUDIES OF DIPHENYLHYDANTOIN

by

Milton Robert Blum
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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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PHARMACOKINETIC STUDIES OF DIPHENYLHYDANTOIN

M. Robert Blum
Department of Pharmaceutical Chemistry
University of California,
San Francisco Medical Center

The effect of plasma protein binding and increasing dose on the disposition kinetics of diphenylhydantoin (DPH) was investigated in dog and man.

DPH kinetics was investigated in dogs after slow infusions of 20, 35, and 50 mg/Kg. Plasma concentrations were analyzed by a GLC method. Immediately after cessation of infusion the log concentration – time curves show what appears to be an initial distributional fall off. After high doses, the post infusion curves appear to be convex descending before a turnover to a log – linear decline. These findings were compatible with capacity limited conversion of DPH to HPPH, where HPPH may be acting as a competitive inhibitor of its own formation, and can be described by Michaelis – Menten kinetics. When identical experiments were carried out with concomitant taurocholate infusion, the post infusion initial fall off phase was not evident and there was a greater tendency towards a convex descending decline. Pharmacokinetic models were proposed which could explain the various findings.

Some indirect evidence was developed which indicated that DPH may have markedly decreased hepatic blood flow. The experimental results were also qualitatively compatible with a perfusion model where clearance is limited by hepatic flow.

Dose dependent elimination of DPH in man was observed in two of the four subjects receiving intravenous DPH doses of 3, 6, and 9 mg/Kg.

Following all intravenous infusions, a plateau or rising plasma concentration of DPH was observed up to 10 hours. These findings could not be rationalized by a distributional or metabolic phenomena, since neither can explain the increases in the DPH plasma concentration post infusion. The plateau effect was not observed in the dog when the drug was infused into the vena cava where good mixing with blood would occur, however, sustained lower levels were produced when DPH was administered by a peripheral infusion mimicing the procedure used in humans. These findings were explained on the basis of the poor solubility of DPH. It was postulated that when the drug enters the blood stream, some may precipitate as the insoluble free acid, then slowly be released into solution.

The half-life in three of the four subjects appeared to be significantly longer when determined after oral rather than intravenous administration. An increasingly prolonged absorption with increasing dose can be expected in light of the pharmacological action of DPH on the gastro-intestinal tract. This effect could be misinterpreted as dose dependent kinetics.

Binding determinations were made by an ultracentrifugation technique at 37°C. There was no correlation between the widely varying half-lives in man and the degree of plasma DPH binding in these individuals. The average unbound fraction at a standard DPH concentration of 10 mcg/ml was 9.67% $\pm$ 1.25 S.D. and 26.44% $\pm$ 5.67 S.D. in twenty normal and twelve drug free uremic patients respectively. The mean binding association constant for the normals and uremics estimated to be 1.5×10^{-4} and 0.57×10^{-4} L/M. The unbound fraction for both groups was inversely related to plasma albumin concentration.

Accumulation of HPPH in uremics was shown not to be responsible for altered binding. Hemodialysis did not increase the ability for uremic plasma to bind DPH or accelerate the removal of the drug.

The calculated volume of distribution of DPH in uremics was double that of normals which would account for the depressed plasma concentrations of DPH seen in these patients. Random screening of patients with various metabolic disorders indicated other disease states may also effect the ability 6or albumin to bind DPH. The clinical implications of decreased binding and increased volume of distribution of the drug were discussed.

ACKNOWLEDGMENTS

To My Family

TABLE OF CONTENTS

vi

CHAPTER I

INTRODUCTION

Diphenylhydantoin (DPH) has been used orally for over
thirty years for controlling various convulsive disorders in
man. Since its introduction by Putnam and Merritt in
1937 (1), it has become one of the most important drugs in
the treatment of grand mal, focal, and psychomotor epilepsy.
Over the years it has been the subject of numerous clinical,
pharmacological, and pharmacokinetic studies. Yet the
clinician still sees many therapeutic failures. Some of
these failures are related to the problem of obtaining and
maintaining proper drug blood levels.

Recently, DPH has found a new use in the treatment of
certain cardiac arrhythmias. In these emergency situations
it is necessary to administer DPH by intravenous route to
maintain higher blood levels than required for the anti-
convulsive therapy. Again the establishment of a dosage
regimen has been difficult. In the last few years this
difficulty has been attributed primarily to dose dependent
kinetics. Other factors implicated in the wide variance
in DPH blood levels after a standard dose include incomplete
absorption or malabsorption, accelerated metabolism and
noncompliance of the patient to take the medication as
prescribed (2). Although the literature reveals a widely
variable half-life of DPH in man, this fact is not well

recognized by many clinicians. Variability in the extent
of drug-plasma protein binding is another important factor
that can introduce differences in drug blood levels after
a standard dose. The present pharmacokinetic studies were
initiated to investigate the effect of the reported dose
dependent kinetics and influence of plasma protein binding
on the disposition of DPH in man and dogs.

Physical and Chemical Properties - DPH is chemically
5,5 diphenyl-2,4,imidizolidinedione. The free acid exists
as needle-like crystals that have a melting point of
295-298° C. The intrinsic solubility in water at room
temperature is about 14 mcg/ml. It is only slightly soluble
in chloroform or ether. The pKa of DPH has recently been
reported to be 8.31 by ultraviolet spectrophotometry and
8.33 by potentiometric titration (3). The sodium salt of
DPH is a white, bitter hydroscopic powder. Due to the pKa
and solubility characteristics, appreciable quantities of
the drug will dissolve in water only when the pH is raised
above pH 11.7. For example, the presently available paren-
teral dosage form contains 50 mg/ml in a hydroalcoholic
solution of pH 12. All commercial oral preparations of DPH
are in the sodium salt form.

Absorption - The absorption of DPH as the sodium salt
from capsules is slow and irregular. Dill et al. (4) found
peak plasma levels occurred between 8 and 12 hours in six
normal adults given a single 400 mg dose of sodium DPH in
gelatin capsules. When DPH was given as the free acid in

the same study, peak plasma levels were reached between 12 and 24 hours. Sodium DPH given orally in a hard gelatin capsule may or may not disperse prior to interaction and precipitation as the free acid in the stomach. If the salt disperses in the gastric fluids prior to reaction with gastric acid, the particle size of the precipitated DPH may well be smaller than the commercial free acid of DPH, and therefore the overall rate of absorption of the drug may be faster due to a more rapid dissolution. However, if the acid reaction occurs on the surface of the impacted powder within the gelatin capsule, the layer of insoluble DPH acid may retard further dispersion and the rate of dissolution of the powder. A more rapidly dispersing and dissolving dosage form of DPH may have peaked prior to 8-12 hours. Other workers have reported that plasma peak levels after oral dosing can be greatly delayed. O'Malley et al. (5) showed maximum levels at 24 hours in two of twelve subjects, after a 500 mg dose, although the average peak levels in this study occurred about 5 hours. Glazko (6) reported peak levels generally were reached between 4 and 8 hours after testing four commercial DPH products. However, in some cases it took as long as 12 hours to peak. Riegelman (7) has replotted the data of Glazko (6) and shown that DPH appeared to be still being absorbed more than 24 hours after a 500 mg dose. An outbreak of DPH toxicity in Australia in 1966 and 1967 was associated with a change from calcium sulfate to lactose as an excipient in the manufacture of DPH capsules (8-9).

Presumably the change affected the rate and extent of absorption.

Distribution - DPH is extensively bound to plasma protein in man (Chapters V and VI). Pharmacokinetic analysis of DPH in man by Suzuki (10) indicates that after IV injection, the drug rapidly distributes out of the vascular compartment. Studies in the rat by Noach et al. (11), Dill et al. (4) and Nakamura et al. (12) indicate that high levels of DPH accumulate in the liver after an oral or IV dose. Intermediate levels were found in the brain by these investigators. Triedman et al. (13) found that CSF concentrations of DPH were equal to the unbound levels of the drug in the plasma. DPH readily passes into the human placenta. Mirkin (14) showed that DPH concentration in the umbilical artery or vein was almost identical to that of the maternal plasma. Studies in this laboratory have shown that 64% of the DPH in whole blood of man or dog is contained in the plasma fraction.

Elimination - The major route of elimination of DPH from man, dogs, and rats is by hepatic metabolism. The urinary excretion of unchanged DPH is a minor route of elimination and generally accounts for less than 5 percent of the dose administered (15). The major urinary metabolite in the three species has been reported by several investigators (16-19) to be the glucuronide conjugate of 5-para-hydroxyphenyl-5-phenyl-hydantoin (p-HPPH). More recently, other minor metabolites have been identified. Chang et al. (20) identified 5-(4-hydroxy-3-methoxyphenyl)-5-phenylhydantoin

from rat urine. The same group (21) isolated 5-(3,4-di-hydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin as a metabolite of DPH from the monkey and rat urine. Horning et al. (22) in 1971 identified this compound as a major metabolite in newborn humans. It exists in urine mainly in the unconjugated form. Upon heating in acid it is converted to a mixture of 3- and 4-hydroxyphenyl derivates of DPH. The 5-meta hydroxyphenyl-5-phenylhydantoin glucuronide was reported as the major metabolite of DPH in the urine of dogs and a minor metabolite in man by Atkinson et al. (23) in 1970. However, this was after acid hydrolysis of the urine, and may in part have been an in vitro conversion to this compound. Borga et al. (24) in 1972 has identified 5(3,4-dihydroxyphenyl)-5-phenylhydantoin as a metabolite of DPH in man and rats. It was found to be mainly in the conjugated form. Despite the recent identification of several new metabolites, the major route of DPH elimination in man is by enzymatic conversion to p-HPPH followed by conjugation with glucuronic acid and excretion into the urine. In man the appearance of p-HPPH in the urine accounts for 60 to 70% of the dose (11,18,25). The only exception may be the newborn human where 5(3,4,-dihydroxy-1,5,-cyclohexadien-1-yl) 5-phenylhydantoin is the major metabolite.

Biliary excretion may play a small role in the elimination of DPH. Noach et al. (11) suggested bile excretion of DPH followed by reabsorption to explain a second peak in plasma and tissue levels 90 minutes after an IV injection in rats.

In these studies small amounts of DPH and metabolites were detected in the bile. Similar findings of DPH in bile were reported by Dill et al. (4). Gerber and co-workers (26) have demonstrated that conjugated p-HPPH is excreted in the bile of the rat, but eventually it appears in the urine under normal conditions after enterohepatic recirculation as originally noted by Noach.

Many drugs have been reported to influence the metabolism of DPH in man. Increased DPH levels have been seen after coadministration of dicoumarol (27), isoniazid with para-aminosalicylic acid or cycloserine (28-29), phenyramidol (30), disulfiram (31), chloramphenicol (32) and sulthiame (33). Inhibition of p-hydroxylation of DPH in the liver has been suggested as the explanation of this phenomenon.

Carbamazepine is believed to accelerate the metabolism of DPH by enzymatic induction (34). In general, it has been found that coadministration of phenobarbital with DPH accelerates the metabolism of the latter. However, this effect is variable (35-37).

Studies by Kutt (2) have shown that some individuals have unusually fast metabolism, while others (15) have unusually slow metabolism. The latter case appears to be related to a specific genetically determined deficiency.

DPH Half-Life in Man - Reports of the half-life of DPH in man are extremely variable. Glazko et al. (18) found an

average plasma half-life of 15.8 ± 7.3[1] hours (range 10.5 to 28.7) after a single IV dose of 250 mg in 6 normal subjects. This is comparable to an earlier study by the same Parke-Davis group. In that study, Dill et al. (4) reported an 18 to 24 hour half-life after a single 400 mg oral dose. Arnold and Gerber (38) followed DPH elimination in a large study of 70 healthy volunteers. Subjects were given 100 mg of DPH orally three times daily for three days. The half-life was determined from plasma concentration measurements starting 12 hours after the last dose. However, since many plots did not appear to show a log-linear slope, the half-life reported was in reality a half time defined as the time for plasma levels to fall to one-half the twelve hour concentration. The average "half-life" for 68 subjects was 22.0 ± 9.0 hours (range 7.0 to 42.0). The mean for 48 Caucasians was 20.5 ± 8.0 hours compared to a mean of 26.5 ± 9.0 hours for 28 Negroes. Two Negro subjects with half-lives of 55.0 and 72.5 were excluded from the mean calculations. Solomon and Schrogie (30) determined the DPH half-life in 5 normal volunteers in a similar oral study. Each subject was given 100 mg 3 times daily for 5 days. Plasma measurements were made 12, 36, 60, and 84 hours after the last dose. The average half-life was 25.8 ± 4.7 hours (range 22-34).

Suzuki et al. (10) reported a biexponential decay of DPH in the plasma after an IV injection. The terminal half-life

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[1]The values noted by ± after a mean reported in this dissertation are standard deviations unless specified otherwise.

in two subjects was 9.1 and 9.5 hours after a 250 and 125 mg dose respectively.

The Scandinavian literature in general reports shorter DPH half-lives in man. Svensmark (39) reported a 4.5 and 7.0 hour half-life after an IV dose of 8.8 and 11.1 mg/kg respectively in two individuals. The analytical procedure used for the DPH determination was a modification of the Dill method (4).

Another group of Danish investigators, Hansen et al. (27,33) found a 9 and a 12 hour half-life in two subjects after a 100 mg IV dose using trace amounts of ^{14}C labelled drug. In another study by the same group (35) involving ten subjects, a mean half-life of 10.2 $\pm$ 3.9 hours (range 6.25 to 22.0) was reported. Again they employed a 100 mg IV dose with radioactive labelled DPH. The half-life was determined twice in each individual at a two week interval. Half-lives in the same individuals varied generally 1 to 2 hours. However, the means of the first and second half-life determinations were identical. In a recent paper by the same group Andreasen et al. (40) studied 13 patients with normal hepatic and renal functions. After an IV dose of 100 mg the average half-life was 11.6 $\pm$ 3.4 hours.

Dose Dependent Kinetics - Classically, most pharmaco-kinetic models have been based on the assumption that the disposition of a drug in the body can be described by a linear system of differential equations. In the case of either the one or two compartment open model, the distribution

and elimination processes are described by pseudo first

order rate processes. Therefore, the overall concentration

time course of a drug in the body can be described by first

order kinetics. In such a system the kinetics of a drug is

not influenced by the dose administered. Thus, the classical

pharmacokinetics presumption of dose independence requires

certain important relationships to hold, i.e.,

(1) the biological half-life is independent of dose,

(2) the fraction of metabolites formed is independent
of dose,

(3) the area under the blood level vs. time curve is
proportional to dose.

In the 1960's the first reports appeared in the literature

of dose dependent kinetics. Levy (41) and Cummings (42) showed

that the half-life of salicylate in man increased with in-

creasing dose. It was demonstrated that one of the metabolic

pathways, the formation of salicyluric acid, became saturated

at high doses. More recently Levy (43) has shown that the

metabolic formation of the phenolic glucuronide of salicylic

acid also becomes saturated after high doses of salicylate.

When saturation of these metabolic pathways of salicylic acid

takes place, the overall elimination of the drug proceeds by

parallel first and zero order processes. The length of time

necessary for 50% of the original dose to be eliminated

increases with increasing dose. However, when drug concen-

trations fall below the levels that affect saturation, the

elimination of salicylate will follow an exponential decline.

The terminal half-life from the plasma curve of all doses of salicylate in the same subject will be the same.

There are other mechanisms besides metabolic saturation that can invoke dose dependent kinetics. These include saturation of active and facilitated transport mechanisms involved in either the absorption or excretion process and saturation of drug binding protein.

The first report of dose dependent kinetics of DPH was made by Dayton et al. (44) in 1967. IV doses of 20 and 50 mg/kg were given to three dogs. The results are summarized in Table I. In all three animals there was a two- to five-fold increase in half-life, going from the low to the high dose. Pretreatment with the hepatic enzyme inducers, phe-tharbital or phenobarbital, eliminated the dose dependent effect. The unique finding in this report is that the elimination after both low and high doses followed an exponential decline. The plasma concentration time curves remained log-linear over the whole time course of the experiment. After the high dose there was no turnover to the faster elimination, as would be expected for "saturation" induced dose dependent kinetics. The authors speculated that the observed dose dependent kinetics might be explained by self-inhibition of metabolism. Dose dependent kinetics as described by Dayton et al. is compared to saturation-induced dose dependent kinetics in Figure 1.

More recently Dayton and Perel (45) have reported dose dependency of DPH and its metabolites in dogs at lower levels.

Using radiolabelled drug, they followed DPH elimination at doses of 0.6 mg and 20 mg/kg. The ^{14}C-DPH activity injected with both doses was the same. In one dog the half-life increased from 110 to 270 minutes and in a second dog the half-life increased from 100 to 135 minutes. The decay curves followed a log-linear decline without turnover to a faster decay during the time course of the experiment. However, in terms of total DPH, the concentration in the plasma after the large dose was always about 450 times greater than the lower dose. The investigators suggested that their data supported the contention that saturation of drug-metabolizing enzymes leads to dose-dependent plasma level decline.

Dose dependency of DPH in mice was reported by Gerber and Arnold (46) in 1969 and in the rat by Gerber et al. (26) in 1971. In both of these studies plasma levels after higher doses followed a non log-linear decline indicative of metabolic saturation.

End product inhibition has been recently suggested as an explanation for dose dependency of DPH elimination. Borondy et al. (47) showed inhibition of DPH metabolism when p-HPPH was added to rat liver homogenates. Ashley and Levy (48) have demonstrated that DPH elimination in rats is increased appreciably by concomitant administration of p-HPPH.

Dose dependence of DPH elimination in man was reported by Arnold and Gerber (38) in 1970. Normal human volunteers received two or three dose levels of DPH ranging on the average from 3.7 to 7.4 mg/kg daily for 3 days. Plasma

samples were collected starting 12 hours after the last dose.
Generally one sample was collected daily for 4 days. The
"half-life" was calculated as the time taken for DPH plasma
concentration to reach one-half the initial plasma level.
Dose dependency of DPH was demonstrated in 10 subjects. The
half-life in these subjects increased an average of 1.9 times,
between the lowest and highest dose. The higher doses in
most cases showed non-exponential decline, turning over to
a faster decay as the drug was eliminated. This gave the
general appearance of metabolic saturation. Gerber and
Wagner (49) had fitted one set of the data following oral
doses of 2.3, 4.7, and 7.9 mg/kg of DPH to the integrated
form of the Michaelis-Menten equation, using the same K_m and
V_m values. However, it should be noted that the correlation
coefficient of the fitted curves was 100 percent or more.
More recently, Atkinson (50) applied a Michaelis-Menten model
to determine K_m, V_m and a volume of distribution from sequen-
tial determinations of DPH plasma concentrations in a patient
with DPH toxicity.

Other reports in the literature indicate that DPH elimi-
nation in man is dose dependent. Garrettson and Kim (51)
reported nearly constant metabolite elimination during
declining high serum levels in two children, thus indicating
saturation of metabolism. Gerber et al. (52) showed in one
subject that the maximum plasma level doubled when a single
oral dose was increased from 600 to 1200 mg, yet the maximum
rate of urinary p-HPPH formation only increased from 110 to

170 mg/day. These results are contrary to the findings of Kutt and McDowell (25), who reported a p-HPPH output proportional to dose.

Statement of Objectives - DPH is the drug of choice in the treatment of many convulsive disorders (53). It is now recognized to have properties that make it unique in the treatment of certain atrial arrhythmias (54). However, daily instances of therapeutic failure or unexpected toxicity might unnecessarily influence the clinician to discontinue its use. A better understanding of the pharmacokinetics of DPH could lead to a more rational use of the drug and to a greater incidence of efficacy in the clinical setting.

Reported dose dependent kinetics of DPH have been implicated as a possible factor related to difficulties in design of a therapeutic regimen. The first objective of these studies was to investigate dose dependent kinetics in dogs with major emphasis on the mechanism of the reported unique dose dependency in this species.

The second objective of these studies was to investigate in man the kinetics of DPH after intravenous infusion at various doses to reconfirm the findings of dose dependent kinetics observed after oral administration.

A final objective was to examine a possible relationship between the previously reported variable plasma protein binding and the widely variable biological half-life among individuals.

Table I

Dose Dependent Kinetics in Dogs

as Reported by Dayton et al. (44)

Dog	Dose mg/kg	Half-Life hrs.
A	20	1.5
	50	7.8
B	20	2.2
	50	6.0
C	20	2.8
	50	5.5
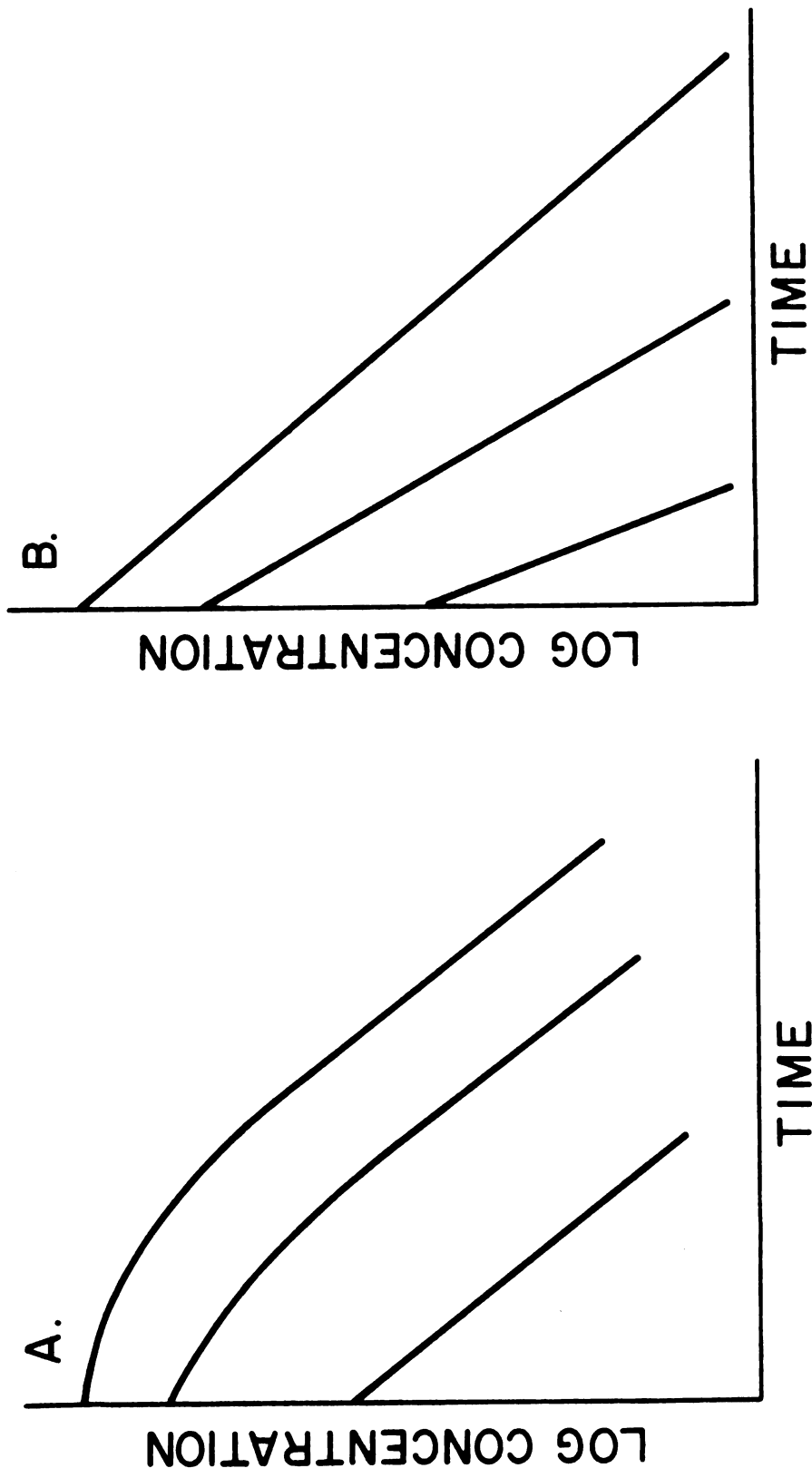
Figure 1. Drug plasma concentration-time curves exhibiting dose dependent kinetics:
A, produced by metabolic saturation; B, as reported by Dayton et al. (42).

CHAPTER II

ASSAY OF DPH IN PLASMA

The first reliable method for the determination of DPH
in plasma was a colorimetric procedure by Dill and associ-
ates (4). This system involves separation of DPH by extrac-
tion from other contaminating drugs, followed by nitration,
diazotization and coupling with Bratton-Marshall reagent.
The method is time consuming, but the major drawback for
kinetic studies in man and experimental animals is that a
large volume of plasma (4-5 ml) is required. Recent reports
of modifications of the Dill method (26,51) have claimed to
improve the specifity and sensitivity of the method, but
these methods still require 2 ml or more of plasma for each
determination. Other widely used spectrophotometric methods
(56-58) have similar disadvantages for kinetic studies.

At the onset of the present studies, two methods for
DPH determination by gas-liquid chromatography (GLC) had
been reported in the literature. Chang and Glazko (59)
published a method based upon solvent extraction, formation
of the trimethylsilyl (TMS) derivative of DPH by the addition
of bis (trimethylsilyl) acetamide (BSA), and GLC measurement
of the resultant product. The method is highly specific and
sensitive and uses only 1 ml of plasma. However, the TMS
derivative is unstable and is subject to rapid hydrolysis in
the presence of moisture. The extraction procedure requires

filtration through filter paper and drying of chloroform with large amounts of sodium sulfate.

Since the analytical procedures for the present studies were developed, numerous other GLC methods have been published. These methods have involved methylation procedures by diazomethane (60-62), or on column methylation with trimethylanilinium hydroxide (63) or tetramethylammonium hydroxide (64). A GLC method for DPH determination without derivative formation has also been published (65).

For the present kinetic studies of DPH, a sensitive and specific method requiring 1 ml samples of plasma was required. A GLC assay was developed based on the method of Chang and Glazko (59) employing the formation of the trimethylsilyl (TMS) derivative.

EXPERIMENTAL

Materials - 5,5-diphenylhydantoin was obtained from
Eastman Kodak Co., Rochester, New York. Bis(trimethylsilyl)
trifluoroacetamide (Regisil ^{R}) was purshased from the Regis
Chemical Co., Chicago, Illinois; N,N, dimethylformamide AR,
Anhydrous Ether AR, and Phosphoric Acid 85% AR, Nanograde
methanol were obtained from the Mallincrodt Chemical Works,
St. Louis. An analytical standard of o,p'-DDT (99 + % purity)
was kindly supplied by Perrine Primate Research Branch, DDA
USPHS, Perrine, Florida. A 3% OV-17 on Gas-Chrom Q column
packing was purchased from Applied Science Laboratories, Inc.,
State College, Pa.

Gas-Liquid Chromatography - GLC analysis was carried out
on a Varian moduline series 204B gas chromatograph equipped
with a flame ionization detector and a varian model 20.1 mv.
recorder. A 1/8" x 6' pyrex glass column was packed with
3% OV-17 on Gas-Chrom Q 100/120 mesh. The analysis was
carried out under isothermal conditions as follows: injector
temperature 250°C, column temperature 230°C, detector temper-
ature 250°C. The carrier gas was nitrogen at a flow of
60 ml/min. Hydrogen flow rate was 40 ml/min. while oxygen
was 360 ml/min.

Extraction of DPH from Plasma - The outline of the
extraction scheme is shown in Figure 1. One ml of plasma,
1 ml of 1M phosphate buffer pH 6.6, and 12 ml of anhydrous
ether was placed in a 16 x 150 mm Kimax culture tube fitted

with a Teflon lined screw cap. The tube was shaken vigor-
ously for 15 minutes on a mechanical shaker. Upon sitting
a minute, the layers separated without centrifugation. The
ether was removed from the aqueous layer by a technique of
freezing, using acetone and dry ice. The extraction tube
was placed up to the solvent interface in an acetone solution
saturated with dry ice contained in a Dewar flask. Within
30-40 seconds the aqueous layer froze and the ether layer
was poured into a 35 ml Kimax centrifuge tube fitted with a
Teflon lined screw cap. The sides of the original tube were
rinsed with 0.5 to 1.0 ml of ether, which was then added to
the initial extract. A 5 ml quantity of 1N NaOH was added
to the ether extract and shaken for 15 minutes. The aqueous
layer was frozen, thus permitting the ether layer to be sep-
arated and discarded. The sodium hydroxide layer was melted
without elevating the temperature of the solution above 30°C,
and then titrated back to pH 6.6 with the addition of 0.5 ml
9 M phosphoric acid. A final 15 ml of ether was added to the
neutralized aqueous layer and the tube was shaken for 15 min-
utes. The final ether extract was removed to a 16 x 125 mm
Kimax culture tube after separation, using the freezing tech-
nique. The ether was evaporated to dryness under a gentle
stream of nitrogen with the tube in a water bath not exceeding
50°C. One-half ml of a 40 mcg/ml solution of o,p'-DDT in
methanol was added to the dry extract and then evaporated.
The tube was removed from the evaporator upon dryness, as
excessive drying time (greater than 10 minutes) caused loss

of internal standard.

The silylation reagent was prepared by mixing 1 ml of bis (trimethylsilyl)-trifluoroacetamide with 0.2 ml of N,N-dimethylformamide. Forty microliters of this reagent was added to dried extract with internal standard. The tube was flushed gently with nitrogen and sealed with a Teflon cap. The tube was placed horizontally and rotated such that the silylation reagent contacted the entire glass surface. The sample was ready for injection in about 15 minutes. One to 3 microliters of the silylated extract was injected into the gas chromatograph. Each time the Kimex tube was opened, it was flushed gently with nitrogen before resealing.

Standard Curve - Standards were prepared from an etherial DPH stock solution of 1 mg/ml. Amounts of 1, 3, 5, 10, 15, 20, and 25 mcg of DPH were added by means of Microcap pipettes to 16 x 125 mm culture tubes. After the addition of 0.5 ml of the o,p'-DDT internal standard solution, the solvents were evaporated and the standards were reacted with the silylation reagent in the same manner as described for the plasma samples. The standard curve was prepared by plotting the peak height ratio (DPH derivative/internal standard) vs. DPH concentration.

RESULTS

Under the GLC conditions described, the retention time for the DPH peak is 4 minutes and the internal standard (o,p'-DDT) is 4.8 minutes. If necessary, adjustment of the carrier gas flow rate was made to achieve these retention times. All standards and samples were injected twice. If the peak height ratio of duplicate injections differed by greater than 5%, an additional injection was made.

The relationship of the peaks of DPH, the major metabolite, p-HPPH, and the internal standard is shown in Figure 2. A typical chromatograph after extraction of DPH from a patient's plasma is seen in Figure 3.

The slight overlap of the DPH and o,p'-DDT peaks does not affect the peak height of either peak. Plasma samples without DPH have shown blank values up to 0.8 mcg/ml of DPH; however, it was generally less than 0.5 mcg/ml. In each dog and human study, the blank value was determined on samples drawn at various times during the day, and it was found to remain constant. Chromatographic determination of phenobarbital under identical GLC conditions showed that there was no interference with either DPH or the o,p'-DDT peak.

The recovery of DPH extracted from plasma is reported in Table I. It was constant at 89-92% over the concentration range of 1 to 50 mcg/ml. The concentration of DPH in a plasma sample was determined by subtracting the blank from the concentration of the sample as determined from the

standard curve, then adding 10% of the difference to correct for incomplete extraction.

DISCUSSION

The method described had sufficient accuracy and sensitivity as needed for the present kinetic studies. The chromatographic analysis time is one-half that of most GLC methods reported in the literature. One exception is a method (60) with a similar analysis time, but which employs diazomethane. Up to sixty samples have been extracted on one day and chromatographed over the following two days.

The average blank peak observed in six normal individuals was 0.5 mcg/ml, and the peak was observed to be constant to $\pm$ 0.1 mcg/ml from samples taken in each individual at various times throughout the day.

TABLE I

Recovery of Diphenylhydantoin from Human Plasma

DPH added, mcg/ml	Average Recovery[*], mcg/ml	Recovery, %
1	0.9 ± 0.1	90
5	4.6 ± 0.1	92
10	9.1 ± 0.1	89
20	17.8 ± 0.1	89
30	27.1 ± 0.2	90
40	38.8 ± 0.2	92

[*]Average of three values ± standard deviation
calculated after subtraction blank value of 0.4 mcg/ml

A. **Extraction Procedure for Plasma**

1 ml Plasma
1 ml Phosphate buffer 6.3   Shake 15 min.→   Acetone
12 ml distilled ether   dry ice
                                               separation   → ether
                                                              → aqueous discard

ether layer   Shake 15 min.→   Acetone   → ether discard
5 ml 1 NaOH   dry ice
                                 separation   → aqueous

Aqueous layer
1/2 ml 8N H₃PO₄   Shake 15 min.→   Acetone   → ether
12 ml distilled ether   dry ice
                                     separation   → aqueous discard

Ether layer   evaporate on N₂ →   dryness
               water bath
               50 C

Add 1/2 ml 40 mcg/ml o, p'-DDT   evaporate 50 C →   dry residue
        (in DCM)   no more than
                                   5 min.

Add 40 μl Regisil-DMF Reagent   React   →   Ready for
                                 5-10 min.   injection

Figure 1:   Schematic Diagram for the Analysis of DPH
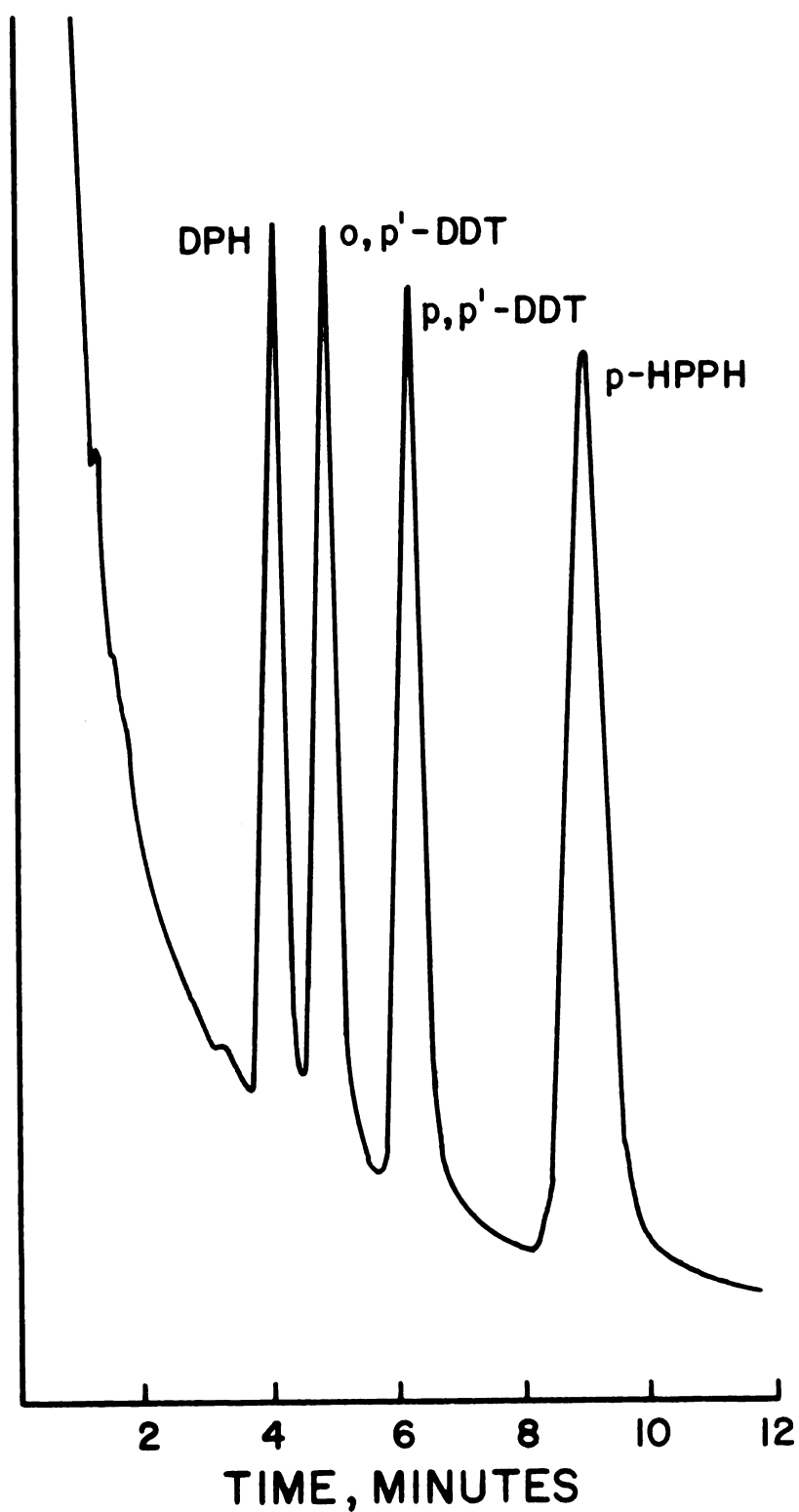
Figure 2. Chromatogram showing relative retention times of DPH, o,p'-DDT, p,p'-DDT, and p-HPPH. Conditions: 3% OV-17 on Gaschrom Q 100/120 mesh, column temperature 230°C, injector and detector temperature 250°C, nitrogen flow rate 60 ml/min.
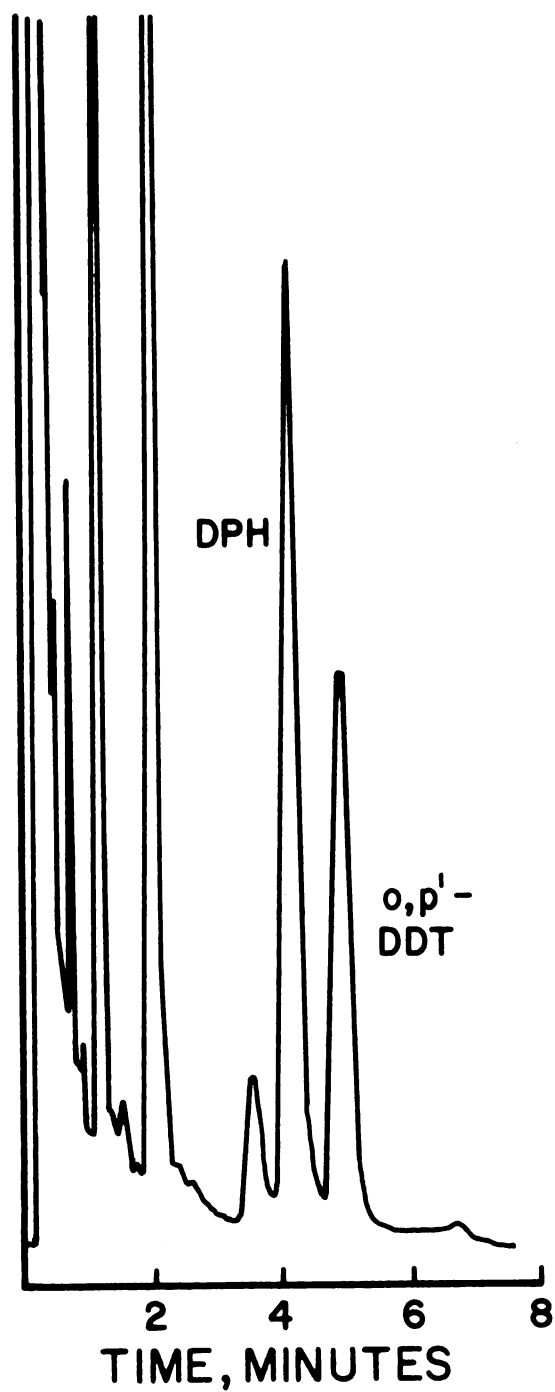
Figure 3. A typical chromatogram of human plasma containing 10 mcg/ml of DPH. Conditions see Figure 2.

CHAPTER III

STUDIES OF DOSE DEPENDENT KINETICS OF DPH IN DOGS

Studies by Dayton et al. (44) in 1967 showed that the half-life of DPH in mongrel dogs increased two to five-fold when the IV dose was increased from 20 to 50 mg/kg. The unusual finding in this investigation was that the plasma concentration declined exponentially over the whole time course of the experiment. The authors suggested possible self-inhibition of metabolism as the mechanism for dose dependent kinetics. Subsequently, Levy (66) explored the possibility that an error in the assay blank value could produce an apparent artifactual dose dependence of the type described by Dayton and co-workers.

The original research plan in this present investigation included kinetic characterization of DPH and its major metabolite in dogs, 5-meta-hydroxyphenyl-5-phenylhydantoin (m-HPPH), after intravenous doses at three levels. The plasma time course of DPH was to be determined at various doses along with the excretion of the unchanged drug and metabolite into urine and bile. However, preliminary control experiments in our dogs did not appear to confirm the finding of dose dependent kinetics as reported by Dayton, in that the terminal log-linear slopes were virtually identical when the dogs were administered doses as high as 50 mg/kg. Further preliminary studies on bile samples indicated very low levels of intact

drug and metabolites, while investigations of the urine showed irregularities in the rate of excretion of drug and metabolites, possibly due to pH dependency. Therefore, the decision was made to focus solely on plasma drug levels. These initial studies in the dogs indicated a terminal half-life of about 100 minutes, yet when the plasma levels exceeded a certain value, a shallow post-infusion decline was seen prior to the conversion to the terminal log-linear slope. Nevertheless, two dogs had been prepared for bile collection by insertion of a Thomas cannula opposite the opening of the common bile duct, and DPH disposition studies were conducted with sodium taurocholate infusion to maintain bile flow. An experiment with indocyanine green was carried out to explore the possibility that DPH may induce hemodynamic alterations that could affect its own liver clearance and thereby its rate of metabolism.

MATERIALS AND METHODS

__Materials__ - 5,5-diphenylhydantoin was obtained from
the Eastman Kodak Co., Rochester, N. Y. The solvent system
used for preparation of the parenteral solution of DPH,
similar to the commercial solvent, was prepared with 40%
propylene glycol, 10% ethanol and adjusted to pH 12. Sodium
taurocholate was purchased from Maybridge Chemical Co.,
Cornwall, U. K. Polyethylene tubing, P.E. 50 and P.E. 190,
was obtained from Clay-Adams, Inc., New York, N. Y. Indo-
cyanine green "Cardio-Green" was obtained from Hyson, Wescott,
and Dunning, Inc., Baltimore, Md.

__DPH Kinetics after Various Doses__ - Male colony bred
labrador retriever dogs weighing 22-24 kg were used for these
experiments. Each dog received 20, 35, and 50 mg/kg doses
of DPH by intravenous infusion with at least a one-week inter-
val between doses. The dogs were placed in a sling harness
during the experiment.

DPH was infused as a 50 mg/ml solution by a Harvard
infusion pump. The infusion catheter was a P.E. 50 tubing
inserted through an 18 gauge thin wall needle into the contra-
lateral femoral vein. The tubing was inserted 30 inches into
the vein which placed the end in all likelihood into the
inferior vena cava. Blood samples were collected through a
similar P.E. 50 tubing placed in the contralateral vein of
of the opposite leg, but inserted only about 10 inches. The
tubing was kept open by a flush with heparin in saline,

25 units/ml. This procedure permitted easy removal of multiple samples. Blood samples were collected into heparinized tubes and centrifuged. The plasma was collected and refrigerated until analysis on the following day.

To minimize any physiological changes, DPH was administered by a slow infusion. For the initial experiment at 35 mg/kg an infusion rate of 12.5 mg/min. was selected. The dogs vomited several times and experienced diarrhea before the end of infusion. Subsequent experiments were carried out at rates of 6.25 or 6.95 mg/min.; however, this did not entirely eliminate these untoward responses.

Indocyanine Green Experiments - The half-life of indocyanine green (ICG) has been demonstrated to be a useful method to evaluate hepatic blood flow since it is virtually cleared in one pass through the liver (67). This property of ICG was utilized to determine if the initial slow decline of DPH concentration post infusion, which was observed in the dose dependent kinetic studies, correlated with a decrease in hepatic blood flow.

The experiment was performed on a 21.5 kg mongrel dog. To serve as a control, the half-life of ICG was first determined the day prior to the DPH infusion. Normal saline was used to dissolve the 12.5 mg of ICG in a 5.0 ml volume. The solution was used immediately due to the liability of the drug to oxidation. This solution was injected into the front leg vein of the dog. Blood samples were withdrawn at 5, 10, 15, 20, and 25 minutes and analyzed for ICG

concentration.

The following day 35 mg/kg of DPH was infused at a rate of 12.5 mg/min. by the same procedure as described for the normal half-life studies. Seventy-five minutes after the start of the DPH infusion, 12.5 mg of ICG was injected as before and samples were collected over the next 30 minutes. Another 12.5 mg of ICG was injected 260 minutes after the start of the infusion, and its plasma half-life was again determined from blood samples taken over the following 25 minutes.

Sodium Taurocholate Experiments - The two dogs used in the dose dependent kinetic studies were surgically prepared for bile collection. This was achieved by cholecystectomy, ligation of the lesser pancreatic duct, and insertion of a Thomas cannula opposite the opening of the common bile duct in the small intestine. Experiments were begun no sooner than one month after surgery. When bile was to be collected, a cork was removed from the Thomas cannula and a polyethylene tube, P.E. 190, was inserted 5-6 cm into the common bile duct through the open duodenal cannula. At the end of the experiment the tubing was removed and the exterior cork replaced in the Thomas cannula, thus enabling the dog to have normal bile circulation.

During an experiment, a 0.5% sodium taurocholate solution in normal saline was infused at a rate of 60 ml/hr. This compensated for the loss of bile acids and maintained a continuous bile flow at a physiological rate. The sodium

taurocholate infusion was begun one hour prior to the DPH
infusion. DPH was infused through the deep catheter pre-
viously described. During the DPH infusion, sodium tauro-
cholate was infused in the shallow rear leg catheter normally
used for withdrawing blood samples. Blood samples were with-
drawn from a front leg vein by a needle and syringe. After
the DPH infusion was completed, the sodium taurocholate in-
fusion was connected to the deep catheter and blood was with-
drawn from the short catheter. Twenty and 35 mg/kg doses of
DPH were infused at a rate of 6.25 mg/min. in each dog. After
the lower dose, the sodium taurocholate infusion was continued
for 3.5 to 4 hours from the start of the DPH infusion, and,
after the higher dose, sodium taurocholate was continued for
6 hours. Plasma samples were obtained frequently up to a few
hours after discontinuation of the sodium taurocholate. Bile
samples were collected over 30 minute intervals.

Peripheral Infusion of DPH in the Dog - The findings of
a rising DPH plasma concentration-time curve post infusion
in the human experiments (Chapter IV) suggested that preci-
pitation of the drug may be occurring in the blood. Similar
results in the dog experiments had not been observed; however,
the method of infusion was different. A peripheral infusion
with dextrose dilution, mimicking the administration procedure
used in man, was carried out in one dog. Infusion was via
a needle inserted in a front leg vein of dog 6180. A dose
of 20 mg/kg was infused at a rate of 6.25 mg/min. into a 5%
dextrose IV drip.

DPH Analysis - The concentration of DPH in plasma was determined by the GLC method described in Chapter II.

Indocyanine Green Analysis - A 0.2 ml volume of plasma was diluted to 2.0 ml with normal saline and analyzed for ICG at 810 mu in a Cary 15 Spectrophotometer. A standard curve was prepared using a 10% plasma in saline solution over the ICG range of 0.5 to 5.0 mcg/ml.

DATA TREATMENT

Diphenylhydantoin is handled in the dog differently
than in man. Atkinson et al. (23) demonstrated that 65-82%
of the drug was eliminated in dog urine as a meta hydroxy-
lated derivative (m-HPPH) with lesser amounts of p-HPPH (18-
33%). Glazko and Chang (68) reported that a bile fistula
dog excreted 34% of the radioactive dose as conjugates of
m- and p-HPPH in the bile and 54% in the urine, while 80%
is recovered in the urine in the normal dog. The biliary
excreted conjugate is probably hydrolyzed and thereafter
reabsorbed since only 1% is excreted in the feces in the
normal dog. Further studies of Chang and Glazko (69) indi-
cate that approximately 6% of the dose is excreted as diphenyl
hydantoic acid (DPHA) and that a dihydrodiol metabolite of DPH
is formed to some extent in all species including the dog (i.e.
9-15%). They postulate that the latter compound forms via an
epoxide reaction and that the epoxide may cleave spontaneously
within the body to form either m- or p-HPPH.

The metabolism of DPH in the dog can, therefore, be
summarized to occur as follows:

DPHA (~6%)
m-HPPH (60%)
m-HPPH (10%) 0-glucoronides (80%)
p-HPPH (10%)
DPH
Epoxide ⟶ Dihydrodiol (15%)
conjugates (15%)
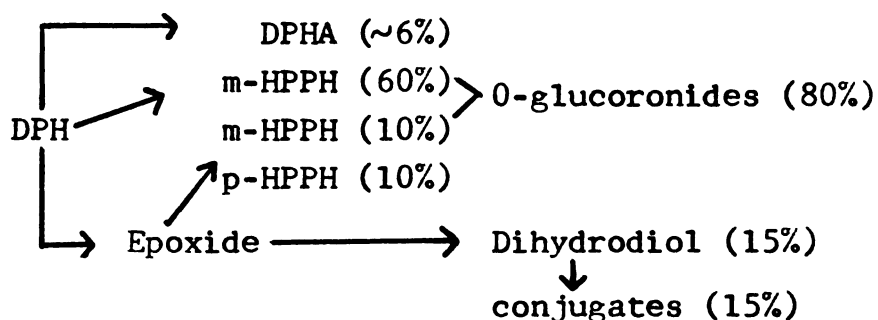
In order to treat the data obtained in our study, we will utilize the symbol HPPH to refer to either meta and the para metabolites since the models to be discussed do not require differentiation of the specific metabolites. We will also ignore the small amount of other metabolites formed by the animal.

If one presumes these processes occur as a catenary chain in a one compartmental body model, the sequence can be depicted as follows:

$$\text{Input} \xrightarrow{\dot{D}} \text{DPH} \xrightarrow{k_1} \text{HPPH} \xrightarrow{k_2} \text{HPPH-GA} \qquad (A)$$

with DPH pointing up to **Other metabolites**, and HPPH going down by k_3, HPPH-GA going down by k_4 to **Biliary and Urinary Excretion**
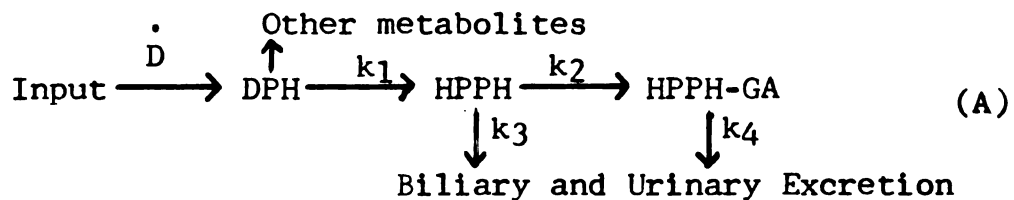
where each of the above symbols for the drug and its metabolites indicate the amount of each formed in the body, $\dot{D}$ is the infusion rate during the infusion period, k_1, k_2, k_3, and k_4 are first order rate constants. Other metabolites are probably formed to a lesser extent but are presumed to take place by simultaneous first order processes. Considering DPH levels in the plasma, the above schematic can be simplified to:

$$\text{Input} \xrightarrow{\dot{D}} \boxed{V^{DPH} C^{DPH}} \xrightarrow{K} \text{Metabolites} \qquad (B)$$

with Metabolites going down to **Excretion**
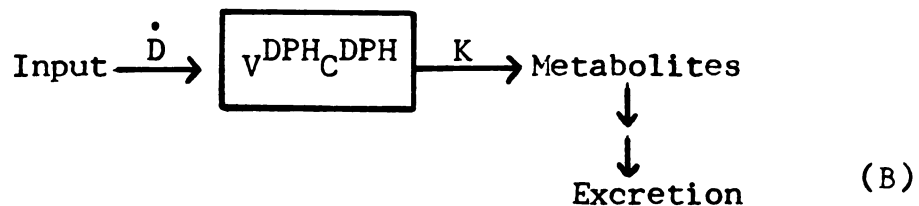
Where V^{DPH} is the volume of distribution of DPH based on a one compartment model, C^{DPH} is the plasma concentration of

DPH, and K is the overall first order elimination rate constant. However, the DPH might distribute within the body in a fashion such that it has to be treated as distributing in a two compartmental body model. This results in schematic (C):

$$\text{Input} \xrightarrow{\dot{D}} \boxed{V_p^{DPH} C_p^{DPH}} \xrightarrow{k_{13}} \text{Metabolites}$$

$$\Big\updownarrow k_{12} \quad \Big\updownarrow k_{21} \qquad\qquad \downarrow \text{Excretion}$$

$$\boxed{V_t^{DPH} C_t^{DPH}} \qquad\qquad\qquad\qquad\qquad (C)$$
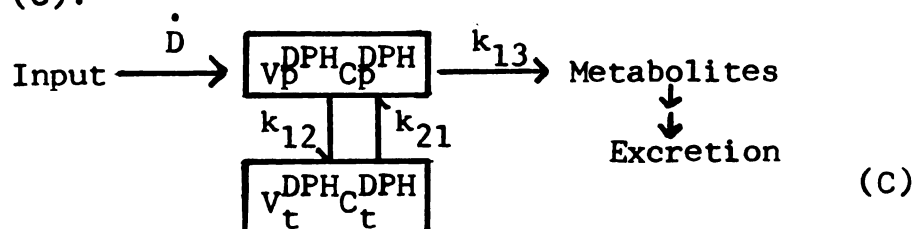
Where subscripts p refer to the central or rapidly equilibrating compartment and t refers to a tissue or more slowly equilibrating compartment, k_{12} and k_{21} are first order distributional rate constants, and k_{13} is the first order elimination rate constant. This model would result in the typical biexponential equation.

Alternatively, one might consider that DPH be converted to its major metabolite, HPPH, in the liver by a capacity limited enzymatically controlled process. The resultant HPPH is further metabolized to its glucuronide and eliminated by subsequent excretion. This sequence can be depicted as shown below in terms of mass transfer where the subscripts p and L refer to the plasma and liver respectively.

$$\text{Input} \xrightarrow{\dot{D}} \boxed{DPH_p} \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} \overset{\text{Liver}}{\boxed{DPH_L \xRightarrow[V_m/K_m]{V_m} HPPH_L \longrightarrow HPPH_L\text{-GA}}} \qquad (D)$$

Biliary and Urinary Excretion
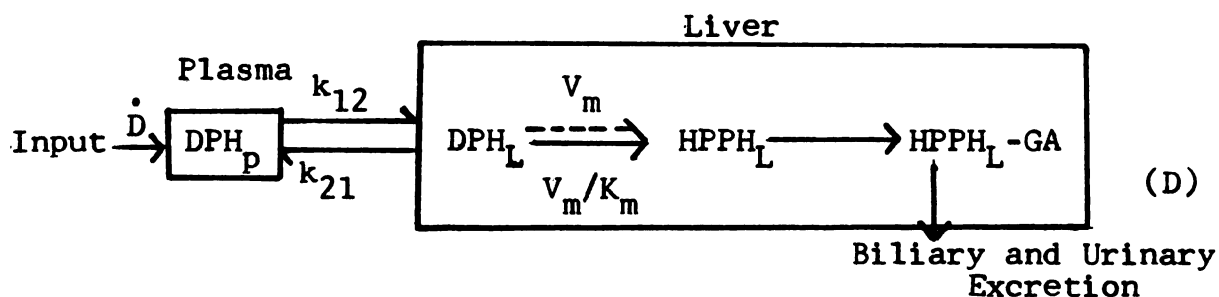
Diphenylhydantoin is depicted as passively transferring

into and out of the liver by first order rate processes,

k_{12} and k_{21}. The enzymatic process of conversion of DPH

to HPPH is interconnected by one directional special symbol

($-------\!\!\rightarrow$) proposed by Krueger-Thiemer to indicate an

irreversible Michaelis-Menten controlled process. Above the

arrow is shown V_m, indicating the zero rate constant (ex-

pressed in units of amount per unit time) for maximal enzy-

matic conversion of the DPH to m-HPPH. Below the arrow is

shown the ratio V_m/K_m where K_m is the Michaelis-Menten enzyme

substrate dissociation constant (expressed in units of amount)

and the ratio is used to denote the first order rate constant

to which the process converges at low DPH levels. Including

appropriate volume terms, this schematic will take the

following form:
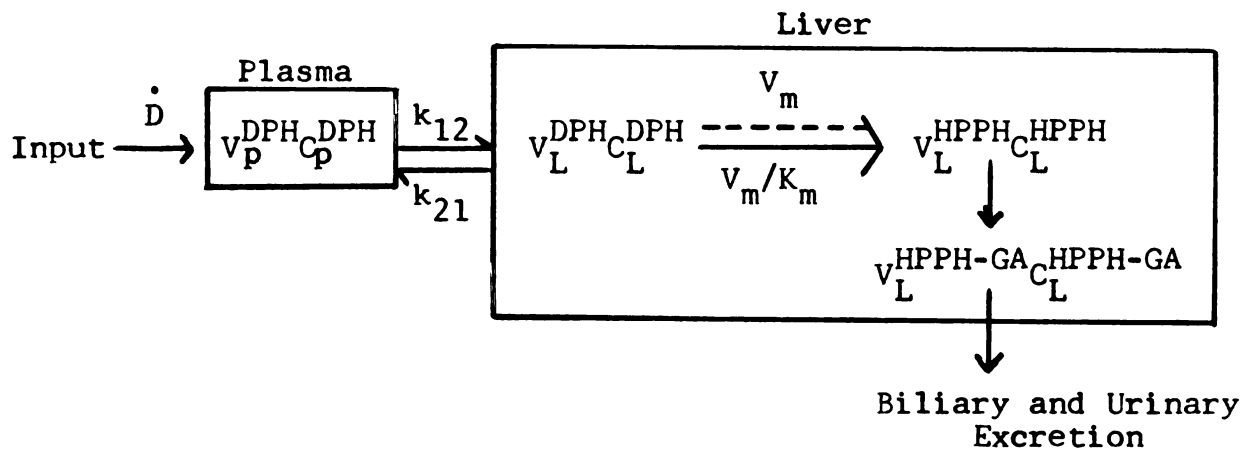
(E)

Mathematical Interpretation of Data - Presuming zero

order infusion up to time τ and first order elimination as

depicted in schematic B, we can describe the plasma concentration time curve as follows:

$$C^{DPH} = \frac{\dot{D}}{-KV^{DPH}} (1-e^{+K\tau})e^{-Kt} \qquad \text{(Equation 1)}$$

Where τ = infusion time and t is clock time from start of infusion ($0 \leq \tau \leq t$).

Similarly for schematic C, we obtain

$$C_p^{DPH} = \frac{\dot{D}(k_{21} - \alpha)(1 - e^{+\alpha\tau}) e^{-\alpha t}}{-V_p^{DPH} \alpha (\beta - \alpha)} + \frac{\dot{D}(k_{21} - \beta)(1 - e^{+\beta\tau}) e^{-\beta t}}{-V_p^{DPH} \beta (\alpha - \beta)}$$

$$\text{(Equation 2)}$$

Where α and β are the fast and slow disposition rate constants of the drug.

Schematic E must be treated in more detail. The differential equation describing the amount of DPH in the plasma compartment vs. time is

$$V_p(\frac{dC_p}{dt}) = \dot{D} - k_{12}V_p C_p + k_{21}V_L C_L \qquad \text{(Equation 3)}$$

Where all the concentration and volume terms refer to DPH. The similar equation for DPH in the liver compartment is

$$V_L(\frac{dC_L}{dt}) = k_{12}V_p C_p - k_{21}V_L C_L - \frac{V_m C_L}{\frac{K_m}{V_L} + C_L} \qquad \text{(Equation 4)}$$

Note: This is a mass balance equation and the units of V_m are, for example, mg/min. and K_m/V becomes mg/L.

If we presume that a sufficient time passes so that $C_L \gg K_m/V_L$, then Equation 4 becomes

$$V_L(\frac{dC_L}{dt}) = k_{12}VpCp - k_{21}VpC_L - V_m \qquad \text{(Equation 4a)}$$

The solution of this equation was developed in the course of studies undertaken on the liver clearance and biliary excretion of iopanoic acid by Staubus and Riegelman (70). During infusion, under the conditions when Equation 4a applies, the following equation results:

$$Cp = \frac{k_{21}(\dot{D} - V_m)}{Vp(k_{12} + k_{21})} t + \frac{\dot{D}k_{12} - V_m k_{21}}{Vp(k_{12} + k_{21})^2} (1 - e^{-(k_{12} + k_{21})t})$$

$$\text{(Equation 5)}$$

When $e^{-(k_{12} + k_{21})}$ approaches 0, Equation 5 reduces to:

$$Cp = \frac{k_{21}(\dot{D} - V_m)}{Vp(k_{12} + k_{21})} t + \frac{\dot{D}k_{12} - V_m k_{21}}{Vp(k_{12} + k_{21})^2}$$

$$\text{(Equation 6)}$$

Differentiation of Equation 6 with respect to time results in:

$$\frac{dCp}{dt} = \frac{k_{21}(\dot{D} - V_m)}{(k_{12} + k_{21})Vp} \qquad \text{(Equation 7)}$$

By rearrangement, Equation 7 may be written as:

$$\dot{D} = Vp (\frac{k_{12} + k_{21}}{k_{21}}) \frac{dCp}{dt} + V_m \qquad \text{(Equation 8)}$$

Where the term Vp $(\frac{k_{12}+ k_{21}}{k_{21}})$ is equal to the volume of distribution at steady state (Vdss) of a two compartmental system. Post infusion, yet during the conditions where Equation 4a applies (i.e., $C_L >> K_m$), the following is obtained:

$$Cp = \frac{Cp'}{Vp} e^{-(k_{12}+ k_{21})t} + \frac{k_{21}(Cp' + Ct')}{Vp(k_{12}+ k_{21})} (1-e^{-(k_{12}+ k_{21})t})$$

$$+ \frac{V_m k_{21} t}{Vp(k_{12}+ k_{21})} + \frac{V_m k_{21}}{Vp(k_{12}+ k_{21})^2} (1-e^{-(-k_{12}+ k_{21})t})$$

(Equation 9)

Where Cp' and Ct' is drug concentration at the end of infusion in the central and tissue compartment respectively. Equation 9 rapidly converges on Equation 10 when $e^{-(k_{12}+ k_{21})t}$ approaches 0.

$$Cp = \frac{V_m k_{21} t}{Vp(k_{12}+ k_{21})} + (\frac{k_{21}(k_{12}+ k_{21})(Cp' + Ct') + V_m k_{21}}{Vp (k_{12}+ k_{21})^2})$$

(Equation 10)

A number of alternative models can be described for inter-relating distribution and dose dependent kinetics. These models can include simultaneous first order excretion and metabolism as well. Yet under suitable specification of conditions, they all appear to take a form similar to

Equation 5, with merely a different definition of the micro constants (70).

One alternative model to Schematic E is a simplified one compartmental equivalent shown below.

(F)
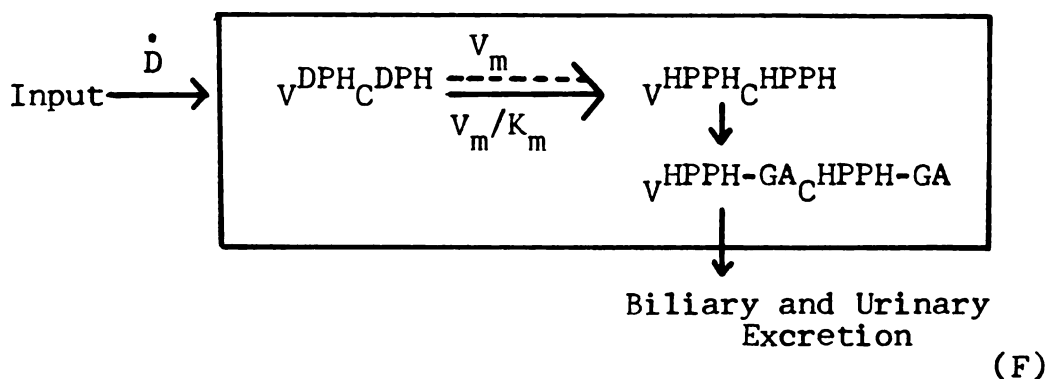
The differential equation describing this model is given below.

$$V^{DPH}\frac{dC^{DPH}}{dt} = \dot{D} - \frac{V_m C^{DPH}}{K_m/V^{DPH} + C^{DPH}}$$

(Equation 11)

Integration of Equation 11 leads to:

$$t = \frac{V_m K_m}{(\dot{D} - V_m)^2} \ln \frac{(\dot{D} K_m/V)}{(\dot{D} K_m/V + (\dot{D} - V_m)C)} + \frac{C V}{(\dot{D} - V_m)}$$

(Equation 12)

Where all the V & C terms refer to the volume of distribution and concentration of DPH respectively. This equation describes the relationship between drug concentration and time during infusion.

When $C \gg K_m/V$, Equation 11 becomes

$$V \frac{dC}{dt} = \dot{D} - V_m$$

(Equation 13)

Rearrangement leads to Equation 14:

$$\dot{D} = V \frac{dC}{dt} + V_m$$

(Equation 14)

Note both Equations 8 and 14 are equations of a straight line. They differ in terms that relate to the slope of the line and, therefore, to the apparent volume of distribution.

Defining the infusion time as Υ and the concentration of DPH at the end of infusion period as C^{Υ} , the post infusion curve will be defined by

$$V \frac{dC}{dt} = \frac{- V_m C}{K_m/V + C}$$

(Equation 15)

which upon integration yields

$$t' = \frac{K_m}{V_m} \ln \frac{C^{\Upsilon}}{C} + \frac{V}{V_m} (C^{\Upsilon} - C)$$

(Equation 16)

Where $t' = t - \Upsilon$.

Analysis of the area under plasma-concentration time curve AUC from $t = 0$ to $t = \infty$ is frequently used to test models. If one presumes that DPH is disposed of by first

order processes such as proposed in Schematic B and C, then
the AUC becomes equal to:

$$AUC = \frac{Dose}{\overset{\bullet}{V}_{cl}}$$

(Equation 17)

Where $\overset{\bullet}{V}_{cl}$ is the average plasma clearance in volume per unit
time. The area can be determined by the trapezoidal rule.
Thus the area should directly increase with dose such that
the system is said to obey dose independent kinetics.

Equation 17 can be alternatively depicted as follows:

$$AUC = \frac{Dose}{(Vd)\ area\ K} = \frac{Dose}{(Vd)\ area\ \beta}$$

(Equation 18)

Where either K or β represent the terminal log-linear slopes
as defined by Equations 1 and 2 respectively and (Vd) area
is the volume of distribution.

The area under the concentration-time curve of a system
including a Michaelis-Menten process is an extremely complex
function. It is not possible to derive a simple relation as
noted for the dose independent kinetic models. Rowland has
published (71) a series of computer simulated curves for a
drug distribution model including elimination by Michaelis-
Menten metabolism. The resultant AUC vs. dose curve is con-
cave ascending and, therefore, increases with dose.

CHARACTERISTICS OF THE ONE COMPARTMENTAL
BODY MODEL VS. MICHAELIS-MENTEN MODEL

In order to clarify the differences between a first
order one compartmental body model (Schematic B) and the
Michaelis-Menten model (Schematic F) during and post infu-
sion, it is instructive to simulate the types of curves that
will result from these two models. Their differences are
subtle, particularly when intermingled with experimental
error. Equation 1 can be used to derive a concentration-
time profile for selected values of the parameters $\dot{D}$, V and K.

Equation 12 describes the ascending (infusion) portion
and Equation 16 the descending (post infusion) portion of
the curve when considering the Michaelis-Menten model. The
data derived from appropriate parameter values are shown on
linear coordinate in Figure 1 and semi logarithmic plots in
Figure 2. Note the difference in the ascending portion of
the curves. In the linear coordinate plots of Figure 1, the
Michaelis-Menten curve is linear while the first order curve
is convex ascending. In contrast, they both are convex
ascending in Figure 2, but as the highest concentrations are
reached, the curves deviate markedly.

The post infusion Michaelis-Menten curve in Figure 1
shows a linear portion, then a conversion gradually to the
exponential decay characteristic of first order decay. The
first order curve shows exponential decay throughout. When
these data are plotted as semi log graphs as in Figure 2,

the first order is typically linear from the end of infusion.

The Michaelis-Menten curve is convex descending throughout.

However, a mid portion of the curve can occasionally be

seemingly fitted to a straight line. Close examination will

show, however, that the data points in this region fall

slightly above and below the straight line. If the curve

cannot be extended to the lower values for experimental

reasons, the terminal fall-off curves may well appear to

be plot log-linear.

It will be shown that our data has an additional physiol-

ogically or biochemically derived complication unexplained

by either model. Namely, immediately after the termination

of infusion, there is a time period where the concentrations

fall off more rapidly than predicted.[1] While this seems like

a so-called α-phase of a two compartment model, the deviation

is greater with higher concentrations and longer infusions

when the α-phase should be smaller under these conditions (72).

---

[1]Staubus and Riegelman have studied the properties of
Model E and related distribution-Michaelis-Menten Models (70).
Under the appropriate conditions, they all take the form noted
in Equations 5 and 9. Note particularly Equation 9 which
refers to the post infusion period. This equation indicates
that a so-called "α" phase should be seen until such time as
$e^{-(k_{12}+k_{21})t} = 0$.

RESULTS

DPH Kinetics after Various Doses - Initial studies at
a moderate infusion rate (12.5 mg/min.) caused physiological
disturbances in the dogs. The plasma concentration-time
curve after a 35 mg/kg dose infused at this rate is shown
on a semi logarithmic plot in Figure 3. The infusion lasted
61 minutes. Near the end of the infusion, the dog vomited
and did so several times up to 100 minutes, as indicated by
the arrows just above the time axis. The dog also showed
ataxia at the end of infusion. Post infusion plasma levels
show a shallow and irregular decline followed by a turnover
to what appears to be a log-linear portion beginning at
about 200 minutes. This terminal portion can be fitted to
first order kinetics which lead to the presumed terminal
half-life of 97.8 minutes. Although it is not obvious from
this graph, the curve shows the tendency for a convex descending
fall-off.

In order to minimize alterations of the normal physiolo-
gical state, the experiment was repeated in the same dog at
one-half the infusion rate or at 6.25 mg/min. No vomiting
occurred. The results are also shown in Figure 3. A short,
shallow period of decline occurred before a turnover to a
more rapid elimination. The terminal portion of fall-off
curve can be fitted to a straight line with an apparent half-
life of 97.3 minutes.

The terminal rate constant, the area under the plasma

concentration-time curve and the volume of distribution can be determined from Equations 1, 17, and 18 on the presumption that drug disposition took place by first order processes.

The plasma concentration-time curves after doses of 20, 35, and 50 mg/kg in dog 6180 are shown in Figure 4, and the experimental parameters estimated on the presumption of a first order model are summarized in Table 1. The low and intermediate dose was infused at 6.25 mg/min. and the high dose at 6.95 mg/min. Even with the slow rate of infusion, pronounced vomiting and diarrhea occurred after the 50 mg/kg dose. Severe ataxia lasted several hours post infusion after this dose.

The DPH plasma concentration appeared to decline exponentially after the end of the 20 mg/kg infusion; however, after both the 35 and 50 mg/kg doses there was a slow or shallow decline before a turnover to what appears to be the terminal exponential decay. The observed, apparent terminal half-lives for the three doses were relatively unchanged, 103.9 minutes for the 20 mg/kg dose, and 97.3 and 98.6 minutes respectively for the 35 and 50 mg/kg doses.

Similar results were obtained in experiments on dog 6181. The results of 20, 35, and 50 mg/kg doses infused at different rates are shown in Figure 5. The solid curves are fitted on the presumption of first order kinetics and the experimental parameters based on this assumption are summarized in Table II. The dashed lines in Figure 5 could represent the fitted curve on the presumption of the Michaelis-Menten kinetics as depicted

in Figure 2. The low dose was infused at 6.25 mg/min., the intermediate dose at 12.5 mg/min., and the high dose at 6.95 mg/min. The post infusion curve showed an initial shallow decay, after which the observed, apparent terminal half-lives again remain unchanged at 95.9, 94.9, and 94.1 minutes respectively. Vomiting and diarrhea occurred during and after the 35 and 50 mg/kg infusions.

The AUC was estimated by the trapezoidal rule and extrapolated beyond the last data point presuming that first order kinetics applied. The area per unit dose was appropriately calculated. The clearance values are calculated from Equation 17 and the apparent (Vd) area values from Equation 18. All these data are included in Tables I and II. The apparent volume of distribution from the 20 mg/kg dose in dog 6180 was estimated to be 32.6 L, and for dog 6181 was 32.5 L. The average clearance of DPH in dog 6180 was estimated to be 218, 169, and 158 ml/min. respectively after doses of 20, 35, and 50 mg/kg. The average clearance in dog 6181 was calculated to be 235, 141, and 149 ml/min. after the same doses.

In all the above calculations, the elimination rate constant was estimated from the terminal data using those points which appeared to obey first order kinetics when fitted by the eye. The presumption of log linearity may be in error since close examination of the data appears to indicate that most of the curves show a slight tendency towards a convex descending curvature which might indicate that the

true log-linear slope had not been reached. This is particularly noticeable in the 35 and 50 mg/kg doses in dog 6181.

Figures 6 and 7 are log plasma concentration-time plots during the infusion time period. They show the comparison of the actual data points with the data generated using Equation 1. The theoretical curves were based on a single compartmental zero order infusion model as depicted in schematic B using the volume of distribution obtained from the 20 mg/kg dose and the terminal first order elimination rate constant for the particular experiment. In both dogs only the low dose appears to follow the predicted curves.

In order to examine the possibility that the system obeys Michaelis-Menten kinetics, the data shown on Figures 4 and 5 are redrawn as linear coordinate plots in Figures 8 and 9. Figures 10 and 11 are close examination of the ascending curves. It is to be noted that the data apparently fits a linear curve throughout the infusion period. The dashed curves in Figure 11 are the theoretical curves generated using Equation 1 and the experimentally derived first order elimination rate constant and volume of distribution. The marked deviation at higher concentrations emphasizes the apparent lack of conformance with the first order kinetics during the infusion period. Another test of conformity is to treat the data obtained during the infusion period as the rate of change of DPH plasma concentration per unit time ($\frac{\Delta c}{\Delta t}$) vs. the mid point of the time interval. This is shown in Figure 12. All curves so analyzed show a positive slope

with good to poor correlation coefficients. The dashed
lines are from theoretical plots of generated first order
elimination data and result in negative slopes.

Table III is a tabulation of the parameter values
obtained from a least mean square analysis of the ascending
curves (during infusion) for both dogs. Figures 13 and 14
are plots of infusion rates vs. the experimentally deter-
mined ascending linear slopes tabulated in Table III.
According to Equation 14, the Y intercept leads to an esti-
mation of the V_m and the slope of the linear curve gives an
estimation of the volume of distribution.

Indocyanine Green Experiment - The plasma concentration-
time curve for DPH in dog 11133 is shown in Figure 15. The
half-life in this dog was found to be unexpectedly long;
namely, 250 minutes. The plasma concentration declined expo-
nentially after the cessation of DPH infusion. A shallow
decline followed by a more rapid decay as seen in the previous
experiments was not demonstrated. The times of ICG injection
are designated by the arrows above the time axis. The average
clearance of DPH was 88.6 ml/min., and the volume of distri-
bution was 32.0 L (Vd area) as calculated by the methods
previously described. The infusion curve based on the intra-
venous infusion equation for a single compartment model is
seen in Figure 16. Figure 17 shows an ICG half-life of 6.5
minutes on the day prior to the DPH study and 12.1 and 9.4
minutes at the 75 and 260 minute injection times. The control
half-life was comparable to findings of Wheeler et al. (73)

after a similar dose in unanesthetized dogs. The plasma protein binding of DPH was shown to be unaltered by the administration of ICG.

Sodium Taurocholate Experiment - Results of DPH elimination with concomitant sodium taurocholate infusions are shown in Figure 18 for dog 6180 and Figure 19 for dog 6181. These semi-logarithmic plots show a remarkable change in form of the fall-off curves just after the infusion. Note that the initial post infusion rapid decline seen in the experiments during the studies done in the absence of taurocholate infusion has been eliminated, and three of the four studies exhibit fall-off curves more like the appearance of the Michaelis-Menten plot shown in Figure 2. Figures 20 and 21 are the linear plots of the data. The arrows indicate when the taurocholate infusion was discontinued.

Peripheral Infusion of DPH in the Dog - In order to investigate whether DPH can precipitate during infusion, two different methods and sites of infusion were compared. In all of the above experiments a vena cava infusion of the undiluted parenteral solution was used. One experiment was done mimicking the human studies (Chapter IV) where the parenteral solution was mixed with a 5% dextrose drip as it was being infused into a peripheral leg vein. No precipitate was observed in the cannula during the 20 second transit time needed for the diluted drug vehicle to enter the vein. Figure 22 shows the results of the peripheral infusion compared to the infusion at the same rate directly into the vena cava.

The concentration at the end of the peripheral infusion was about 4.5 mcg/ml compared to 9.0 mcg/ml at the point in time after deep infusion.
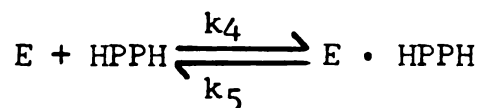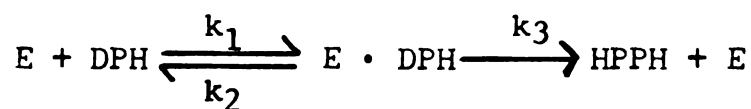
DISCUSSION

When DPH was infused in our dogs at doses of 20, 35, and 50 mg/kg, dose dependent kinetics as described by Dayton et al. (44) was not observed. Dayton had reported a log-linear decline of DPH plasma concentrations over the time course of the experiment when doses of 20 and 50 mg/kg were injected IV. The two doses resulted in a different half-life without a turnover to a faster elimination as might be expected when simple Michaelis-Menten kinetics are anticipated. Neither the plasma curves nor the blood level data were reported in Dayton's study, and only the resultant half-lives were listed. In our investigations, the infusion of 50 mg/kg per dose lasted almost three hours. Even at this low rate of infusion the animals showed pronounced physiological effects (vomiting, diarrhea, and severe ataxia lasting for several hours post infusion). After administration of identical doses as an IV bolus, it is not unreasonable to expect that major physiological and hemodynamic alterations might have occurred in the Dayton studies, especially in the light of a reported lethal dose of 66-69 mg/kg for DPH in dogs (74). However, our data are not without support for the contention of the capacity limiting processing.

Interpretation of Data by Michaelis-Menten Kinetics - Consider Schematic E or F, discussed in earlier data treatment section, which are based on the presumption that Michaelis-Menten kinetics apply. If the conversion of DPH to HPPH is

capacity limited and follows this scheme, the resultant plasma concentrations for DPH after different doses by bolus injection would be expected to show the characteristic curves depicted in Figure 23. Thus, the curves will appear to be convex descending and only when the concentration falls below the K_m will the terminal slopes tend to become parallel.

It has been recently reported from *in vitro* (47) and *in vivo* (48) studies in rats that HPPH inhibits the metabolism of DPH. The competitive inhibition may be shown as follows:

$$E + DPH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot DPH \xrightarrow{k_3} HPPH + E$$

$$E + HPPH \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E \cdot HPPH$$

Where $K_m = \dfrac{k_2 + k_3}{k_1}$ and $K_I^{HPPH} = \dfrac{k_4}{k_5}$.

The resultant Michaelis-Menten equation now takes the form:

$$\frac{dC^{DPH}}{dt} = \frac{V_m C^{DPH}}{C^{DPH} + K_m \left(1 + \dfrac{C^{HPPH}}{K_I^{HPPH}} \right)}$$ (Equation 19)

If $C^{HPPH} > K_I^{HPPH}$, then Equation 17 reduces to:

$$\frac{dC^{DPH}}{dt} = \frac{V_m C^{DPH}}{C^{DPH} + K_m \left(\dfrac{C^{HPPH}}{K_I^{HPPH}}\right)}$$

(Equation 20)

If the elimination of HPPH is slow such that C^{HPPH} is ostensibly constant, Equation 18 can be rewritten as:

$$\frac{dC^{DPH}}{dt} = \frac{V_m\ C^{DPH}}{C^{DPH}\ K_m{'}}$$

(Equation 21)

Where $K_m{'} = K_m \left(\dfrac{C^{HPPH}}{K_I^{HPPH}}\right)$.

Perrier (75) has proposed that the system described by Equation 19 under certain conditions will be virtually mono-exponential as depicted in Figure 24. Now, if the total amount of DPH and HPPH were sufficient to totally occupy the available enzyme, the system would become zero order in kinetic behavior, the rate of metabolism dependent on the amount of enzyme present in the system.

Figures 25 and 26 include the data from experiments on dog 6180 and dog 6181 wherein equivalent doses were infused in the presence and absence of taurocholate infusion. Taurocholate was discontinued in each of the experiments at the time indicated by the arrow.

In the experiments with concomitant taurocholate the rapid so-called " α " phase following discontinuance of DPH

infusion has been eliminated. The resultant curves appear
to be compatible with the Michaelis-Menten curve shown in
Figure 2.

The existence of an "α" phase under similar conditions
has been observed, but not commented upon by Wheeler et al.
(76). Figure 3A of the Wheeler study shows an initial rapid
decline post-infusion before an apparent zero order elimi-
nation of the drug. Staubus and Nelson (77) have observed
a similar "α" phase and zero order kinetic elimination
data for iopanoic acid. They have confirmed that when the
rate of a concomitant infusion of taurocholic acid is
increased, the "α" phase for iopanoic acid is obliterated.
They have developed evidence that this may be due to changes
in drug transport into the cell. Further studies are needed
to confirm this concept.

Figures 27 and 28 include two experimental studies and
data simulated by using Equations 12 and 15. The theoretical
down curves are parallel to but displaced from the experi-
mental down curve. However, by shifting the zero time con-
centration for the fall-off curves to exclude the "α" phase,
it is possible to fit the fall-off curves rather precisely
to the Michaelis-Menten generated post-infusion curve shown
by the dashed line.[2]

The V_m and K_m and V_d values chosen for these computer
fits are given in the legends to the figures. These curves

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[2]Staubus and Riegelman will report separately on the
fitting of Equations 5 and 9 which generate the so-called
"α" phase.

were generated by the use of the IBM 360-50 computer,

using a CPS generated program and an empirical fitting

procedure. The data at hand does not warrant a more detailed

attempt at computer fitting. Obviously, the values obtained

by this procedure are probably poor estimates of the para-

meters. Nevertheless, the V_m values are in the range of those

estimated in Figures 13 and 14 where all the data were used

to obtain the appropriate values for substitution into Equa-

tion 14. This equation allows an estimate of V_m from the

Y-intercept and V_d from the slope. They indicate that the

enzyme system is apparently saturated with a maximum velocity

of 2-4 mg/min. in these dogs. The relatively low K_m values

are in conformity with the early saturation of the system,

and the suggestion that the terminal first order log-linear

decline has not been reached is indicated by the fall-off

curves.

Hemodynamic Considerations - Capacity limited metabolism

and end product inhibition described by the Michaelis-Menten

model in the previous discussion is not the only reasonable

explanation that is compatible with the findings in the

present studies. The findings of altered DPH kinetics in

these studies after high doses and/or rapid infusion might

be explained by hemodynamic changes induced by the drug

itself. The hemodynamic effects of DPH in both man and dog

have been extensively studied. Hypotension is a well-recog-

nized side effect of DPH therapy in man. Harris and Kokernot

(78) demonstrated significant hypotension when doses of

12.5 mg/kg were injected rapidly into dogs. The same dose
was given by slow injection produced only slight alteration
of blood pressure. Louis et al. (79) have shown that rapid
infusions of DPH in cats result in severe hypotension due
to the additive effects of DPH and propylene glycol from
the drug vehicle. Gupta et al. (80) in 1967 reported that
the cardiac output was reduced 20% for periods over 20 minutes
when DPH was administered to dogs at 5 mg/kg doses. In the
same year, Rowe (81) reported similar findings of significantly
reduced cardiac output and stroke volume after 5 mg/kg injec-
tions of DPH in anesthetized dogs. He also demonstrated an
average 18% decrease of coronary blood flow in 10 dogs. Conn
et al. (82) have reviewed the literature on the cardiovascular
effects of DPH in dogs and conclude that the depression of
myocardial function and peripheral vasodilation induced by
the drug is related to the dose and to the rapidity of admin-
istration.

The liver receives a large fraction of the cardiac out-
put. It seems reasonable to expect a marked decrease of
hepatic blood flow when large doses of DPH are infused slowly
to dogs. Rowland (83) has pointed out that if the extraction
ratio of an eliminating organ approaches unity, then the
clearance by that organ will be equal to the blood flow of
the organ. Thus, the clearance of a drug, with a high hepatic
clearance approaching liver blood flow, would be expected to
decrease with decreasing hepatic blood flow.

In the present studies, decreases in the mean clearance

of DPH were seen and appeared to be related to the dose and to the rapidity of administration. For example in dog 6181 the mean clearance after a 35 mg/kg dose infused at 12.5 mg/min. was 141 ml/min. while a 50 mg/kg dose infused at about one-half the rate had an average clearance of 149 ml/min. A dose of 20 mg/kg infused at the slow rate was cleared at an average of 235 ml/min. (see Table II). When all doses were infused at a slow rate, the average clearance decreased with increasing dose. The average clearance for dog 6080 (Table I) was 218, 169, and 158 ml/min. after doses of 20, 35, and 50 mg/min. respectively.

The present findings quantitatively fit a perfusion limited drug clearance model. When the clearance of DPH is high, it is affected by hepatic blood flow and when it is low, it is unaffected by this parameter.

A quantitative estimate of the effect of a decreased blood flow can be made by applying the equation derived by Rowland (83) for mean clearance.

$$\bar{\dot{V}}_{cl} = \dot{V}_B \left(\frac{K_L K_e V_e}{\dot{V}_B + K_L K_e V_e} \right) \qquad \text{(Equation 22)}$$

Where $\bar{\dot{V}}$ is the average clearance, $\dot{V}_B$ is the blood flow through the eliminating organ, K_L is the apparent partition coefficient of the drug between the eliminating organ and the emergent venous blood, K_e is the first order rate constant for drug elimination, and V_e is the volume of the eliminating

organ. The quantity in the parenthesis of Equation 22 is the extraction ratio. The term $K_L K_e V_e$ can be evaluated by substituting experimental drug clearance and estimated blood flow in the rearranged Equation 22:

$$K_L K_e V_e = \frac{\dot{V}_{cl}}{\frac{(1 - \dot{V}_{cl})}{\dot{V}_B}}$$

(Equation 23)

Calculations were made based on whole blood clearance as suggested by Rowland (84). The whole blood clearance for Experiment 8 was found to be 250 ml/min., and the DPH extraction ratio in this experiment was 0.29, assuming the average hepatic blood flow of 860 ml/min. (39 ml/kg/min.) as reported by Drapanas et al. (85). $K_L K_e V_e$ was calculated to be 352 ml/min. for this experiment. If, for example, the blood flow to the liver is diminished by 50% over the whole time course of the experiment, then the calculated average clearance of DPH would be 195 ml/min. Even though a 50% decrease in blood flow was invoked over the entire experiment for the calculation, the resultant average clearance was greater than experimentally seen (Tables I and II). However, the blood flow value used in the calculation was an average value of a widely varient parameter. Drapanas reported hepatic blood flow rates in dogs to vary from 26 to 60 ml/kg/min., and studies by Kettering et al. (67) showed a similar variation from 14.4 to 50.2 ml/kg/min. If the

hepatic blood flow in dogs 6180 and 6181 was on the low side of the average value, then a greater decrease in DPH clearance would be calculated from decreased blood flow.

It is obvious, therefore, that there might be a hemodynamic effect of DPH removal. When DPH clearance is approaching blood flow in the dog, as the above data suggest, then the system may be blood flow dependent. An experiment was designed to establish whether the non log-linear elimination of DPH might correspond to a period of decreased liver perfusion. The experiment was based upon the linear relationship between ICG clearance and hepatic blood flow established by Kettering et al. (67). In a single experiment ICG clearance was determined at different times after an infusion of 35 mg/kg of DPH.

At the end of the DPH infusion, the plasma clearance of ICG was 87.9 ml/min., and the DPH level was 21.5 mcg/ml. Over three hours later, when the DPH level had dropped to 13.7 mcg/ml., the ICG clearance was 121 ml/min. (Figures 15 and 17). The ICG clearance in this dog prior to DPH infusion was 217 ml/min. Therefore, if one presumes that the ICG clearance is directly related to blood flow to the liver, the results appear to indicate the hepatic flow was reduced 40-55% of normal. However, one might propose that when the test is done in the presence of DPH that it no longer responds appropriately. If ICG and DPH are competing for binding sites, it might well be that the liver uptake of ICG is reduced. At high DPH levels it, therefore, appears to indicate reduced

clearance. The result will remain ambiguous unless veri-
fied by a direct measure of blood flow. Unfortunately,
time did not permit similar studies in dogs known to have
high DPH clearance. Additional studies will be required to
clarify the hemodynamic contribution to the non-linear
kinetic behavior of DPH in dogs.

Effect of the Site of Infusion - The results reported
for the human studies in Chapter IV show that the DPH plasma
concentration actually continued to rise for a rather pro-
longed period post-infusion. This led to the suggestion that
the drug may have precipitated in these studies and only
gradually redissolved. Similar findings were not observed
in the dog studies; however, a different method of drug
administration was utilized. In these experiments, the
drug in the commercial hydroalcoholic vehicle was infused
into the vena cava directly. Thus, even though the vehicle
was not prediluted, it was mixed into a high volume, rapidly
moving blood stream. In the human studies the drug solution
was infused into the end of an IV drip set, inserted into
the antecubital vein, and mixed with a rapid flow of 5%
dextrose. This supposedly allowed dilution of the alkaline
vehicle prior to interaction with the blood constituents.
However, the volume and movement of the fluid in the ante-
cubital vein is undoubtedly much less than that of the vena
cava. Whether or not this caused any precipitation of the
drug was uncertain but warranted some further study. It
was, therefore, decided to mimic the human infusion experiment

by injecting the drug into a fore leg vein of the dog and diluting with a rapidly flowing 5% dextrose IV drip.

Evidence of the precipitation of DPH in the blood after peripheral infusion with 5% dextrose is shown in Figure 22, where the identical infusions of DPH were administered via the two routes. It can be seen that when DPH is infused at a slow rate in the vena cava, the infusion curve is convex ascending throughout the infusion period. When administered via the IV drip, while the points are few in number, the curve clearly deviates from the expected shape. More important, the concentration achieved at the end of the peripheral infusion was only about one-half that of the deep infusion at the same time point. Also, plasma concentrations did not drop for about 50 minutes post infusion after the peripheral infusion. Even after the plasma concentration has dropped, it is apparent that the drug is still being redissolved beyond 300 minutes as indicated by a rise in the curve at 360 and 420 minutes and a reduced area under the concentration-time curve out to the end of the period of sampling.

CONCLUSIONS

DPH disposition in dogs was investigated after slow infusions of 20, 35, and 50 mg/kg. Immediately after the cessation of infusion the log concentration-time curves show what appears to be an initial fall-off followed by a shallow decay and then a turnover to a more rapid elimination. A distributional explanation for the initial fall-off which is compatible with experimental findings will be reported elsewhere. At lower concentrations, the curves follow first order elimination. However, after high doses the post infusion curves appear to be convex descending over the time course of the experiment. These findings are compatible with capacity limited conversion of DPH to HPPH, where HPPH may be acting as a competitive inhibitor of its own formation, and can be described by Michaelis-Menten kinetics. When identical experiments were carried out with concomitant taurocholate infusion, the post infusion, initial fall-off phase was not evident, and there was a greater tendency towards a convex descending decline.

Some indirect evidence was developed which indicates that DPH may have markedly decreased hepatic blood flow. The experimental results are also qualitatively compatible with a perfusion model where clearance is limited by hepatic blood flow.

It was demonstrated that after peripheral infusion with dextrose dilution sustained plasma levels of DPH occurred,

indicating precipitation of drug in the blood. This
supported speculation of drug precipitation in the blood
as an explanation for similar findings in human experiments
after intravenous infusion.

Pharmacokinetic models generally assume a physiological
homeostasis. Considerations of the physiologic and hemo-
dynamic state and the effects such changes have on drug
disposition should not be overlooked when interpolating
experimental kinetic data.

TABLE I

Summary of Various Doses of DPH
Infused in Dog 6180

Experiment	20 mg/kg exp. 9	35 mg/kg exp. 11	50 mg/kg exp. 13
Dose, mg	440	750	1112
Rate of Infusion, mg/min.	6.25	6.25	6.95
Length of Infusion, min.	70.6	120	160
Terminal Half-Life[a], min.	103.9	97.3	98.6
Terminal Elimination[a] rate constant, min.$^{-1}$	.00667	.00712	.00703
AUC , mg x min./L[b]	2016	4445	7021
AUC /Dose, min./L	4.58	5.92	6.31
Average Clearance, ml/min.	217.6	168.7	158.4
Vd area L	32.6	---	---
Concentration where rapid elimination begins, mcg/ml	---	12.8	15.0

[a]Calculated by fitting the terminal points by presuming first order kinetics apply in this region. However, see text for further discussion.

[b]The area reported includes a value obtained by extrapolating to infinity by presuming the following applies: $A_{t \to \infty} = C_t/K$ where C_t is the last data point, and K is the elimination rate constant obtained in (a) above.

TABLE II

Summary of Various Doses of DPH
Infused in Dog 6181

Experiment	20 mg/kg exp. 8	35 mg/kg exp. 6	50 mg/kg exp. 12
Dose, mg	440	770	1200
Rate of Infusion, mg/min.	6.25	12.5	6.95
Length of Infusion, min.	70.6	61.0	173.0
Terminal Half-Life[a], min.	95.7	95.0	94.1
Terminal Elimination[a] rate constant, min.$^{-1}$	.00724	.00729	.00737
AUC , mg x min./L[b]	1869	5497	8034
AUC /Dose, min./L	4.25	7.14	7.00
Average Clearance, ml/min.	235.4	140.8	149.4
Vd area, L	32.5	---	---
Concentration where rapid elimination begins, mcg/ml	---	15.0	17.5

[a]Calculated by fitting the terminal points by presuming
first order kinetics apply in this region. However, see
text for further discussion.

[b]The area reported includes a value obtained by extrapolating
to infinity by presuming the following applies: $A_{t\to\infty} = C_t/K$
where C_t is the last data point, and K is the elimination
rate constant obtained in (a) above.

TABLE III

Linear Least Square Fit of Plasma Concentration-
Time Curve During Infusion Period

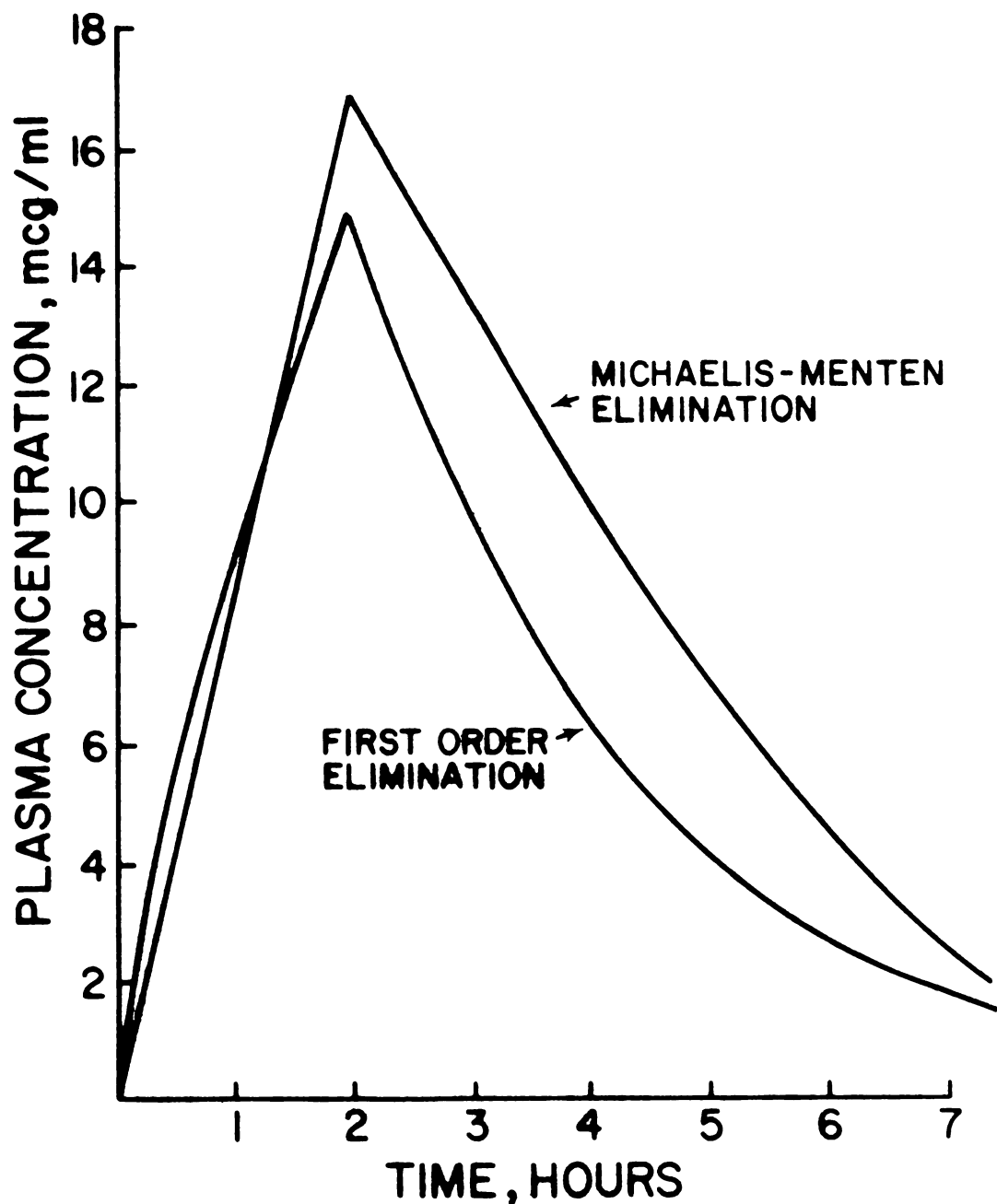| Dog 6180	Dose mg/kg	Rate of Inf. mg/min.	Slope mg/L/min.	Y-intercept mg/L	Correlation Coefficient
Exp. 7	35	12.5	.404	-1.39	.994
Exp. 9	20	6.25	.158	.414	.994
Exp. 11	35	6.25	.157	.749	.995
Exp. 13	50	6.95	.179	-1.06	.994
Dog 6181					
Exp. 8	20	6.25	.150	.124	.995
Exp. 6	35	12.5	.461	-1.77	.999
Exp. 12	50	6.95	.174	.381	.999

Figure 1. Comparison of first order and Michaelis-Menten
elimination during and after infusion as plotted on linear
coordinates. First order elimination curve parameters:
D = 6 mg/min., V = 32.6 L, K = .00700 min.$^{-1}$. Michaelis-
Menten elimination curve parameters: D = 6 mg/min.,
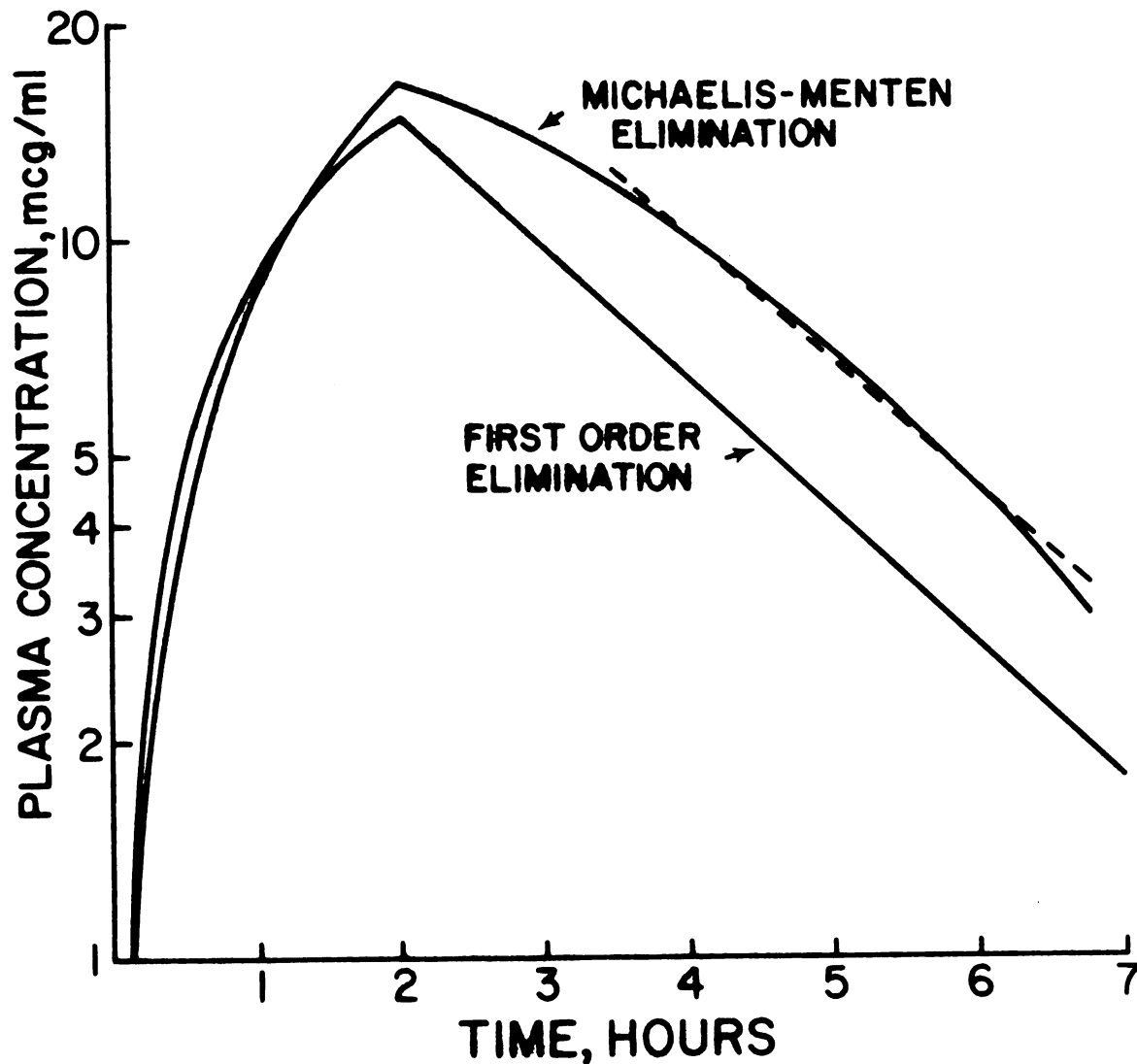K_m/V = 5 mg/L, V = 27.5 L, V_m = 2.2 mg/min.

Figure 2. Comparison of first order and Michaelis-Menten elimination during and after infusion as on semi-logarithmic coordinates. Dashed line shows Michaelis-Menten curve fitted to first order elimination. Parameter values as in Figure 1.
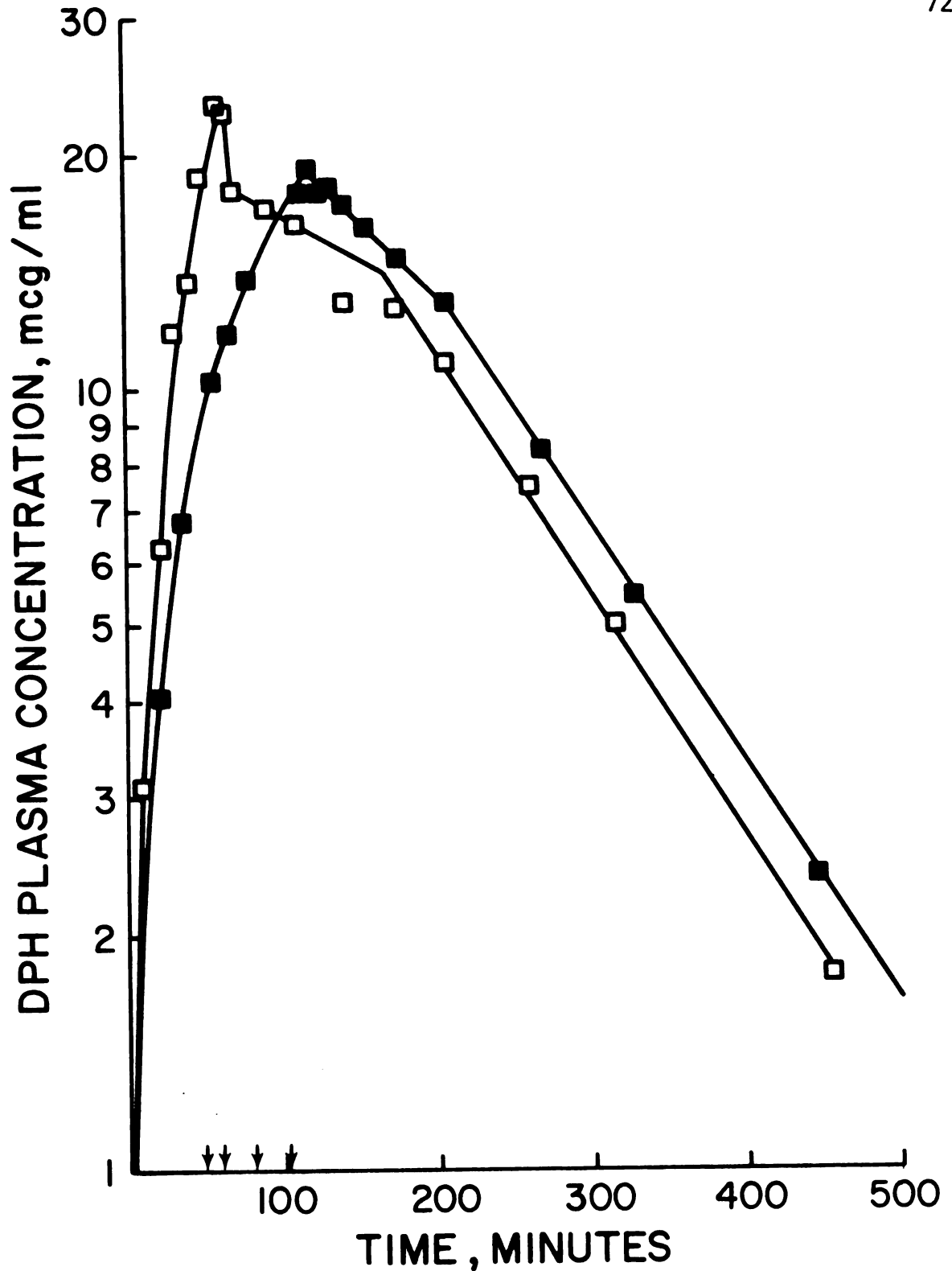
Figure 3. Plasma concentration of DPH during and after the infusion of 35 mg/kg in dog 6180. Key □, infused at 12.5 mg/min. ■, infused at 6.25 mg/min. Arrows above time axis indicate when vomiting occurred during rapid infusion.

Figure 4. Plasma concentrations of DPH during and after infusion of various doses in dog 6180. Key: ●, 20 mg/kg at 6.25 mg/min.; ■, 35 mg/kg at 6.25 mg/min.; ▲, 50 mg/kg at 6.95 mg/min.
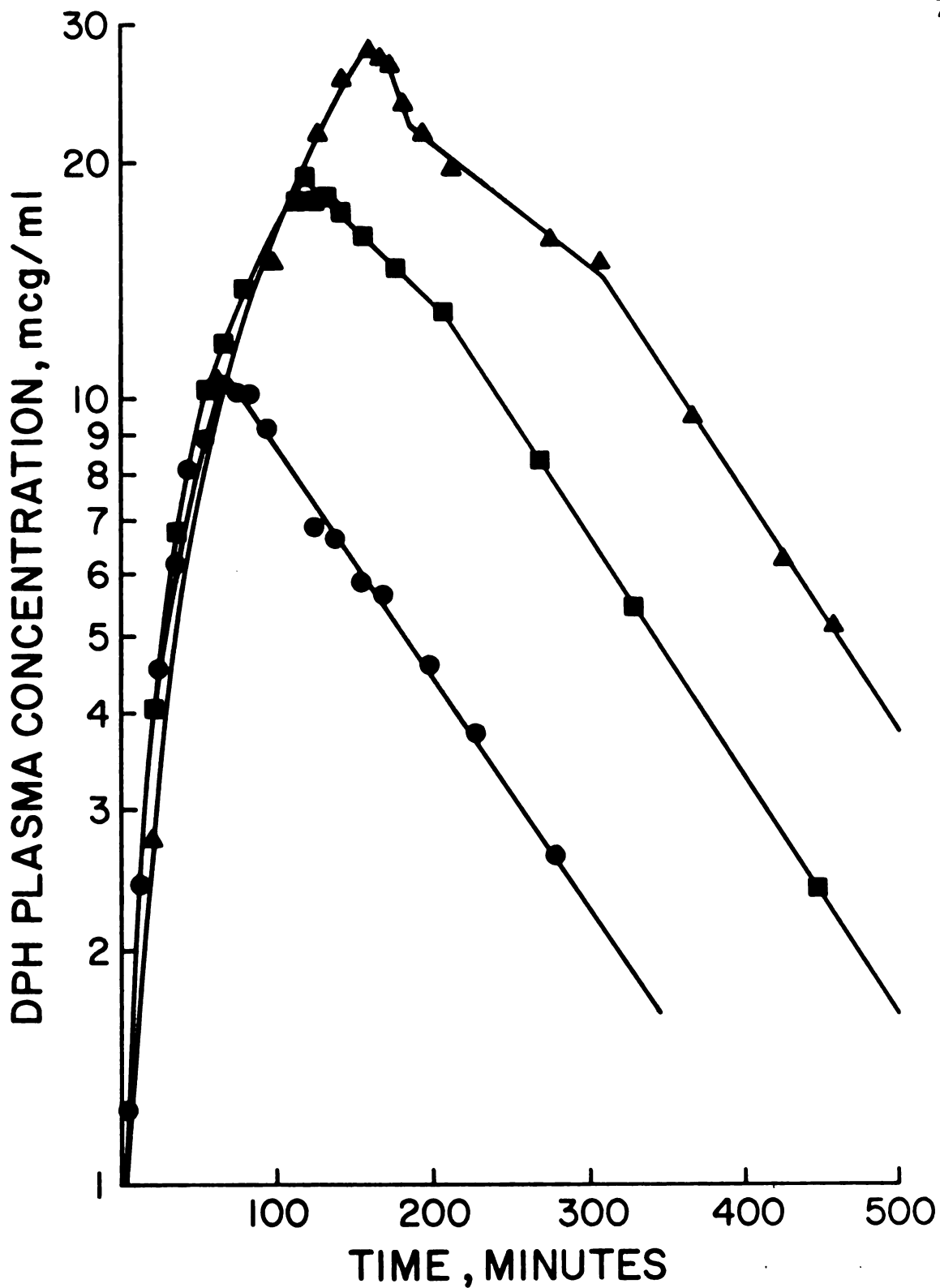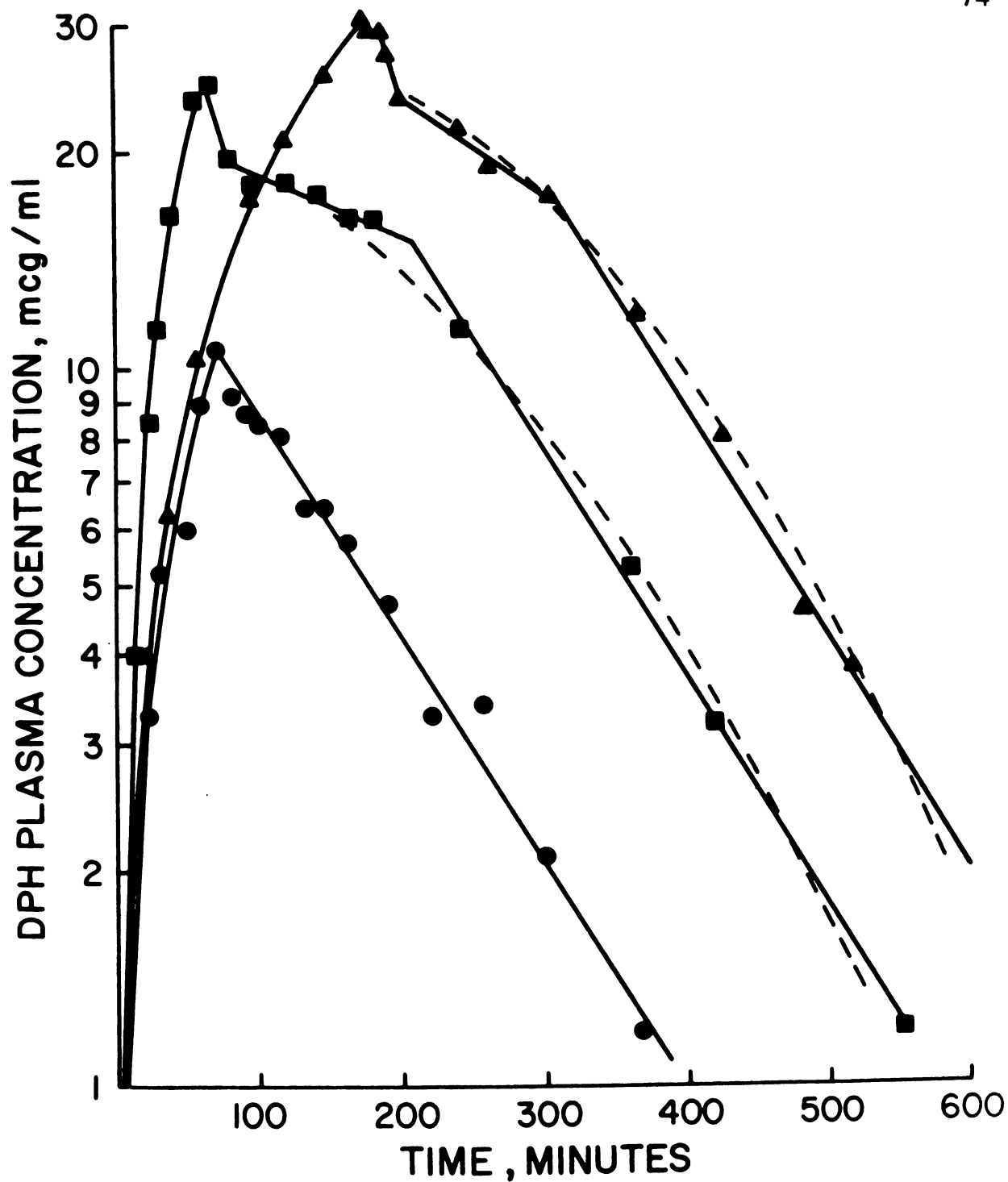
Figure 5. Plasma concentrations of DPH during and after infusion of various doses in dog 6181. Key: ●, 20 mg/kg at 6.25 mg/min.; ■, 35 mg/kg at 12.5 mg/min.; ▲, 50 mg/kg at 6.95 mg/min. Dashed lines represent curve fitted on the presumption of Michaelis-Menten kinetics.

Figure 6. Comparison of theoretical plasma concentration-
time curve and experimental points during infusion of DPH
in dog 6180. Key: ●, 20 mg/kg and ■, 35 mg/kg doses
infused at 6.25 mg/min.; ▲, 50 mg/kg dose infused at
6.95 mg/min.

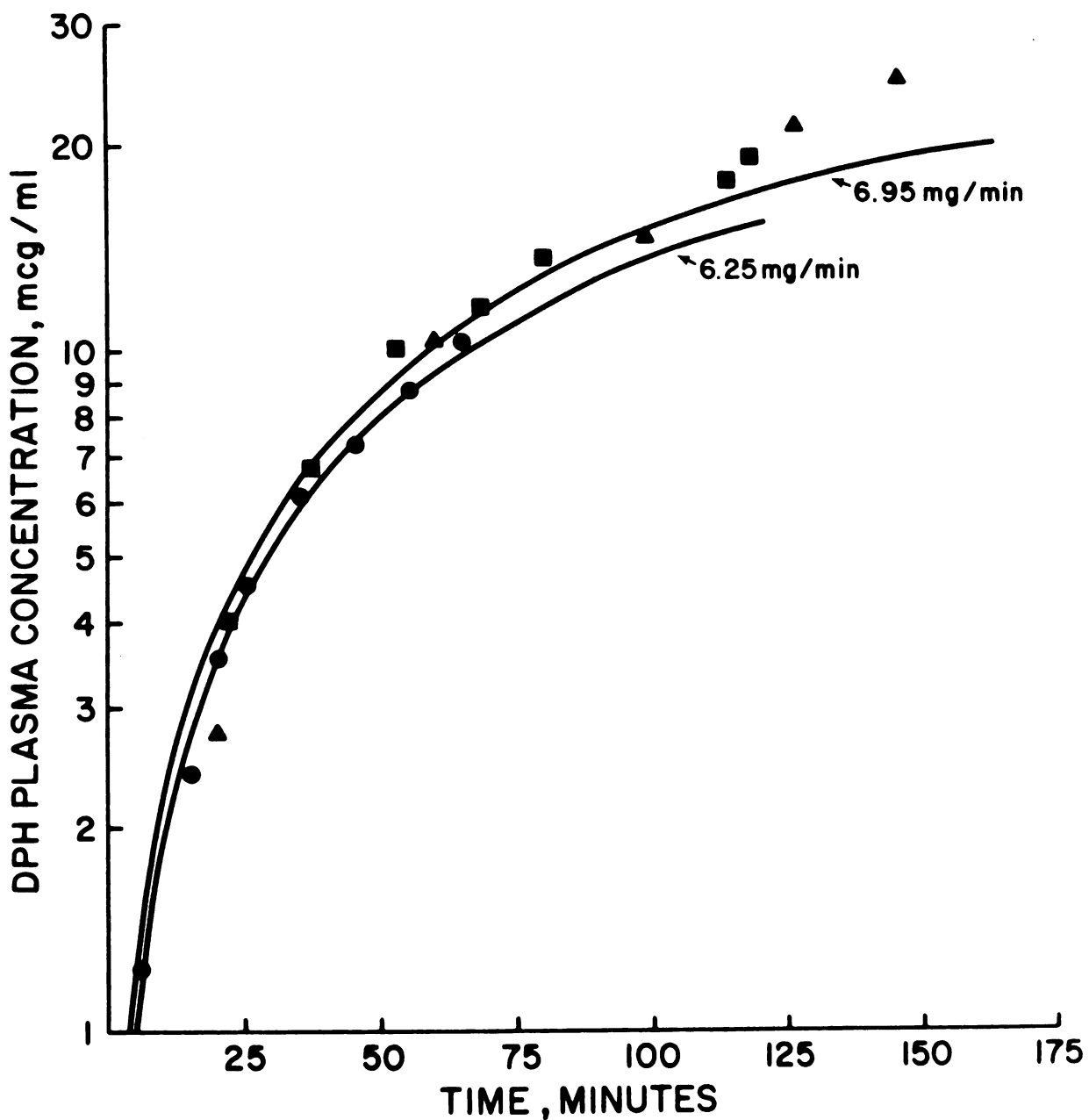Figure 7. Comparison of theoretical plasma concentration-time curve and experimental points during infusion of DPH in dog 6181. Key: ●, 20 mg/kg at 6.25 mg/min.; ▲, 35 mg/kg at 12.5 mg/min.; ■, 50 mg/kg at 6.95 mg/min.

Figure 8. Linear coordinate plot of data shown in Figure 4. Plasma concentrations of DPH during and after various doses in dog 6180. Key: ●, 20 mg/kg at 6.25 mg/min.; ■, 35 mg/kg at 6.25 mg/kg; ▲, 50 mg/kg at 6.95 mg/min.
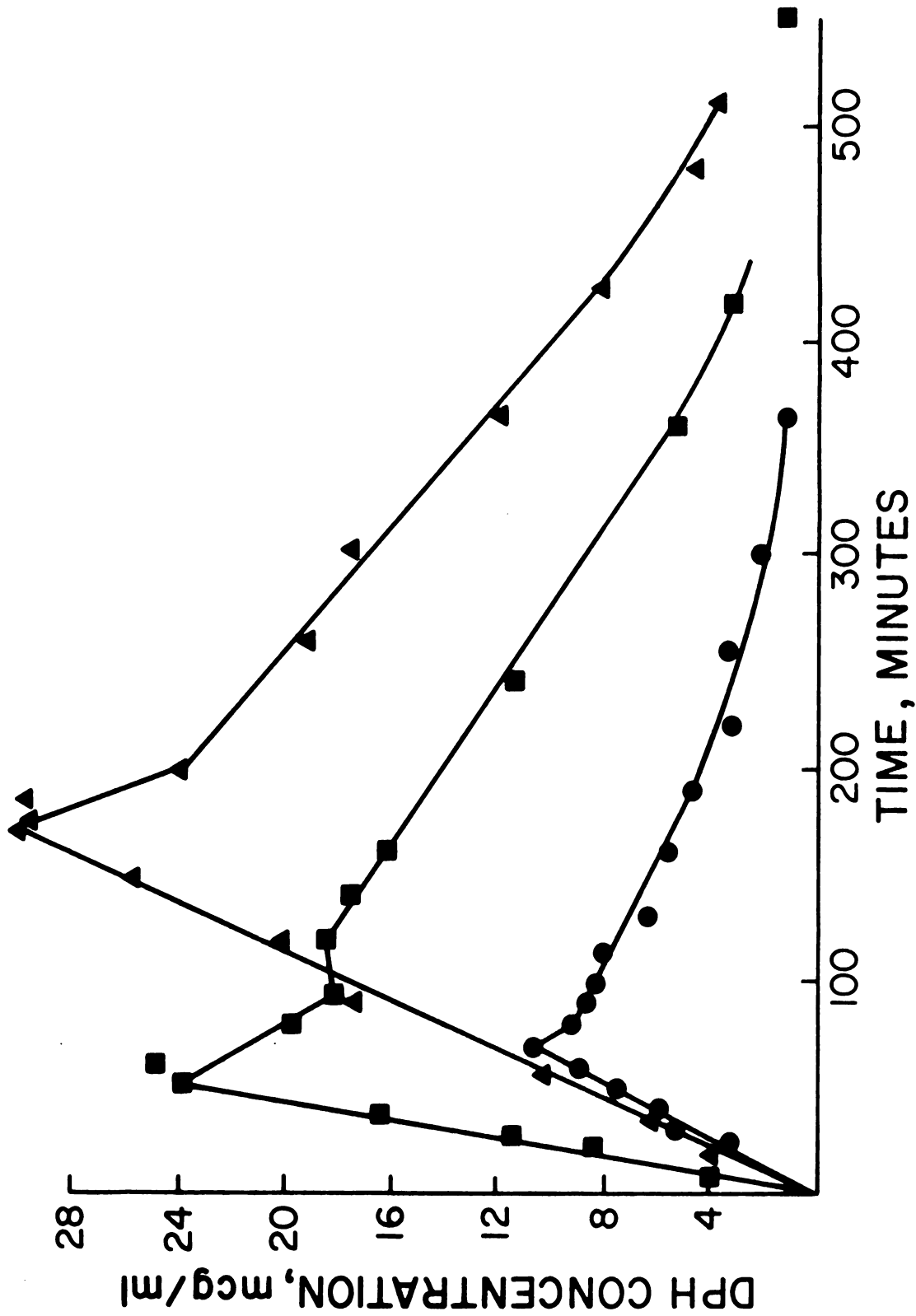
Figure 9. Linear coordinate plot of data shown in Figure 5. Plasma concentrations of DPH during and after various doses in dog 6181. Key: ●, 20 mg/kg at 6.25 mg/min.; ■, 35 mg/kg at 12.5 mg/min.; ▲, 50 mg/kg at 6.95 mg/min.
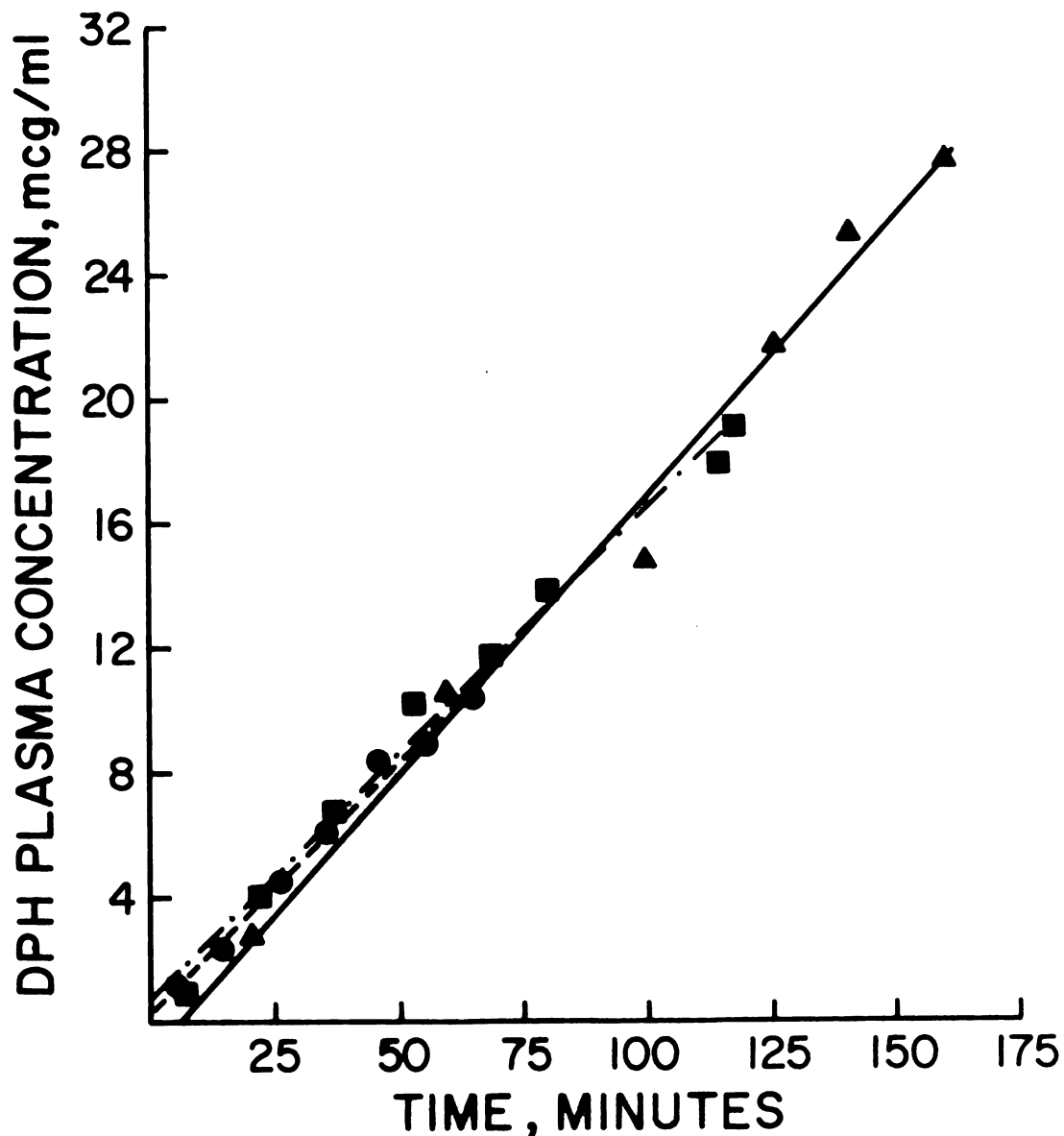
Figure 10. Linear coordinate plot of data shown in Figure 6. Plasma concentration-time curves during infusion of DPH in dog 6180. Key: ●, 20 mg/kg and ■ , 35 mg/kg doses infused at 6.25 mg/min.; ▲ , 50 mg/kg dose infused at 6.95 mg/min. Least square fit of data represented by dashed, dot-dashed, and solid lines for the 20, 35, and 50 mg/kg doses respectively.
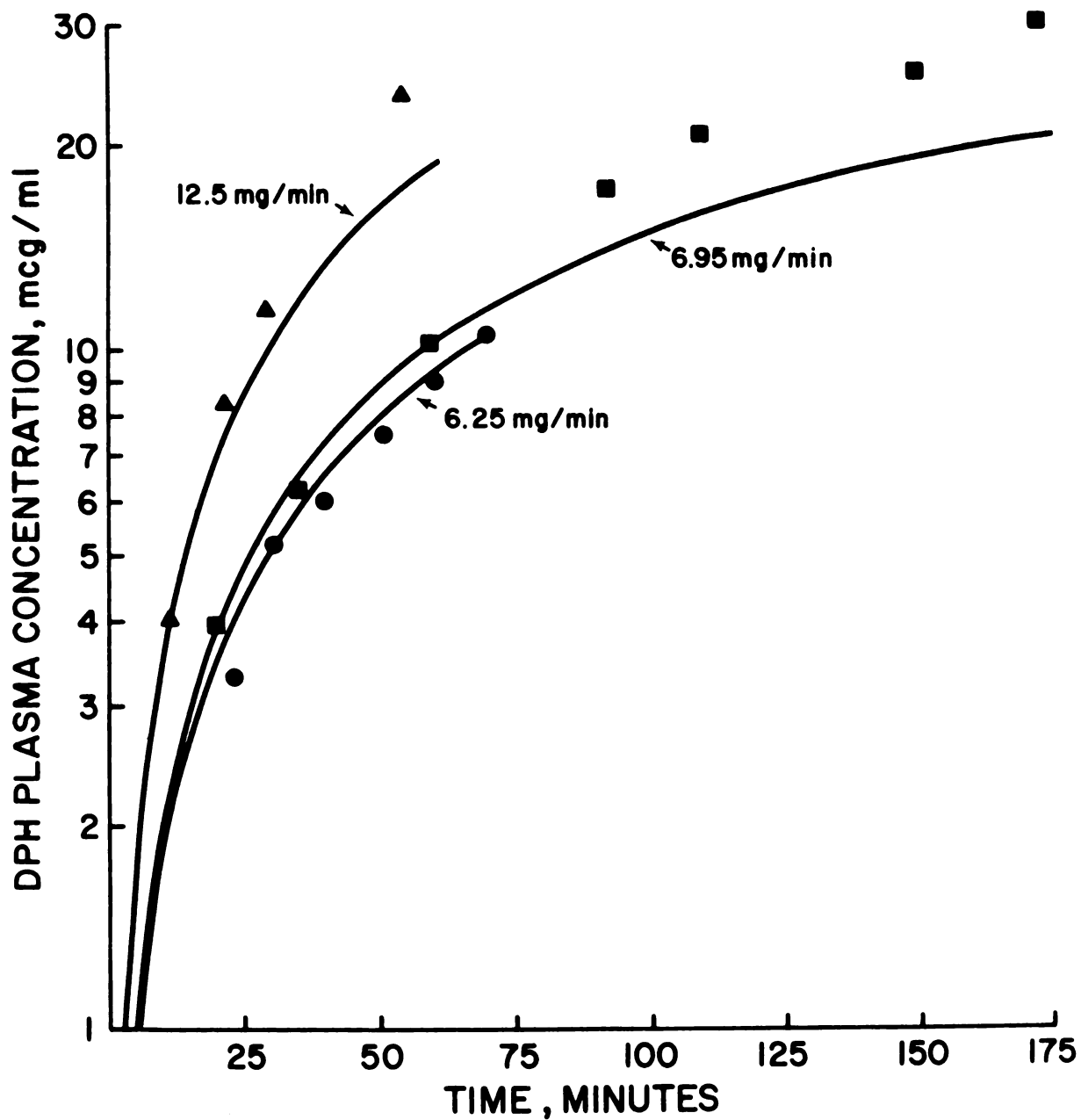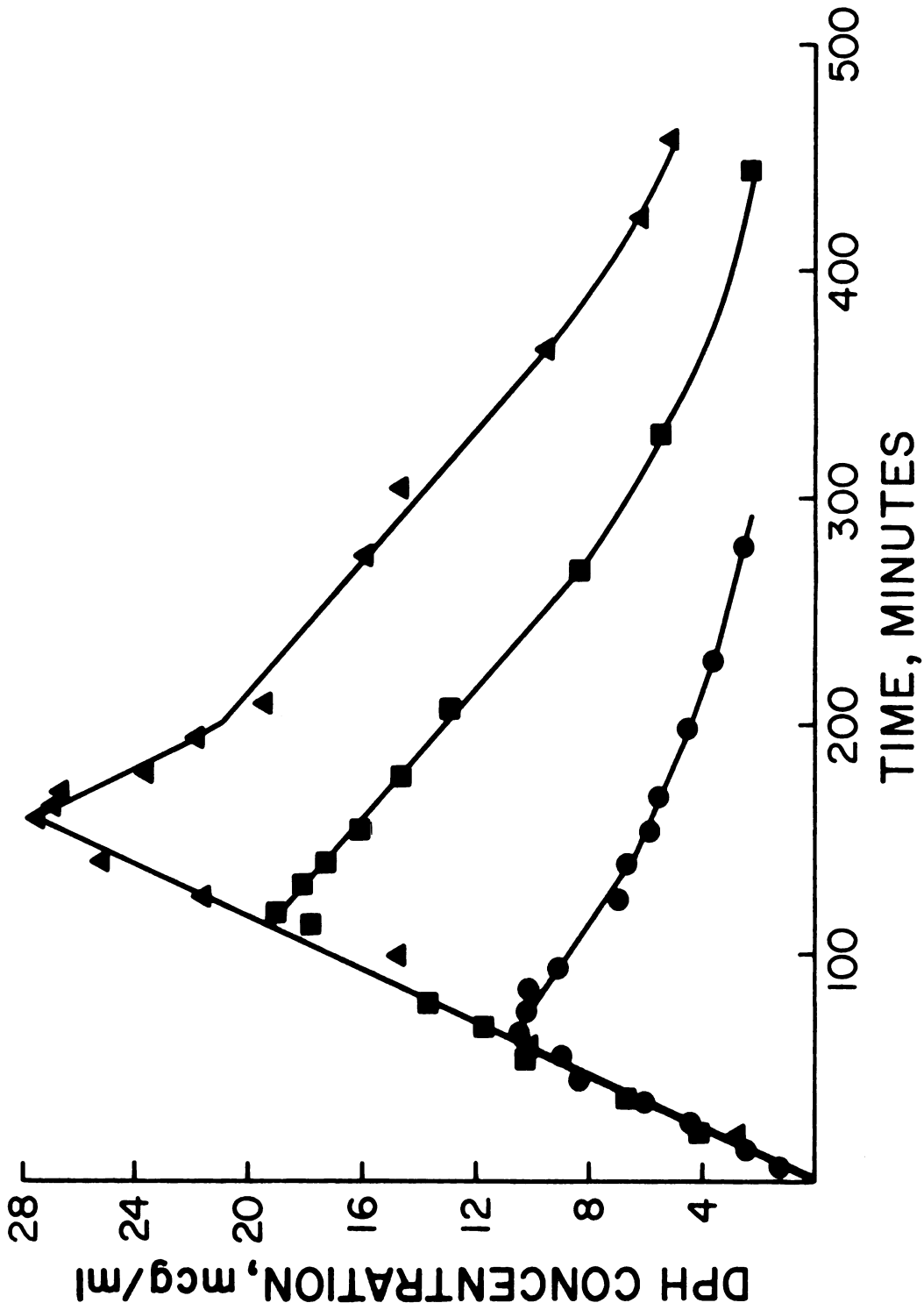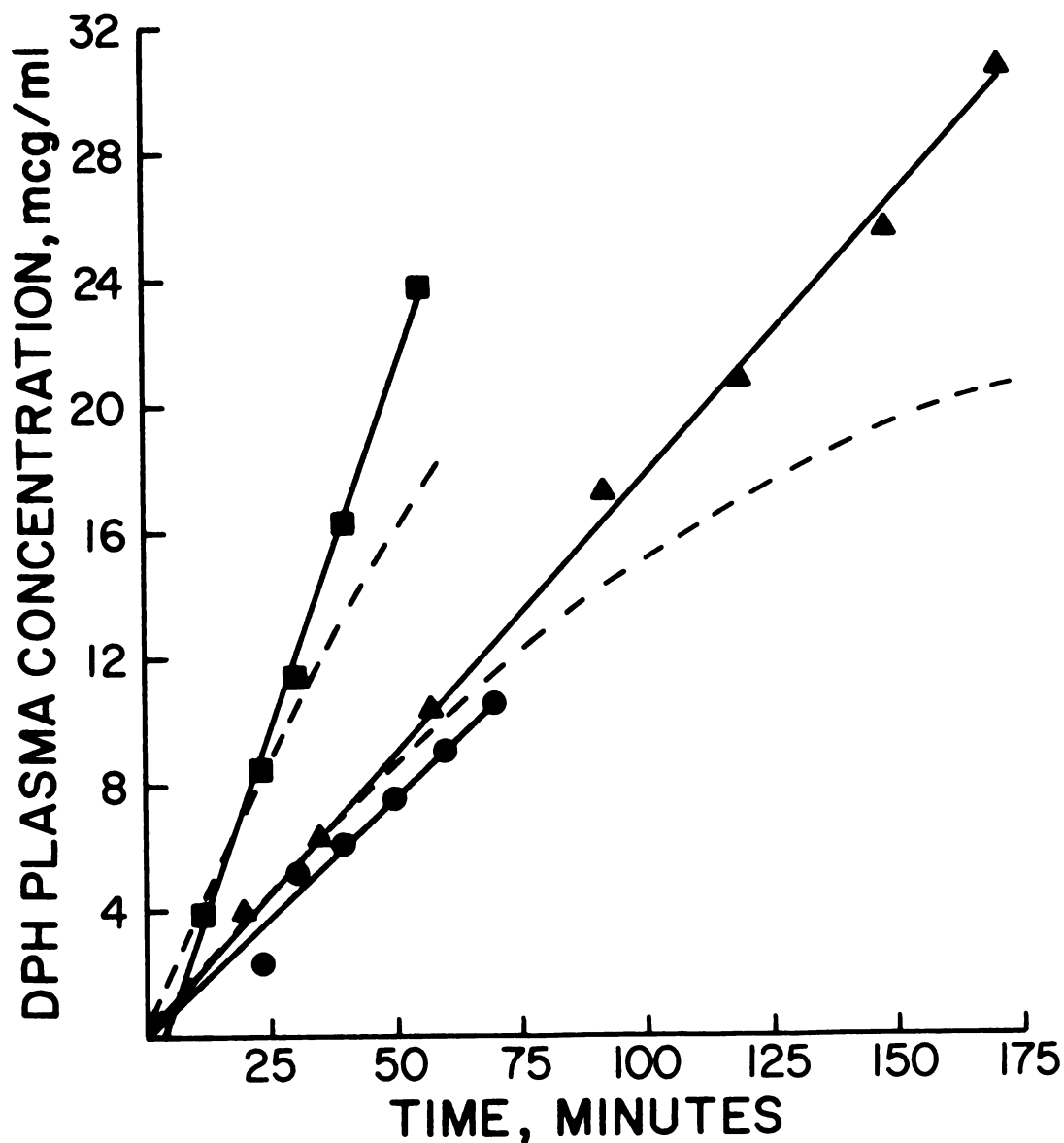
Figure 11. Linear coordinate plot of data shown in Figure 7. Plasma concentration-time curves during infusion of DPH in dog 6181. Key: ●, 20 mg/kg at 6.25 mg/min.; ■, 35 mg/kg at 12.5 mg/min.; ▲, 50 mg/kg at 6.95 mg/min. Theoretical infusion curves for rates of 12.5 and 6.95 mg/min. and first order elimination are shown as upper and lower dashed lines respectively.
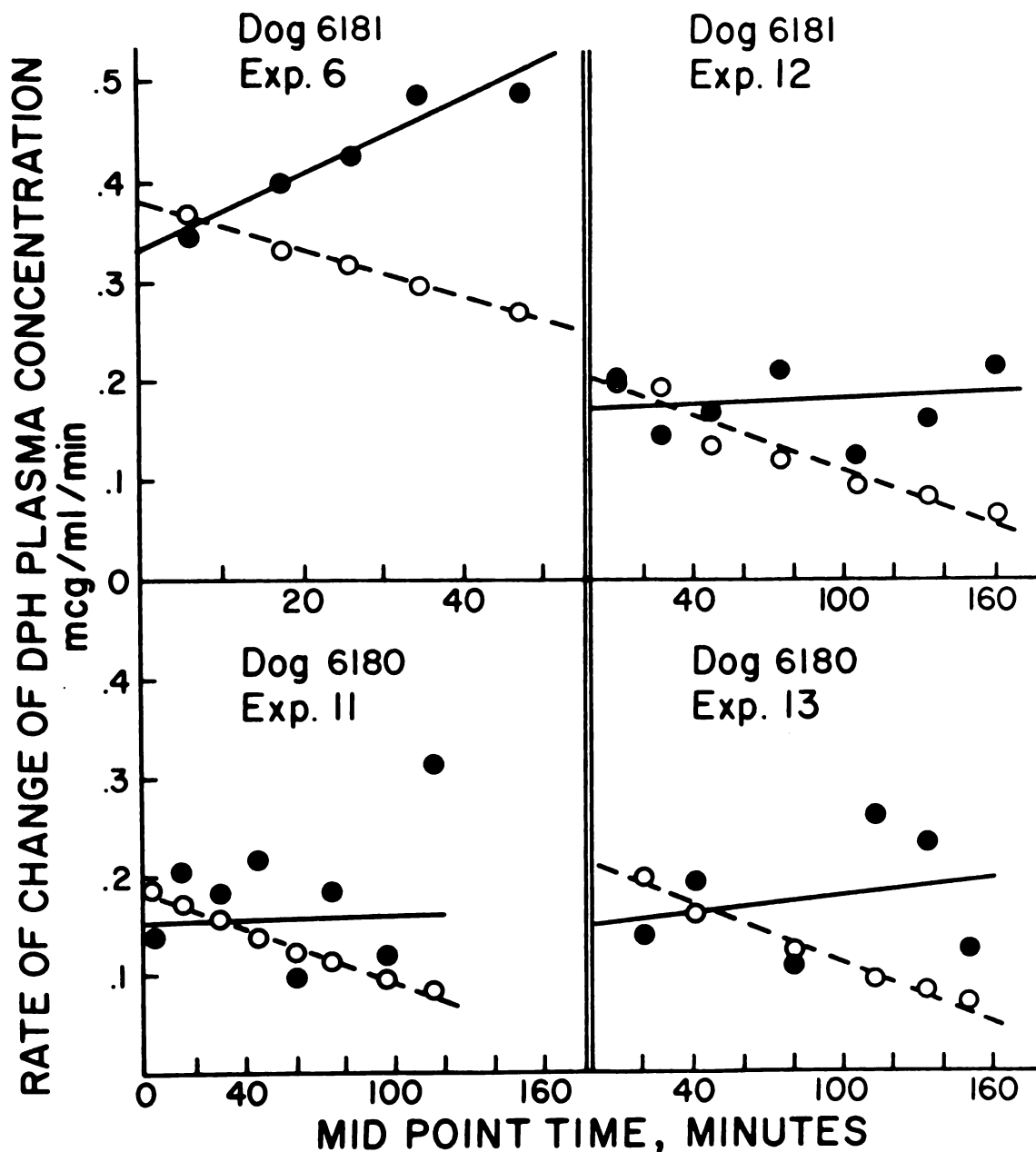
Figure 12. Rate of change of DPH concentration during infusion vs. time. Solid circles and solid line calculated from experimental data. Open circles and dashed lines calculated from theoretical data generated at experimental time points on the presumption of first order elimination (see text).
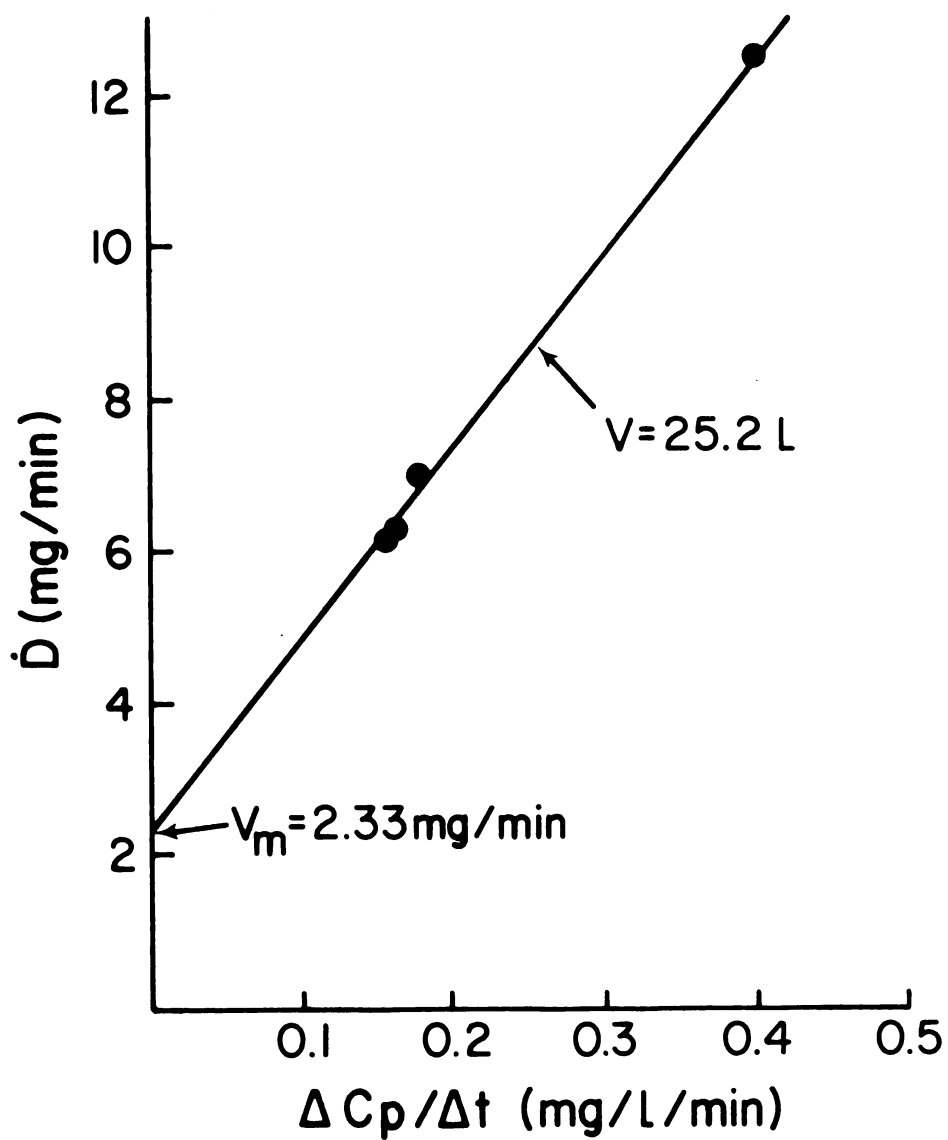
Figure 13. Rate of infusion vs. ascending linear slope for dog 6180 plotted according to Equation 14 (see text and Table III).
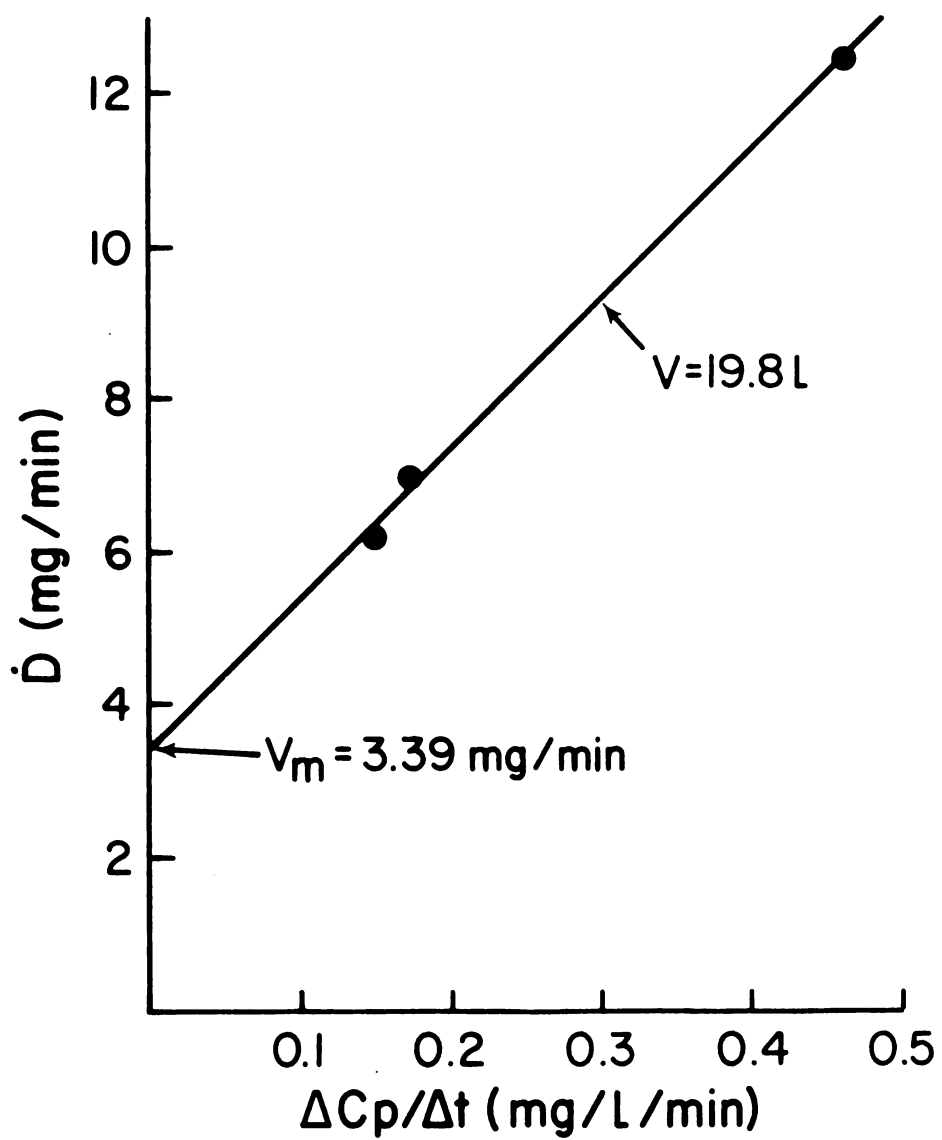
Figure 14. Rate of infusion vs. ascending linear slope for dog 6181 plotted according to Equation 14 (see text and Table III).
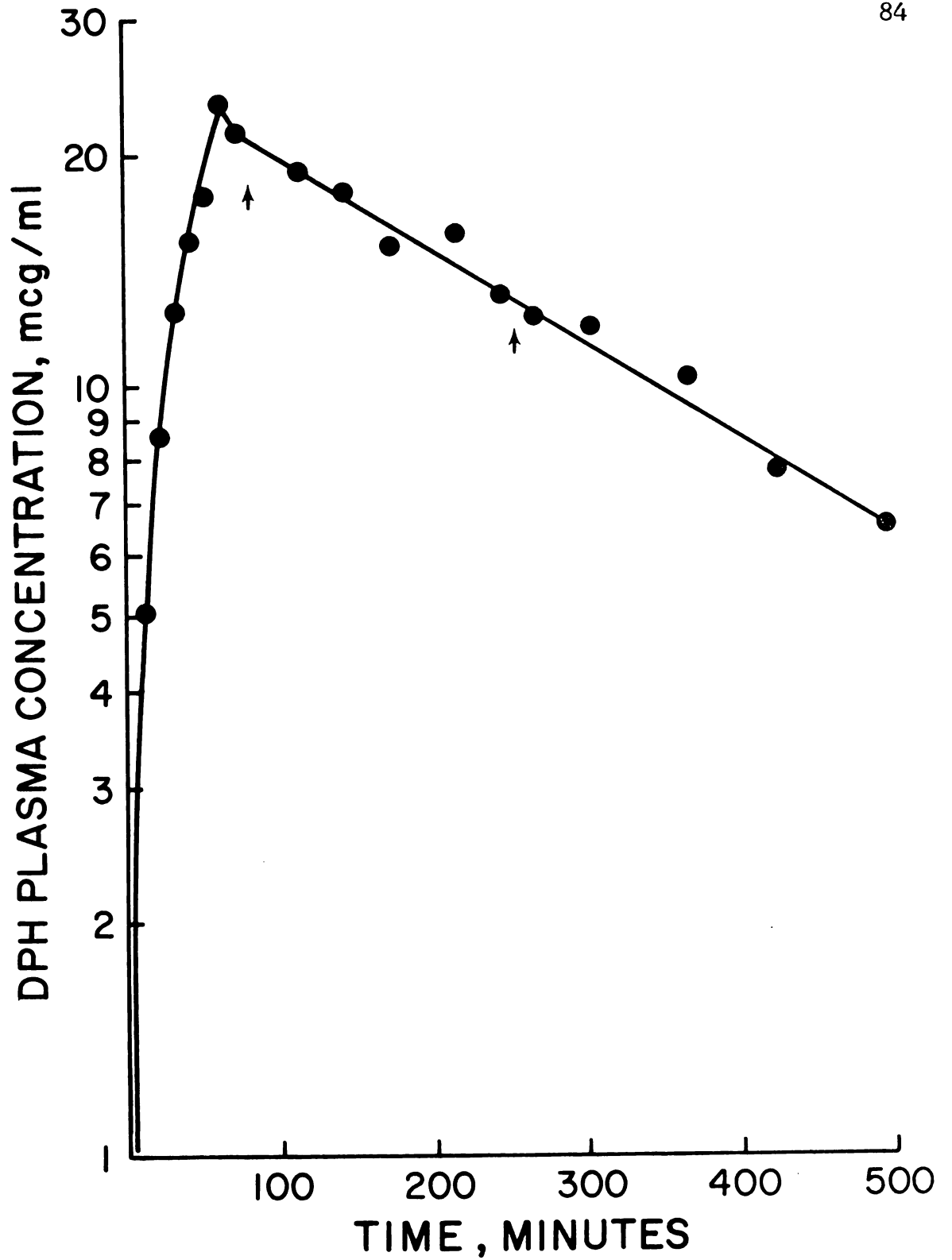
Figure 15. Plasma concentrations of DPH during and after the infusion of a 35 mg/kg dose in dog 11133. Rate of infusion 12.5 mg/min. Arrows indicate time of ICG injection (see text).
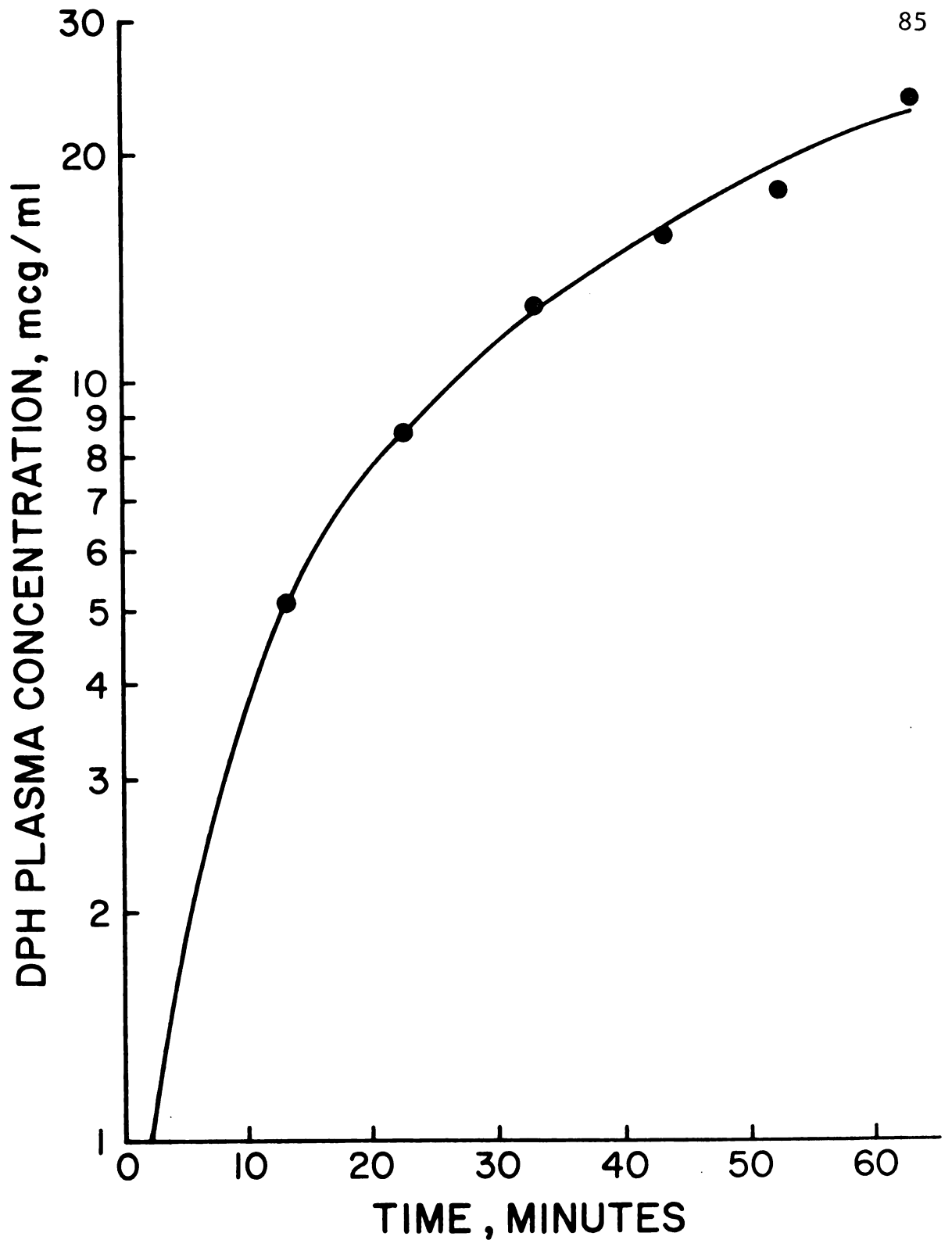
Figure 16. Comparison of theoretical plasma concentration curve based on first order elimination (solid line) and experimental points during infusion of a 35 mg/kg dose of DPH at a rate of 12.5 mg/min. in dog 11133.
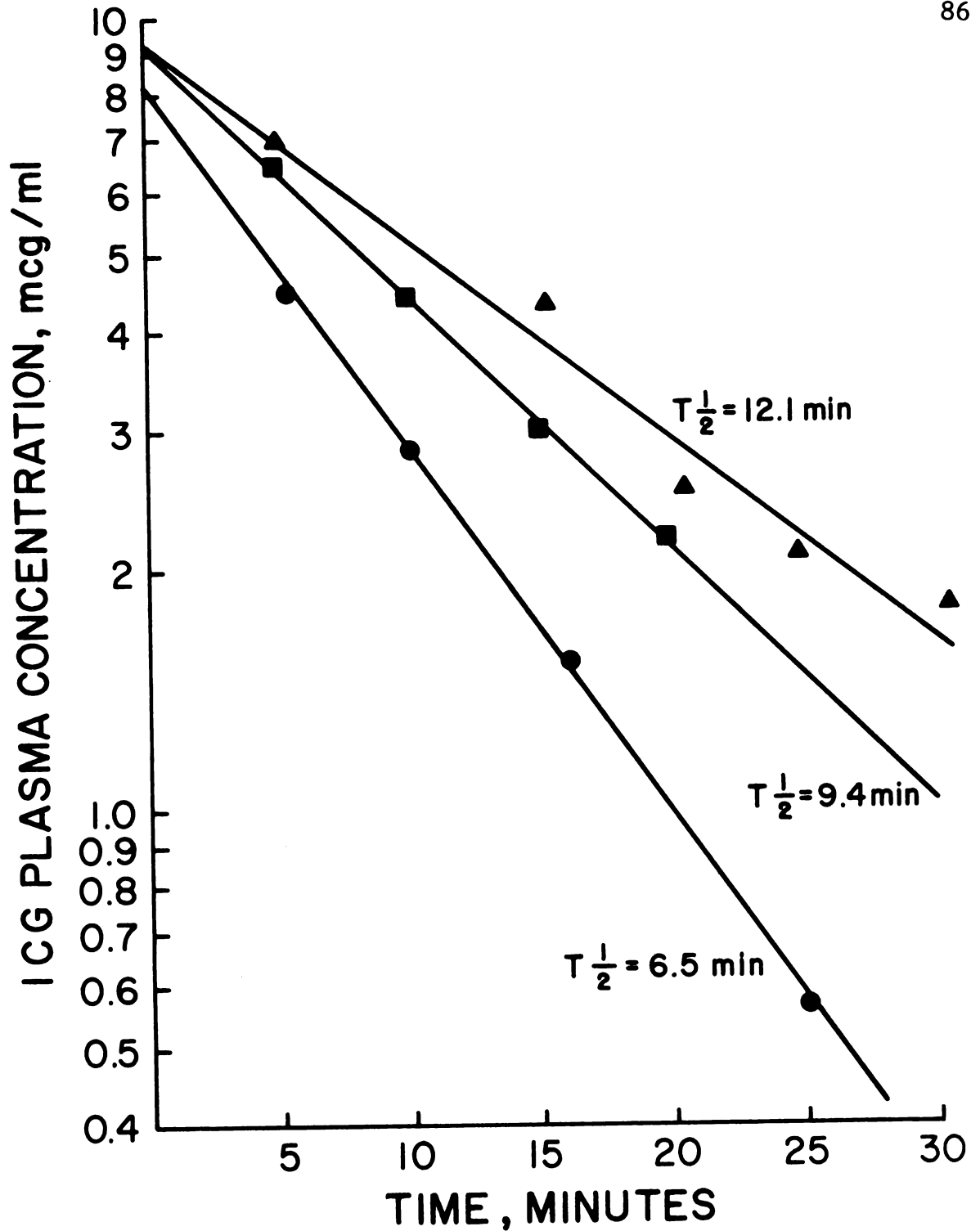
Figure 17. Half-life of ICG in dog 11133 at various times.
Key: ●, prior to DPH infusion; ▲, 75 min. and ■, 260 min. after start of infusion.
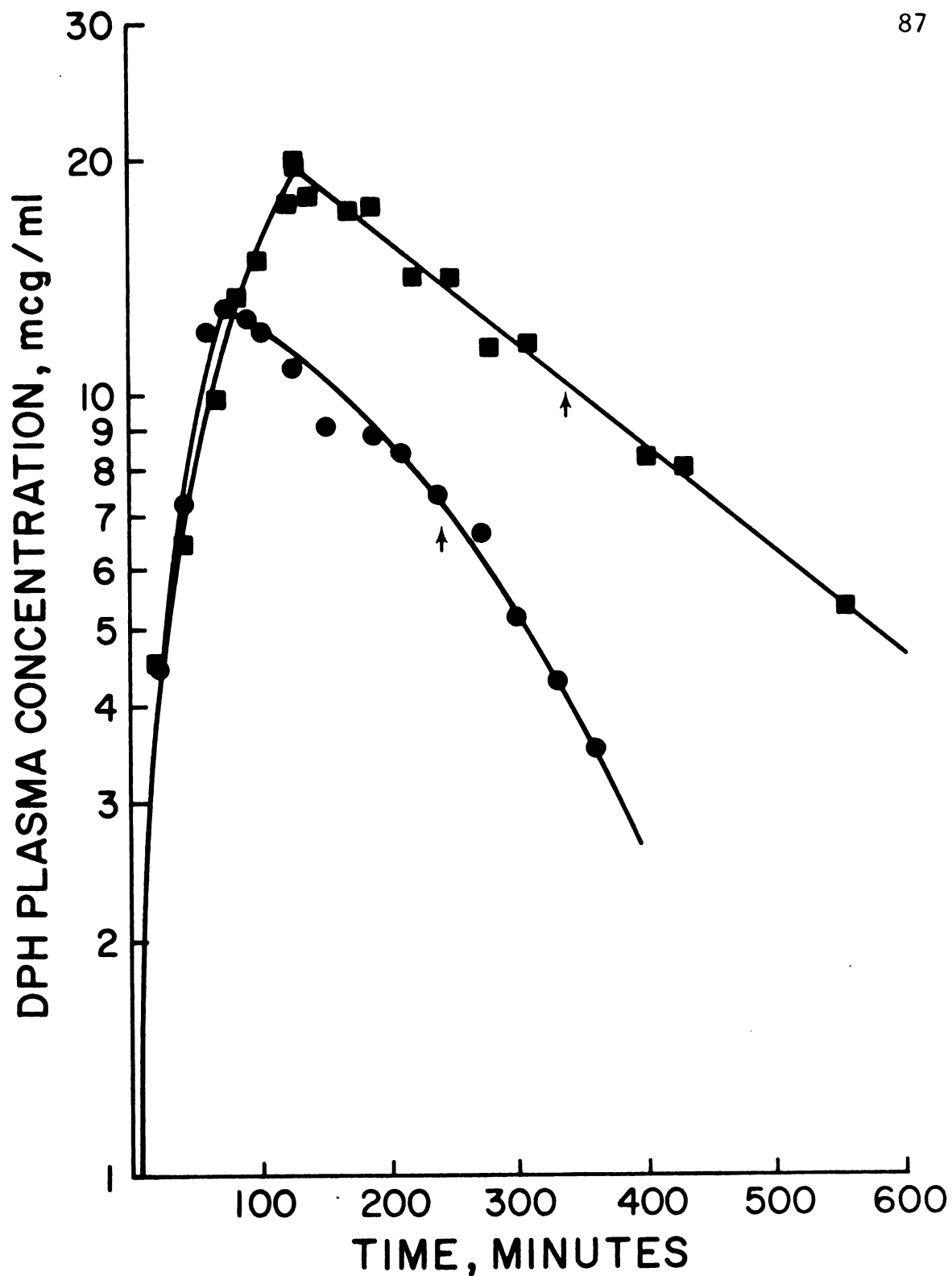
Figure 18. Plasma concentrations of DPH during and after infusion with concomitant sodium taurocholate in dog 6180. Arrow marks end of sodium taurocholate infusion. Key: ●, 20 mg/kg, and ■, 35 mg/kg infused at 6.25 mg/min.
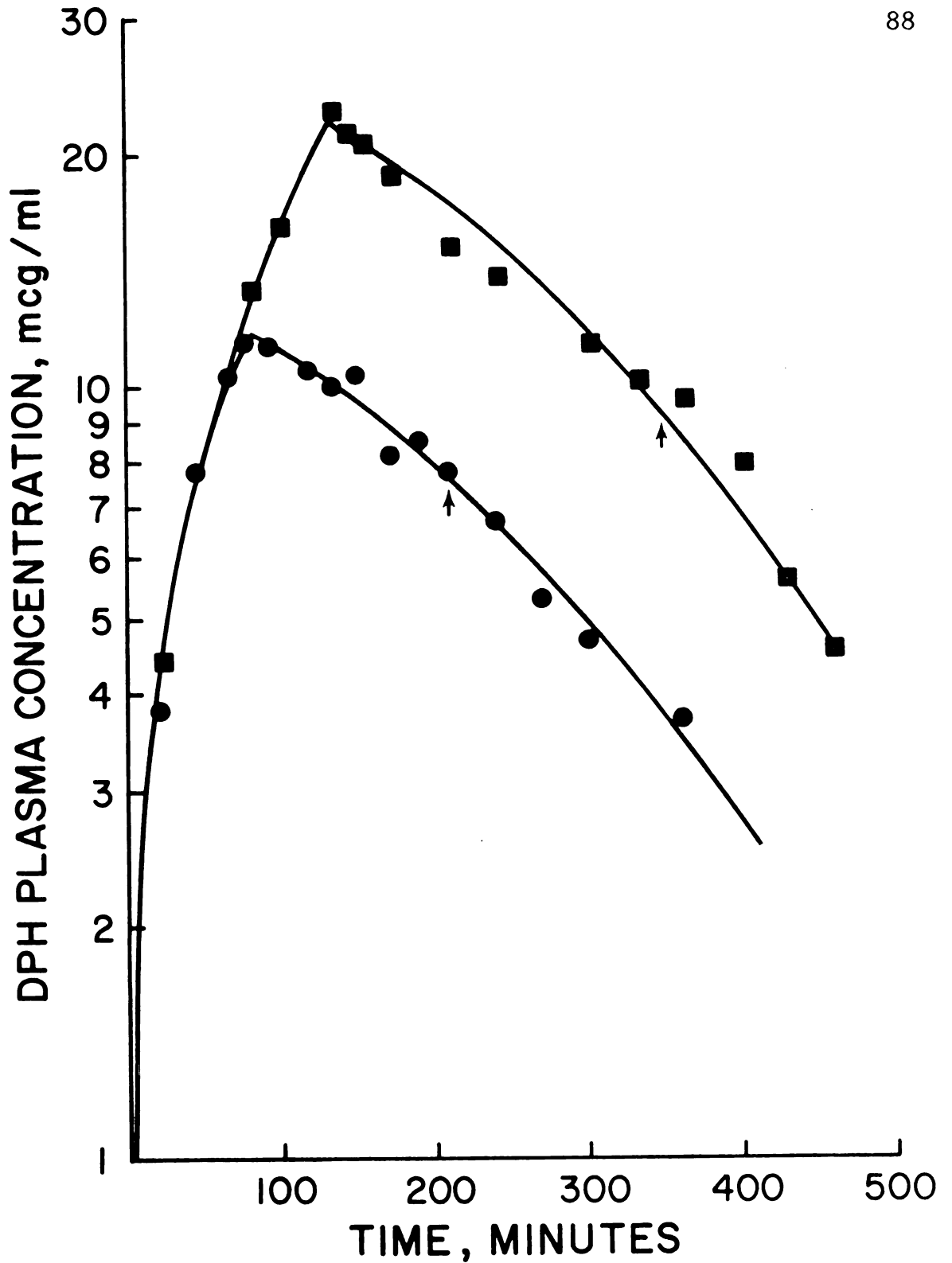
Figure 19. Plasma concentrations of DPH during and after infusion with concomitant sodium taurocholate in dog 6181. Arrow marks end of sodium taurocholate infusion. Key: ●, 20 mg/kg and ■, 35 mg/kg infused at 6.25 mg/min.
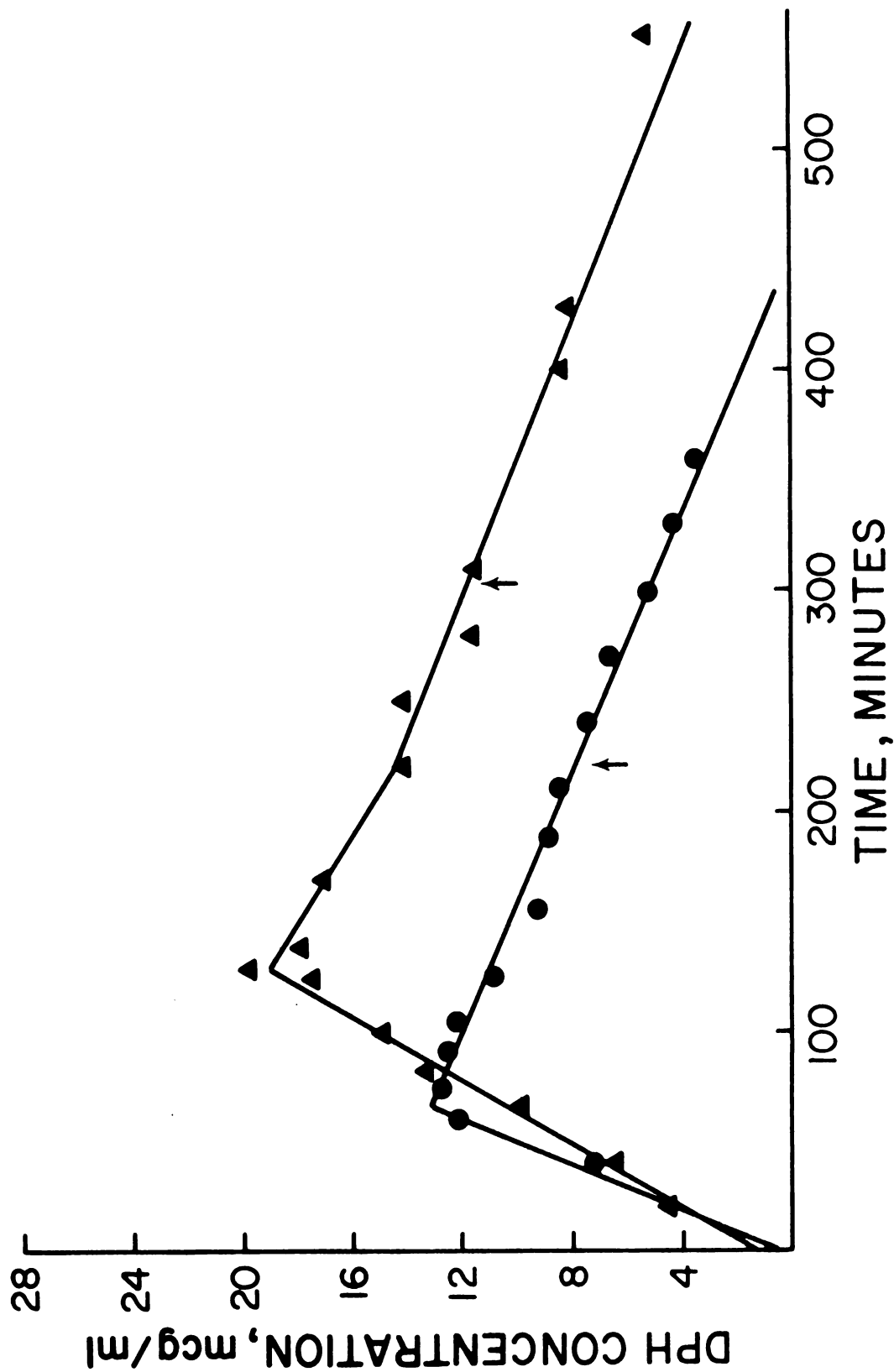
Figure 20. Linear coordinate plot of data shown in Figure 18. Plasma concentrations of DPH during and after infusion with concomitant sodium taurocholate in dog 6180. Arrow marks end of sodium taurocholate infusion. Key: ●, 20 mg/kg and ▲, 35 mg/kg infused at 6.25 mg/min.
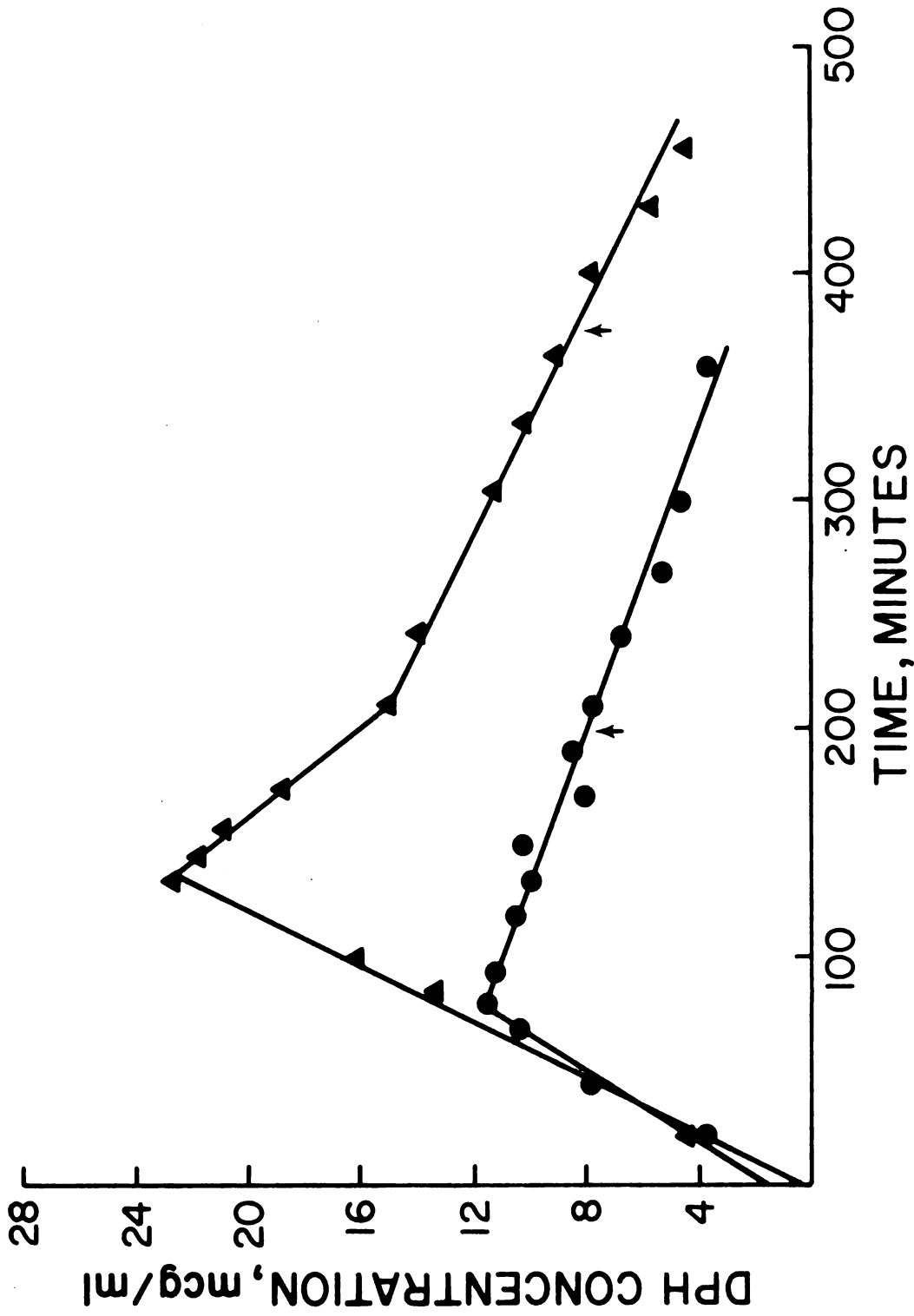
Figure 21. Linear coordinate plot of data shown in Figure 19. Plasma concentrations of DPH during and after infusion with concomitant sodium taurocholate in dog 6181. Arrow marks end of sodium taurocholate infusion. Key: ●, 20 mg/kg and ▲, 35 mg/kg infused at 6.25 mg/min.
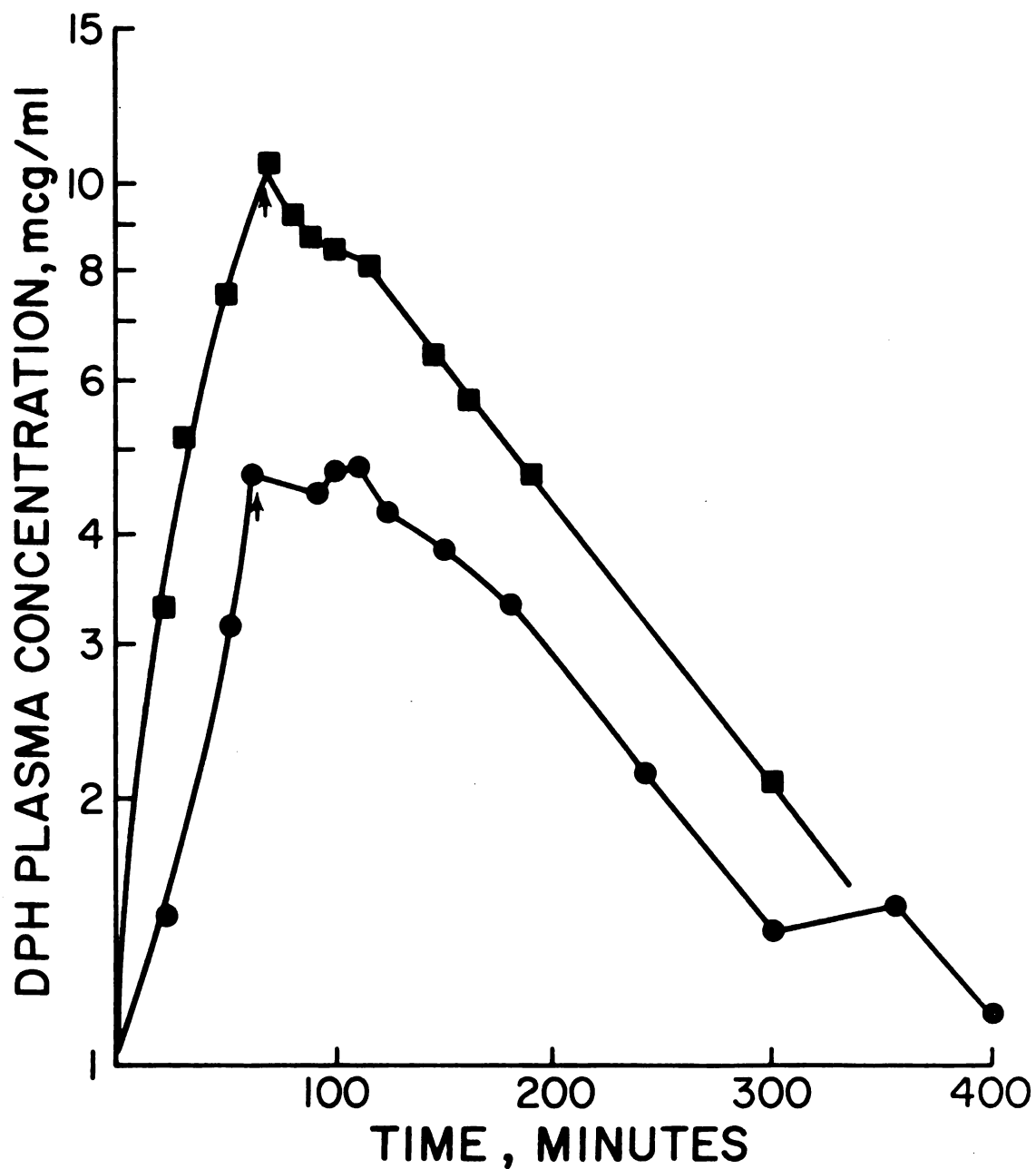
Figure 22. Infusion of DPH at different sites in dog 6180. Rate of infusion 6.25 mg/min. Arrow marks the end of infusion. Key: ●, peripheral infusion with dextrose dilution; ■, vena cava infusion.
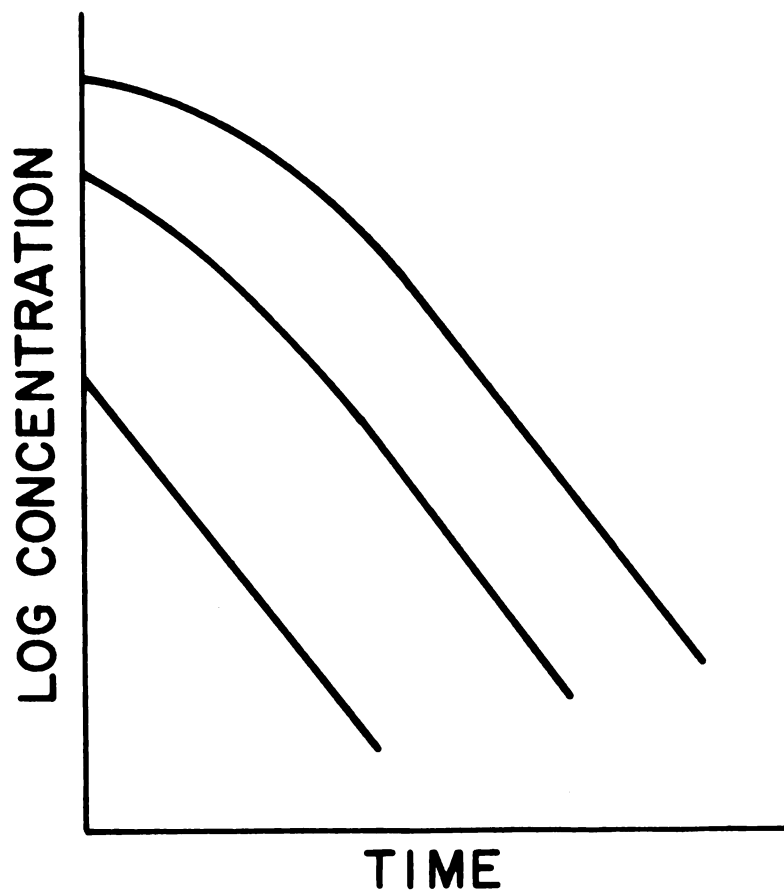
Figure 23. Drug plasma concentration-time curves after IV injection produced by metabolic saturation as described by simple Michaelis-Menten kinetics.
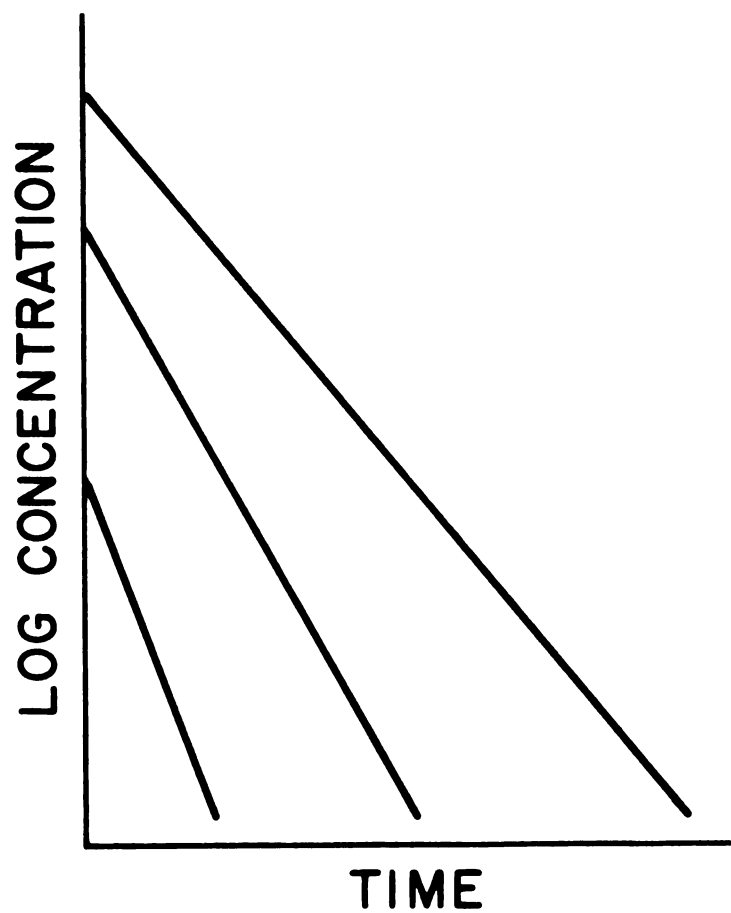
Figure 24. Drug plasma concentration-time curves after
IV injection produced by metabolic saturation and end-
product inhibition according to Equation 19 and conditions
described in text.
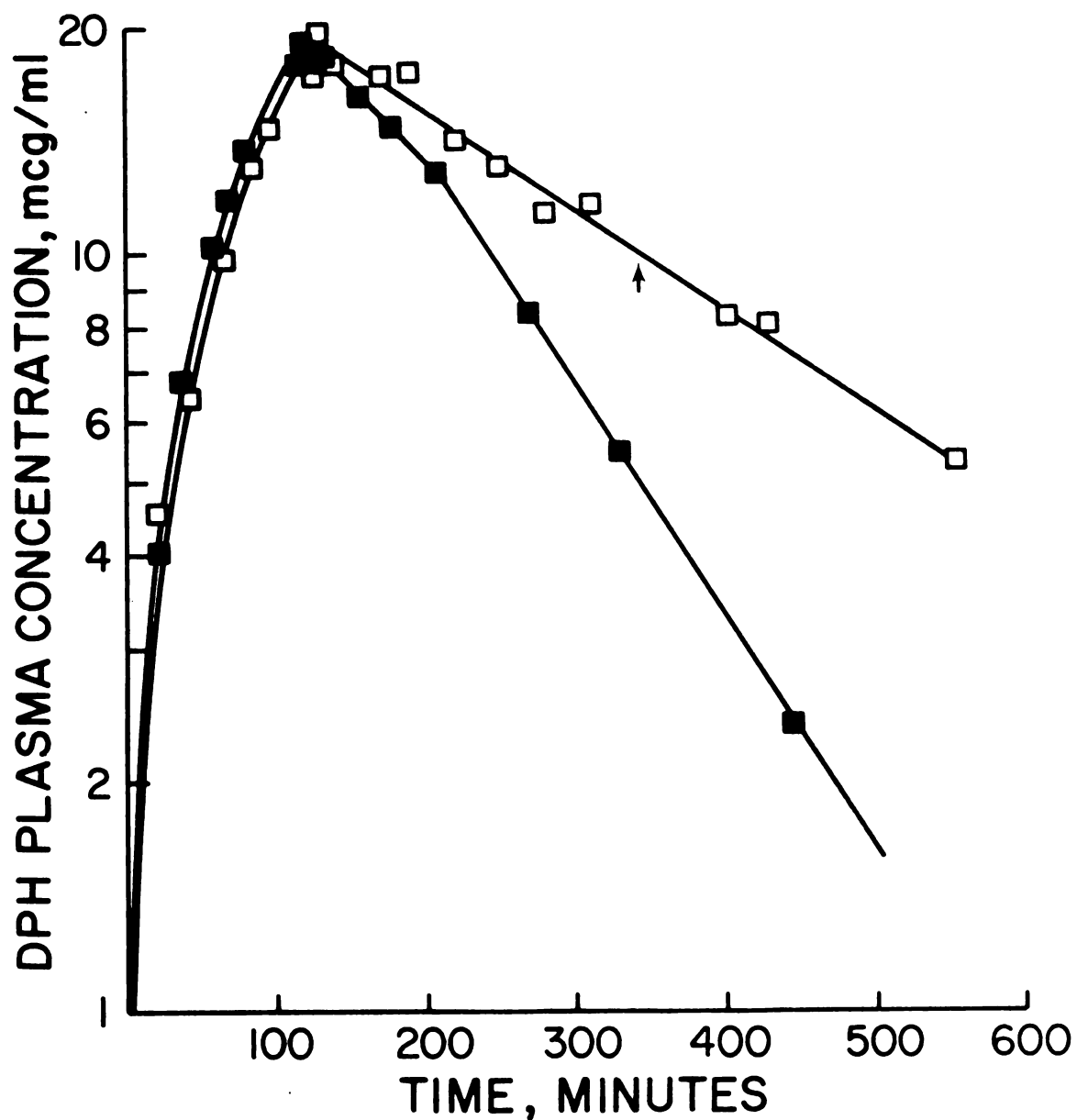
Figure 25. Plasma concentrations of DPH during and after infusion of 35 mg/kg at a rate of 6.25 mg/min. in dog 6180. Key: ■, without sodium taurocholate infusion; □, with sodium taurocholate. Arrow marks end of taurocholate infusion.
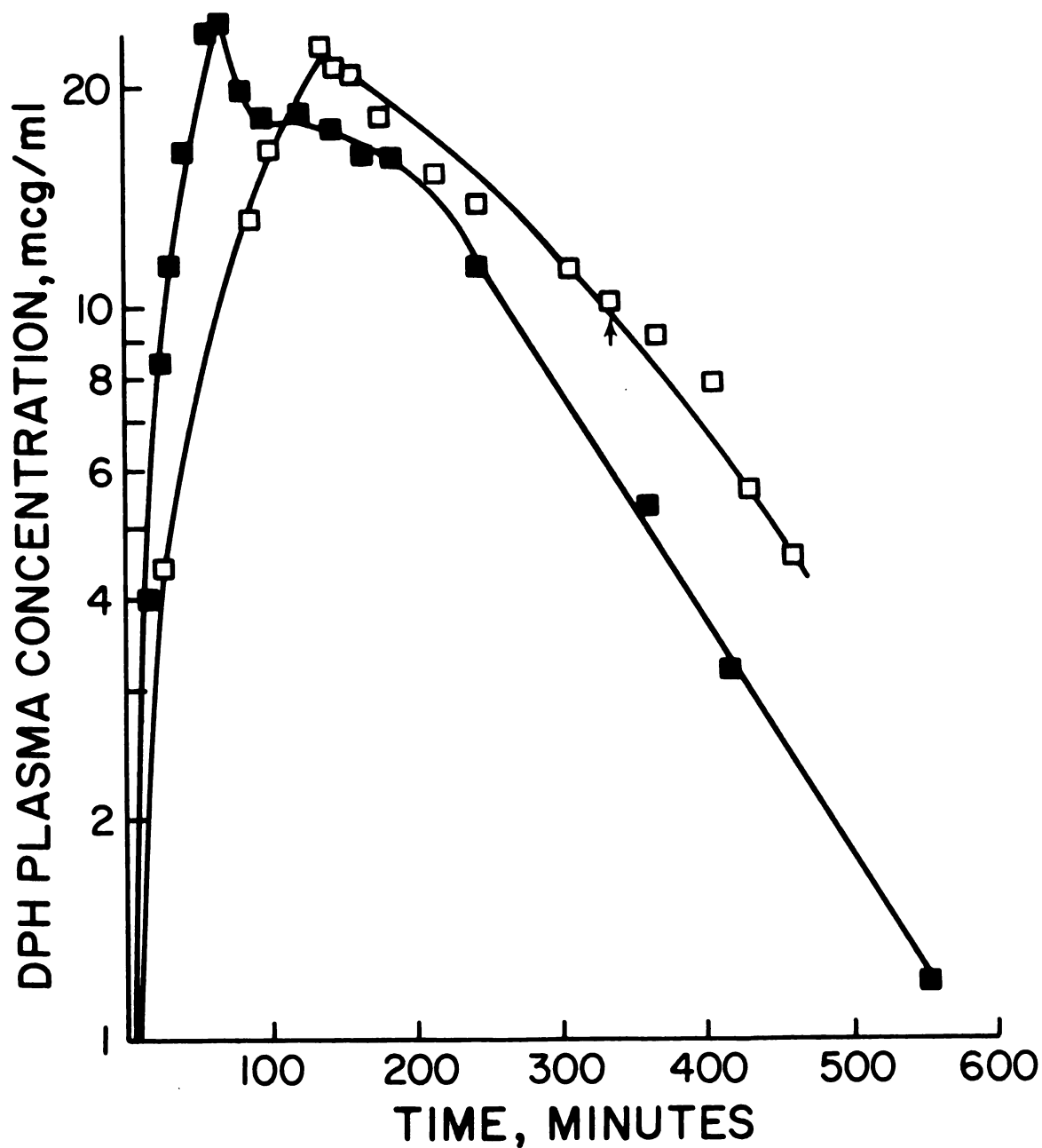
Figure 26. Plasma concentrations of DPH during and after
infusion of 35 mg/kg in dog 6180. Key ■, DPH rate
12.5 mg/min. without sodium taurocholate □, DPH rate
6.25 mg/min. with concomitant sodium taurocholate. Arrow
marks end of taurocholate infusion.
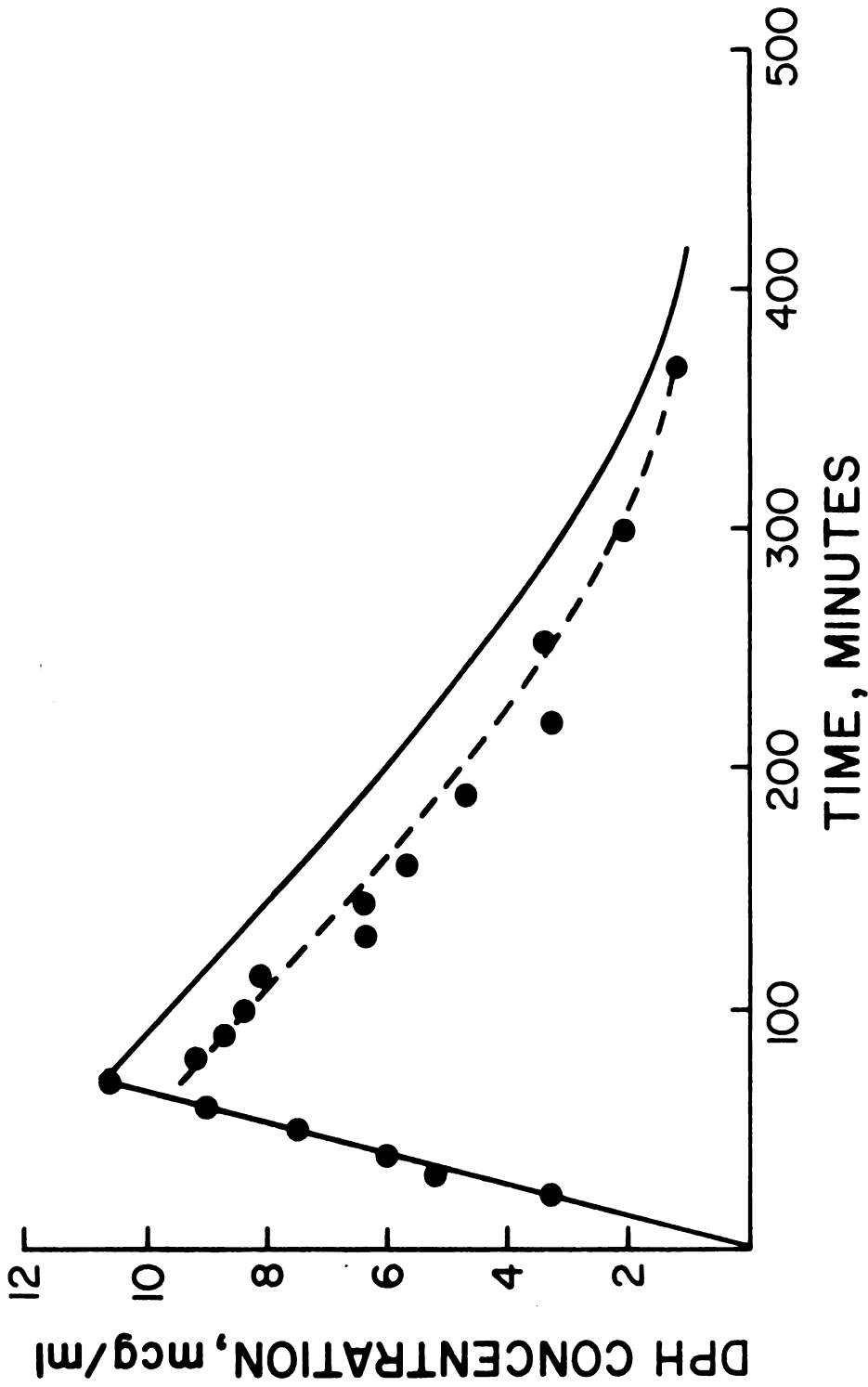
Figure 27. Comparison of experimental data (exp. 8) and theoretical Michaelis-Menten curves generated during and after infusion with D = 6.25 mg/min., V_m = 3.43 mg/min., K_m = 80 mg, and V = 20 L. Key: ●, experimental data points, solid curve generated for total experiment, and dashed curve generated for post-infusion period when C^r = 9.44 mcg/ml. (see text).
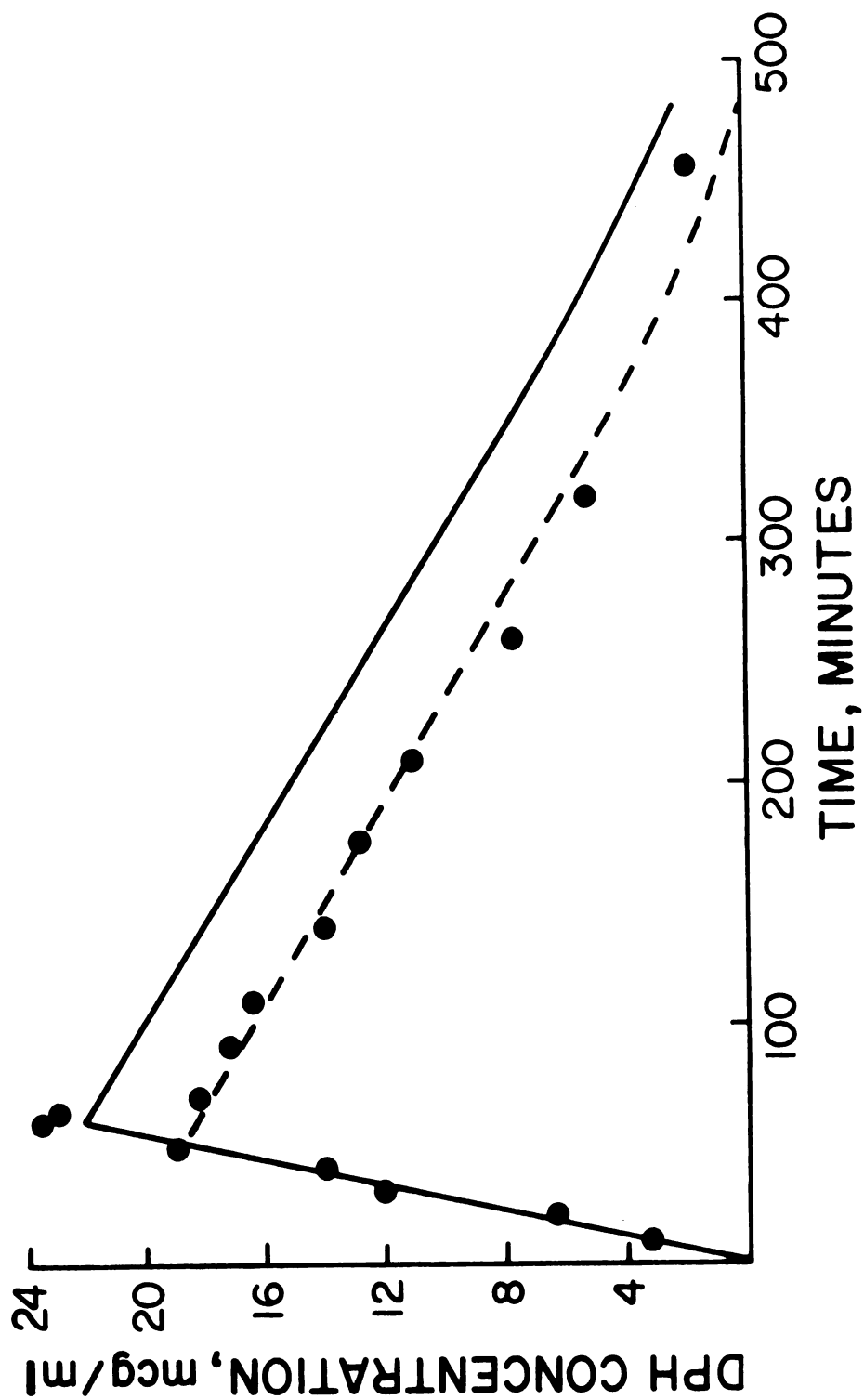
Figure 28. Comparison of experimental data (exp. 7) and theoretical Michaelis-Menten curves generated during and after infusion ,with V_m = 3.0 mg/min., K_m = 12.5 mg, and V = 25 L. Key: ●, experimental data points, solid curve generated for total experiment, and dashed curve generated for post-infusion period when C^r = 18.2 mcg/ml. (see text).

CHAPTER IV

STUDIES OF DOSE DEPENDENT KINETICS OF DPH IN MAN

Dose dependent kinetics of DPH in man was first investi-
gated by Arnold and Gerber (38) in 1970. In this study they
reported dose dependent elimination in 10 normal volunteers
who had received different total doses of DPH during a three
day loading period in two or three experiments. At the
higher doses, the log plasma concentration versus time plots
showed convex descending curvature with resultant increase
in the apparent half-life. The authors have suggested
saturation, autoinduction, or product inhibition of the
drug-metabolizing enzyme system as the possible mechanism
in the dose dependency and nonexponential decline of DPH
plasma concentration curves. Subsequently, Gerber and
Wagner (49) fit the dose dependent data from one subject
of the Arnold and Gerber study to the integrated form of
the Michaelis-Menten equation, thus implying an enzymatic
saturation mechanism for the observed DPH elimination.

Although Arnold and Gerber gave sodium DPH in their
investigation, the free acid could be precipitated in the
gut and be absorbed slowly along much of the intestine.
The prolonged absorption can lead to an overestimate of the
elimination half-life (86), and with increasing dose the
results could be interpreted as dose-dependent kinetics.
The objective of the present investigation was to study the

elimination kinetics of DPH after intravenous administration of the drug at various doses, in order to establish whether or not the earlier report of dose dependent kinetics was an artifact of prolonged absorption and to lead to a better understanding of DPH disposition in humans.

EXPERIMENTAL

<u>Subjects</u> - The subjects were four male volunteers between the ages of 25 to 30 years and in good health as determined by medical history, physical examination, and routine blood and urine analysis.

<u>Methods of DPH Administration</u> - An oral DPH dose of four 100 mg capsules[1] was given to the fasting subjects two weeks prior to the first intravenous study to establish whether any hypersensitive response or unusual metabolic disorder was present. It also served to determine the elimination half-life in these subjects after an oral dose. Blood samples (5 ml) were collected from the antecubital vein for the oral study at approximately 1, 2, 3, 5, 7, 9, 24, 30, 33, and 48 hours after ingestion.

For the intravenous studies, DPH was given as a commercial solution[2] (50 mg/ml) at a rate of 20 mg/min. For the 3 mg/kg study in two subjects (R.R. and J.O.), the DPH solution was infused into the antecubital vein directly from a Harvard infusion pump. Since the subjects complained of the intense burning pain caused by the very alkaline (pH 12) solution, subsequent doses were infused from the Harvard pump to the flow line of a 5% dextrose (saline in one experiment) intravenous drip set running into the subject

[1]Dilantin Kapsules[R], Parke-Davis & Co.
[2]Dilantin Steri-Vial, Parke-Davis & Co.

at rates shown in Table I. Virtually all of the pain disappeared. The dextrose drip system was tested in vitro and no precipitation was observed.

During infusion the subjects were continuously monitored by EKG. Aside from a slight increase in pulse rate, no abnormal physiological events were observed during or immediately following infusion. The two subjects undergoing direct infusion of the undiluted commercial solution experienced local pain, vasovagal weakness, and one also experienced nausea. These symptoms were not apparent with IV dextrose drip. After the 9 mg/kg dose, all subjects experienced dizziness and slight ataxia for about 3 hours post infusion.

Approximately twenty, 6 ml samples of heparinized blood were drawn the day of infusion, and four samples were obtained daily up to four days after the dose. As many as five individual blood samples were drawn at various times during the day prior to DPH infusion to verify the consistency of the assay procedure including the GLC response.

DPH Analysis - The concentration of DPH in plasma was determined by the GLC method described in Chapter II.

Calculations - The half-life of DPH was determined by a log-linear least square fit of the terminal portion of the plasma concentration time curve from the 24-hour sample to the last concentration value exceeding 1.2 mcg/ml.

The average clearance of DPH was calculated from the following equation:

$$\bar{V}cl = \frac{Dose}{AUC} \qquad \text{(Equation 1)}$$

where $\bar{V}cl$ is the average clearance and AUC is the area under
the plasma concentration-time curve from t = 0 to t = ∞
which is evaluated by the trapezoidal rule method.

The volume of distribution was calculated by the
clearance method:

$$(Vd)\ area = \frac{\bar{V}cl}{K} \qquad \text{(Equation 2)}$$

where (Vd) area is the volume of distribution and K is the
first order elimination rate constant.

RESULTS

The plasma concentration-time curves for the four
subjects at various doses are shown in Figures 1-4. The
experimental and estimated parameter values obtained for
these experiments are summarized in Tables II-V. Figures 5-6
show the expanded early period post infusion of the subjects.
The striking observation is seen at the end of infusion,
namely, the plasma concentration did not drop as would be
expected, but instead it rose or remained steady for several
hours before declining. This phenomenon was apparent after
intravenous administered to all the human subjects, whether
a direct infusion or dilution with an IV drip.

Two subjects showed relatively unaltered elimination
of DPH after the three intravenous doses, as indicated by a
consistency of their average plasma clearance values shown
in Tables II and III. The clearances ranged from 18.7 to
22.0 ml/min. for M.M. and 18.2 to 20.9 ml/min. for J.O. Also
there was no appreciable change in the terminal half-lives
(18.1 to 22.8 hrs. for M.M. and 28.1 to 32.3 hrs. for J.O.).

The other two subjects, W.M. and R.R., showed a decreasing
average clearance with increasing dose as reported in Tables
IV and V. In subject R.R., the average plasma clearance
values were estimated to be 42.9, 32.0, and 29.6 ml/min.
after the 3, 6, and 9 mg/kg doses respectively. In this
subject the plasma concentration-time curves of DPH appeared
to decay exponentially after the decline began for all doses.

The terminal half-lives were 9.0, 13.6, and 13.4 hours
after the 3, 6, and 9 mg/kg doses respectively.

The decline of plasma concentrations of DPH appear
to be non-exponential for the 6 and 9 mg/kg doses in sub-
ject W.M. The average plasma clearance in this subject
decreased from 34.9 ml/min. after the 3 mg/kg dose to
25.3 and 21.4 ml/min. respectively after the 6 and 9 mg/kg
doses.

In three of the four subjects, the half-life determined
after oral administration was longer than an equivalent dose
given intravenously, and area divided by oral dose was
appreciably less than found in the IV studies in three of
the four subjects (Table VI). Figures 7 and 8 include
comparative plots of oral and similar IV doses in the same
subject.

DISCUSSION

Post-Infusion Plateau Effect - The reasons for the
unusual findings of rising or plateau blood levels of DPH
several hours post infusion were explored. The possibility
was considered that the postural changes from the reclining
position during and after infusion to a normal standing
position could cause distributional changes; however, it
was difficult to rationalize that such changes could main-
tain blood levels up to seven or ten hours. The more rea-
sonable explanation seems to be that the drug, with its poor
solubility, was precipitating in the blood, and the particles
were being trapped in the reticular endothelial of the lung
and possibly the liver. They would then redissolve slowly
from these sites, thereby prolonging the blood levels. This
would particularly explain the rising, post-infusion blood
levels of DPH.

A review of the literature revealed that sustained DPH
levels post intravenous administration was not unique to the
present studies; however, this has never been pointed out in
published investigation. In a study by Bigger et al. (86a)
six patients received intravenously 300 mg of DPH dissolved
in the same commercial vehicle used in the present study.
Each dose was followed by an injection of sterile saline
to prevent pain and inflammation at the injection site.
After an initial rapid drop of plasma concentration over
the first twenty minutes, the published curves indicate that

the plasma DPH levels remained constant or increased up to twelve hours. A study conducted by Jensen and Grynderup (86b) include four experiments where doses of 10 mg/kg were given intravenously. In the reported data, one notes that plasma levels rose or remained constant after an initial decline. Mirken and Wright (87) gave 150 mg intravenous doses of DPH over a five-minute period. Once again, the plasma DPH levels rose for a period up to four hours post infusion and did not decline until after twelve hours. In the study by Bigger, an initial decline of DPH levels was seen before a plateau or a rise. In that study, the drug was injected between 100 and 300 mg/min. and sampling was begun at 5 minutes after injection. The decline over the first 20 minutes was presumably due to tissue distribution. In the present study, a slow rate of infusion (20 mg/min.) would mask the rapid distribution phase seen after a fast infusion (72).

Neither a rising nor a plateau effect was observed in the dog experiments reported in Chapter III. The only major experimental difference was the means of infusion. In the dog, the drug was infused at one-third of the rate used in the human experiments and by a cannula placed in the vena cava which, of course, exposes the infusion stream to a fast flow and large volume of blood. One might expect a more intensive mixing of the semi-aqueous alkaline vehicle containing the drug, and thereby reducing the possibility of drug precipitation.

An attempt was made to produce the plateau effect in dogs by infusing the drug via a 5% dextrose drip into a peripheral vein, thereby mimicking the administration procedure used in man. The resultant data are shown in Figure 22, Chapter III. At the end of the peripheral infusion in the dog, the DPH concentration was only about one-half of that achieved by the vena cava infusion. The blood levels did not drop for 50 minutes. This is significant when considering that the half-life in the dog was only about 95 minutes, compared to the much longer half-life in man. Further, no metabolic or distributional explanation can account for the unusual secondary peaks seen post infusion in the curve shown in Figure 22.

Analysis of IV Data - In two subjects, M.M. and J.O., one can readily accept the postulate that dose dependent kinetics was not observed, since in these subjects the curves appear to be fitted by a log-linear slope with reasonably consistent half-lives and clearance values after all of the three IV doses. Even though plateau or rising plasma concentrations were observed in these individuals, the average clearance remained almost constant. The area under the plasma concentration-time curve was proportional to the dose despite the fact that some apparent precipitation of the drug in the blood had presumably occurred. This should be expected, as the area under the curve is independent of the rate of availability. The terminal half-life of each experiment in these subjects was determined from plasma concentrations beyond

twenty-four hours to avoid an overestimation caused by prolonged redissolution. In most instances the half-life was nearly identical to that which was computed from the time when the plasma concentration began to decline (ca. 5-10 hours). However, from our dog experiments we note that even though the data appears at first glance to indicate first order kinetics, the fall off curves are convex descending. If this were the case in these subjects, the assumption of first order elimination would lead to an overestimation of the area under the plasma concentration time curve and an underestimation of plasma clearance.

A dose dependent elimination of DPH was observed in the other two subjects, R.R. and W.M. The average clearance declined from 42.9 ml/min. after the 3 mg/kg dose to 29.6 ml/min. after the 9 mg/kg dose for R.R. The elimination appears to follow first order elimination after all doses with a 9.0, 13.6, and 13.4 hour half-life after the 3, 6, and 9 mg/kg doses respectively.

The clearance of DPH in W.M. declined from 34.9 ml/min. to 21.4 ml/min. as the dose was increased from 3 to 9 mg/kg. The 6 and 9 mg/kg doses show a nonexponential decline until plasma concentrations dropped below 5 mcg/ml. The terminal portion of the log concentration time curve appears to be parallel with a half-life of about 12 hours.

In Chapter III, it was noted that HPPH might be functioning as a competitive inhibitor of the enzyme system involved in the hydroxylation of DPH. This can be expressed

by a modified Michaelis-Menten equation as follows:

$$\frac{dC^{DPH}}{dt} = \frac{V_m C^{DPH}}{C^{DPH} + K_m \left(1 + \frac{C^{HPPH}}{K_I^{HPPH}}\right)}$$

as defined in Chapter III. If the ratio C^{HPPH}/K_I^{HPPH} is greater than 1, and the concentration of the metabolite remains essentially constant, this equation becomes

$$\frac{dC^{DPH}}{dt} = \frac{V_m C^{DPH}}{C^{DPH} + K_m'}$$

$$\text{where } K_m' = K_m \left(1 + \frac{C^{HPPH}}{K_I^{HPPH}}\right)$$

If the situation existed with subject R.R. that $K_m \gg C^{DPH}$, then the resultant elimination curves for the various doses should be linear but possess different apparent half-lives depending on the changes of K_m' with the relative concentration of HPPH. Similarly, the convex descending curves seen in the studies on subject W.M. can be explained as being in the ideal M concentration ranges (i.e., $C^{DPH} \geq K_m$).

The volume of distribution was determined by the area method. For the two subjects, M.M. and J.O., who have been proposed to demonstrate dose independent kinetics, the average Vd area calculated from the three intravenous doses was 0.52 ± 0.09 and 0.54 ± 0.02 L/kg respectively. For the

subjects demonstrating dose dependency, the volume of distribution was calculated from the 3 mg/kg dose, and was found to be 0.44 L/kg for R.R. and 0.51 L/kg for W.M. Three of the four subjects showed a very similar DPH volume of distribution adjusted for weight. The calculation of volume of distribution from clearance data is dependent on a good estimation of the elimination rate constant. In subject R.R., the estimate could only be made from four points because of the plateau effect and the unexpected short half-life. If the half-life was estimated to be 10.2 rather than 9.0 hours, then the volume of distribution would have been calculated to be 0.51 L/kg. It is interesting to note that the volume of distribution of DPH in dog (Chapter III) is approximately three times greater than in man. This discrepancy is probably related to differences in plasma protein binding between man and dog.

Individual differences in the ability to metabolize DPH is reflected in the variability of half-lives reported in the literature. A wide range of half-lives has been observed (18,38). In the present study of only four subjects, a range of 9 to 32 hours was found. However, it is interesting to note that reports from the Scandinavian literature (33-35) indicate a narrow range of DPH half-life among individuals, generally with a mean of about 10 hours. A low metabolic capacity for DPH reported by Kutt et al. (15) appears to be a specific genetically determined deficiency.

Oral Studies - The intravenous studies of DPH in man

were carried out because of the possibility that the findings of dose dependent kinetics shown by Arnold and Gerber (38) after repeated oral dosing with DPH were artifically produced by prolonged absorption. It should be emphasized that their results were obtained after multiple dosing and using a different DPH analysis. The absorption of DPH is known to be irregular. Peak level times from two to more than twenty-four hours after a single oral dose have been reported by several investigators. In the present studies, each of the fasted subjects were administered 400 mg of DPH in four, 100 mg capsules of Dilantin^R. The oral data listed in Tables II-V and summarized in Table VI indicate the apparent terminal half-life observed in three of the four subjects was longer than when an equivalent dose of DPH was given IV. The fourth subject (J.O.) had a similar half-life after oral and IV doses. However, this subject had the longest half-life (ca. 32 hrs.) and the effects of prolonged absorption on the terminal portion of the curve would be minimal.

It is probably more informative to attempt to estimate the fraction of administered dose available by computing the area/dose administered from IV and oral studies, which are tabulated in Table VI. It can be seen that the ratio of the IV doses remains essentially constant for subjects J.O. and M.M. The results from the latter subject are shown in Figure 7. It is clear that subjects J.O. and M.M. must have only absorbed approximately 90 and 55% respectively of the orally administered dose. Subject W.M. shows a

decreasing value for area/dose with increasing intravenous doses. However, when this subject was given 400 mg orally, the area/dose ratio was virtually identical with that seen after a 250 mg IV dose, indicating that approximately 60% of the oral dose was absorbed. This estimate is still liable to error since the oral curve does not superimpose on the IV data. The results obtained from the studies on subject R.R. shown in Figure 8 are even more difficult to analyze. Yet one must conclude from the area analysis that absorption is not complete.

One can postulate both physical and pharmacological explanations for the apparent reduction and prolonged absorption after oral administration. The physical chemical bases for prolonged absorption were discussed in Chapter I. Other pharmacological aspects need to be reviewed. Druckman and Moore (88) have shown that DPH has a direct effect on the isolated rabbit gut. They have shown in vitro that solutions of 20 mcg/ml of DPH rapidly reduce tone, amplitude, and the rate of contraction of the rabbit duodenum. This pharmacological action in vivo would decrease intestinal motility. Frey and Kampmann (89) have demonstrated delayed DPH absorption in mice when amphetamine is co-administered. This effect was attributed to the action of amphetamine to delay gastric emptying and decrease intestinal motility. However, the authors could not exclude the possibility that part of the effect may be due to decreased intestinal blood flow induced by amphetamine.

It is interesting to note that studies reporting DPH half-life show a lower average value when determinations were made after intravenous administration. The present studies show that the terminal half-life of DPH can be overestimated when evaluated after oral dosing. Dill et al. (4) reported an 18 to 24 hour half-life in six subjects after a 400 mg oral dose. Arnold and Gerber (38) reported an average half-life at 22 ± 9.0 hours in their oral study. Solomon and Schrogie (30) found an average half-life of 25.8 ± 4.7 hours after five normal volunteers had received 100 mg orally, three times daily for five days. On the other hand, Glazko et al. determined the plasma half-life to be 15.8 ± 7.3 hours after a single intravenous dose of 250 mg in six normal subjects. Suzuki et al. (10) reported that after an initial distributional phase, the DPH half-life in two subjects was 9.1 and 9.5 hours following a 250 and 125 mg dose respectively. In several intravenous studies from the Scandinavian literature (33-35) the average half-life reported was never greater than 12 hours, even though doses up to 11.1 mg/kg were given.

Despite the possibility that prolonged absorption of DPH may give the appearance of dose dependent elimination, the results of the present study confirm the findings of Arnold and Gerber in at least subject W.M. where the IV curves were convex descending. Ten subjects of the 70 subjects studied by Arnold and Gerber were reported in detail and demonstrated dose dependent kinetics with convex descending curves 12 hours after withdrawal of the drug.

In the present studies it was felt that collection of metabolite excretion data would not aid in the understanding of the mechanism of dose dependency because of several complicating factors. First, there is an initial delay in the excretion of p-HPPH-glucuronide. Glazko et al. (18) reported that maximum excretion of the metabolite occurred generally 6 to 8 hours after an intravenous dose. Secondly, in man the metabolite is probably recycled by biliary excretion (11,12); thus, its excretion pattern would be subject to the influence of extrinsic factors such as diet and eating patterns.

CONCLUSIONS

The finding of dose dependent elimination of DPH in
man was confirmed in at least two of the four subjects
receiving intravenous DPH doses of 3, 6, and 9 mg/kg.
The elimination of DPH in the other two subjects may not
have been influenced by the dose administered to the extent
necessary to show dose dependent kinetics.

Following all intravenous infusions, a plateau or
rising plasma concentration of DPH was observed up to 10
hours. A survey of the literature showed similar findings
in several studies. These findings could not be rationalized
by a distributional or metabolic phenomenon, since neither
can explain the increases in the DPH plasma concentration
post infusion. Rather, the findings may be explained on
the basis of the poor solubility of DPH. When the drug
enters the blood stream, some may precipitate as the insoluble
free acid, phagocytized, and then slowly be released into
solution. The plateau effect was not observed in the dog
when the drug was infused into the vena cava where good
mixing with blood would occur; however, sustained levels
were produced when DPH was administered by a peripheral
infusion with dextrose mimicking the procedure used in
humans. The clinical implications of DPH precipitation are
unknown.

The half-life in three of the four subjects appeared to
be significantly longer when determined after oral rather than

intravenous administration. An increasingly prolonged absorption with increasing dose can be expected in light of the pharmacological action of DPH on the gastro-intestinal tract. This effect could be misinterpreted as dose dependent kinetics.

Although only four subjects were studied, it is apparent that multiple dose therapy with DPH is likely to lead to unpredictable body stores. The half-life in the above subjects varied from 9 to 32 hours. Also the extent of absorption on an empty stomach varied from 50 to 90%. These unpredictable variables, coupled with a narrow therapeutic blood level range and the possibility of dose dependent kinetics, lead to the conclusion that far better therapeutic results might be obtained if routine blood monitoring was instituted for subjects administered DPH. It is obvious that the complexity of possible mechanisms which can alter pharmacokinetics may confound the physician making quantitative decisions with regard to the therapeutic regimen. Clearly the present results serve to strengthen the conviction that individualization of DPH kinetic parameters and computer assists to quantitative decision-making are mandatory.

TABLE I

Dosage and Dilutions of DPH for Intravenous
Infusion*

Subject	DPH Dose mg/kg	Total DPH, mg	Length of Infusion, min.	5% Dextrose, ml
M.M.	3	210	10.8	480
	6	420	21.6	500
	9	630	32.5	600
J.O.	3	279	14.4	0
	6	558	28.8	500
	9	837	43.1	450
R.R.	3	225	11.6	0
	6	449	23.1	350
	9	674	34.8	250+
W.M.	3	225	11.6	500
	6	450	23.2	650
	9	675	34.8	900

*Infusion rate 0.39 ml/min. = 19.4 mg DPH/min.

+Saline was used as diluent instead of dextrose.

TABLE II

Summary of Various Doses of DPH in Subject M.M.

Experiment	5.7 mg/kg Oral	3 mg/kg IV	6 mg/kg IV	9 mg/kg IV
Dose, mg	400	210	420	630
Half-Life, hr.	35.7	20.8	22.8	18.1
Elimination rate constant, hr.$^{-1}$	.019	.033	.030	.038
Average Clearance, ml/min.	----	20.0	22.0	18.7
Vd_{area}, L	----	36.1	43.5	29.9
Vd/kg, L/kg	----	.52	.62	.43

mean Vd=.52 $\pm$ .09 L/kg

TABLE III

Summary of Various Doses of DPH in Subject J.O.

Experiment	4.3 mg/kg Oral	3 mg/kg IV	6 mg/kg IV	9 mg/kg IV
Dose, mg	400	279	558	837
Half-Life, hr.	34.0	30.6	28.1	32.3
Elimination rate constant, hr.$^{-1}$	.020	.023	.025	.021
Average Clearance ml/min.	----	18.2	20.9	18.7
Vd$_{area}$, L	----	48.3	50.8	52.5
Vd/kg, L/kg	----	.52	.55	.56

mean Vd = .54 $\pm$ .02 L/kg

TABLE IV

Summary of Various Doses of DPH in Subject R.R.

Experiment	5.3 mg/kg Oral	3 mg/kg IV	6 mg/kg IV	9 mg/kg IV
Dose, mg	400	225	449	674
Half-Life, hr.	20.7	9.0	13.6	13.4
Elimination rate constant, hr.$^{-1}$	.034	.077	.051	.052
Average Clearance ml/min.	----	42.9	32.5	29.6
Vd_{area}, L	----	33.4	38.0	34.1
Vd/kg, L/kg	----	.45	.51	.45

mean Vd = .47 L/kg

TABLE V

Summary of Various Doses of DPH in Subject W.M.

Experiment	5.3 mg/kg Oral	3 mg/kg IV	6 mg/kg IV	9 mg/kg IV
Dose, mg	400	225	450	675
Terminal Half-Life, hr.	20.2	12.6	11.9	12.5
Terminal elimination rate constant, hr.$^{-1}$	.034	.055	.058	.055
Average Clearance, ml/min.	----	34.9	25.3	21.4
Vd$_{area}$, L	----	37.9*	----	----
Vd/kg, L/kg	----	.51	----	----

*Based on the assumption of first order elimination.

TABLE VI

Analysis of Area Under Plasma Concentration-Time
Curve per Dose

Subject	Intravenous		Oral Area/Dose hr./ml	Percent Absorbed Orally
	Dose mg/kg	Area/Dose hr./ml		
M.M.	3	.81	.45	55
	6	.75		
	9	.89		
	mean	.82		
J.O.	3	.91	.79	89
	6	.82		
	9	.90		
	mean	.88		
W.M.	3	.48	.50	--
	6	.65		
	9	.78		
R.R.	3	.39	.26	--
	6	.52		
	9	.56		

Figure 1. Plasma concentration of DPH after infusion of various doses in subject M.M. Key: ●, 3 mg/kg; ■, 6 mg/kg; ▲, 9 mg/kg.

Figure 2. Plasma concentration of DPH after infusion of various doses in subject J.O. Key: ●, 3 mg/kg; ■, 6 mg/kg; ▲, 9 mg/kg.
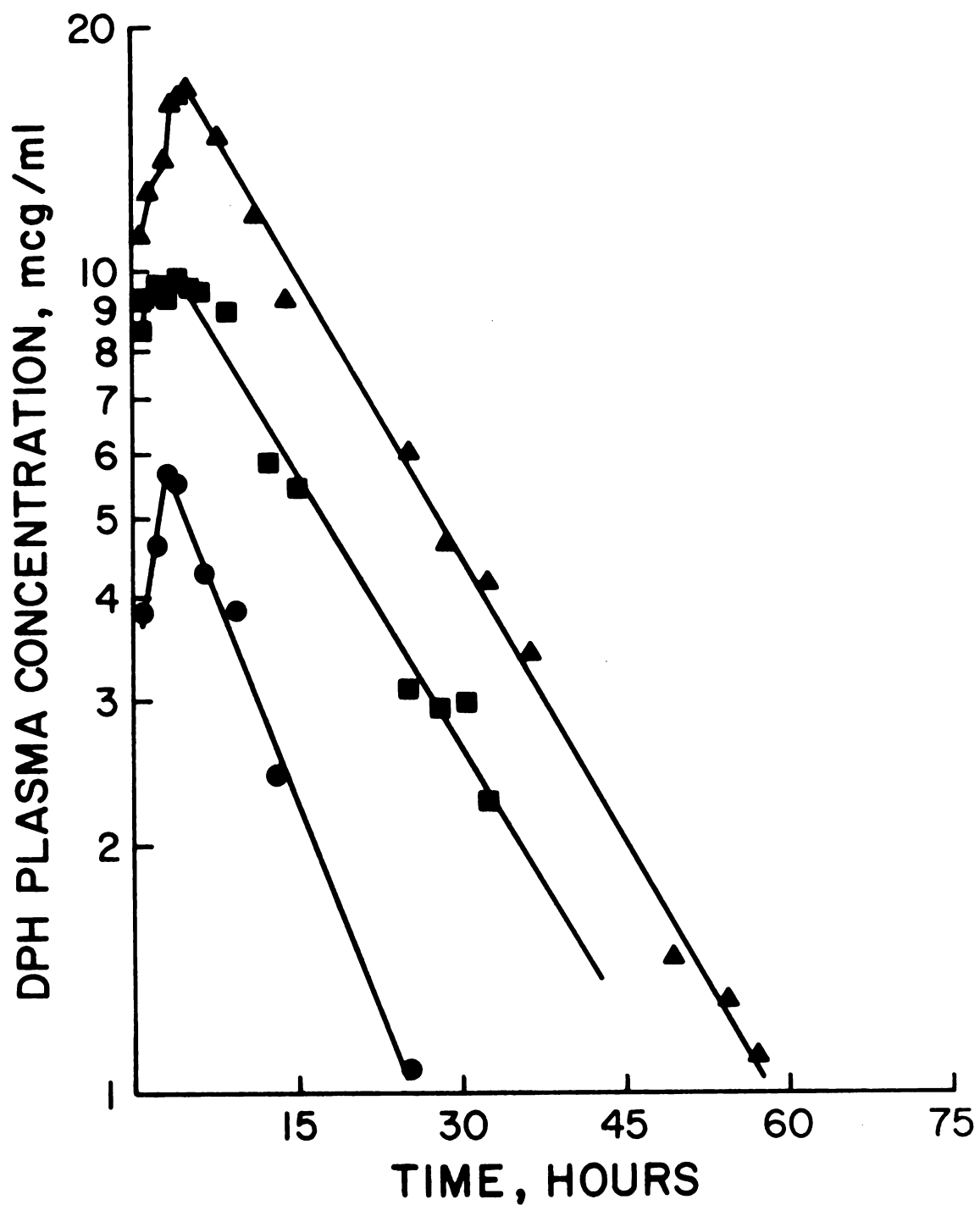
Figure 3. Plasma concentration of DPH after infusion of various doses in subject R.R. Key: ●, 3 mg/kg; ■, 6 mg/kg; ▲, 9 mg/kg.
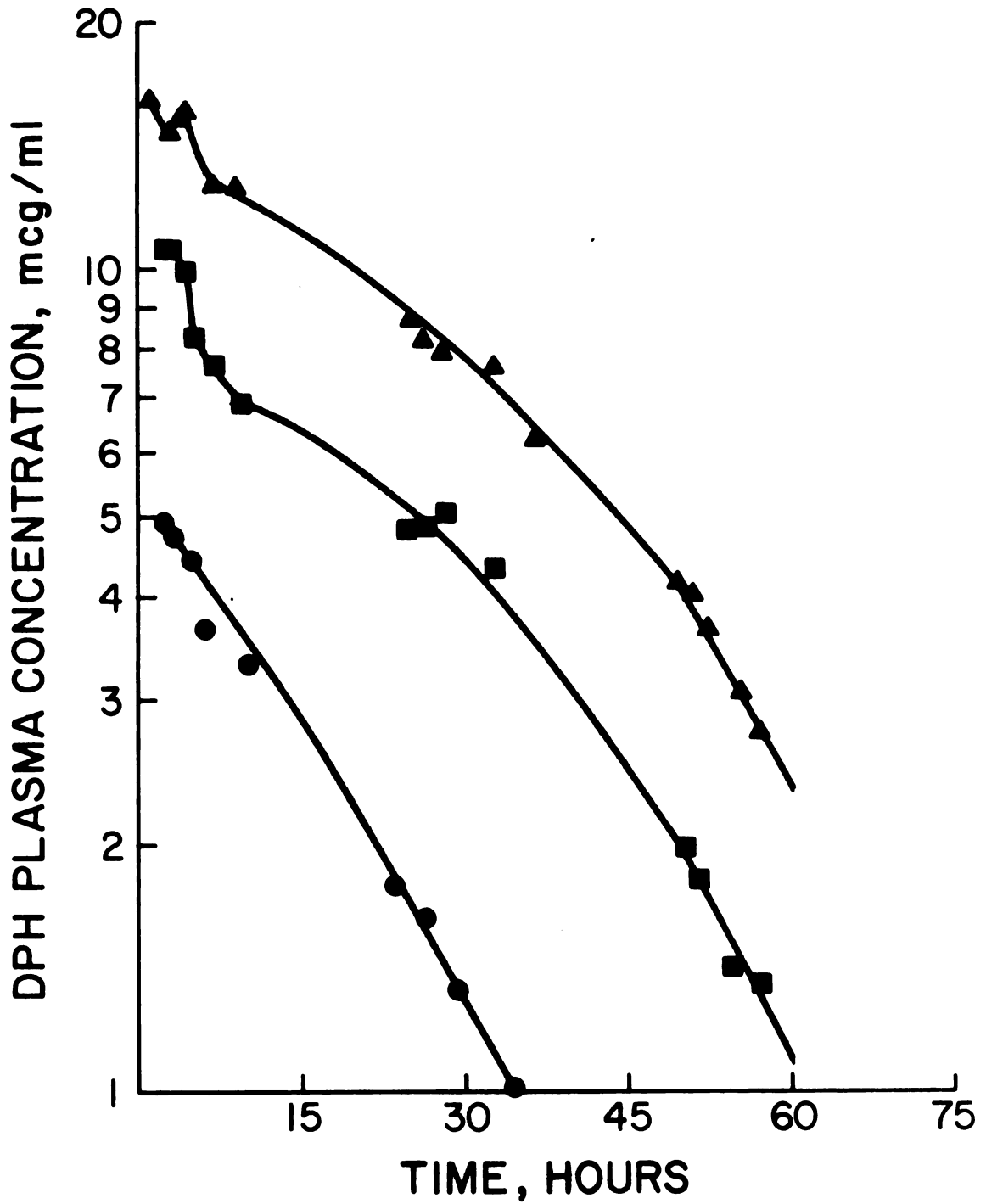
Figure 4. Plasma concentration of DPH after infusion of various doses in subject W.M. Key: ●, 3 mg/kg; ■, 6 mg/kg; ▲, 9 mg/kg.
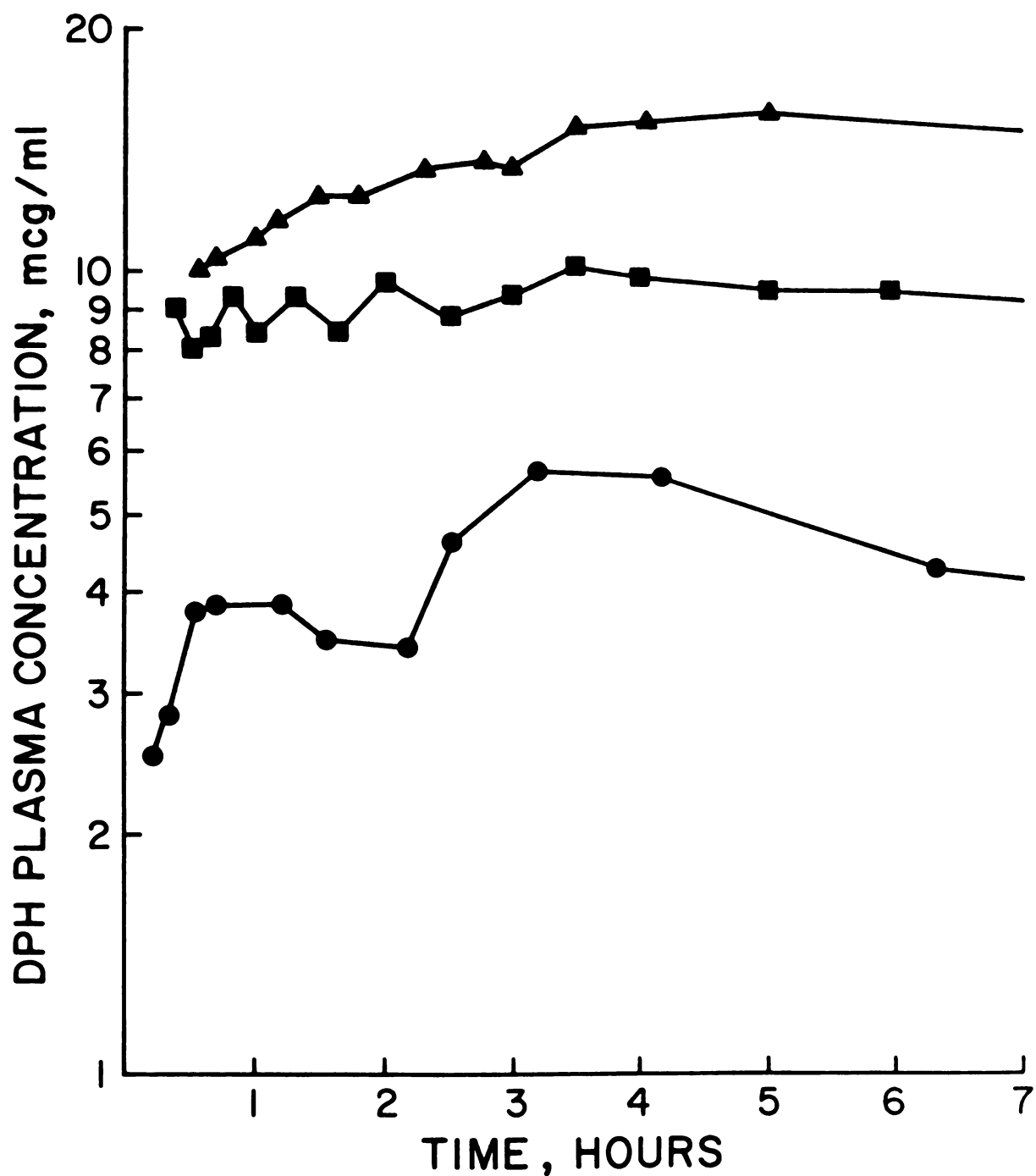
Figure 5. Post infusion plasma concentration of DPH for seven hours after various doses in subject R.R. Key: ● , 3 mg/kg; ■ , 6 mg/kg; ▲ , 9 mg/kg.

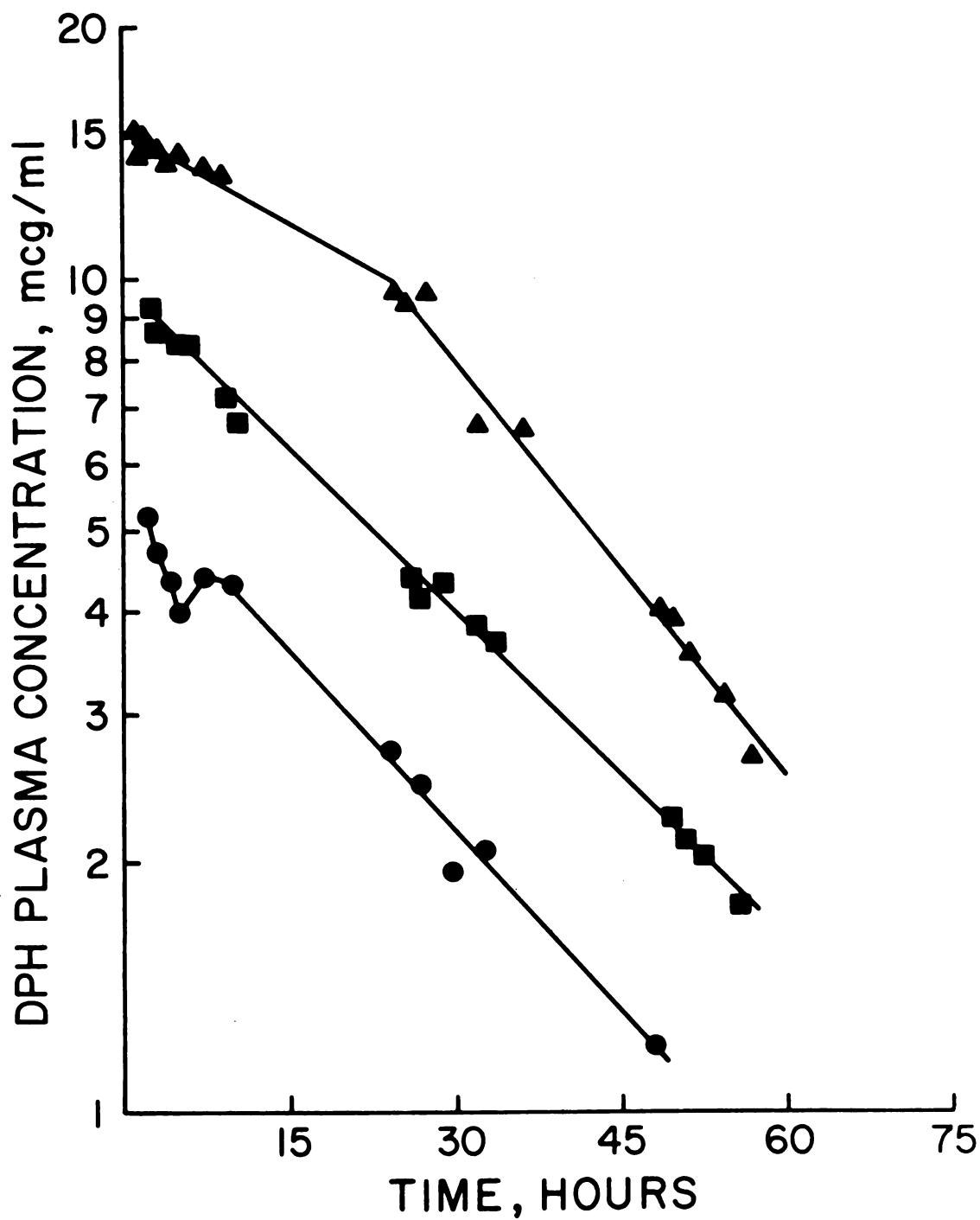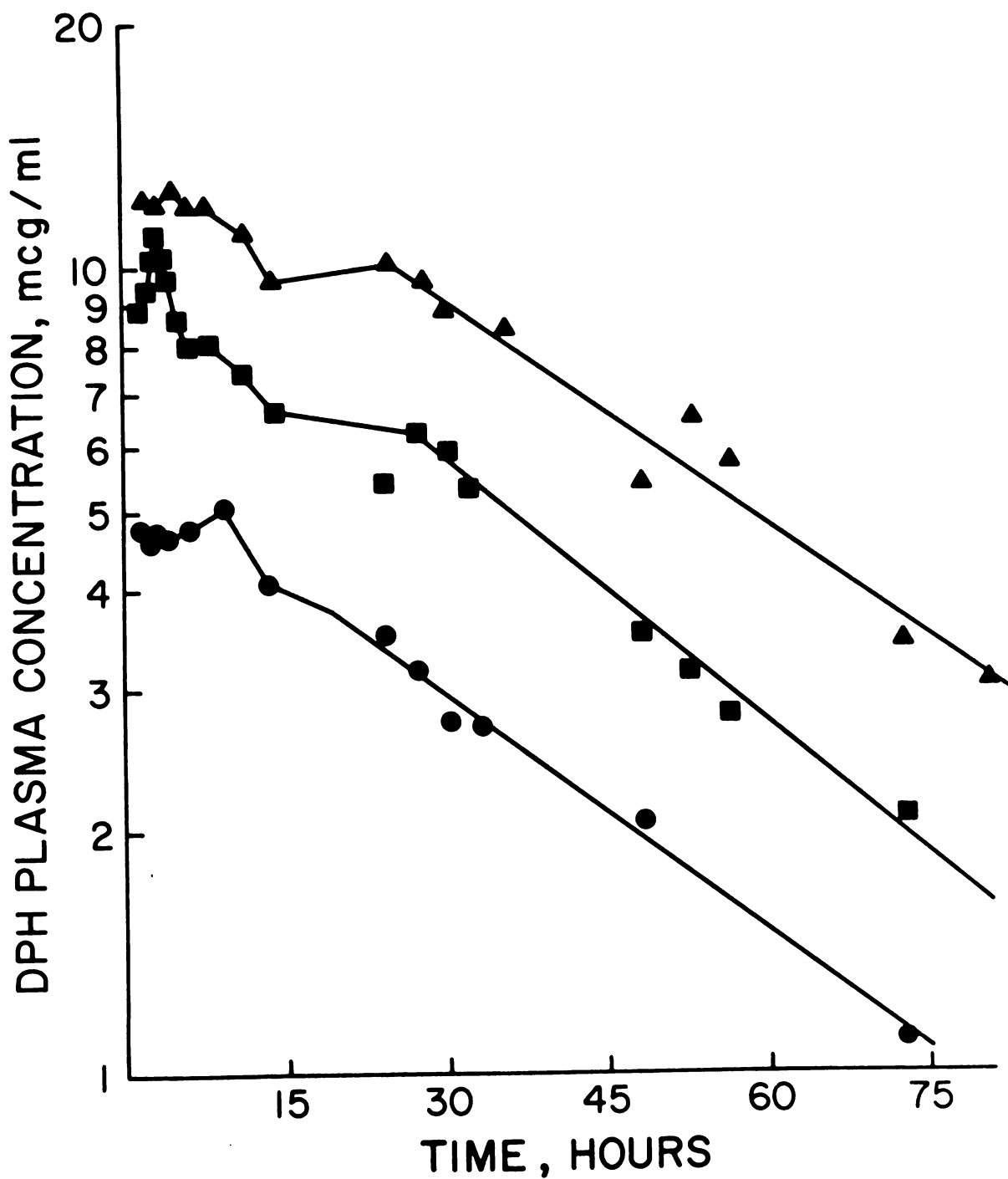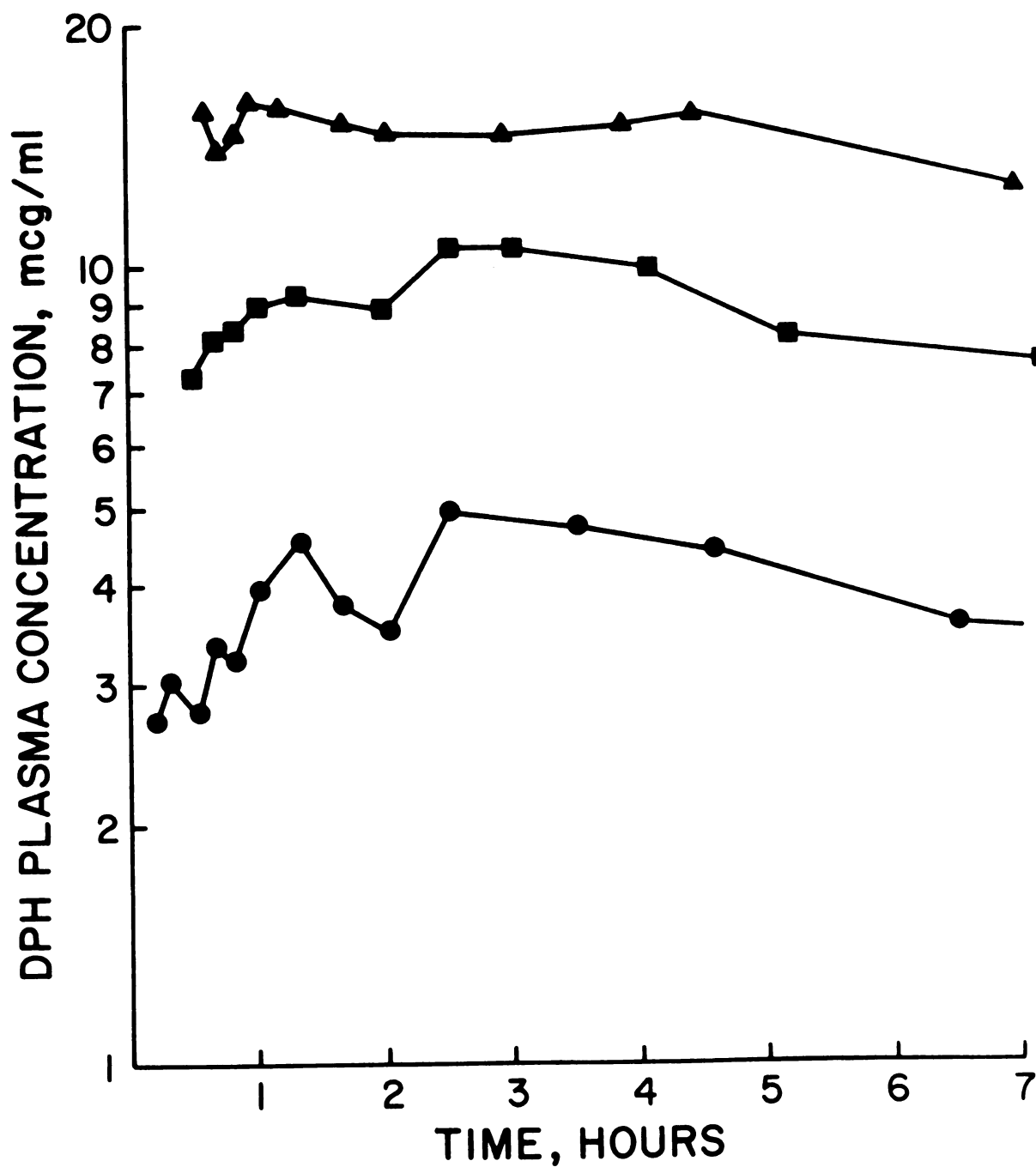Figure 6. Post infusion plasma concentration of DPH for seven hours after various doses in subject W.M. Key: ●, 3 mg/kg; ■, 6 mg/kg; ▲, 9 mg/kg.
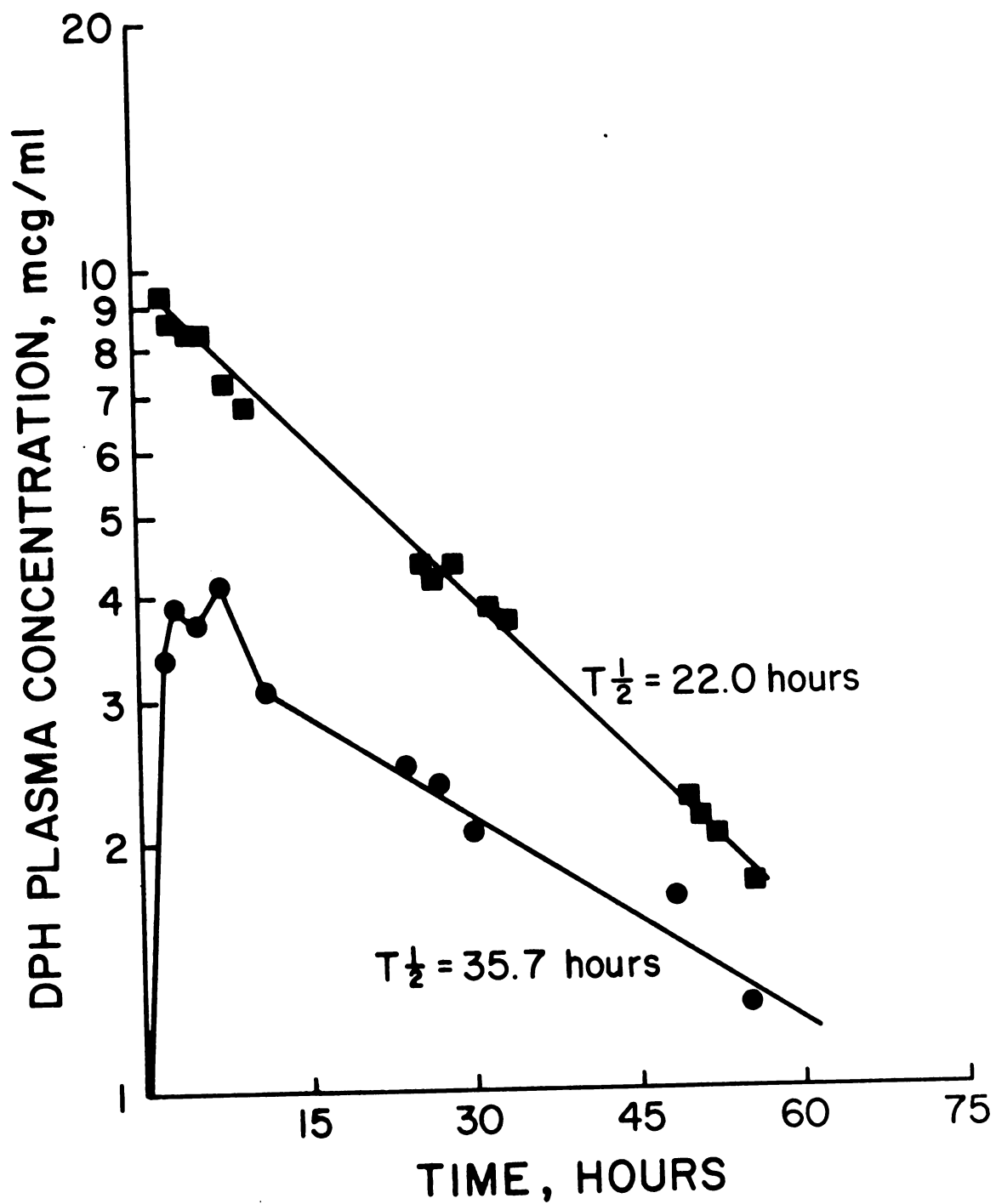
Figure 7. Plasma concentration of DPH after oral and intravenous administration in subject M.M. Key: ●, 400 mg oral; ■, 420 mg IV.
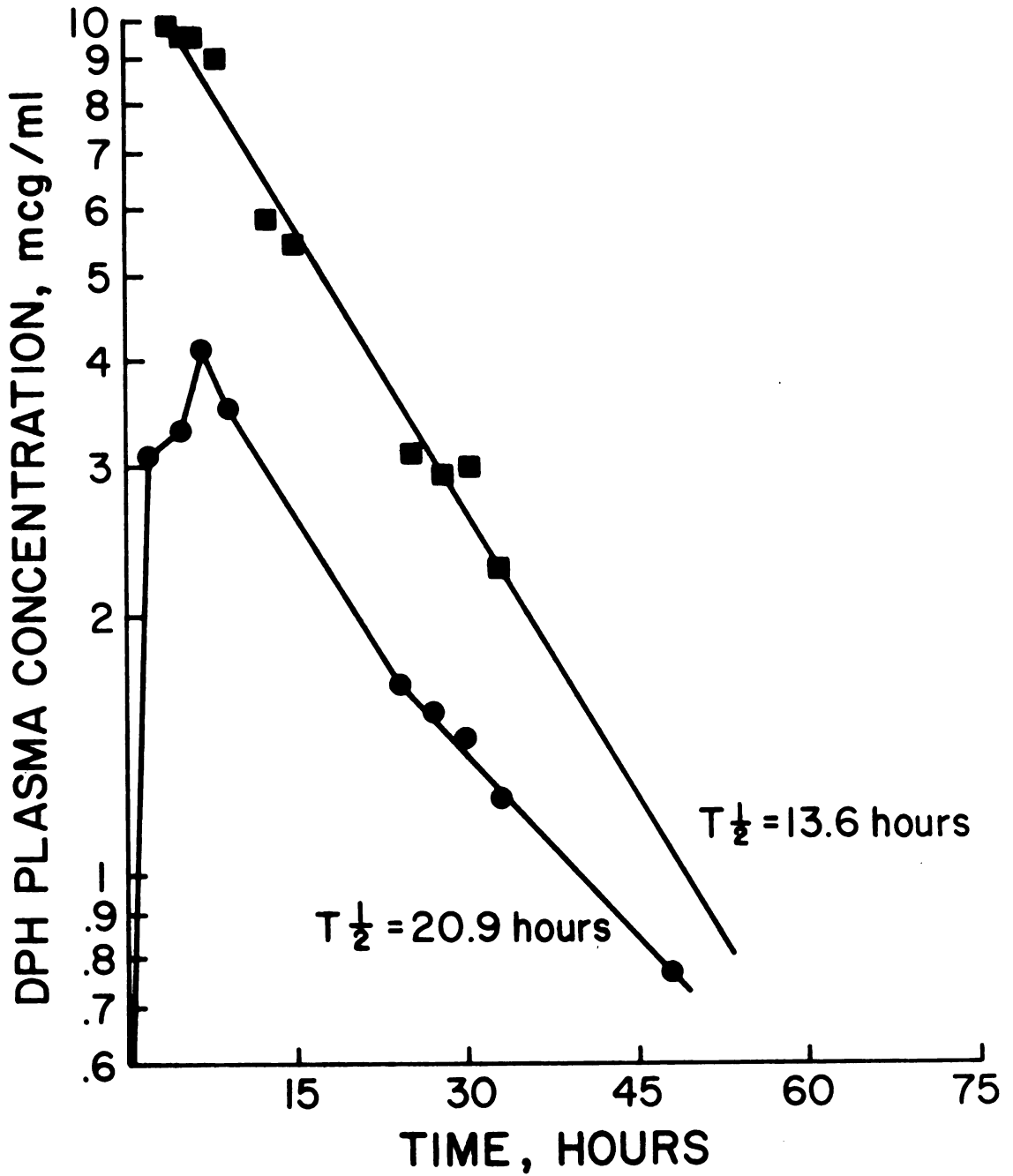
Figure 8. Plasma concentration of DPH after oral and intravenous administration in subject R.R. Key: ●, 400 mg oral; ■, 449 mg IV.

CHAPTER V

METHODOLOGICAL STUDIES OF DPH PROTEIN BINDING

Plasma protein binding is a factor that can significantly affect drug distribution and elimination. Passage of a drug across cell membranes, glomerular filtration, and possibly hepatic metabolism are limited to the unbound or free species of a drug. Drug binding is also very important from a therapeutic point of view as it is the unbound drug levels that often correlate with a pharmacologic or toxic response. Differences in therapeutic response of a drug may be related in some degree to variations in protein binding among individuals.

Plasma protein binding studies of DPH were undertaken for the following reasons. Initially, it was thought desirable to see if any correlation existed between the widely variable half-life and the degree of drug binding in the normal volunteers for the dose dependent kinetic studies in man (Chapter IV). It was felt that DPH binding might be a factor affecting DPH elimination.

Secondly, it was reported in the literature by Mellk et al. (90) that uremic patients exhibited a decreased half-life for DPH. It was suggested by the investigators that the increased elimination of DPH in these patients was due to increased metabolism by "uremic" induction of the parahydroxylating enzyme systems. Altered protein binding affecting

DPH elimination was seen as an alternate explanation for the Mellk et al. findings. Therefore, DPH binding studies in uremic patients were undertaken in an effort to add to the pharmacokinetic information on this drug.

A third area of interest of DPH binding was concerned with artificial plasma systems and the possibility that other components besides albumin might be significantly involved as carrier proteins. These studies were of interest in making correlations between free drug levels and pharmacologic response.

The principle technique employed in the studies of DPH binding in plasma was ultracentrifugation. This method is considered one of the more ideal techniques for studying protein binding (91). It has the distinct advantage over equilibrium dialysis in that smaller volumes of biological fluids can be handled and the analysis time is generally shorter. Ultracentrifugation also has a major advantage over equilibrium dialysis and ultrafiltration techniques in that the latter two methods require the passage of drug through a membrane. Many drugs will bind to a dialysis or filtration membrane, thus introducing errors in binding results. The ultracentrifuge technique, however, has a major disadvantage in that it requires expensive instrumentation that can process only a limited number of samples per run.

For these studies it was desired to use the ultracentrifugation techniques as a standard reference method to

determine protein binding and then to develop an ultra-filtration technique using commercially available "Centriflo" membrane ultrafilters for routine analysis.

MATERIALS AND METHODS

Materials - 5,5-diphenylhydantoin as the free acid
was purchased from Eastman Kodak Company, Rochester, New
York. 5,5-diphenylhydantoin-4-C^{14} with a specific activity
of 5.49 mc/mg and "Aquasol" liquid scintillation counting
solution was obtained from New England Nuclear Corporation,
Boston, Massachusetts. Centriflo membrane ultrafilters
CP50A, conical supports and centrifuge tubes were obtained
from the Amicon Corporation, Lexington, Massachusetts.
Polyallamer tubes, 4.2 ml capacity, for the ultracentrifu-
gation were purchased from Spinco Division of Beckman Instru-
ments, Palo Alto, California.

Sample Preparations - Fresh or frozen plasma was used
for most of the binding studies. Plasma samples were obtained
after centrifugation of heparinized blood (approximately 20 I.U.
heparin per ml whole blood) collected from normal volunteers.
Binding of serum was compared to binding in plasma. Serum
samples were collected after centrifugation of clotted blood.

A standard concentration of 10 mcg/ml DPH in plasma or
serum was used for all the binding studies. Sample sizes of
4.5 ml and 5.0 ml were used for ultracentrifugation and ultra-
filtration respectively. An ethanolic solution of ^{14}C-DPH
was made by dissolving 1 mg ^{14}C-DPH in 5 ml ethanol. This
gave a solution with an activity of approximately 9600 dpm/ml.
Solutions of cold DPH were made by dissolving 41 mg DPH in
20 ml of ethanol for the ultracentrifugation studies and

46 mg DPH in 20 ml of ethanol for the ultrafiltration studies. For either the ultracentrifugation or ultrafiltration study 20 μl of the ^{14}C-DPH solution and 20 μl of the appropriate cold DPH solution were added to the plasma or serum sample. In either case the final concentration of total DPH was 10 mcg/ml. Lunde (92) has shown that ethanol concentration below 2 percent v/v did not cause displacement of DPH from plasma protein. In these experiments the ethanol concentration was below 1 percent. After the addition of DPH, the samples were incubated and sealed in Kimex tubes on a water bath of 37°C for 2 hours. Changes of pH in the sealed tubes were small, 7.4-7.8, and the addition of an atmosphere of 5 percent CO_2 did not seem to minimize this change. Lunde (92) has reported that the binding of DPH is unaffected by changes of pH over this range. At the end of the incubation period, 100 μl of sample was removed and placed in a counting vial for radioassay.

Ultracentrifugation - Determination of protein binding by ultracentrifugation was carried out in a Beckman L2-65B Ultracentrifuge with high temperature modification for temperature control to 60 $\pm$ 0.5°C. Six determinations were carried out simultaneously in a SW56 Swinging Bucket titanium rotor. A 4.2 ml quantity of the plasma sample was placed in a polyallomer tube which was loaded into the bucket and sealed air tight by a lubricated metal cap. Runs were made at 55,000 rpm for 10 hours at 37 $\pm$ 0.5°C. At this speed the

maximum centrifugal force achieved was 349,000 g, while the average centrifugal force was 297,000 g. At the end of the run, 0.3 ml of supernatent fluid was removed by means of a "Biopipet" and analyzed for radioactivity or protein concentration.

Ultrafiltration - Binding determination by ultrafiltration utilized Centriflo ultrafilters. The conical filter was soaked in distilled water for one hour at 37°C. Excess water was shaken off the filter in a systematic way so that the amount of water remaining on the filter was predictable within a few milligrams. The presoaked filter was placed in a conical support mounted in a collecting centrifuge tube, both of which had been equilibrated at 37°C. The filter was then filled with 4.9 ml of prepared sample and covered with parafilm. The samples were spun in an International centrifuge model no. UV at 600 g. The centrifuge was controlled at 37 ± 1°C by placing a commercial hair dryer at the air intake on the top of the centrifuge door. By adjusting the distance of the hair dryer from the air intake, the chamber temperature could readily be controlled after an initial warm-up period of about 15 minutes. The chamber temperature could be accurately monitored by insertion of a thermometer through the air outlet in the bottom of the centrifuge. In the initial experiments the samples were centrifuged for 6 minutes each. After the first centrifugation the ultra-filtrate was discarded, as this represented the water content of the filter. The second centrifugation produced 230-250 μl

of ultrafiltrate. A 200 μl sample of this filtrate was used for determination of the DPH content and a 20 μl portion was used for the protein determination. Subsequent experiments were carried out by six consecutive centrifugations of the same samples. The time of centrifugation was gradually increased in order to achieve uniform volumes of filtrate in each sample. The fraction of drug bound was determined for each consecutive sample.

Radioassay - The radioactive samples, 100 μl for the pre-spin determinations and 300 μl or 200 μl for the post ultracentrifugation and ultrafiltration respectively, were placed in polyethylene scintillation vials. A 10 ml quantity of "Aquasol", a sylene-based, protein-dissolving, liquid scintillation-counting fluid, was added to each vial and the fluid was shaken until homogeneous. The pre-spin samples which contained protein were allowed to stand about 10 to 12 hours with occasional shaking before they were counted. The radioactivity in each sample was measured in a Packard liquid scintillation spectrometer, Model 3375. The counting efficiency was determined by an internal standard method. No corrections were made, as the counting efficiency of both the pre-spin and post-spin samples were in the same range of 92-94%.

Calculations - The fraction of drug bound to protein was calculated from the ratio of the activity per unit volume in the supernatant or filtrate to the activity in the original solution. A correction was made for the volume displaced by

protein in the sample by utilizing the method of McLean
and Hasting (93). For plasma or serum samples the corrected
activity of protein (CPM corr) pre-spin sample is:

$$\text{CPM corr} = \frac{\text{CPM}}{\text{PW}} \quad \text{Where PW} = 99.0-(.75 \times \% \text{ protein})/100.$$

Studies where purified proteins in the saline solutions are
used, the established correction factor is:

$$\text{PW} = 99.6 - (.75 \times \% \text{ protein})/100.$$

<u>Protein Determination</u> - Total protein concentration
in original plasma or serum samples and post-spin supernatant
or ultrafiltrate was determined by the biuret method. Samples
of 20 μl were needed for the determination. Once the conditions
were established for the protein-free supernatent after ultra-
centrifugation, routine protein measurement was no longer
necessary.

RESULTS AND DISCUSSION

ULTRACENTRIFUGATION

Establishing Conditions - Initially ultracentrifugation
conditions had to be established which produced a reasonable
volume of protein-free supernatant for the radioassay. Figure 1
shows protein distribution in the various layers of sample
after centrifugation for 6 and 10 hours at 55,000 rpm and
37°C. After 6 hours the second 0.1 ml of sample from the
top of the tube contained 0.18 gm/100 protein or approxi-
mately 0.25% of the original 7.4 gm/100 ml protein. After a
10 hour centrifugation the top 0.4 ml of the sample was vir-
tually free of protein. Ten-hour centrifugation was selected
as the standard time for all binding studies. Only the top
0.3 ml of sample was used for DPH analysis. This insured a
margin of safety. The 10-hour centrifugation time was con-
siderably longer than the 3-hour spin time used by Van der
Kleijn (91). The difference was due to smaller sample size
and lower centrifugation force achieved in the ultracentrifuge
used in this study.

Precision of the Method - The precision of the method
was evaluated by determining the binding of DPH in the plasma
of a normal volunteer analyzed five times during a two-month
period. The first determination was made on plasma that had
been freshly drawn. All other runs were made on the same
plasma but stored in a freezer at -10°C. Table I shows the
results. Five determinations on the same plasma had a range

for unbound drug from 8.43 to 8.94% with a mean and standard deviation of 8.67 ± .21. The low standard deviation, which reflects both variability in the ultracentrifugation technique and radioassay, indicates good precision in the combined methodology. From this and other experiments no significant difference was observed in the binding results obtained from fresh or frozen plasma. Also no significant difference was found in the binding of DPH as determined from serum or plasma.

ULTRAFILTRATION

Drugs Adsorption on the Filter - Adsorption of DPH on the filtration membrane was determined by finding the ^{14}C activity in excess of that which would be expected from the ultrafiltrate retained in the membrane after a run. In two determinations the calculated amount of total DPH adsorbed was .288 mcg and .335 mcg. This represented 0.58 and 0.67% of the total amount of drug in the sample. In light of the generally good correlation of the ultrafiltration method with the ultracentrifugation method, the small adsorption of DPH on the filtration membrane does not significantly affect the results.

Precision of the Method - The reproducibility of the ultrafiltration method was tested in a similar manner and on the same plasma as used for the ultracentrifugation precision studies. The mean and standard deviation for the percent free of five determinations was 8.43 ± .65, as shown on Table II. Although the mean values of the two methods were comparable,

8.67 and 8.43%, the standard deviation for the ultrafil-
tration method was greater than three times that of the
ultracentrifugation method. It is evident that a signifi-
cantly greater precision is achieved by ultracentrifugation.

Reasons for the greater scatter in the binding results
by ultrafiltration were considered. Lower values might be
the result of drug adsorption on the filtration membrane or
dilution of the ultrafiltrate by incomplete removal of water
from the membrane remaining after pre-wetting. Higher values
by ultrafiltration could be caused by protein leak into the
ultrafiltrate. To investigate these possibilities, samples
were filtered six times consecutively, and the apparent per-
cent free DPH and protein content was determined for each
ultrafiltrate. If adsorption or dilution were causing low
values, these effects would be overcome after enough filtrate
had passed through the membrane.

The results of two samples run in duplicate are shown on
Tables III and IV. The first ultrafiltrate shows low binding
as expected, as this is primarily water from the wetted filter.
The estimated volume of water in the filter was 0.375 ml. The
calculated binding in the other ultrafiltrates progressively
increased. However, there was also a progressive increase in
protein content of the ultrafiltrate. The apparent percent
free was plotted vs. protein concentration in the ultrafil-
trate in Figures 2 and 3. The extrapolated value to the
y axis where protein content of the ultrafiltrate is equal
to zero should give the true value for the unbound drug.

Using a least squares fit of the data, Figure 2 shows the extrapolated values of 7.94 and 8.00 percent free. The duplicate runs differ by less than 1%. They correlate within 6.5% of the binding results of 8.46 and 8.59 obtained by ultracentrifugation of this sample. However, results of 10.35 and 9.09 percent free would have been obtained using the normal method of ultrafiltration, that is the results of the second spin (Table III, spin 2). In this case the duplicate samples differed by more than 12% and averaged 14% greater than the ultracentrifugation results. The extrapolated values for sample PD were 8.28 and 9.50 percent free. The results were not as encouraging, as the extrapolated results differ by 13%, whereas the normal second spin results 10.15 and 10.00 (Table IV, spin 2) differ by 1.5%. The binding of sample PD as determined by ultracentrifugation was 9.76% free.

The inconsistent reproducibility of binding values resultant from extrapolation to zero protein concentration after consecutive ultrafiltrations was probably due largely to insensitivity of the biuret method for protein analysis at low levels. The use of a more sensitive protein assay procedure may give more reproducible results.

The extrapolation procedure could most likely be applied to ultracentrifugation studies. The major disadvantage of ultracentrifugation is the long spin time required to obtain a protein free supernatant. The long centrifugation not only limits the number of samples that can be processed, but it is

also expensive when depreciation of the limited life of the rotor is considered. The ultracentrifugation procedure might be modified by a short centrifugation time of two to three hours, then consecutive layers could be removed and measured for apparent binding and for protein by a sensitive method. A plot of apparent binding vs. protein extrapolated to zero protein concentration should give reliable measurements. In this way rotor life could be extended four to five fold.

Comparison of Results with Ultracentrifugation - Table V shows the results of seven different samples run by both ultracentrifugation and ultrafiltration. Paired analysis of the data from the two groups showed no difference at the 95% confidence level. The mean and standard deviation by ultra-centrifugation was 8.80 $\pm$ .43 and 8.98 $\pm$ .89 by ultrafiltration. The difference between methods as percent of ultracentrifugation was 5.5 or less for five of the seven samples. The other two samples differed by 10.8 and 16.4%. The results reported in Tables I and II also show that the two methods are comparable. The mean of five repeated samples by ultracentrifugation was 8.67% free compared to 8.33% free by ultrafiltration. The slightly lower mean value found by ultrafiltration might be expected from the small adsorption of DPH on the filter. However, the ultrafiltration results are not consistently lower. The mean value for ultrafiltration reported in Table V is greater than the mean value by ultracentrifugation. A much larger sample size would be necessary to distinguish any differences between the two methods.

CONCLUSIONS

Determination of DPH binding by the ultracentrifugation technique was shown to be highly reproducible. Ultracentrifugation was ideally suited for these studies where often only enough plasma for a single determination was available from clinical patients. A modification of the ultracentrifugation method was suggested for future study which would reduce centrifugation time by 70% or more.

The ultrafiltration method using the Centriflo membrane gave binding results that compared well with the ultracentrifugation technique. However, the variability in the results by this method was large. It was obvious that protein leakage through the filtration membrane into the ultrafiltrate caused artifacts in the calculated percent free. The unpredictable nature of the protein leak was probably primarily responsible for the greater variability in the results obtained by this method. Attempts for better reproducibility by ultrafiltration were not pursued. Instead, it was decided to use the ultracentrifugation analysis in all the binding studies in this work.

Precision of the Methods

Percent free DPH in the same human plasma determined at different times

TABLE I

Ultracentrifugation

Experiment Number	Date	DPH % Free
6	5-11-71	8.43
9	6-18-71	8.49
10	6-29-71	8.94
10	6-29-71	8.68
11	7-02-71	8.79

Mean 8.67 $\pm$ .21 S.D.

TABLE II

Ultrafiltration

Experiment Number	Date	DPH % Free
9	6-18-71	8.06
11	7-02-71	8.23
11	7-02-71	9.58
18	10-22-71	9.06
18	10-22-71	8.23

Mean 8.43 $\pm$ .65 S.D.

TABLE III

DPH BINDING AFTER SIX CONSECUTIVE
ULTRAFILTRATIONS

Duplicate samples of plasma AS

Spin no.	Spin Time (min.)	AS-A			AS-B		
		vol.* (ml)	protein (gm/100 ml)	Apparent DPH % free	vol.* (ml)	protein (gm/100 ml)	Apparent DPH % free
1	6	.290	.00	4.72	.360	.00	4.24
2	6	.234	.12	10.35	.286	.07	9.09
3	6	.208	.33	13.24	.232	.18	11.28
4	7	.170	.49	16.52	.208	.25	12.90
5	8	.201	.52	17.17	.229	.31	14.19
6	10	.207	.58	18.44	.259	.38	14.48

*Volume determined by weight using a density factor of 1.00

TABLE IV

DPH BINDING AFTER SIX CONSECUTIVE
ULTRAFILTRATIONS

Duplicate samples of plasma PD

Spin no.	Spin Time (min.)	PD-A			PD-B		
		vol.* (ml)	protein (gm/100 ml)	Apparent DPH % free	vol.* (ml)	protein (gm/100 ml)	Apparent DPH % free
1	6	.394	.00	6.42	.425	.00	5.67
2	6	.307	.10	10.15	.333	.08	10.00
3	6	.256	.19	12.60	.251	.12	11.50
4	7	.228	.25	14.33	.235	.25	13.21
5	8	.248	.31	15.62	.232	.37	13.69
6	10	.283	.41	17.02	.277	.39	14.68

*Volume determined by weight using a density factor of 1.00

TABLE V

Comparison of Binding Results from
Ultracentrifugation and Ultrafiltration

Subject	DPH % Free		Difference as % of UC
	UC	UF	
BR	9.03	9.41	4.2
JO	8.67	7.73	10.8
MM	8.75	8.73	2.3
RB	8.43	8.06	4.3
ASA	8.46	8.93	5.5
ASB	8.59	10.0	16.4
PDA	9.76	10.0	2.4

mean and standard deviation
Ultracentrifugation 8.80 ± .43
Ultrafiltration   8.98 ± .89
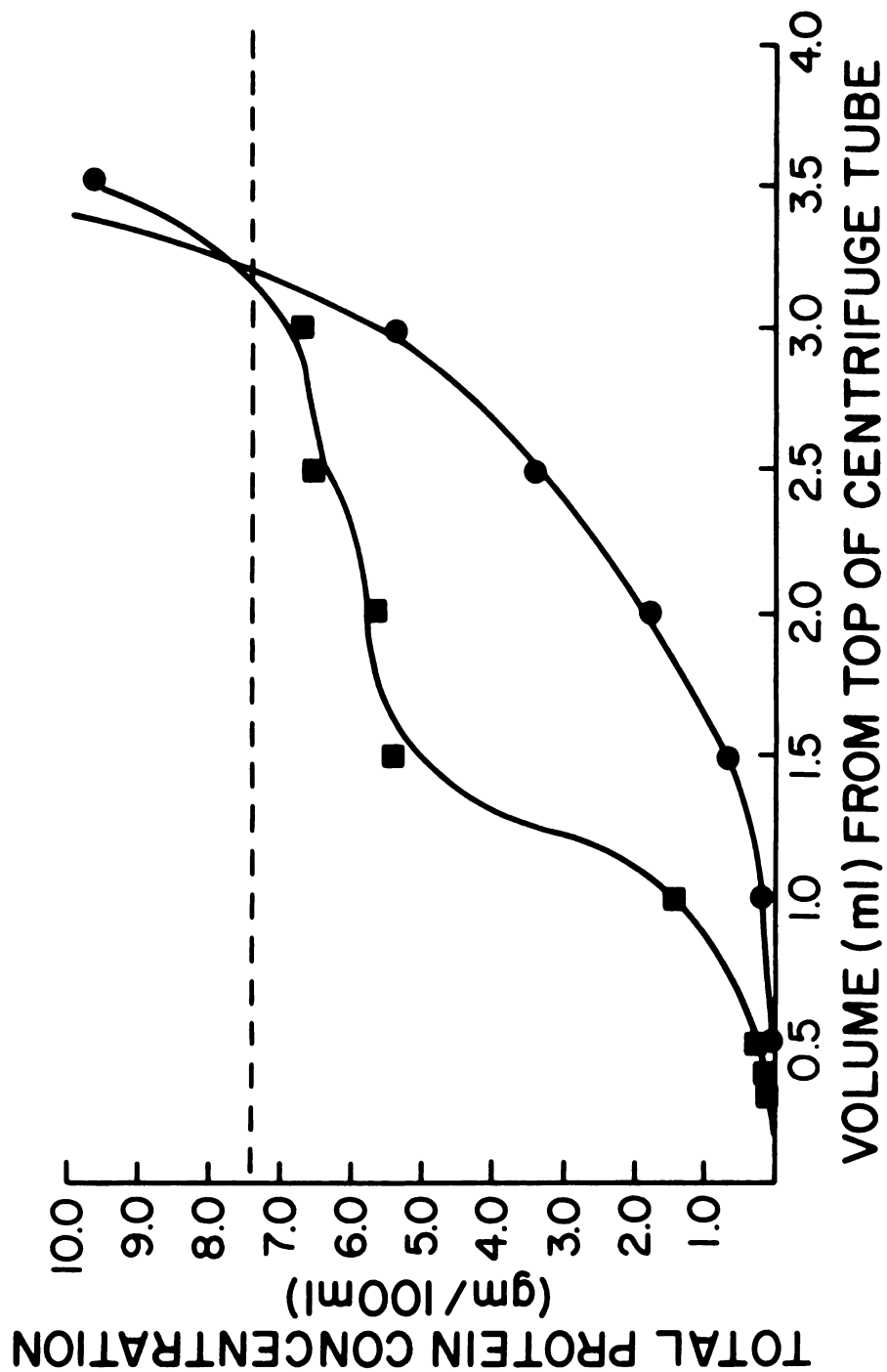
Figure 1. Distribution of protein in centrifuge tube after centrifugation at 37°C and 55,000 rpm. Key: ■, after a 6-hour run; ●, after a 10-hour run; ---, prior to centrifugation.
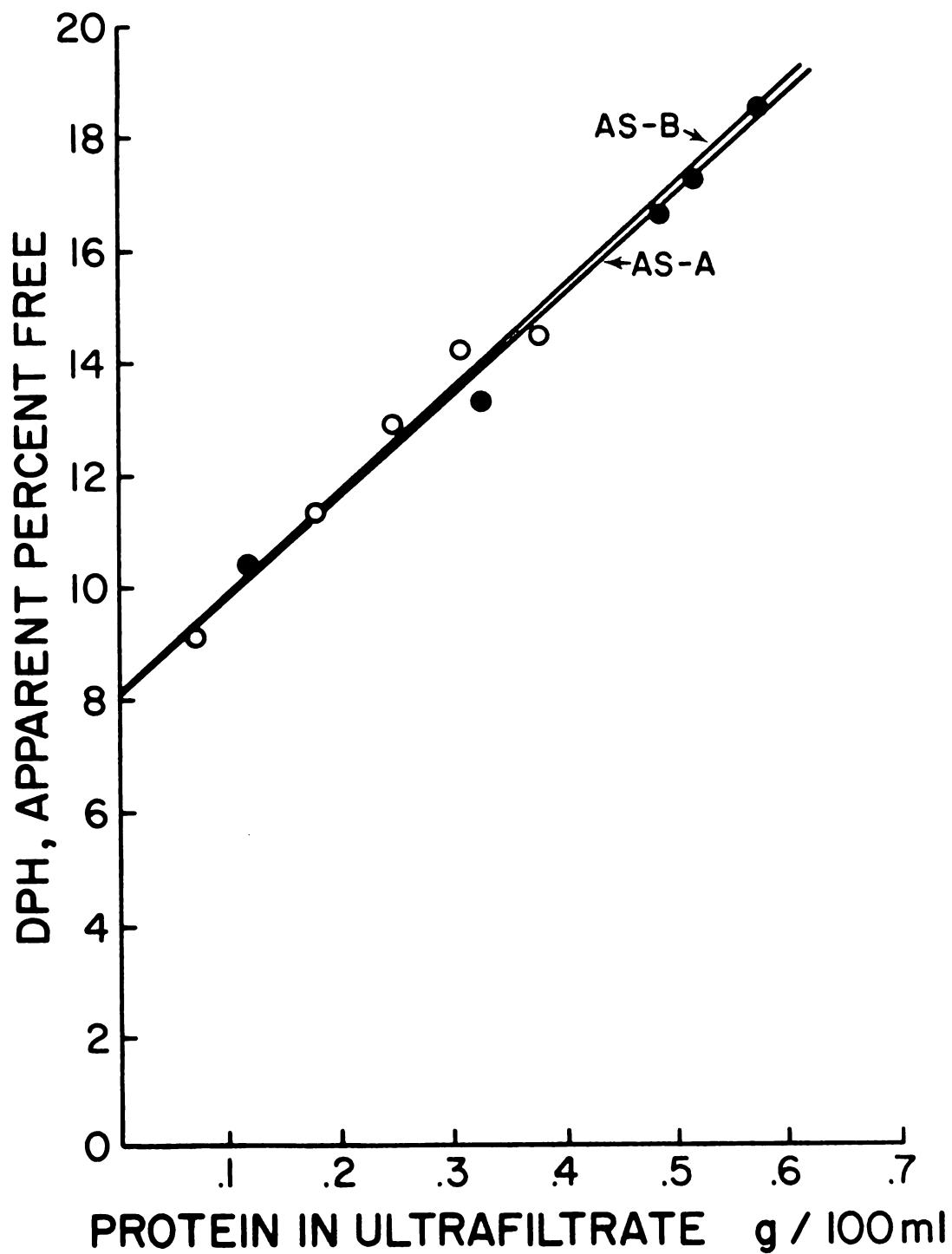
Figure 2. Apparent DPH binding vs. protein in ultrafiltrate.
Sample AS-A & AS-B.
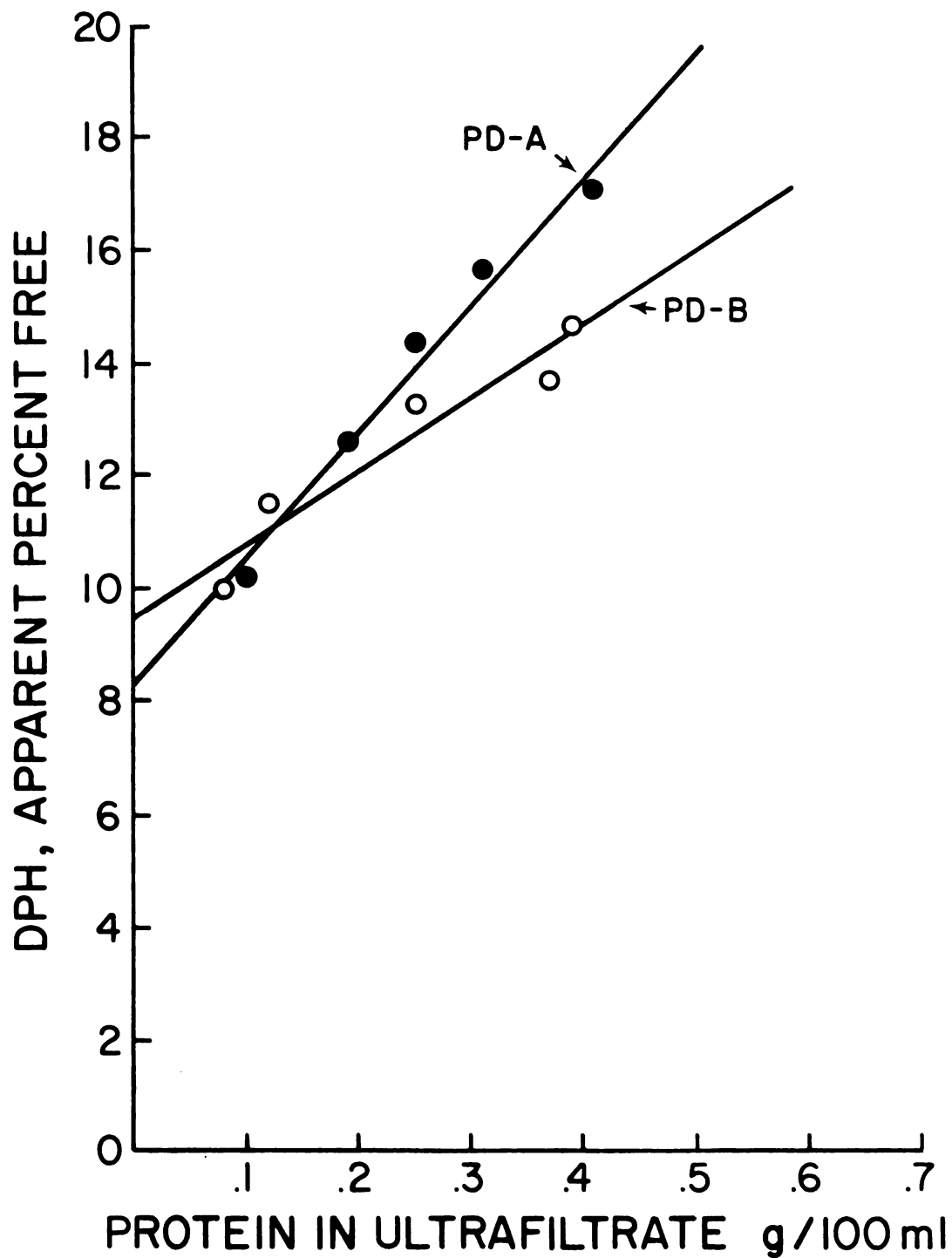
Figure 3. Apparent DPH binding vs. protein in ultrafiltrate. Sample PD-A & PD-B.

CHAPTER VI

PROTEIN BINDING STUDIES OF DIPHENYLHYDANTOIN
IN NORMAL, UREMIC AND OTHER DISEASE STATES

INTRODUCTION

It is well recognized that the degree of plasma protein
binding can influence the distribution and elimination char-
acteristics of a drug from the body. Drug binding is also
very important from a therapeutic point of view as it is
the unbound drug levels that often correlate with a pharma-
cologic or toxic response.

Recent reports (90,94,95) indicate abnormally rapid
elimination of DPH in uremic patients, compared to normals
(18,38). Accelerated elimination of DPH in uremic patients
was first reported in 1970 by Mellk et al. (90). Six patients
had an average half-life of 7.5 hours compared to 15.4 hours
in five normals. In another series of five uremic patients
studied by the same group, Letteri et al. (94) reported an
average elimination half-life of 8.1 ± 1.7 hours after an
intravenous dose of 250 mg. A proposed explanation for the
increased DPH elimination was "uremic" induction of the para-
hydroxylating enzyme systems responsible for the conversion
of DPH to 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH).

A review of the literature on protein binding points to
discrepancies in the extent of DPH binding in human plasma.
One of the earliest studies was done by Triedman et al. (13)

in 1960. Employing an equilibrium dialysis technique, these workers found the average unbound fraction of DPH in the plasma from 10 epileptic patients to be 10.7% with a wide range of 4.1% to 34.1%. Unfortunately, neither the temperature at which dialysis was carried out or the plasma levels of DPH were stated. The concentration of DPH in the cerebrospinal fluid in these patients was also measured; it was reported to be 12% (expressed as percent of plasma concentration) with a range of 1.2% to 34.3%. This corresponded approximately to the free concentration of DPH in the plasma. Subsequent studies have used the concept of DPH levels in the cerebrospinal fluid to estimate unbound levels in the plasma.

In 1961, Loeser (96) showed a log-linear relationship between the fraction bound and albumin concentration. Utilizing the equilibrium dialysis method at unstated temperature and concentration of the drug, he found 23% of the DPH was unbound at albumin concentrations comparable to that of normal serum.

Lunde et al. (92) utilized a modified Scatchard plot to analyze DPH binding data and indicated that several sites of plasma proteins were involved in binding; however, at the therapeutic concentrations only one or two sites seemed to be involved. Rudman (97) examined the binding of DPH in solutions prepared from crystalline human serum albumin in 0.05 M sodium phosphate buffer at pH 7.4. DPH binding could only be studied over a relatively narrow drug concentration range because of limited solubility of DPH. Within the

concentration range examined, the reproducibility of binding measurements did not permit reliable determination of the number of binding sites (n) and the affinity constant (K). However, in replicated experiments, the intercept on the ordinate (nK) had a value of 1.4 x 10^4 with a low coefficient of variation.

In 1968 Ganshorn et al. (98) reported a small inter-individual variation of binding. They found the percent of the unbound DPH in human plasma to be 14.2% $\pm$ 1 in adults and 25.7% $\pm$ 1 in newborns, using equilibrium dialysis in unstated temperature and a total DPH concentration of 50.4 mcg/ml. Lunde et al. (92) in 1970 employed an ultrafiltration technique and a standard concentration of 16 mcg/ml. He found 6.3% $\pm$ 0.4 unbound at room temperature and 10.4% $\pm$ 0.3 at 37°C in five donor samples. When binding was determined by equilibrium dialysis at room temperature in the same plasma samples, the percentage free was found to be 9.3% $\pm$ 0.7. In 1972 the same group (99) reported an average of 6.3% $\pm$ 0.9 unbound DPH in 8 epileptic patients using the ultrafiltration method at room temperature. This corresponded to 10.3% $\pm$ 1.4 unbound at 37°C. The mean DPH plasma concentration of this group of patients was 11.2 $\pm$ 7.5 mcg/ml. Data presented by Conard et al. (100) indicated a 5% unbound fraction of DPH at a total level of 16 mcg/ml, when human plasma was dialyzed at 4°C.

Some uncertainty exists as to the sites of binding to plasma protein. Albumin was suggested by Loeser (96) as the

major carrier protein for DPH. Lightfoot et al. (101)
showed by radioimmunoelectrophoresis that DPH bound to
albumin and two alpha-globulins associated with thyroid
binding globulin. Quantitatively, the amount of thyroid
binding globulin is low compared to albumin (102) (approxi-
mately 0.015 mg/ml vs. 40 mg/ml) and, therefore, probably
only contributes slightly to the overall binding properties
of DPH. Bilirubin, a compound which binds avidly to albumin,
can displace DPH. Rane (103) found improved correlation
between DPH binding in hyperbilirubinemic infants when the
percent unbound was plotted against the ratio of bilirubin
over albumin rather than against bilirubin. In normal infants,
Rane showed an inverse correlation (r = -0.66) between percent
unbound and plasma albumin concentration.

An abnormally high percent of unbound DPH was reported
in one uremic patient by Odar-Cederlof et al. (95). Free
levels of 30% of the total plasma concentration were found,
whereas in normal subjects an average free fraction of 6.8%
was reported (92) using the same methodology.

Subsequently , Reidenberg et al. (104) reported a two
to three-fold increase in the unbound levels of DPH in a
series of fifteen uremics. In vitro dialysis of uremic
plasma did not increase DPH binding. This indicated the
abnormal binding in uremics was not due to dialyzable sub-
stances in the plasma. As one explanation for altered binding
in the uremic plasma, Letteri et al. (94) suggested that the
accumulation of p-HPPH in blood may displace DPH from binding

sites on the carrier proteins.

In some institutions, DPH is given routinely to uremic patients on hemodialysis to prevent seizures that sometimes complicate this treatment. The effect of hemodialysis on the removal of DPH is not clear from the past literature reports. There are no kinetic studies of DPH elimination during hemodialysis. However, there are a few instances reported where hemodialysis has been employed to treat acute DPH poisoning. Schreiner (105) reported clinical improvement when hemodialysis was used in one patient treated for toxic ingestion of DPH, but reliable blood concentrations of the drug were not obtained. Theil (106) reported a drop of plasma DPH levels from 50 to 4 mcg/ml during a six-hour hemodialysis treatment for acute toxic poisoning due to Dilantin and pheno-barbital ingestion, but unfortunately an ambiguous method of assay was used. Despite this reported drop in plasma drug concentrations, there was little clinical improvement and symptoms of DPH toxicity such as nystagmus still persisted. Repeated attempts to measure DPH in the dialysis bath were unsuccessful.

The present investigations include reports of additional studies of the binding characteristics of DPH in plasma obtained from normal subjects, patients with uremia and other patients with a spectrum of disease states.

MATERIALS AND METHODS

Determination of DPH Binding by Ultracentrifugation -
See Chapter V.

Protein Determination - Total protein concentration in
the plasma samples was determined by the biuret method. Albu-
min concentration was determined by electrophoresis on cellu-
lose acetate. A Beckman Microzone Electrophoresis System
#32433 was used. The protein was stained with Ponceau-S and
quantitatively determined on a Clifford densitometer Model #345.

Binding in Artificial Plasma Systems - Binding to human
serum albumin (HSA) was determined in a Krebs Ringer buffer
and a 0.15 M phosphate buffer both at pH 7.4. The human
serum albumin was obtained in a 25% solution from Cutter
Laboratory, Berkeley, California. The purity of the sample
was determined using the electrophoresis noted above. It was
found to be greater than 95% pure. The Krebs Ringer system
was prepared as described by Curry et al. (107). The final
concentrations of HSA in both systems ranged from 2 to 6
gm/100 ml. Binding was also determined in 4% HSA-Krebs Ringer
solution when physiological quantities of other fractions of
plasma proteins were added. Gamma globulin was added in
amounts to produce a concentration range from 0.3 to 1.2
gm/100 ml. Krebs Ringer - 4% HSA systems were also made
containing 1.8 gm/100 ml of Cohn's fraction II + III and
0.6 gm/100 ml Cohn's fraction IV-1. The fractions of plasma
proteins were purchased from the Nutritional Biochemical

Company. They were repurified before use by dialysis, followed by freeze drying.

Subjects - Normal subjects included sixteen male and four female volunteers who were in good health and not taking any medication. Twelve uremic subjects were patients on the dialysis ward at San Francisco General Hospital. All but two patients received no medication 48 hours prior to sample collection. The two patients on medication received only DPH. The binding determinations in other disease states reported were made from samples received from the Metabolic Unit, University of California Medical Center and the Herbert C. Moffitt Hospital, San Francisco, California.

Kinetic Studies - The half-life of DPH in a uremic patient was determined during and after hemodialysis. An IV dose of 6 mg/kg was given by slow injection into the AV shunt for hemodialysis hook-up. Samples were collected hourly during the six-hour dialysis period. A similar experiment was carried out when the patient was off dialysis.

Plasma concentrations of DPH were analyzed by the GLC procedure described in Chapter II. The half-life of DPH on and off hemodialysis was determined by a log-linear least squares fit of the data.

RESULTS AND DISCUSSION

Plasma Protein Binding of DPH in Artificial Plasma

Systems and Normal Subjects - The effect of increasing DPH

concentration on plasma protein binding over the therapeutic

range is shown in Figure 1. There is a linear increase of

unbound DPH from 8.80% at 10 mcg/ml to 9.91% at 40 mcg/ml.

The average increase was 0.35% for each 10 mcg/ml of plasma

concentration. This factor was used to correct the binding

determination of clinical samples to the standard concentra-

tion of 10 mcg/ml.

These data were treated by the method of Scatchard (108)

to determine the binding parameters of DPH in normal plasma.

Calculations of $\bar{v}$ (moles drug bound per mole of protein) were

based upon the albumin content of the plasma. Figure 2 shows

a Scatchard plot $\frac{\bar{v}}{D_f}$ vs. $\bar{v}$. (D_f = concentration of free drug

in moles/liter). The least square fit of the data has a

correlation coefficient of 0.995. The results show an apparent

association constant of 1.17×10^4 liters/mole and 1.35 binding

sites per mole of albumin. Several limitations are intrinsic

in the manner in which the association constant was measured.

First, the concentration range over which binding was measured

was narrow, as it was limited to the therapeutic concentrations

of DPH. The concentration range of DPH in plasma is also

limited because of the low solubility of the drug. Other

limitations of this calculated binding constant include a

four point determination and the assumption that only albumin

is responsible for binding DPH. Despite these various con-
siderations, the calculated binding parameters are in good
agreement with the scant information reported previously in
the literature. Rudman et al. (97) measured the binding of
DPH to crystalline human serum albumin dissolved in a 0.05
sodium phosphate buffer at pH 7.4 by equilibrium dialysis at
25°C. Because the concentration range examined was too narrow
to permit reproducible calculations of n and K, just the
reproducible intercept on the ordinate (nK) of the Scatchard
plot was reported. The value found was 1.4 x 10^4 binding
sites liters/mole. This compares favorably with the ordinate
intercept of 1.58 x 10^4 binding site liters/mole found in
this study. Lunde et al. (92) made a modified Scatchard plot
from binding determinations of DPH in normal human plasma.
Binding parameters were not calculated. The authors only
state that DPH appears to be bound to several sites on plasma
proteins at a concentration of 20 mcg/ml or less, only one or
a few sites seem to be involved in binding.

In the treatment of data obtained at constant total DPH
levels, but variations in total protein, one should consider
the mass law equation:

$$\frac{D_b}{(D_f)(C_{alb})} = K \qquad \text{(Equation 1)}$$

where K = the association constant.

D_b = the equilibrium concentration of the drug albumin
complex.

D_f = the equilibrium concentration of the unbound drug.

C_{alb} = the equilibrium concentration of the umcomplexed albumin.

This equation can be rearranged as follows:

$$\frac{D_b}{D_f} = K \cdot C_{alb} \qquad \text{(Equation 2)}$$

It is important to consider how much albumin would be complexed if all of the DPH were bound in a 1:1 complex. The concentration of DPH was $3.96 \times 10^{-5}M$ and the albumin ranged from $22-87 \times 10^{-5}M$. Assuming all the DPH was found in a 1:1 complex, only 6-18% of the albumin would be complexed. This would mean that 82-94% of the albumin was not involved in the complex. Therefore, as a first approximation, we may presume that $(C_{alb})_{total} = (C_{alb})_{free}$, which means we can express Equation 2 as:

$$\frac{D_b}{D_f} = K(C_{alb})_{total} \qquad \text{(Equation 3)}$$

This is, of course, an equation of a straight line with a zero intercept. Equation 3 is useful in treating data where the drug concentration is held constant and protein concentration is varied.

The results of the HSA studies are shown in Figure 3 as the ratio of the bound to free DPH (as determined by the radio-active-ultracentrifugation method) vs. the total albumin added. The data appears to follow Equation 3. The correlation coefficients for the two sets of data are 0.994 and 0.998. The

slope of the curve can be used to estimate the binding

constant K. It was found to be 0.44×10^4 L/M for the

Krebs-Ringer buffer system and 0.31×10^4 L/M for the phos-

phate system. It is interesting to note that the two buffer

systems result in different binding constants. At 4 gm/100 ml,

the unbound DPH in the phosphate buffer and Krebs Ringer system

is 31.9% and 21.4% respectively. The expected free fraction

in human plasma at this protein concentration would be approx-

imately 10% (see below). This illustrates the problem of

extrapolating the results of binding in artificial systems

to human plasma. The possibility that other protein fractions

of plasma influence DPH binding were considered. Kutz (109)

has presented evidence that the binding properties of albumin

are affected by the presence of other plasma proteins. Several

experiments where various other fractions of plasma protein

were added in physiological amounts to the Krebs Ringer HSA

system did not significantly alter the binding. Cohn's frac-

tion IV-4, which contains some beta and alpha 1 + alpha 2

globulins, could not be investigated because of low solubility

in the HSA-Krebs Ringer system.

The results of binding determinations of DPH in 20 normal

subjects at a standard concentration of 10 mcg/ml is shown in

Table I. The average percent free DPH for this group is

$9.67 \pm 1.25\%$. The average total protein and albumin concen-

tration is 6.75 ± 0.06 and 4.16 ± 0.4 gm/100 ml respectively.

The results of plotting (D_b/D_f)   vs. $(C_{alb})_{total}$ for normal

volunteers are shown as the upper curve of closed circles in

Figure 4. The data for these 20 volunteers can be fitted to a least square line forced through the zero intercept, resulting in an estimate of the mean association constant for normal subjects of 1.5×10^{-4} L/M.

Previous work by Lunde et al. (92) found an average unbound DPH in 15 normal plasmas of 7.4% $\pm$ 0.7 using an ultrafiltration technique at room temperature and a standard DPH concentration of 16 mcg/ml. Correcting this average concentration to 37°C (1.64 x percent free at room temperature) (99) and a standard concentration of 10 mcg/ml (percent free + .035 x (10-16)), the average free level would be 11.9 mcg/ml. This is somewhat higher than the 9.67% average value found in this study. Loeser (96) reported a log-linear relationship between the fraction of DPH bound and albumin concentration found in 3 subjects with a range of albumin concentrations from 1.1 - 6.5 gm/100 ml. Rane et al. (103) reported an inverse linear relationship between the percentage of unbound DPH in newborn infants and plasma albumin with a correlation coefficient of only 0.66. Our studies in normal volunteers showed an intraindividual variation in DPH binding of from 8.46 - 13.5% free drug DPH. This is a larger variation than reported by Lunde (92), but not as great as the range of 4 - 34.1% free DPH reported by Triedman (13). The intraindividual variation in binding in the present work can be generally explained on the basis of differences in albumin concentration. These findings appear to indicate that the law of mass action applies when comparing the binding of DPH in relationship to

albumin concentration for normal subjects. In certain clinical conditions, such as nephrotic syndrome, plasma albumin concentrations can drop to 1 to 2 gm/100 ml. The unbound levels of DPH in these patients can be expected to reach twice that of individuals with normal albumin levels.

<u>Plasma Protein Binding of DPH in Uremic Patients</u> - The results of binding studies in 12 uremic subjects are shown in Table II. The average unbound DPH level was 26.44 $\pm$ 5.67%. The average total protein and albumin concentrations were 6.56 $\pm$ 1.03 and 3.43 $\pm$ 0.83 gm/100 ml respectively. Figure 4 also includes a plot for the data obtained from uremic plasma samples and is shown as the open circles in the bottom portion of the figure. Once again the data can be fit to a least square line forced through the zero intercept, resulting in a mean association constant for the uremics of 0.57 x 10^{+4} L/M. Clearly, there is a qualitative change in the binding. This is in agreement with Reidenberg <u>et al</u>. (104), who found a two to three-fold decreased ability for patients with poor renal function to bind DPH. These authors observed only a weak correlation between serum albumin or total protein to decreased binding. However, they found a strong correlation ($r = 0.8$, $P < 0.001$ binding to serum creatinine). This correlation included five nonuremic subjects, eleven uremics who were not dialyzed, and four uremics who were dialyzed. The present study shows the opposite results - a strong correlation between albumin concentration $r = 0.805$ and a weak correlation to serum creatinine $r = -0.355$ for the 9 patients where this information

was available. However, the uremic patients all were dialyzed twice weekly, thus creatinine levels between dialysis procedures may not necessarily reflect each steady state to the true degree of renal impairment.

General attempts were made to determine the nature of the binding changes in the uremic patients. The binding of several samples was determined before and after hemodialysis treatment. No change in binding occurred. This agrees with the findings of Reidenberg et al. (104) which has subsequently been published showing no change in DPH binding in three uremic plasma samples after in vitro dialysis in hemodialysis bath fluid. Recently Shoeman and Azarnoff (110) have isolated albumin from uremic patients by curtain electrophoresis and determined the association constant in 0.05 M phosphate buffer, pH 7.4. The mean value was 0.280×10^4 L/M, while the binding constant for albumin from normal subjects prepared in an identical manner was 0.598×10^4 L/M.

Letteri et al. (94) have suggested that the accumulation of the HPPH in the uremic plasma may be displacing DPH from binding sites on the carrier proteins. This possibility was examined by determining DPH binding in uremic plasma from patients who never received the drug and, therefore, had no metabolites present (10 of the 12 uremic patients studied). All subjects studied in this way showed free levels two to four times greater than the normal subjects. These findings suggest that displacement of DPH from plasma proteins by HPPH is not responsible for the altered DPH binding in the uremic

patient.

Uremic subjects are actually anemic and require blood transfusions. Typical values for the hematocrit for uremics may be 15-30% (46% is a normal value) and their blood urea nitrogen (pre-dialysis) is in the order of 70-90 mg% (13 mg% is a normal value). Many other pathological conditions exist in uremic subjects. These have recently been reviewed in a symposium on uremic toxins (111). Protein electrophoresis of plasma from uremics shows mild to marked hypoalbuminemia and some depression of total proteins. Because of the anemic state of the uremic subjects, they require frequent transfusions. Our studies indicate that both uremic and normal subjects show predictable changes in binding with changes in HSA content of the plasma in accordance with the law of mass action. At 4% HSA and 10 mcg/ml DPH the uremic plasma will bind DPH to approximately 70% while in normal plasma DPH is bound to 90%. Note that 86-95% of the albumin molecules are not involved in the drug protein complex at this drug level. The data plotted in Figure 4 leads to a smaller mean binding constant in the uremic subjects (0.57×10^4 L/M) than in the normal subjects (1.5×10^4 L/M). In order for the observed change in DPH binding to be due to a modification of the structure of the albumin molecules, extensive conversion of the albumin to a different chemical and/or physical form with reduced binding characteristics would have to have taken place. Unless virtually all the molecules of albumin are changed to this new form, a mixture of "normal and modified" albumin

would be present in the binding studies and should have led to results indicating two different types of proteins (or binding sites) of markedly different affinities. Also, electrophoresis would probably show two different albumin peaks. One would still have to reason that albumin with the stronger binding affinity would take precedence in reacting with the 10-15 mole percent of DPH (relative to moles of albumin) available to bind with the albumin.

While there are many diverse changes which take place within the plasma of uremics, all studies to date appear to indicate that plasma of uremic subjects shows similar binding properties to that of the controls. Anton and Corey (112) reported on the binding characteristics of the anephric patients with sulfamethazine. The anephric (uremic) subjects showed similar numbers of binding sites as did the normal control subjects. However, the percent of binding of the sulfa drug was greatly reduced. The normal controls showed a mean of 77% while the anephric patients showed 36-68% bound sulfa when studied post-dialysis. These results are compatible with a postulate that an undialyzable or poorly dialyzable compound competes for binding. Plasma nonesterified fatty acids (NEFA) have high affinity for albumin as reported by Rudman (97) and may act as an inhibitor of DPH binding. Another potential competitive binding inhibitor of DPH is bilirubin. Bilirubin is tightly bound to albumin. Rane et al. (103) have demonstrated a positive correlation of unbound DPH and the quotient between total plasma bilirubin

and the albumin concentrations. Whether abnormal binding
of DPH in uremic patients can be partially or completely
attributed to competitive binding of an undialyzable or
poorly dialyzable compound awaits further studies.

Random Screening of Various Metabolic Diseases for

Abnormal Binding - The binding of ten patients with various
metabolic disorders is shown on Figure 5 and tabulated on
Table III. Four of these patients had binding which was
higher than the established range. Of these four patients,
two were clinically classified as obese, one was diabetic,
and the fourth was hypoglycemic. Two of the ten patients,
one diabetic and one with multiple endocrine adenomas, had
plasma protein binding that fell below the established normal
range. The latter patient exhibited free levels of DPH 50%
greater than the expected value for this patient's albumin
concentration, and 100% greater than the average binding of
9.67% established for normal subjects.

Four patients with idiopathic epilepsy all exhibited
expected binding for their serum albumin level. However, the
serum albumin concentration in two patients was below 3.00
gm/100 ml, and the unbound fraction was greater than 13.50%
(Table III). Lund et al. (99) have reported that DPH binding
in five epileptic patients did not differ from their normal
controls.

The binding results of three patients with nephrotic
syndrome are also shown in Figure 5. The criteria for diag-
nosis of the nephrotic syndrome includes proteinuria (over

3.5 gm/24 hours) and hypoalbuminenia. Some patients with

chronic nephrosis may eventually become uremic. Two of

the three nephrotic patients show DPH binding which appears

between the normal and uremic regression lines, possibly a

transition stage in protein alternation. The third nephrotic

falls into the uremic binding group.

DPH Half-Life of a Uremic Patient on and off Hemodialysis -

The plasma concentration curves on and off dialysis of a patient

given 6 mg/kg DPH by IV injection are shown in Figure 6. The

unbound DPH level in this patient was 35.5% compared to 9.67%

for normals. The serum albumin concentration was 2.10 gm/100 ml.

DPH plasma concentrations were determined hourly for six hours

during a hemodialysis treatment and during a control run off

dialysis. The half-life of DPH in the plasma was 9.2 hours

off dialysis and 13.5 hours during dialysis. These results

indicate that DPH removal from the body is not accelerated

by hemodialysis treatment. An unexplicable feature of this

data is that the half-life appears to be shorter off dialysis.

Whether this is real or due to experimental error cannot be

determined from this data. Ideally, for an accurate assess-

ment of half-life, the plasma should be sampled over two to

three half-lives. In these experiments, samples were collected

over a six-hour period - or approximately one-half of the half-

life. The log-linear least square fit of the data from the

off dialysis run had a correlation coefficient of 0.93 com-

pared to the on dialysis run of 0.98. The greater scatter

of the control run was probably due to the fact that blood

samples were drawn through the same A-V shunt tubing into which the IV injection was given and, therefore, may have been contaminated. Blood samples were not drawn from the site of injection for the on dialysis study.

The findings of these half-life studies are in conflict with the findings of Theil (106) who reported a drop of plasma DPH concentration from 50 to 4 mcg/ml during the course of the six-hour hemodialysis treatment. This would be consistent with a 1.4 hour half-life during dialysis. In that study no drug could be recovered in the dialysate, and signs of DPH toxicity still persisted despite the low plasma level reported post dialysis. Although the assay procedure used to determine DPH was not referenced, it is presumed to have been a colorimetric procedure commonly used at that time, one that is especially unreliable in the presence of phenobarbital. Additional well controlled half-life studies should be carried out to verify this initial finding that DPH removal is apparently not accelerated by hemodialysis treatment.

Volume of Distribution of DPH in the Uremic Patient - DPH elimination apparently follows Michaelis-Menton kinetics, and, therefore, calculations of volume of distribution and half-life based on first order kinetics are somewhat inappropriate. However, the presumption of first order kinetics will suffice for comparison purposes. The volume of distribution of a uremic patient (Figure 6) was determined by the extrapolation method:

$$Vd = \frac{D}{Cp^o} \qquad \text{(Equation 4)}$$

where Vd = volume of distribution

Cp^o = plasma concentration extrapolated to zero time.

and by the clearance method:

$$Vd = \frac{D}{K(AUC)} \qquad \text{(Equation 5)}$$

where K is first order elimination rate constant and AUC is
the area under the plasma concentration-time curve which is
evaluated by the trapezoidal rule method from t = 0 to t =∞.
Corrections were made for the drug present in the plasma
prior to the IV dose. The calculated volume of distribution
from the on dialysis experiment was 83.8 and 87.3 liters by
extrapolation and clearance respectively. The volume of
distribution from the off dialysis run was 77.4 and 78.4
liters calculated by the extrapolation and clearance method
respectively. The average volume of distribution by the
clearance method is 1.18 liters/kg for this patient. The
volume of distribution determined by the same method for
four normal subjects (Chapter IV) ranged from 0.468 to 0.548
liters/kg. An increase in the volume of distribution is
expected when there is an increase in the fraction of un-
bound drug in plasma. The expected increase in apparent
volume of distribution can be estimated by considering both
the change in the unbound plasma protein fraction and the
change in the drug plasma water concentrations in equilibrium
with tissue water. The magnitude of the latter factor may be

approximated by considering shift of drug from plasma

proteins to tissues when binding is altered. The percent

of drug in the total body bound to plasma proteins can be:

% drug in body bound to plasma proteins = $\frac{3\beta}{Vd}$ x 100

where 3 is the plasma volume in liters and β is the fraction

of drug in plasma bound to plasma proteins. For normal sub-

jects approximately 8.2% of the drug will reside on the plasma

proteins. In this uremic patient the unbound fraction in

plasma was 35%. The estimated drug bound to plasma proteins

was 2.4%. This implies an additional 5-6% of the dose is in

the tissues of this patient. This shift would only be expected

to have a slight increase in the unbound concentration of DPH

in the tissue at any given dose. Consider the 425 mg IV dose

of DPH given to the uremic patient in the dialysis study.

The initial total and unbound concentrations expected after

a similar dose in a normal individual of the same weight and

drug distribution characteristics (Vd = 35L) and normal binding

(9.6% free) would be 12.1 and 1.16 mcg/ml respectively. The

expected total concentration in a patient with 35% unbound

levels would be 3.3 mcg/ml based on the approximation that

free levels would be unchanged. This total concentration

would give a calculated volume of distribution which would

be 129 L. However, the experimental volume of distribution

(80L) was less than expected. The initial free plasma concen-

tration of DPH in the uremic was 1.95 mcg/ml instead of the

estimated 1.16 mcg/ml. A decrease in tissue binding could

explain the higher free concentrations and the smaller than expected increase in the volume of distribution in this uremic patient. Little is known about the factors involved in the tissue binding of DPH, but it is not unreasonable to expect that a factor which would alter the plasma binding of a drug would also affect tissue binding.

The findings by Letteri et al. (94) show that uremic plasma levels of DPH were lower than normal after chronic administration of the drug. This is readily explained by the increased volume of distribution. Letteri's data shows that the extrapolated concentration of DPH to time = 0 in five uremics was 3.48 mcg/ml compared to 7.49 mcg/ml for three normals receiving the same 250 mg IV dose. This data also shows that the volume of distribution has more than doubled in the uremic patients.

Implications of Altered Protein Binding of DPH - The pharmacological and toxic activity of DPH is probably a function of the unbound concentration in plasma and tissue water. Viukari and Tammisto (113) have suggested that individual variation of DPH binding might explain an occasional discrepancy between serum levels and expected therapeutic or toxic effects of the drug. Although the exact clinical importance of altered binding is not conclusive, experience by Odar-Cederlof (95) shows adequate seizure control at plasma concentrations well below the accepted therapeutic levels of 10 to 20 mcg/ml. One uremic patient investigated in these studies had well controlled focal seizures despite

a low DPH plasma concentration of 3 mcg/ml. The patient

was found to have an unbound fraction of 35.48% as compared

to the 9.67% average for the normal subjects of this study.

This would mean that the free DPH plasma level was 1.1 mcg/ml,

which would be the equivalent of a free level resultant from

a total plasma concentration of 11 mcg/ml with normal binding.

A recent report from the Boston Collaborative Drug Surveil-

lance Program (114) has shown an increased DPH toxicity in

patients with low serum albumin levels. This supports the

contention that therapeutic success of toxicity of DPH will

most likely correlate best with unbound drug concentrations.

Letteri et al. (94) have reported accelerated metabolism

of DPH in 5 uremic subjects. This finding would be expected

if free DPH concentrations are elevated, assuming that hepatic

metabolism is dependent on the unbound drug fraction.

There is possible reason to question the half-life deter-

minations made by Letteri and the conclusion that uremics have

an accelerated metabolism of DPH. The small variation of half-

life for the three normals, 13.0 ± 0.15 hours, is not consis-

tent with the findings of others. Arnold and Gerber (38)

reported a mean plasma half-life of 22.0 ± 9.0 hours for 70

normal volunteers. Glazko et al. (18) reported a mean of 15

hours (range 11 to 29 hours) for five normal adults given

250 mg IV in the same manner as Letteri. The half-life after

a similar IV dose in four normal volunteers (Chapter IV)

showed an average half-life of 18.8 hours with a range of

8.8 to 31.0 hours. The five uremic patients studied by

Letteri and co-workers showed an average half-life of 8.1 ± 1.7 hours. In that study the range of concentrations for all subjects fell between approximately 2.8 and 0.2 mcg/ml. The colorimetric assay procedure (55) used to determine DPH concentrations is probably not reliable at such low levels. Individual data for the uremic half-life studies was not published, and, therefore, it is not possible to determine how well the plasma concentrations followed the calculated exponential decline. Additional DPH half-life studies should be made in uremic patients to clearly establish if drug elimination is accelerated. A more sensitive assay procedure at low levels should be used. Because of the large variability of DPH half-life in normals, a larger sample of uremic patients should be studied.

Finally, one needs to examine if any correlation exists between DPH half-life and plasma protein binding. Table IV summarizes the finding half-life and protein binding data of four normal subjects (Chapter IV) after the administered 6 mg/kg DPH. The half-life listed was taken from the terminal log linear portion of the plasma concentration-time curve. The lack of correlation is obvious.

SUMMARY AND CONCLUSIONS

The average unbound fraction of DPH in the plasma of twenty normal drug-free volunteers is found to be 9.67 ± 1.25 of the total standard concentration of 10 mcg/ml. Binding determinations were made by an ultracentrifugation technique at 37°C. The calculated mean association constant for the normal subjects was 1.5 x 10^{-4} L/M. An inverse relationship with a correlation coefficient of -0.903 is observed between the unbound fraction of DPH and the plasma albumin concentration. In normal subjects the range of the unbound fraction (8.46% to 13.55%) is greater than recent reports. There is no correlation between the widely varying half-lives of the dose dependent studies and the degree of plasma DPH binding in these individuals. The free fraction of drug increases an average of 0.35% for each 10 mcg/ml increase in concentration over the range of 10 to 40 mcg/ml. The binding of DPH to HSA in Krebs Ringer solution or phosphate buffer does not compare to the binding in human plasma. The addition of other plasma protein fractions does not enhance the binding in the Krebs Ringer system.

The average free fraction of 26.44% ± 5.67 is found for twelve uremic patients. These patients exhibited an unbound fraction that ranged two to four times higher than the normal average. The mean association constant was estimated to be 0.57 x 10^{-4} L/M. The unbound fraction is inversely related to plasma albumin concentration with a correlation coefficient

of -0.805. Accumulation of p-HPPH in uremics is shown not to be responsible for altered binding. Hemodialysis treatment does not increase the ability for uremic plasma to bind DPH. Altered binding in the uremics may be due to qualitative changes in drug binding protein or to competitive binding by nondialyzable substances. The binding in two of three nephrotics falls between the normal and uremic regression lines. This may represent a transition stage in protein alteration.

The volume of distribution of DPH in uremics appears to be double that of normals, which would account for the depressed plasma concentrations of DPH seen in these patients. Hemodialysis does not appear to accelerate the removal of DPH.

Drug binding is increased in two of three obese patients. One patient with multiple endocrine adenomas has a markedly depressed binding. Four epileptics showed normal binding for their albumin concentration, although two of these patients had elevated binding because of albumin levels below 3.0 mg/ 100 ml.

DPH binding may be depressed by two mechanisms:

(1) an excessively low plasma albumin concentration with normal binding ability, and

(2) alteration of the binding ability of albumin.

In the former case, if plasma albumin levels fall below 2.5 gm/100 ml, the free DPH levels should be expected to be elevated by 50% or more. This may occur in many disease states. The latter case is exemplified by the uremic patient

where there is depressed DPH binding with normal albumin levels. Random screening of patients with various metabolic disorders indicates other disease states may also affect the ability for albumin to bind DPH.

The clinical implications of the increased free fraction of DPH must be considered in conjunction with the volume of distribution of the drug. In the case of the uremic patients there is an increased volume of distribution concomitant with the increased free fraction of drug. At a given dose, total DPH levels in the uremic will be decreased compared to the normal individual. However, it was seen in one uremic subject that the free concentration of the drug was elevated considerably more than expected by decreased plasma binding. This implies that tissue binding sites may also be affected by the uremic state. Dosage adjustments may be necessary in the uremic patient. Additional clinical observation must be made before any definite conclusion can be drawn on this point. However, when DPH plasma protein binding is altered by either low protein or the uremic state, the therapeutic concentration range probably should be considered in terms of unbound rather than total drug levels.

TABLE I

DPH Binding in Plasma of Twenty Normal Subjects

Subject	Total Protein gm/100 ml	Albumin gm/100 ml	DPH % Free
RR	7.10	4.45	9.03
MM	7.40	4.26	8.75
JO	7.60	4.70	8.67
WM	7.40	4.33	8.50
CM	7.30	4.23	8.87
RB	7.30	4.73	8.75
DA	6.33	4.36	9.73
SA	6.76	3.90	10.13
PA	6.72	4.15	9.22
RW	7.08	4.24	9.47
BV	6.84	4.29	8.29
MC	5.30	2.92	13.55
NI	6.20	3.96	10.74
SW	6.79	4.28	9.22
DV	6.03	4.22	10.04
RU	6.40	3.88	11.34
AS	7.29	4.47	8.46
FG	6.80	4.18	9.75
HO	6.40	3.87	10.20
BA	6.48	3.86	10.63
mean and S.D.	6.78 ± 0.567	4.16 ± 0.385	9.67 ± 1.25

TABLE II

DPH Binding in Twelve Uremic Patients

Subject	total protein gm/100 ml	albumin gm/100 ml	DPH % free	Serum Creatinine mg/100 ml
ME	5.12	2.10	35.48	-----
CS	7.20	4.00	25.18	-----
WM	6.29	3.69	22.15	17.2
PN	7.37	4.31	26.96	14.0
ST	6.84	3.04	29.11	10.8
LM	6.29	3.70	17.92	8.3
DH	7.22	3.79	23.62	7.0
JS	7.74	3.89	26.51	15.0
SM	6.57	3.55	29.12	11.3
AS	7.50	4.11	20.31	7.3
LS	6.42	3.41	23.92	12.0
TN	4.20	1.55	37.09	-----

mean and S.D. 6.56 ± 1.03 3.43 ± 0.837 26.44 ± 5.67

TABLE III

DPH Binding in Plasma of Patients with Various
Metabolic and Neurological Disorders

Subject	Diagnosis	Total Protein gm/100 ml	Albumin Conc. gm/100 ml	DPH %.free
BK	diabetes	6.00	3.00	10.83
MU	diabetes	6.29	4.08	11.94
CO	diabetes	6.72	3.90	10.19
EV	hypoglycemia	6.09	3.65	11.30
PS	hypoglycemia	5.82	3.61	9.10
EF	insulinomas	6.08	3.61	11.22
CP	multiple endo-crine adenomas	7.30	3.27	17.85
AM	obesity	5.95	3.54	9.21
BC	obesity	6.29	3.64	11.30
BA	obesity	6.48	3.68	9.39
NAA	idiopathic epilepsy	6.42	2.96	14.39
GZ	idiopathic epilepsy	6.69	4.17	10.54
NM	idiopathic epilepsy	6.42	2.70	13.53
HG	idiopathic epilepsy	5.15	3.45	11.04

TABLE IV

DPH Half-Life and Plasma Protein Binding
in Four Normal Volunteers

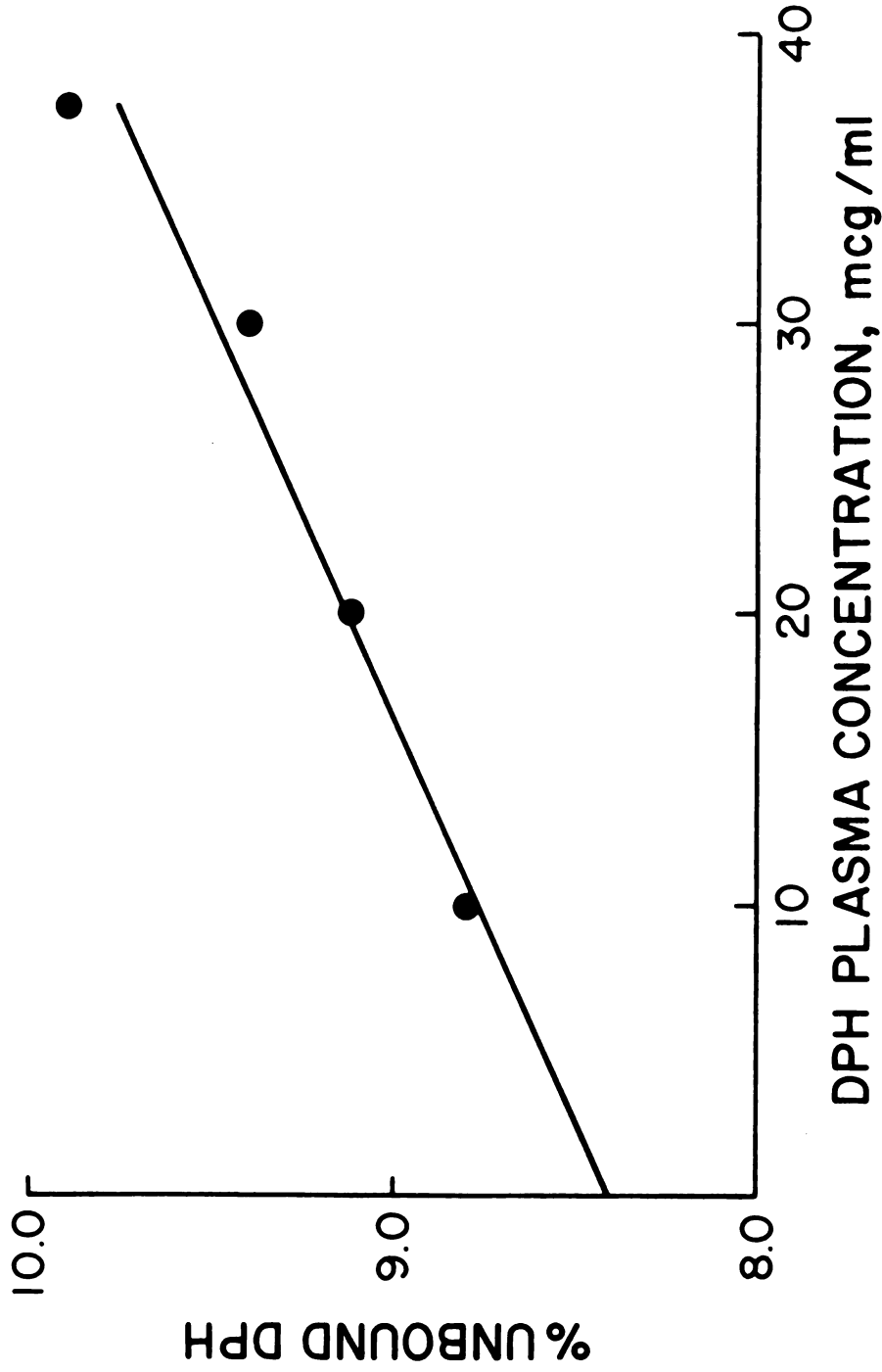| Subject	DPH % Free	Half-Life* (hours)
RR	9.03	13.3
MM	8.75	21.4
WM	8.50	14.8
JO	8.67	32.5

*after IV infusion of 6 mg/kg

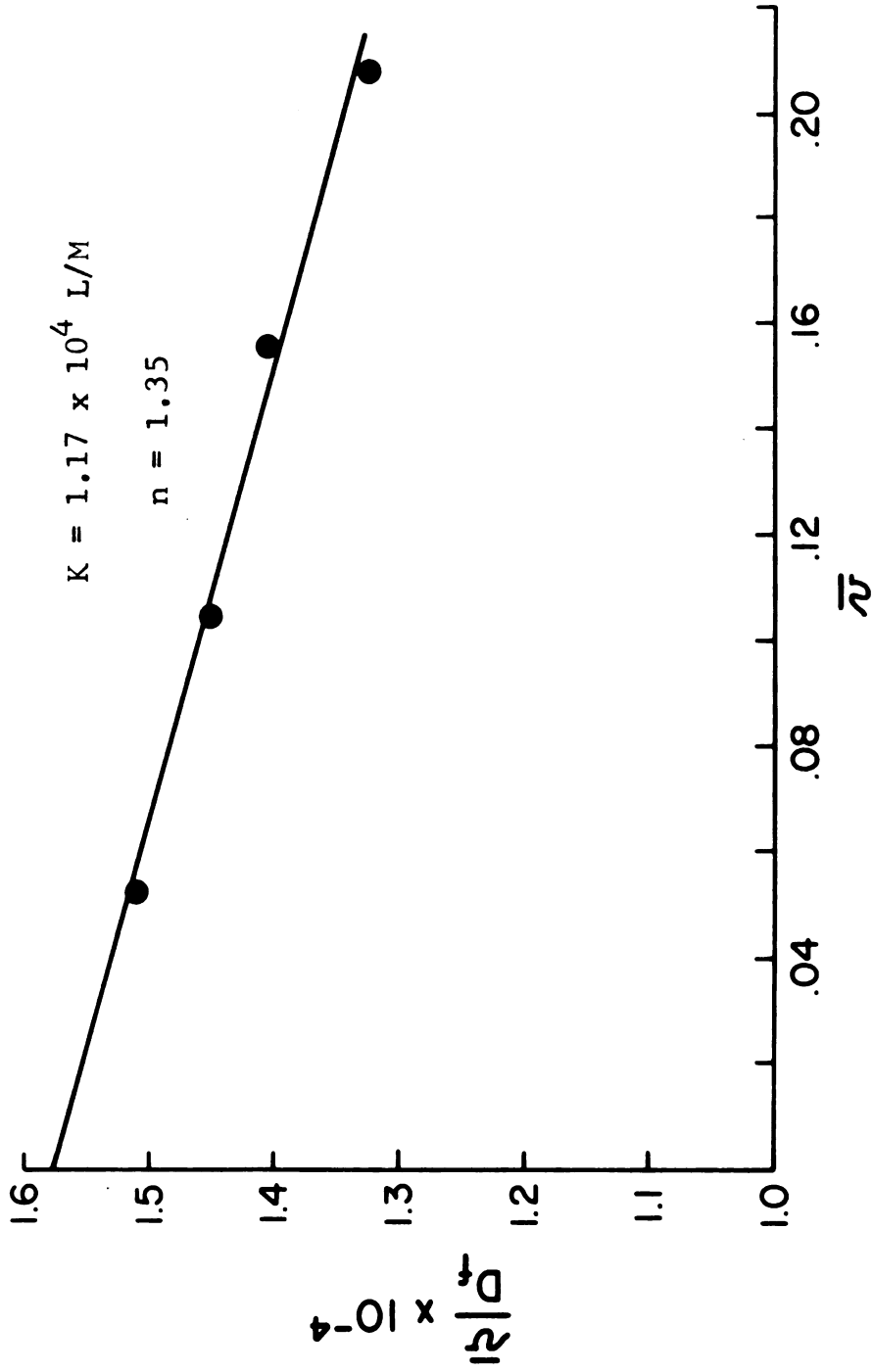Figure 1. Effect of DPH concentration on binding in normal plasma.

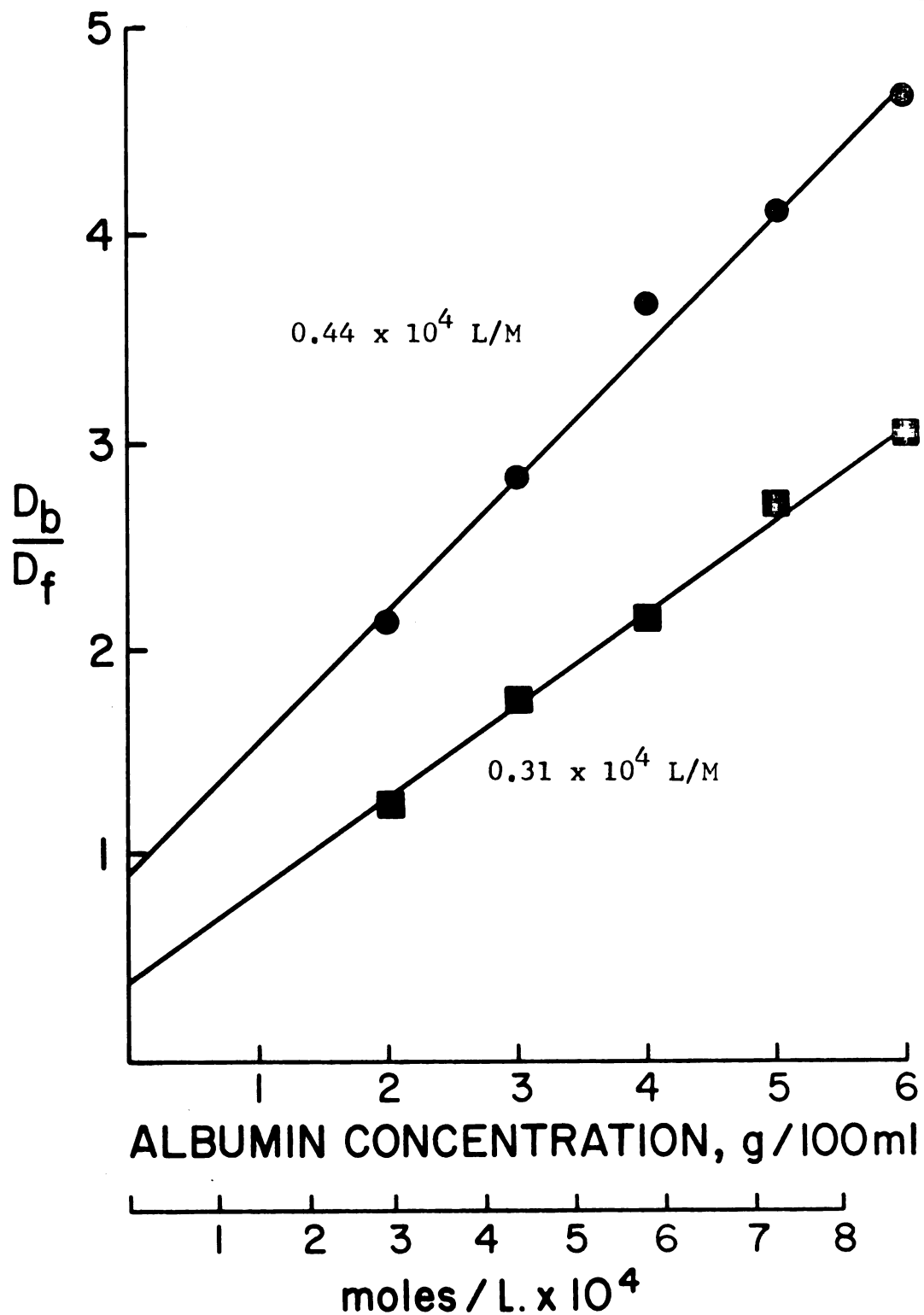Figure 2. Scatchard plot for binding of DPH to normal plasma.

Figure 3. Binding of DPH in purified human serum albumin systems.
● Krebs-Ringer buffer
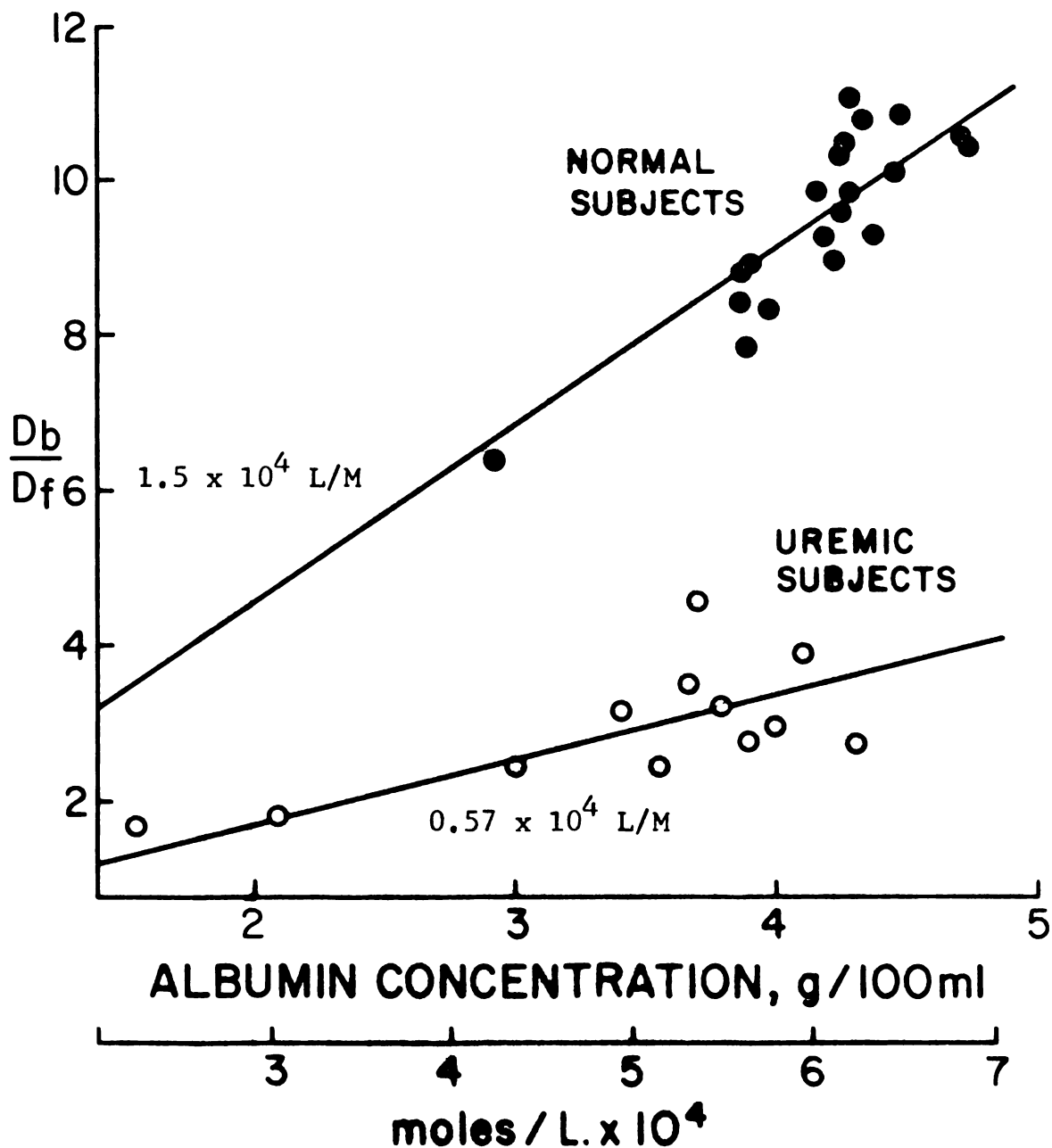■ .15 M phosphate buffer pH 7.4

Figure 4. Binding of DPH in 20 normal subjects (●) and 12 uremic patients (O). Solid lines represent least square fit forced through the zero intercept for each set of data.
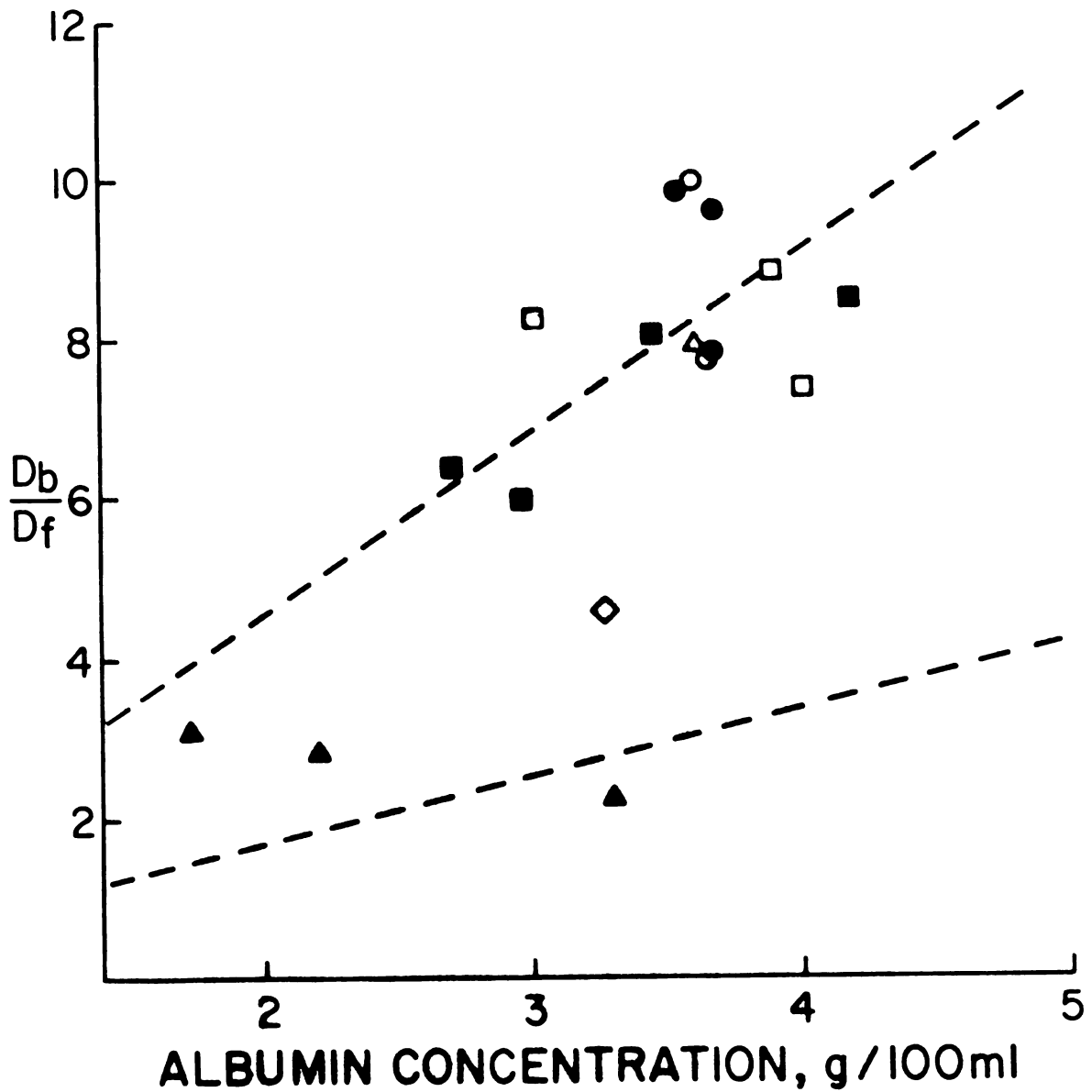
Figure 5. Binding of DPH in patients with various disorders.
Key: □, diabetes; O, hypoglycemia; △, insulinoma;
◇, multiple endocrine adenomas; ●, obesity; ■, idio-
pathic epilepsy; ▲, chronic nephrosis. The upper and
lower dashed lines represent the least square fit forced
through the zero intercept for normal and uremic subjects
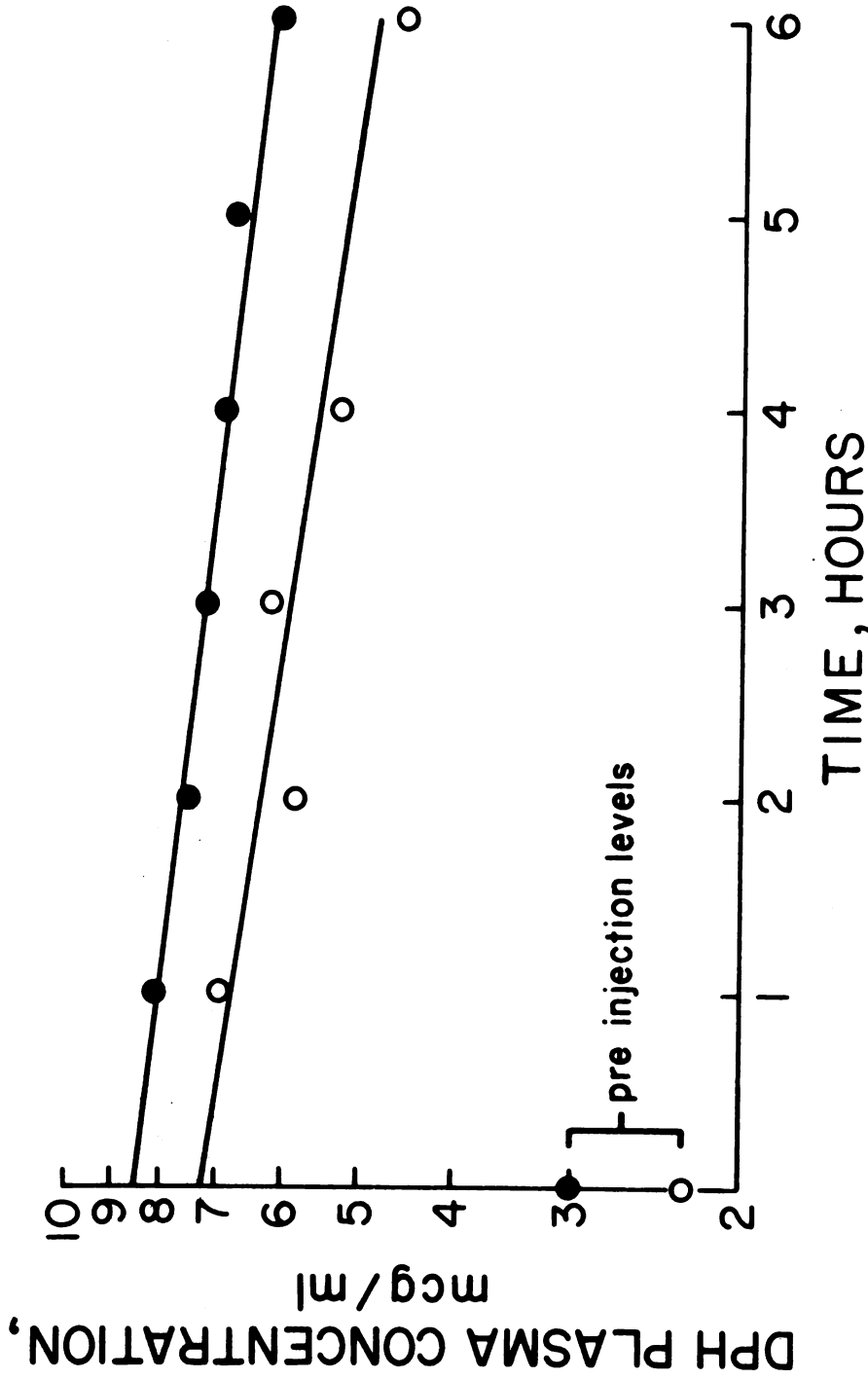respectively (see Figure 4).

Figure 6. DPH half-life in a uremic patient on and off dialysis.
● off dialysis $t_{1/2}$ = 9.2 hours.
○ on dialysis $t_{1/2}$ = 13.5 hours.

REFERENCES

1. Putnam, T. J., and Meritt, H. H., Science 85, 525 (1937).

2. Kutt, H., Haynes, J., and McDowell, F., Arch. Neurol. (Chicago) 14, 489 (1966).

3. Agarwal, S. P., and Blake, M. I., J. Pharm. Sci. 57, 1434 (1968).

4. Dill, W. A., Kazenko, A., Wolf, L. M., and Glazko, A. J., J. Pharmacol. Exp. Therap. 118, 270 (1956).

5. O'Malley, W. E., Denckla, M. B., and O'Doherty, D.S., Trans. Amer. Neurol. Assoc. 94, 318 (1969)

6. Glazko, A. J. "Bioavailability of Drugs" Brodie, B. B., and Heller, W. M. (Eds.), S. Karger, New York, 1972, pp. 163-177.

7. Riegelman, S., Drug Information Bulletin, 3, (1969).

8. Rail, L., Med. J. Aust., 2, 339 (1968).

9. Eadie, M. J., Med. J. Aust., 2, 515 (1968).

10. Suzuki, T., Saitoh, Y., and Nishihara, K., Chem. Pharm. Bull. 18, 405 (1970).

11. Noach, E. L., Woodbury, D. M., and Goodman, L. S., J. Pharmacol. Exp. Therap. 122, 301 (1958).

12. Nakamura, K., Masuda, Y., Nakatsuji, K., and Hiroka, T., Naunyn-Schmiedebergs Arch. Pharmak. u. Path. 254, 406 (1966).

13. Triedman, H. M., Fishman, R. A., and Yahr, M. D., Trans. Am. Neurol. Assoc. 85, 166 (1960).

14. Mirkin, B. L., J. Pediatr. 78, 329 (1971).

15. Kutt, H., Wolk, M., Scherman, R., and McDowell, F., Neurology (Minneap.) 14, 542 (1964).

16. Maynert, E. W., J. Pharmacol. Exp. Therap. 130, 275 (1960).

17. Panalaks, T., Clin. Chim. Acta, 8, 968 (1963).

18. Glazko, A. J., Chang, T., Baukema, J., Dill, W. A., Goulet, J. R., and Buchanan, R. A., Clin. Pharmacol. Ther. 10, 498 (1969).

19. Butler, T. C., J. Pharmacol. Exp. Therap. 119, 1 (1957).

20. Chang, T., Okerholm, R. A., and Glazko, A., Res. Commu. Chem. Path. Pharmacol. 4, 13 (1972).

21. Chang, T., Savory, A., Glazko, A. J. Biochem. Biophys. Res. Commun. 38, 444 (1970).

22. Horning, M. G., Stratton, C., Wilson, A., Horning, E. C., and Hill, R. M., Analyt. Letters 4, 537 (1971).

23. Atkinson, A. J., Jr., MacGee, J., Strong, J., Garteiz, D., and Gaffney, T. E., Biochem. Pharmacol. 19, 2483 (1970).

24. Borga, O., Garle, M., and Gutova, M., Pharmacology 7, 129 (1972).

25. Kutt, H., McDowell, F., J.A.M.A. 203, 969 (1968).

26. Gerber, N., Weller, W. L., Lynn, R., Rangno, R. E., Sweetman, B. J., and Bush, M. T., J. Pharmacol. Exp. Ther. 178, 567 (1971).

27. Hansen, J. M., Kristensen, M., Skovsted, L., and Christensen, L. K., Lancet 2, 265 (1966).

28. Kutt, H., Winters, W., and McDowell, F. H., Neurology (Minneap.) 16, 594 (1966).

29. Kutt, H., Brennan, R., Dehejia, H., and Verebely, K., Am. Rev. Resp. Dis. 101, 377 (1970).

30. Solomon, H. M., and Schrogie, J. J., Clin. Pharmacol. Ther. 8, 554 (1967).

31. Olesen, O. V., Arch. Neurol. 16, 642 (1967).

32. Christensen, L. K., and Skovsted, L., Lancet 2, 1397 (1969).

33. Hansen, J. M., Kristensen, M., and Skovested, L., Epilepsia 9, 17 (1968).

34. Hansen, J. M., Siersboek-Nielsen, K., and Skovsted, L., Clin. Pharmacol. Ther. 12, 539 (1971).

35. Kristensen, M., Hansen, J. M., and Skovsted, L., Acta Med. Scand. 185, 347 (1969).

36. Diamond, W. D., and Buchanan, R. A., J. Clin. Pharmacol. 10, 306 (1970).
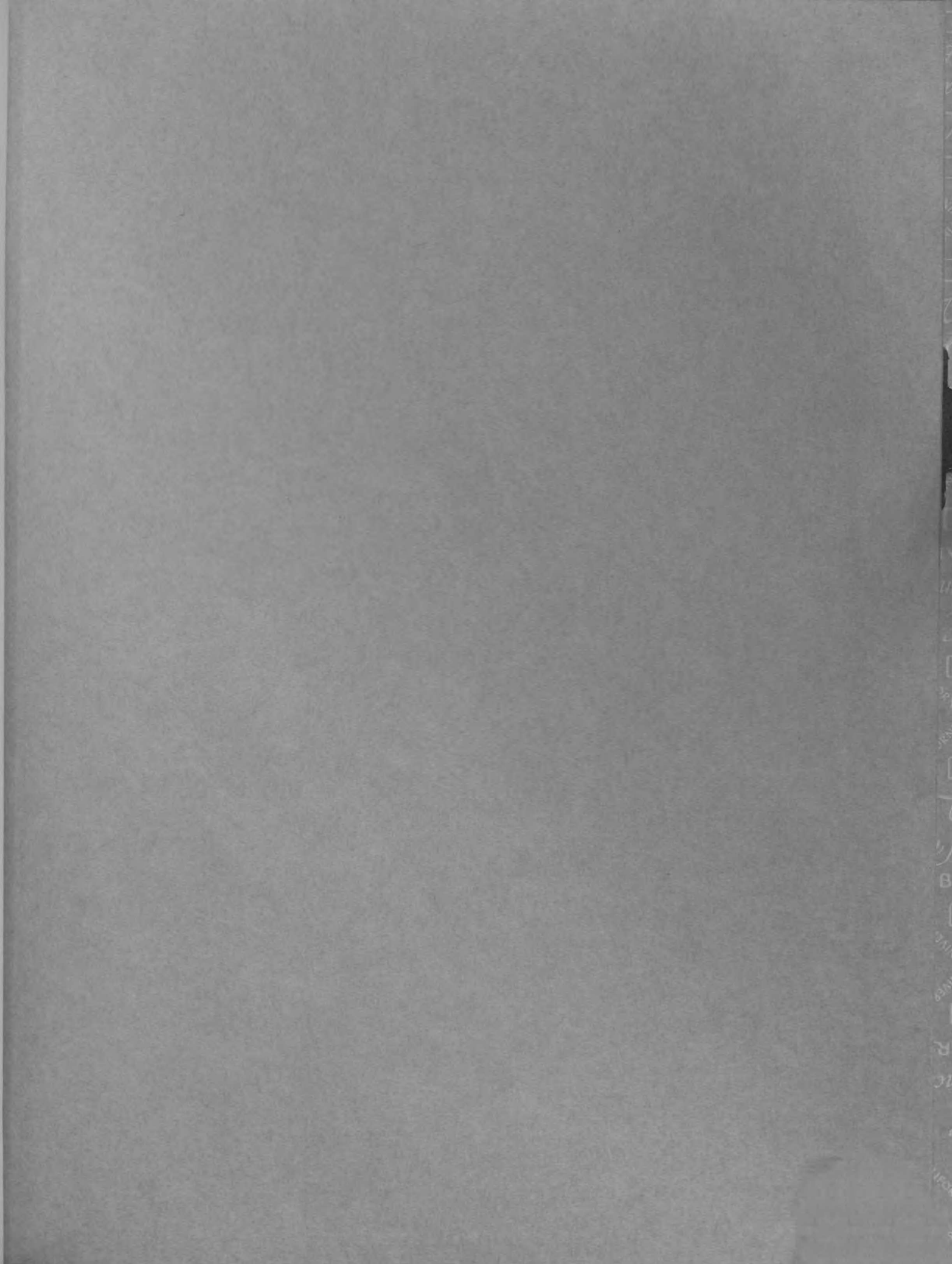
37. Morselli, P. L., Rizzo, M., and Garattini, S., Ann. N.Y. Acad. Sci. 179, 88 (1971).

38. Arnold, K., and Gerber, N., Clin. Pharmacol. Ther. 11, 121 (1970).

39. Svensmark, O., Schiller, P. J., and Buchthal, F., Acta Pharmacol. et Toxicol. 16, 331 (1960).

40. Andreasen, P. B., Hansen, J. M., Skovsted, L., and Siersbaek-Nielsen, K., Acta Neurol. Scand. 47, 117 (1971).

41. Levy, G., J. Pharm. Sci. 54, 959 (1965).

42. Cummings, A. J., Martin, B. K., and Renton, R., Brit. J. Pharmacol. 26, 461 (1966).

43. Levy, G., Tsuchiya, T., and Amsel, L. P., Clin. Pharmacol. Ther. 13, 258 (1972).

44. Dayton, P. G., Cucinell, S. A., Weiss, M., and Perel, J. M., J. Pharmacol. Exp. Ther. 158, 305 (1967).

45. Dayton, P. G., and Perel, J. M., Ann. N. Y. Acad. Sci. 179, 67 (1971).

46. Gerber, N., and Arnold, K., J. Pharmacol. Exp. Ther. 167, 77 (1969).

47. Borondy, P., Chang, T., and Glazko, A. J., Fed. Proc. 31, 582 (1972).

48. Ashley, J. J., and Levy, G., Res. Commun. Chem. Path. Pharmacol., 4, 297 (1972).

49. Gerber, N., and Wagner, J. G., Res. Commun. Chem. Path. Pharmacol. 3, 455 (1972).

50. Atkinson, A. J., Shaw, J. M., Clin. Pharmacol. Therap. 14, 521 (1973).

51. Garrettson, L. K., and Kim, O. K., Pediatr. Res. 4, 455 (1970).

52. Gerber, N., Bush, L. M., and Oates, J., Clin. Pharmacol. Ther. 13, 139 (1972).

53. Buchthal, F., and Lennox-Buchthal, M. A., "Antiepileptic Drugs," Woodbury, D. M., Penry, J. K., and Schmidt, R. P., Eds. Raven Press, New York, 1972, p. 193.

54. Helfant, R. H., Scherlag, B. J., and Damato, A. N., Circulation 36, 108 (1967).

55. Dill, W. A., Baukema, J., Chang, T., and Glazko, A. J., Proc. Soc. Exp. Biol. Med. 137, 674 (1971).

56. Svensmark, O., and Kristensen, P., J. Lab. Clin. Med. 61, 501 (1963).

57. Wallace, J., Biggs, J., and Sahl, E. V., Anal. Chem. 37, 410 (1965).

58. Wallace, J. E., Clin. Chem. 15, 323 (1969).

59. Chang, T., and Glazko, A. J., J. Lab. Clin. Med. 75, 145 (1970).

60. Sandberg, D. H., Resnick, G. L., and Bacallao, C. Z., Anal. Chem. 40, 736 (1968).

61. Sabih, K., and Sabih, K., Anal. Chem. 41, 1452 (1969).

62. Grimmer, V. G., Jacob, J., and Schafer, H., Arzneim. Forsch. 19, 1287 (1969).

63. Kupferberg, H. J., Clinica Chimica Acta 29, 283 (1970).

64. MacGee, J., Anal. Chem. 42, 421 (1970).

65. Evenson, M. A., Jones, P., and Darcey, B., Clin. Chem. 16, 107 (1970).

66. Levy, G., J. Pharm. Sci. 59, 1847 (1970).

67. Ketterer, S. G., Wiegand, B. D., and Rapaport, E., Am. J. Physiol. 199, 481 (1960).

68. Glazko, A. J., and Chang, T., "Antiepileptic Drugs," Woodbury, D. M., Penry, J. K., and Schmidt, R. P., Eds., Raven Press, New York, 1972, p. 129.

69. Chang, T., and Glazko, A. J., Ibid, p. 147.

70. Staubus, A., and Riegelman, S., Personal Communication.

71. Rowland, "Current Concepts in the Pharmaceutical Sciences - Dosage Form Design and Bioavailability," Swarbrick, J. S., Ed., Lea and Febiger, Philadelphia, 1973, pp. 184-222.

72. Loo, J. C. K., and Riegelman, S., J. Pharm. Sci. 59, 53 (1970).

73. Wheeler, H. O., Cranston, W. I., and Meltzer, J. I., Proc. Soc. Exp. Biol. Med. 90, 11 (1958).

74. Scherf, D., Bull. N. Y. Med. College Flower and Fifth Ave. Hosp. 6, 82 (1943).

75. Perrier, D., Ashley, J. J., and Levy, G., J. Pharmacokin. Biopharm. 1, 231 (1973).

76. Wheeler, H. O., J. Clin. Invest. 39, 1131 (1960).

77. Staubus, A., and Nelson, J., Personal Communication.

78. Harris, S., and Kokernot, R. H., Am. J. Physiol. 163, 505 (1950).

79. Louis, S., Kutt, H., and McDowell, F., Am. Heart J. 74, 523 (1967).

80. Gupta, D. N., Unal, M. O., Bashour, F. A., and Bebb, W. R., Dis. Chest 51, 248 (1967).

81. Rowe, G. G., McKenna, D. H., Sialer, S., and Corliss, R. J., Am. J. Med. Sci. 254, 534 (1967).

82. Conn, R. D., Kennedy, J. W., and Blackman, J. R., Am. Heart J. 73, 500 (1967).

83. Rowland, M., Benet, L. Z., Graham, G. G., J. Pharmacokin. Biopharm. 1, 123 (1973).

84. Rowland, M., J. Pharm. Sci. 61, 70 (1972).

85. Drapanas, T., Kluge, D. N., and Schenk, W. G., Jr. Surgery 48, 1017 (1960).

86. Wagner, J. G., J. Pharm. Sci. 52, 1097 (1963).

86a. Bigger, J. T., Jr., Schmidt, D. H., and Kutt, H., Circulation 38, 363 (1968).

86b. Jensen, B. N., and Grynderup, V., Epilepsia 7, 238, (1966).

87. Mirkin, B. L., and Wright, F., Neurol. (Minneap.) 21, 1123 (1971).

88. Druckman, R., and Moore, F. J., Proc. Soc. Exp. Biol. Med. 90, 173 (1955).

89. Frey, H. H., and Kampmann, E., Acta Pharmacol. Toxicol. 24, 310 (1966).

90. Mellk, M., Letteri, J. M., Durante, P., Louis, S., Kutt, H., and Glazko, A., Ann. Intern. Med. 72, 801 (1970).

91. Van der Kleijn, E., Arch. Int. Phamacodyn. 179, 225 (1969).

92. Lunde, P. M., Rane, A., Yaffe, S. J., Lund, L., and Sjoqvist, F., Clin. Pharmacol. Therap. 11, 846 (1970).

93. McLean, F. C., and Hastings, A. B., J. Biol. Chem. 108, 285 (1935).

94. Letteri, J. M., Mellk, H., Louis, S., Kutt, H., Durante, P., and Glazko, A., N. Engl. J. Med. 285, 648 (1971).

95. Odar-Cederlof, I., Lunde, P., and Sjoqvist, F., Lancet 2, 831 (1970).

96. Loeser, W. W., Neurol. 11, 424 (1961).

97. Rudman, D., Bixler, T. J., and Del Rio, A., J. Pharmacol. Exp. Therap. 176, 261 (1971).

98. Ganshorn, A., and Kurz, H., Arch. Pharm. Exp. Path. 260, 117 (1968).

99. Lund, L., Berlin, A., Lunde, P. K., Clin. Pharmacol. Therap. 13, 196 (1972).

100. Conard, G. J., Haacik, C. O., and Finger, K. F., J. Pharm. Sci. 60, 1642 (1971).

101. Lightfoot, R. W., and Christian, C. L., J. Clin. Endocrin. Met. 26, 305 (1966).

102. Oppenheimer, J., N. Engl. J. Med. 278, 1153 (1968).

103. Rane, A., Lunde, P. M., Jalling, B., Yaffe, S. J., and Sjoqvist, F., Ped. Pharmacol. Therap. 78, 877 (1971).

104. Reidenberg, M. M., Odar-Cederlof, I., von Bahr, C., Borga, O., and Sjoqvist, F., N. Engl. J. Med. 285, 264 (1971).

105. Schreiner, G. E., Arch. Int. Med., 102, 896 (1958).

106. Theil, G. B., Richter, R. W., Powell, M. R., and Doolan, P. D., Neurol. 11, 138 (1961).

107. Curry, D. L., Bennet, L. L., and Grodsky, G. M., Endocrin. 83, 572 (1968).

108. Scatchard, G., Coleman, J. S., and Shen, A. L., J. Am. Chem. Soc. 79, 12 (1957).

109. Kurz, H., Arch. for Pharmac. 269, 426 (1971).

110. Shoeman, D. W., and Azarnoff, D. L., Pharmacology 7, 169 (1972).

111. Welt, L. G., Black, H. R., and Krueger, K. K., Arch. Internat. Med. <u>126</u>, 773 (1970).

112. Anton, A. H., and Corey, W. T., Acta Pharmacol. Toxicol. <u>29</u>, Supp. 3, 134 (1971).

113. Viukarc, N. M. A., and Tammisto, P., J. Mout. Defic. Res. <u>13</u>, 212 (1969).

114. The Boston Collaborative Drug Surveillance Program, Clin. Pharmacol. Therap. <u>14</u>, 529 (1973).