MEASUREMENT OF THE LEVELS OF DIPHENYLHYDANTOIN AND PHENOBARBITAL IN PATIENTS WITH EPILEPSY

by

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TO MY FAMILY

PREFACE

Frequently encountered questions in recent years have concerned themselves with just how valuable our research efforts are to mankind; has too great a portion of our resources been expended on pure research which outwardly seems to have no relevance to life, or have we just reached a suitable balance in our priorities? Not even under the present day stress of decreased availability of funds has an answer to this challenge arisen.

The specific aspect of life encompassed by "medicine" has its own similar issues — given general acceptance of the need for medical services by society, how do we balance both completeness and economic efficiency in our health care system? Besides research, a very major item of concern here is the use of laboratory tests, one type of which (drug assays) forms the basis for the present work.

This thesis represents an attempt at producing, from laboratory research, work which will have immediate use and application in the clinical neuropharmacology and therapeutics of the epilepsies. In effect perhaps, a "middle of the road" approach has been followed. The author has felt reasonably at ease in such a situation, although this certainly should not be taken to represent the best approach to all projects. It is a beginning.

The work summarized in this thesis was carried out during the period in which the Clinical Neuropharmacology

Laboratory of the Montreal Neurological Institute was initiating a program to make blood anticonvulsant drug levels readily available as an aid in the medical treatment of the epilepsies.

I am indebted to my research director, Dr. Allan L. Sherwin, for the opportunity to share in this development and am particularly thankful for his allowing me a relatively free hand in the determination of the exact direction of various aspects of this work — an attitude which I have found to be very conducive to productive research. Aid in matters of liaison and clinical interpretation was available from Professors J. Preston Robb and Theodore B. Rasmussen.

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'Appreciation of the "pharmacological individuality" of each person will not simplify the problems of using drugs correctly, but it helps us to understand why formulas for dosage based upon body weight or the <u>average</u> therapeutic dose are so inadequate.'

(La Du, 1969)

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I

INTRODUCTION AND OBJECTIVES OF THE WORK PROPOSED

Approximately one-half of one percent of the total population suffers from epilepsy, a neurological disorder characterized by the paroxysmal occurrence of seizures. "An epileptic seizure is a state produced by an abnormal excessive neuronal discharge within the central nervous system." (Penfield and Jasper, 1954)

Two drugs very widely used for the medical management of "the epilepsies" are phenobarbital and diphenylhydantoin (5,5-diphenylhydantoin, phenytoin). These pharmaceutical agents are the prime concern of this thesis.

Within most patient populations, the ratio of the diphenylhydantoin (DPH) level achieved to dose administered is extremely variable (Loeser, 1961; Svensmark and Buchthal, 1964; Jensen and Grynderup, 1966). This "level-to-dose ratio" additionally is subject to idiosyncrasies of metabolic origin which may occur when DPH is administered alone (Kutt et al., 1964a and 1964b; Arnold and Gerber, 1970), or in combination with other drugs (Hansen et al., 1966; Solomon and Schrogie, 1967; Brennan et al., 1970) notably phenobarbital (Kutt et al., 1969) as it often is in epilepsy (Schmidt and Wilder, 1968). So the magnitude of dosage is a poor scale for evaluating appropriateness of DPH

therapy: in the individual patient, the result of a given dosage — success, failure, or toxicity — is often unpredictable. In the proper management of many patients, then, knowledge of the amount of drug actually reaching the tissues via the plasma is considered to be a necessity (Frey et al., 1967; Gordon, 1969; Vajda, 1970). The aspect of the prolonged use of any drug which would most lead the physician to monitor its level over an extended period of time is the possibility of slow, occult accumulation of the drug, ultimately producing side effects. Even when apparently not related to co-administration of another agent or to "known provocative factors", such toxicity due to DPH can appear clinically at two to twelve months following initiation of (or increase in) a dosage rate, or occasionally not until several years later (Roseman, 1961; Husby, 1963; Patel and Crichton, 1968; Behrman, 1969). Periodic drug determinations in plasma would allow prevention of many of these toxic reactions.

Levels should also be monitored whenever a change is made in a patient's overall therapy. The rate of administration of the anticonvulsant may then be altered, if necessary, before a change in the status of the patient occurs clinically.

Assays for anticonvulsants may also be employed to estimate the relative efficacy of a drug in any particular patient with epilepsy. In the past, in some centers, the

dosage rate of an anticonvulsant drug was increased to just below clinical toxicity routinely (Putnam and Rothenberg, 1953; Livingston, 1956; Roseman, 1961); the frequent occurrence of side effects makes this poor pharmacological practice (Patel and Crichton, 1968; Ang et al., 1969; Goodman and Gilman, 1970). By themselves, records of seizure control may be misleading in specific seizure disorders which are partially drug-resistant or otherwise difficult to control. So although a therapeutic range of drug concentration is probably person-specific (Buchthal et al., 1960; Kutt et al., 1964a; Cromie, 1966), the ability to compare a given level with those observed in a large patient population should increase the safety and effectiveness of drug administration (Gordon, 1969; Buchthal and Svensmark, 1971). A greater individualization of the total therapy would result from the proper use of assay data, and over-medication could be prevented.

Because DPH is one of the most frequently used drugs in the treatment of generalized convulsive and partial seizures (Sutherland and Tait, 1969; Rosenblum and Shafer, 1970), its quantitation in biological fluids is therefore of prime importance in neurological practice. The first objective of this work was to develop an assay method for DPH which would be rapid, specific, accurate, and yet inexpensive to set up and use. For this to be useful clinically, a "normal" range of values for our own patients would have to be established, correlating drug level with clinical status. This is always

necessary because the different combinations of drugs used in various parts of the world, and the effects of local genetic and dietary factors, make the interpretation of drug levels somewhat laboratory-specific.

Phenobarbital is of course a widely used anticonvulsant drug and very frequently it is administered concurrently with DPH (Schmidt and Wilder, 1968; Goodman and Gilman, 1970). The availability of a dependable assay method for phenobarbital is probably the second requirement for an anticonvulsant drug laboratory, especially in North and South America. Simultaneous use of DPH and phenobarbital results in a combination of the effects of the two drugs with respect to the degree of control of seizures and also the side effects and toxic reactions. It is therefore important to characterize fully each clinical case which is presenting therapeutic problems since it may be true that only one of the administered drugs is at fault. High anticonvulsant drug levels and certain neurological disorders, especially those in which there are tumors or increased intracranial pressure, can present similarly (Frantzen et al., 1967; Patel and Crichton, 1968). As well, patients with some degree of brain damage may also be more sensitive to the side effects of these drugs. Such diagnostic problems would best be considered as emergency situations with the determination of circulating drug levels done as one of the initial tests, prior to more complex investigations which have their attendant risks. Hence, assay procedures must be relatively rapid.

Development of such a method for determining phenobarbital in biological fluids was the second objective of this work.

Specific factors influencing the level-to-dose ratio, besides the pharmacological abnormalities and drug interactions mentioned above, include the metabolic capability of the liver for transforming these drugs, and the degree of protein binding of the drugs in the plasma. DPH crosses the blood-brain barrier easily (Firemark et al., 1963; Kutt et al., 1968; Woodbury, 1969) so the variations observed in its protein binding (Svensmark et al., 1960; Triedman et al., 1960; Viukari, 1969) have potentially great effects both upon the distribution and body content of the drug (Desgrez and de Traverse, 1966; Kutt et al., 1969), as well as upon its biological activity (Taylor et al., 1954; Brodie and Hogben, 1957; Kane and Smith, 1959). Although the binding of phenobarbital is smaller and less-variable (Lous, 1954b; Svensmark et al., 1960), it is still a significant consideration. Development of methodology to allow measurement of the protein binding of these drugs would aid in the characterization of the pharmacodynamics in selected patients, so that total plasma drug levels, and elimination half lives (Martin, 1965a and 1965b), could be properly interpreted.

A preliminary investigation of the correspondence between plasma and intracellular DPH, as related to protein binding, was to be attempted.

The other major aspect of this project was a controlled cross-over study of the DPH levels achieved in patients with epilepsy, along with the resultant degree of seizure control and patient well-being, when different dosage schedules were followed: the total daily dose was administered once daily, after breakfast, and then in later months by the more traditional divided-dose regimen (t.i.d. or q.i.d.). The results have special implications for patients receiving only DPH for the control of epilepsy, and those experiencing seizures during only a certain portion of the day or night. In many cases, individualization of the dosage schedule for DPH could be of benefit to the patient.

II

HISTORICAL REVIEW

A. ASSAY METHODS FOR DIPHENYLHYDANTOIN

From 1938, when diphenylhydantoin (DPH) was introduced as an anticonvulsant drug (Merritt and Putnam, 1938), up until 1956, DPH was used in neurology without the benefit of practical methods for its measurement in biological fluids (Dill et al., 1956; Plaa and Hine, 1956). The applicability of and need for DPH assays have been published widely. Yet even today, many clinical laboratories do not offer this service, and in this field generally there is "a pitiful failure of application of current information" (Rose et al., 1971). Frequently in the laboratory, the reason may be that the balance between the expense and potential use of such a drug assay is not attractive.

Each of the approximately twenty-five methods which have been proposed over the years for the determination of DPH in biological materials has its own advantages and disadvantages. Paper and thin-layer chromatographic techniques are highly specific, many even for several drugs simultaneously, but these often are not very quantitative. Typical colorimetric and spectrophotometric techniques are quite accurate but suffer greatly from interfering substances. And many of the newer, more sophisticated methods are more expensive or require greater time for the completion of an assay.

Tables I and II summarize and compare the important aspects of original techniques proposed for the determination of DPH.

TABLE I

ASSAYS AVAILABLE FOR THE DETERMINATION OF DIPHENYLHYDANTOIN
(THE CHARACTERISTICS OF EACH ARE LISTED IN TABLE II)

No. in Table II	Method: Author(s) and Year	Reagents / (Reactions)	Wavelength of Analysis, millimicrons (∓)
1.	Dill et al., 1956.	(Diazotization)	550
2.	Plaa & Hine, 1956.	Buffer of pH = 11.0	235/260
3.	Schiller & Buchthal, 1958; and Svensmark et al., 1960.	(modified No. 2 by No. 1)	235/260
4.	Svensmark & Kristensen, 1963.	(modified No. 3)	235/260
5.	Cucinell et al., 1965.	Alcohols/NH_4OH	(UV light)
6.	Olesen, 1965 & 1967a.	Piperidine	(UV light)
7.	Huisman, 1966.	(TLC, then No. 1)	550
8.	Vedsö et al., 1969.	(TLC)	-
9.	Simon et al., 1971.	(TLC, then No. 1)	550
10.	Sandberg et al., 1968.	Diazomethane (Methoxylation)	(GLC)
11.	Chang & Glazko, 1968 & 1970.	(Trimethylsilylation)	(GLC)
12.	Sabih & Sabih, 1969.	--	(GLC)
13.	Pippenger & Gillen, 1969.	--	GLC for DPH plus other drugs.
14.	Evenson et al., 1970.	--	
15.	Kupferberg, 1970.	(Methylation)	
16.	Wallace et al., 1965.	Bromine (oxidation)	257
17.	Wallace, 1966.	$NaOH/KMnO_4$ (oxidation)	257
18.	Wallace, 1968 & 1969a.	(modified No. 17 using hexane)	247
19.	Lee & Bass, 1970.	(micro-No. 17)	257
20.	Bock & Sherwin, 1971.	(micro-No. 17)	257

(∓) "GLC" indicates that gas-liquid chromatography, instead of spectrophotometry, is employed in the final step of the method. Only several representative methods are listed here for comparative purposes.

TABLE II

CHARACTERISTICS OF THE ASSAYS FOR DIPHENYLHYDANTOIN†

Cf. No.	Washes or Purifications required	Number of extractions employed	Reactions	Reported Sensitivity, ug DPH (in ml volume noted)		Approx. Δ O.D. per 100 ug DPH	Reported S.D., or (Error)	Serum blank, ug/ml
1.	1	3	+	0.5	(5)	*	*	*
2.	1	2	0	20.	(5)	0.39	(≤8% for >9ug/ml)	1.5
3.	1	2	0	10.	(5)	0.095	4-20% (2.4 ug/ml)	1.-2.7
4.	2	2	0	*	(2)	0.32	(1.0 ug/ml)	1.5
5.	1	1	+	*		(Paper Chrom.)	5.%	~1.0
6.	0	2	+	3.	(3)	(TLC)	(1.-2.ug/ml)	~0.(*)
7.	2+TLC	1	+	~10.	(3)	*	*	*
8.	0	1	+	0.3	(1)	(TLC)	(1. ug)	~0.(*)
9.	TLC	1	+	1.0	(1)	2.0	(2.1 ug)	?
10.	0	1	+	0.4	(1)	(GLC)	3.% (2.2 ug)	*
11.	1	3	+	*		(GLC)	~3.%	*
12.	0	1	0	0.4	(2)	(GLC)	>1.5% (*)	*
13.	0	1	0	1.0	(2)	(GLC)	0.8/5.(=16%)	*
14.	0	1	0	0.3	(1)	(GLC)	~6.%	*
15.	1 incl. in:	3	+	~1.5	(1)	(GLC)	≤8.%	*
16.	0	2-3	+	≤25.	(10)	0.53	3.-7.%	≫0.4(*)
17.	0	2-3	+	10.	(10)	0.65	1.-5.%	0.2
18.	0	2	+	3-5.	(10)	0.70	2.-6.%	~0.6(*)
19.	0	2-3	+	2.	(2)	2.6	**	~1.
20.	0	1	+	0.6	(1)	2.0-2.8	7.% (0.7ug)	≤0.5

†Abbreviations: "ug" = microgram (10^{-6} gram); "ml" = millilitre; "O.D." = optical density; "S.D." = standard deviation.

*Signifies that the information is not given in the report.

**Only the standard error of the mean (3.6%) is noted, without the number of determinations ("n").

Almost all of the reports noted strongly support the proposition that rapid quantitation of DPH should be readily available to the physician treating patients for epilepsy. However, most especially in the ever-increasing numbers of papers concerning gas-liquid chromatography (GLC), criticism of previously published methods has frequently been used to justify the development of that given technique. It is certainly true that most methods have their own specific strong points, and in some cases a particular method is the one best suited to the needs and resources of an individual laboratory. The methods listed in Tables I and II will therefore be considered further only by mentioning relative attributes and disadvantages of general techniques, in relation to specific situations.

Most spectrophotometric methods are relatively rapid and easy to perform. But attempts at shortening them to true emergency status have been accompanied by warnings against misinterpretation of the results clinically when no back-up method is available — because obtuse values can be obtained due to interfering substances (Kristensen et al., 1967). Despite such warnings, clinical studies have been published in which interpretation was apparently not done carefully enough (e.g., Gibberd et al., 1970; Viukari, 1969); other authors have noted that levels given in such reports are too high to be considered as being totally valid (Taylor and Jamieson, 1970; Buchanan and Allen, 1971). However, for screening purposes, these methods are valuable when a more

sophisticated method, although available, is not considered to be practical for routine use.

In the more complex spectrophotometric methods, distributions employing several buffer solutions are used to separate some of the interfering substances. These selective extraction processes, although removing phenobarbital efficiently (Svensmark and Kristensen, 1963), have been shown to allow ultimate interference by drugs such as ethosuximide, acetanilide, dicoumarol, and barbiturates other than phenobarbital (Svensmark and Kristensen, 1963; Frantzen et al., 1967). When such limitations of these techniques are recognized, and information concerning individual medication patterns is sent to the laboratory, then they are quite sufficient to yield clinically valuable data.

Chromatographic techniques are very specific, but also rather slow (Garrettson and Dayton, 1970); many are not quantitatively accurate (Simon et al., 1971). However, the paper or thin-layer plate does often give a good picture of the entire drug pattern; qualitative determinations (identifications) are done by measuring R_f values (Cucinell et al., 1965; Pippenger et al., 1969). Quantitation of specific drugs in some cases is then done by another technique such as colorimetric reaction (Huisman, 1966; Olesen, 1967a; Simon et al., 1971). Even the use of chromatography may not eliminate the non-zero blank value for the determination of some drugs (Cucinell et al., 1965).

The present ultimate in methodology is gas-liquid

chromatography; it is very sensitive, and accuracy is achieved by employing internal standards. The recent development of technology for assaying a number of different drugs simultaneously on a single column (e.g., Alber, 1969; Street, 1969) promises to make the GLC technique more useful in the future.

The final category of spectrophotometric methods (16-20 of Tables I and II) employs a chemical transformation to achieve outstanding specificity in the determination of DPH (Wallace, 1966; Lee and Bass, 1970). Bruce et al. (1968) have used the same reaction to prepare samples for the gas chromatographic determination of several other drugs.

Reserving the GLC techniques as best for routine determination of two or more drugs, the above mentioned method of Wallace (1966) was chosen as the one most promising specifically for modification to a rapid, inexpensive, and convenient assay for the single drug DPH. The methodology eventually developed proved to be reproducible in numerous other laboratories.

B. CLINICAL STUDIES ON DIPHENYLHYDANTOIN

Previous to 1956, determination of diphenylhydantoin (DPH) for other than purely toxicologic or forensic purposes was seldom done due to the inconvenience or inapplicability of the methods available; but upon the introduction of practical assay techniques for DPH (Dill et al., 1956; Plaa and Hine, 1956), various laboratories began to measure blood levels in patient populations. Initial studies reported only fragmentary data on rates of rise and fall of blood levels, and attempted correlation of peak levels with dosage (e.g., Schiller and Buchthal, 1958). Subsequently, the literature became more clinically oriented.

Table III summarizes the findings of the reports specifically concerned with DPH and the treatment of epilepsy. Figures noted include estimates of the therapeutic range of concentration for this drug, as well as the average levels observed in various groups of treated patients. Tables IV, V, and VI summarize the literature concerned with levels observed in patients showing toxic signs while receiving DPH therapy.

Throughout this thesis, the following abbreviations are employed for weights and concentrations of drugs:

mg = milligram (10^{-3} gram).
ug = microgram (10^{-6} gram).
ml = millilitre (10^{-3} litre).

TABLE III

CLINICAL DIPHENYLHYDANTOIN (DPH) LEVELS

Report, Author(s) and Year	S.T.*	LEVELS, ug DPH/ml sample			
		Range	Average	Level/dose**	Therapeutic
Buchthal & Svensmark, 1960.	S	--	--	1.0 (dose<2) 3.3 (dose>2)	10 - 50 (mean, 15)
Buchthal et al., 1960.	S	0-48	18.	3.4 (n=17)	>(10 - 15)
Plaa & Hine, 1960.	B	2-16	--	1.8 (females)	
Svensmark et al., 1960.	S	5-30	--	3.3 (dose>1.8)	
Triedman et al., 1960.	P	3-52	24.	--	
Loeser, 1961.	B	2-48 in-patients= out-patients=	9.5 10.5 9.2	(Level/dose line and group differences not significant.)	
Husby, 1963.	S	--	16.4 (n=151)	3.5 (range, 3-4)	
Tveten, 1963.	B	2.6-22	10.5	--	
Stensrud & Palmer, 1964.	S	10-25 (for 85% of n=180)	--	3.5 (±1.4)	
Svensmark & Buchthal, 1964.	S	--	--	(Variable)	10 - 20
Cucinell et al., 1965.	P	4-21 (200 mg/day)= (300 mg/day)=	9.6 (±2.2) 11.4 (±1.6)	(n = 6) (n=10)	
Jensen & Grynderup, 1966.	S	7-33	--	(all on dose=5)	
Kutt & McDowell, 1968.	B	--	--	--	10 - 15
Haerer & Grace, 1969.	S	--	10.4 (n=282)	2. to 3.	
Viukari, 1969; and Viukari & Tammisto, 1969.	S	12-37	22 (±7.)	3.7 (±1.2) (n=40)	
Buchanan & Allen, 1971.	P	0-46	8.2	~2. (children)	0 - 46 (mean, 6.7)
Simon et al., 1971.	B	0-54	23. (n=30)	--	

*(S.T.) sample type: B=blood; P=plasma; S=serum.

**(Level as ug DPH/ml, dose as mg DPH per day/kg weight).

TABLE IV

TOXIC LEVELS OF DIPHENYLHYDANTOIN REPORTED

Report, Author(s) & Year	Sample Type*	Levels, ug DPH/ml, and explanation
Buchthal et al., 1960.	S	14 - 60
Triedman et al., 1960.	P	30 - 70
Theil et al., 1961.	P	50 (level at 138 hours after consumption of 21.5 grams of DPH.)
Husby, 1963.	S	31 - 58 (mean = 39; median = 37)
Kutt et al., 1964a.	B	23 - 89 (n = 32)
Kutt et al., 1964b.	B	87 (n = 1)
Svensmark & Buchthal, 1964.	S	>25
Kokenge et al., 1965.	B	75 (n = 1) DPH >>300 mg/day.
Laubscher, 1966.	B	94 (fatal), level at 24 hrs. after intake.
Cantu & Schwab, 1966.	B	50 (1 out of 35 persons receiving 300 mg DPH/day)
Jensen & Grynderup, 1966.	S	12 - 43 toxicity was variable.
Frantzen et al., 1967.	S	35 (DPH and ethosuximide, 1 mo.)
		54 ("mononucleosis hepatitis")
		63 (⟶ severe anorexia)
Andia et al., 1968.	B	>58 (level at 12 hrs. after peritoneal dialysis begun.)
Blair et al., 1968.	B	60 (level at 1 day after overdose.)
		>>130 (peak level, at 2 days after the consumption of about 12 grams of DPH.)
Tenckhoff et al., 1968.	B	112 (n = 1; child).
Logan & Freeman, 1969.	S	40 - 69 (n = 4)
Gibberd et al., 1970.	S	40 (n = 1), ⟶ nystagmus.
		50 (n = 1), ⟶ gum hypertrophy.
Prensky et al., 1971.	S	>45 (due to dosage increase.)

*Sample type: B = blood, P = plasma, S = serum.

TABLE V

VARIABLE CORRELATION OF TOXICITY WITH LEVEL OF DIPHENYLHYDANTOIN (DPH)

Report, Author(s) and Year	S.T.*	Levels, ug DPH/ml, and explanation.
Buchthal et al., 1960.	S	<14 → No side effects.
		10-30: 85% had no side effects. 15% had mild side effects. (No "pronounced" side effects were observed.)
		>30: 50% had "pronounced" side effects. 24% had mild side effects. 26% had no side effects.
Triedman et al., 1960.	P	<30 No toxicity.
		>30 Extreme personal variation.
Husby, 1963.	S	~30 =Limit for all toxicity seen.
		BUT
		>30 Only 50% showed "pronounced" side effects.
Stensrud and Palmer, 1964.	S	>25 19 out of 35 showed toxicity.
		40-50 → "pronounced intoxication".
Viukari, 1969.	S	(No toxicity seen even at the maximum level observed, 36.5)
Haerer and Grace, 1969.	S	<5 → 2% have nystagmus.
		10-15 → 20% have nystagmus.
		20-25 → 65% have nystagmus.
		BUT
		≤30 90% have no other side effects. besides possible nystagmus.

*(S.T.) sample type: B = blood; P = plasma; S = serum.

TABLE VI

TOXIC SIGNS AND AVERAGE LEVELS OF DIPHENYLHYDANTOIN

Report, Author(s) & Year	S.T.*	Picture of Toxicity	Average levels, ug/ml.
Kutt et al., 1964a.	B	Nystagmus on lateral gaze....	20's (n = 32)
		Ataxia, and nystagmus on 45° gaze.....	30's
		"mental slowing".....	40's
		Nystagmus on forward gaze....	>50
		BUT there was variation of clinical picture for a given level within each group.	
Kutt & McDowell, 1968.	B	Nystagmus.....	20's
		Gait ataxia.....	30's
		"constant lethargy".....	40's
Haerer & Grace, 1969.	S	Marked nystagmus.....	42.2 (n = 12)
		Of all patients with "GOOD" (<1/month) seizure control, 32% had "significant nystagmus".	
		OTHER SIDE EFFECTS:	
		Mild or Questionable.....	14.5 (n = 32)
		Moderate or Marked.....	36.6 (n = 15)
Buchanan & Allen, 1971.	P	Vomiting.....	45.5 (n = 1)
		Nystagmus.....	21.8
		Anorexia.....	16.4 (n = 3)
		Ataxia.....	11.9
		Gingival hyperplasia.....	7.8

*Sample type (S.T.): B = blood, P = plasma, S = serum.

All of these reports can probably be summarized well in three statements:

1) Patients on DPH therapy show great variation of the level-to-dose ratio, and there is some variation in the clinical picture seen at a given drug level.

2) Despite such variability, approximate guidelines can be made for DPH —

200 mg $\leq$ daily therapeutic dose $\leq$ 400 mg, generally.

5 ug $\leq$ therapeutic level $\leq$ 20 ug/ml, generally.

20 to 30 ug/ml = the lower limit for the appearance of toxic signs.

3) There are exceptions. The exact pharmacology of DPH is specific to the individual patient.

The best organized format for the study of therapeutic and toxic levels of DPH would be one conforming to the criteria of Buchthal and Svensmark (1971): an incremental change in DPH level allows the determination of exact levels at which given effects begin to appear, and those above which no further change in clinical status occurs. This is in effect a "titration" of the patient with the drug (Goldstein et al., 1968; Goodman and Gilman, 1970). Bigger et al. (1968) very nicely employed just such a procedure relative to the use of DPH for the treatment of cardiac arrhythmias.

This incremental procedure would also remove the "group selective processes" referred to by Buchthal et al. (1960) which have affected most clinical studies for DPH done so far.

C. THE DYNAMICS OF DIPHENYLHYDANTOIN LEVELS IN THE CIRCULATION

Aside from the changes of diphenylhydantoin (DPH) concentration in the body resulting from initiation of therapy, or other alterations of the rate of dosage (see Table VII), an important concern relative to the anticonvulsant action of DPH is the magnitude of variation of its level hour-by-hour during the day, while a given dosage format is being followed. Triedman et al. (1960) report that administration of the total daily dosage of DPH in two or more (divided) doses yields an almost constant plasma level throughout the day, whereas administration only once per day allows fluctuation of that level. In quantitative terms, the DPH level falls from the peak value at a rate of about 5% in 6 hours, so that the overall variation during the day is about 10% for the two-dose schedule, and about 25% when the total daily dosage is administered as only a single dose (Buchthal and Svensmark, 1960; Buchthal et al., 1960; Svensmark et al., 1960). On the other hand, Buchanan et al. (1972) have concluded that the average values of the peak-to-minimum level differences for single (q.d.) and divided (t.i.d.) dose schedules are essentially equal.

As for the actual levels resulting from each dosage regimen, an uncontrolled study showed that (at 1300 hours, eighteen hours after the administration of the evening dose) nineteen patients on a q.h.s. schedule had an average level

of 11.7 ug DPH/ml of serum, while 138 patients on a thrice-daily schedule had levels averaging only 9.6 ug/ml (Haerer and Grace, 1969). But earlier reports state that there is no difference in level achieved whether DPH is administered in one or two or three doses per day (Svensmark et al., 1960; Loeser, 1961); and a very recent study, with twenty-four volunteers divided equally between once-daily and thrice-daily schedules, found an insignificant difference (0.6 ug/ml of plasma) between the average DPH levels for the same 300 mg/day dosage rate in the two groups (Buchanan et al., 1972).

Given the value for the biologic half life of DPH in humans (see Table VII), and considering the information summarized above, the conclusion seems to be that there is greater variation of level hour-by-hour when the total daily dosage of DPH is taken in only a single dose, but the actual levels during much of the day do not differ significantly from those achieved by a divided-dose regimen. Except for a greater frequency of nystagmus, which the authors considered to be overinterpreted, nothing adverse was observed in male volunteers when 300 milligrams of DPH was given in a single dose daily (Buchanan et al., 1972). However, the clinical effects of such an alteration in dosage regimen have not been determined at all.

TABLE VII

THE DYNAMICS OF DIPHENYLHYDANTOIN LEVELS IN THE CIRCULATION

Report, Author(s) and Year	RATES OF RISE	RATES OF FALL; and Biologic Half-life ($t_{\frac{1}{2}}$).
Buchthal et al., 1960; and Buchthal & Svensmark, 1960.	Plateau at 6 to 10 days after initiation of oral DPH.	40 to 55% decrease/day upon withdrawal.
Svensmark et al., 1960.	Plateau in 11 days.	35 to 55% decrease/day.
Triedman et al., 1960.	Single oral dose⟶ Maximum level at 2 to 8 hrs. Chronic Oral dosage initiated ⟶Plateau after 4 to 8 days.	$t_{\frac{1}{2}}$ = 9.5 hours. (Eliminated by 5 days.)
Hansen et al., 1966.	--	$t_{\frac{1}{2}} \simeq$ 9 hours (n=2).
Solomon & Schrogie, 1967.	--	mean $t_{\frac{1}{2}}$ = 26 hours; range, 22 to 34 (n=5).
Chang et al., 1968; and Glazko et al., 1969.	--	mean $t_{\frac{1}{2}}$ = 15 hours; range, 10 to 29 (n=6). (Eliminated by 5 days.)
Kutt & McDowell, 1968.	Normal oral dosage schedule ⟶therapeutic level at 5 to 15 days. 1000 mg oral dose⟶therapeutic level in 3 to 10 hrs.	--
Kater et al., 1969.	"normals" (n=76)... vs. "alcoholics" (n=15)...	$t_{\frac{1}{2}}$ = 23.5 ±11 hours (41% fall/day). $t_{\frac{1}{2}}$ = 16.3 ± 7 hours (68% fall/day).
Kristensen et al., 1969.	--	$t_{\frac{1}{2}}$ = 6.6 to 26.7 hours (n=29).
Arnold & Gerber, 1970.	--	$t_{\frac{1}{2}}$ = 22.0 ± 9 hours (n=70); range, 7 to 42 for (n=68/70).
Baughman & Randinitis, 1970.	--	Human neonate $t_{\frac{1}{2}} \simeq$19 hrs.
Mirkin, 1971a & 1971b.	--	Human neonate $t_{\frac{1}{2}} \simeq$60 hrs (n=7).
Buchanan et al., 1972.	Plateau after initiation of oral DPH—8 to 10 days.	mean $t_{\frac{1}{2}}$ = 29 hours (n=24).

D. THE DISTRIBUTION OF DIPHENYLHYDANTOIN IN VIVO

The effects of protein binding phenomena upon the biological activity and metabolic rate of any drug have been described by a number of authors (e.g. Brodie and Hogben, 1957; Kane and Smith, 1959). Such pharmacological implications of protein binding are considered in the General Discussion.

Table VIII summarizes the various studies which have been reported on the protein binding of diphenylhydantoin (DPH); the techniques of, and materials for, such investigations have varied. Recent data (Lunde et al., 1970; Reidenberg et al., 1971), obtained by employing ultrafiltration which some authors* feel cannot be depended upon for quantitatively correct values, showed much less interpersonal variation in the percentage of total DPH which is unbound than did earlier reports (Svensmark et al., 1960; Triedman et al., 1960; Tveten, 1963; Viukari, 1969).

The consensus at the present time is that DPH is protein bound to a great degree (approximately 80 to 90%) in human plasma. This makes the distribution and biological activity of the drug very susceptible to dramatic changes when even only minor alterations in the protein binding mechanism occur (Desgrez and de Traverse, 1966; Palmer, 1971). The degree of protein binding "must be known to interpret pharmacological phenomena" (Taylor et al., 1954).

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*Grollman, 1926; Goldstein, 1949; Oppenheimer and Surks, 1964; Scholtan, 1964.

TABLE VIII

PROTEIN BINDING OF DIPHENYLHYDANTOIN (DPH) IN BLOOD

Report, Author(s) and Year	DPH FREE (UNBOUND)	EXPLANATION OF TECHNIQUE*
Svensmark et al., 1960.	10.% to 30.%	(n=4 of 5) CSF/serum level.
	45 %	(n=1 of 5).
Triedman et al., 1960.	11 %	(avg.) CSF/plasma level.
	4 % to 35 %	(CSF DPH ≅ dialysate DPH).
Loeser, 1961.	23 %	Dialysis of 5 gram% albumin.
Barlow et al., 1962.	23 %	Sephadex gel filtration.
Firemark et al., 1963.	22 %	Ultrafiltrate ≅ CSF value in animals: 25% at pH = 6.5 18% at pH = 7.5
Tveten, 1963.	14 % to 25 %	CSF/blood level.
Kutt et al., 1964b.	6.7 %	CSF/blood level (5.8/87).
Ang et al., 1969.	14 %	CSF/serum level (n=1).
Kristensen et al., 1969.	6 % to 7 %	Ultrafiltrate of serum.
Viukari, 1969; and Viukari & Tammisto, 1969.	25 % to 100 %	(overall, 3 x 40 CSF/serum ratios),
	31 % to 70 %	(for 40 averages of 3 serial ratios).
	48(±11)%	GRAND AVERAGE (±S.D.) for 3 x n=40.
Lunde et al., 1970.	7.4(±0.7)%	Ultrafiltrate of plasma (n=15).
		For n=5 blood donor plasmas:
	9.3(±0.7)%	Equilibrium dialysate, at room temp.
	6.3(±0.4)%	Ultrafiltrate, at room temp.
	10.4(±0.3)%	Ultrafiltrate, at 37°C. (Values Constant, 7.4≤pH≤8.2)
Rane & Jalling, 1970; and Rane et al., 1971.	7.4(±0.7)%	Normal adults; Ultrafiltrate.
	10.6(±1.4)%	Infants; Ultrafiltrate**.
Reidenberg et al., 1971.	5.8% to 7.3%	Ultrafiltrate, "normal subjects" (n=5).
	8. % to 25 %	Ultrafiltrate, "patients with poor renal function" (n=15).

*Abbreviations: CSF = Cerebrospinal fluid, S.D. = standard deviation.

**The unbound fraction of DPH was even greater in "hyperbilirubinemic" infants.

Although the thermodynamics (including affinity constants) of the DPH binding mechanism have not been studied, there are data on the transfer and equilibration of this drug between body compartments.

The high degree of protein binding of DPH in plasma would tend to slow its distribution from the circulation (Kane and Smith, 1959). Nevertheless, the drug does rapidly diffuse into the brain and other tissues (Kutt et al., 1968; Woodbury, 1969; Lee and Bass, 1970). Levels in the cerebrospinal fluid and brain water are reported to approximate the concentration of unbound DPH found in the plasma (Triedman et al., 1960; Firemark et al., 1963).

The binding of some drugs is responsible for their "extensive and reversible tissue localization" (Brodie and Hogben, 1957). The DPH concentration in the brain equals the total (i.e., unbound plus bound) level in plasma (Dill et al., 1956; Buchthal and Svensmark, 1960; Lee and Bass, 1970). Various reports place the DPH concentration in erythrocytes at 45% of that in serum (Grimmer et al., 1969), or 50% and upwards depending upon the dynamics of the plasma level at the time (Nakamura et al., 1967), or about equal to the plasma level (Dill et al., 1956). The adrenal glands, liver, kidney, salivary glands, and fat all show a concentration of DPH exceeding that of the perfusing plasma (Noach et al., 1958; Nakamura et al., 1967; Woodbury, 1969).

A few reports concerned themselves with the transfer of DPH across the placenta. While DPH injected intravenously

into the mother appears in fetal tissues in significant amounts early within the hour, plasma levels in mother and child are equal at term following more lengthly use of the drug (Baughman and Randinitis, 1970; Mirkin, 1971a and 1971b). The presence of DPH in fetal tissues may be an etiological factor in certain congenital abnormalities (particularly cleft lip and cleft palate) which have been observed in some children born to mothers receiving DPH therapy during most of pregnancy (Mirkin, 1971b). However, no causation has been proven. For clinical DPH intoxication during pregnancy, the one case reported occurred during the third trimester and was followed by the delivery of a normal child (Theil et al., 1961).

In summary, although DPH is approximately ninety percent protein bound in human plasma, the drug crosses biological membranes freely. Reversible binding of DPH throughout the body tends to stabilize the free drug concentration over time, and hence between doses, so that the duration of action of the drug is prolonged considerably (Brodie and Hogben, 1957). It is the free, unbound DPH in the plasma which is the biologically active portion and that ultimately subjected to biotransformation (Taylor et al., 1954; Brodie, 1965).

E. THE SPECTROPHOTOMETRIC DETERMINATION OF BARBITURATES

The literature dealing with the spectrophotometric techniques available for the determination of barbiturates has been summarized in Table IX. For each method listed, the optical density (O.D.) resulting per microgram of drug present depends upon the structure of the barbiturate. There have been two specific proposals for the spectrophotometric identification of which barbiturate drug is present in a sample: the exact shape of the spectrum of difference in O.D. between two pH values (10,13) is characteristic for each of the barbiturate drugs (Goldbaum, 1952); and their rates of hydrolysis, in alkaline solution at 80°C, are likewise characteristic when followed spectrophotometrically (Bjerre and Porter, 1965). To maintain the ability for providing this qualitative aspect, without investing excessive laboratory time either routinely or when a single such determination might be requested, the spectral recording technique is more suitable. In a laboratory assaying anticonvulsant drugs, where phenobarbital (administered as such or else being the metabolite of primidone) is practically the sole barbiturate routinely encountered, this identification procedure should need to be done very seldom.

As regards to the quantitative methods, Bjerre and Porter (1965) have compared the three main prototypes (see Table IX); they all rely upon the existence of various barbiturate species in aqueous solution:

At pH ≃ 2, the drug is ~100% un-ionized (acid form).
At pH ≃ 10, the drug is ~100% in its first dissociation.
At pH ≃ 13, the drug is ~100% in its second dissociation*.
The O.D. of a given solution of barbiturate at each of the above pH values is different; so, the difference in O.D. observed at one wavelength, between two different pH's, is proportional to the concentration of drug. This principle has been used in all of the methods noted.

These techniques are of course subject to interference from the natural and drug constituents of biological samples. Salicylates may appear in as many as twenty percent of the samples submitted for the quantitation of barbiturates, and all three assay prototypes suffer interference from these drugs (Bjerre and Porter, 1965); however, these same authors, as well as Guzak and Caraway (1963), have found a rapid way to detect and then correct for the presence of salicylates in any given case (by measuring difference in O.D. also at 296 mu). The situation with other drugs is less convenient. Bemegride, glutethimide, and sulfathiazole and other sulfonamides interfere in every case (Bjerre and Porter, 1965). Additional interference with the measurement of barbiturate concentration when using O.D. at 240 mu is given by diphenylhydantoin (Schiller and Buchthal, 1958), sulfadiazine (Guzak and Caraway, 1963), ethoxybenzamide and the metabolite

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*Except for the N-substituted barbiturates, e.g. hexobarbital.

of the anticonvulsant drug trimethadione (Olesen, 1967c), plus the anticonvulsants ethotoin and mephenytoin (Svensmark and Kristensen, 1963). Specifically, at 240 mu, greater than about 9 ug of diphenylhydantoin causes significant disturbance with the quantitation of barbiturates even when purification steps are included (Svensmark and Buchthal, 1963b). The frequent co-administration of diphenylhydantoin with phenobarbital makes this a particularly important aspect to be considered.

For the techniques at 260 mu, there are reasonably low blanks for biological samples. Wallace (1969a) has stated that the 260 mu spectrophotometry eliminates interference from almost all "acid-extractable drugs", including diphenylhydantoin up to at least 100 ug/ml, but not the salicylates; although he adjusted the pH of the sample prior to its extraction in order to reduce this interference from salicylates, Guzak and Caraway (1963) have found that this procedure is unwise since it leads to about twenty percent lower recovery of barbiturate.

In conclusion, for an anticonvulsant drugs laboratory, spectrophotometric determination of barbiturates should be done at 260 mu, between pH=10 and pH $\geq$ 13. Further spectral analysis will expose the presence of salicylates and other interfering drugs or metabolites.

TABLE IX

SPECTROPHOTOMETRIC ASSAY METHODS FOR BARBITURATES

Report, Author(s) and Year	Wavelength, Millimicrons	pH values	Other specifics*
Lous, 1950.	240	2, 10	Sample volume = 3 ml Best accuracy = ± 5%
Goldbaum, 1952.	260	10.5, >12	Sample volume = 1 to 5 ml Sensitivity = 10. ug Blank ≃ 1 ug/ml
Plaa & Hine, 1956.	260	10, 13	Sample volume = 5 ml Sensitivity = 25. ug Blank ≃ 0.5 ug/ml
Svensmark et al., 1960.	240	1.5, 9.0	Sample volume = 5 ml Sensitivity = S.D. = 1.4 ug/ml CSF Blank = 1.6 ug/ml
Guzak & Caraway, 1963.	239	2, 10	Sample volume = 3ml Blank ≃ 5 ug/ml, occasionally 10 or 15 ug/ml
Svensmark & Kristensen, 1963.	240	1.5, 9.0	Sample volume = 2 ml Blank ≃ − 0.2 ug/ml S.D. ≃ ± 0.3 ug/ml
Bjerre & Porter, 1965.	259 253 239	10, 13 2, 13 2, 10	(Sample volume = 3 ml Sensitivity ≃ 10. ug)
Olesen, 1967c.	240	1.5, 8.8	Blank = 0.1 ± 0.2 ug/ml S.D. ≃ ± 1.5%
Wallace, 1969a.	260	10.5, 12.0	Sample volume = 5 ml S.D. ≃ 3% (for >50. ug)

*S.D. denotes standard deviation reported.

F. CLINICAL STUDIES ON THE USE OF PHENOBARBITAL IN THE EPILEPSIES

Phenobarbital was the first effective organic anticonvulsant drug to be discovered; in this capacity, its sedative side effects limit the dosage rate so that true toxicity is seldom seen (Goodman and Gilman, 1970).

The level-to-dose relationship for phenobarbital is significantly linear, with quite good correlation (Lous, 1954b; Plaa and Hine, 1960). Overall, 1 mg/kg administered daily to adults and children gives a level of 6 to 12 ug/ml of serum (Buchthal and Svensmark, 1960; Melchior, 1965), so levels within a treated study population are generally 10 to 60 ug/ml (Lous, 1954b; Buchanan and Allen, 1971). The concentration of phenobarbital achieved by long-term therapy depends upon the function of both liver and kidney—not only is the drug hydroxylated in the liver but about 30% is eliminated from the body by direct excretion in the urine as unchanged drug (Lous, 1954b; Schmidt and Wilder, 1968). This urinary excretion of phenobarbital can be augmented several-fold by increasing the pH of the renal tubular fluid (Schmidt and Wilder, 1968).

In grand mal epilepsy, Buchthal and Svensmark (1960) reported clinical improvement at 20 ug phenobarbital/ml of serum. Generally, the therapeutic range for epilepsy has been considered to be 10 to 20 ug/ml (Svensmark and Buchthal, 1964). "Toxic" levels reported by Melchior (1965) ranged from 8 to 100 ug/ml of serum. In a group of sixty-one patients being

treated with phenobarbital, Plaa and Hine (1960) observed five patients who showed signs of drug toxicity. Although the levels for these five were 36 to 77 ug/ml of blood, the other seven patients who also had levels greater than 35 ug/ml showed no toxic signs at all. So, phenobarbital does in fact show variable clinical pictures for a given drug level. This behaviour resembles that emphasized for diphenylhydantoin.

Single oral doses of phenobarbital have been reported to yield maximal serum levels at about 6 hours (Lous, 1954a), or not until 10 to 12 hours after the dose is consumed (Schmidt and Wilder, 1968). In the case of continuous oral administration of phenobarbital, its plateau level is not reached until about 20 days after the initiation of therapy, with 60% of this level being achieved by the end of the first week (Buchthal and Svensmark, 1960; Svensmark and Buchthal, 1963b). Upon cessation of therapy, the level of phenobarbital falls at a rate of 10 to 25% per day (Lous, 1954a; Svensmark et al., 1960). The sum total effect of these dynamic aspects is an approximately 10% variation in level of phenobarbital when the total drug dosage is administered in only one dose per day (Buchthal and Svensmark, 1960).

The degree of protein binding of phenobarbital (50%) was measured by determining the ratio of the drug concentration in cerebrospinal fluid to that in serum, once the cerebrospinal fluid content had been shown to approximate the level in an ultrafiltrate of the corresponding serum (Lous, 1954b). As in the case of diphenylhydantoin however, there

is variation of this binding percentage; the range in humans is about 41 to 52% bound (Svensmark et al., 1960). The implications in this case are somewhat the same as those previously noted relative to the protein binding of diphenylhydantoin.

Much has been written about the interaction of diphenylhydantoin and phenobarbital when they are administered to the same patient at the same or different times. This aspect of the pharmacology of these anticonvulsant drugs will be considered in the General Discussion.

III

MATERIALS

AND

METHODS

A. ASSAY OF DIPHENYLHYDANTOIN*

A micromodification (Lee and Bass, 1970) of the technique of Wallace (1966) was to be employed for the determination of diphenylhydantoin (DPH) in the clinical studies planned for this work. However, a separate glassware unit and a separate condenser with its own cold water supply are required for each determination so done. This makes these and similar methods cumbersome for routine laboratory use (Wallace, 1969b). Additionally, in our hands, the (expensive) glassware recommended by Lee and Bass (1970) proved to be somewhat difficult to use and resulted in rather high blank determinations. So the initial aspects of this work were then directed towards developing a spectrophotometric method for the quantitative determination of DPH — one which would be both rapid for emergency use and yet also immediately adaptable for the uncomplicated assay of numerous experimental and routine clinical samples per day.

The basic concept of Wallace et al. (1965) was retained — a final quantitation step which measures the oxidation product** of DPH. The full procedure and laboratory technology finally developed are described in the following pages, and the laboratory characteristics of the method are noted in Tables X and XI. This method was then employed in all subsequent studies.

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*Published in abbreviated form (Bock and Sherwin, 1971).

**The reaction product of diphenylhydantoin in this method is benzophenone (diphenyl ketone); see Figure 1.

METHOD FOR THE RAPID

SEMIMICRO QUANTITATION OF 5,5-DIPHENYLHYDANTOIN (DPH)

a) MATERIALS & EQUIPMENT

1) Silicone-treated phase-separating filter paper, 11 cm diameter (Whatman No. 1 PS).
2) Fused aluminum oxide anti-bumping granules (British Drug Houses).
3) Syringe-pipettors, 2 ml and 10 ml sizes ("Mini-Pet", Manostat Corp.). Plus disposable 1.5-inch 18 guage needles for use with the smaller pipettor.
4) Glass-stoppered test tubes:
   20 mm x 100 mm, for extraction purposes (Quickfit MF24/2);
   20 mm x 150 mm, for the reaction-distillation step of the procedure (Quickfit MF24/2/6).
   Both types of tubes use B19-size glass stoppers.
5) Shaker, manual or electric.
6) Spectrophotometer capable of measuring optical density in the ultraviolet region around 257 mu (nm). Preferably, semimicro cuvettes with a working volume of approximately 1.5 ml are employed.

b) APPARATUS

1) Connecting bulbs with splash baffle, modeled after the (large) Iowa State type Kjeldahl connecting bulb (Figure 2B).
2) Combination condensing-collection tubes for the steam

distillate, with glass stoppers to fit (Figure 2D-F-G). An adhesive metric scale may be attached to each in order to facilitate reading of the height of distillate columns within the tubes.

3) Standard micro Kjeldahl heating unit (gas or electric) or any other similar source of heat.

4) Specially designed Lucite (Plexiglas) water bath to fit into or alongside the heating unit (Figure 3).

Figure 4 shows the entire apparatus assembled, during the reaction-distillation step of the procedure.

c) REAGENTS

1) For extraction of plasma, serum, and aqueous solutions: Chloroform, reagent grade, filtered but not necessarily redistilled. (Fisher C-298 or J.T. Baker 9180).

2) For extraction of whole blood or erythrocyte lysates: Ethylene dichloride (1,2-dichloroethane), reagent grade, filtered. (Fisher E-175 or J.T. Baker H076 or Matheson, Coleman, Bell DX800).

3) Hydrochloric acid solution, approximately 1 N.

4) Alkalinic permanganate solution — a 2:3 mixture of commercial 50% sodium hydroxide low in carbonate (Fisher So-S-254) with 1% aqueous potassium permanganate ($KMnO_4$).

5) Acid diphenylhydantoin* (HDPH) in absolute ethanol, 1-5 mg/ml, as a primary standard solution.

d) PROCEDURE

(SAMPLE PREPARATION)

1) A 1.0 ml volume of sample, dispensed from the 2 ml syringe-pipettor, plus three drops of the 1 N HCl solution are placed into a 20 mm x 100 mm test tube.
2) A 10 ml volume of chloroform is carefully added into each tube using the larger syringe-pipettor.
3) The tubes are then stoppered, using a few drops of distilled water to seal the ground glass joint so as to ease disassembly later.
4) The tubes, held in the upright position only, are shaken gently by machine for five minutes. (One minute's manual shaking of each tube is as satisfactory.)
5) The organic phase of each tube is filtered into the corresponding 20 mm x 150 mm reaction tube; only one or two tubes should be run at a time:
   (a) Each silicone-treated filter paper cone is soaked with a small volume of pure chloroform.
   (b) The total content of each tube is then poured into the corresponding filter.

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* Kindly supplied by Parke, Davis and Company, Detroit, Michigan (U.S.A.).

(c) When filtration has almost ceased, the aqueous phase held back is returned to its extraction tube; this is done by pouring it from the filter paper cone, which is then rinsed with a 1 ml volume of fresh cholorform to maintain flow through it.

(d) The aqueous phase is then re-extracted with a 4 ml volume of fresh chloroform, by hand-shaking for ten seconds.

(e) The two phases are emptied into the same filter, as is a 2 ml chloroform rinse of the extraction tube.

(f) Finally, before filtration has stopped, two rinses of the entire filter paper cone are made, each with a 1 to 2 ml volume of fresh chloroform.

(g) Just after filtration again appears to be complete, the 20 mm x 150 mm test tube is removed to a water bath heated to 75 to 85°C.

6) The entire volume of organic solvent collected as the filtrate is evaporated from these tubes under a fume hood. To lessen the time requirement for this step, a rapidly flowing stream of air, filtered through glass wool, is directed into each tube.

(REACTION-DISTILLATION)

7) Several anti-bumping granules and 10 to 12 ml of the alkalinic permanganate solution are placed into each dry 20 mm x 150 mm test tube. A generous amount of distilled water is used to "seal" the B19 joint in the glassware as it is assembled (to allow easy disassembly later). The permanganate solution must still be purple when the distillation is begun over the heating unit (Figure 4).

8) A 2 to 4 ml volume of steam distillate is collected in the combination condensing-collection tube (corresponding to a height of 4 to 7 cm for the glassware specified). The exact height of this distillate column in the combination tube is measured and recorded (being a gauge of distillation volume to within ± 1%).

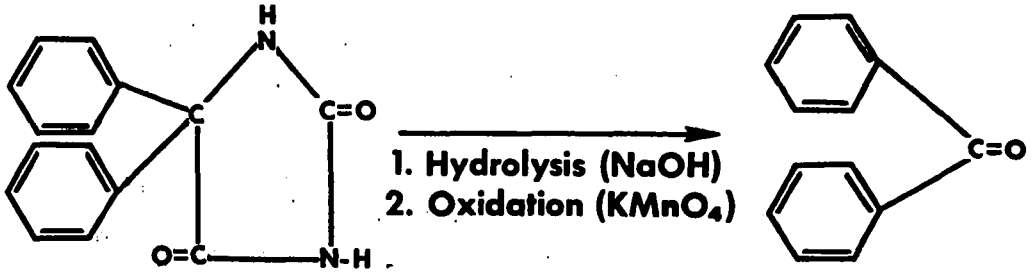
(SPECTROPHOTOMETRY)

9) The distillate solution is mixed thoroughly by inverting the stoppered combination tube several times, keeping the side with the vent tube on top. The optical density (O.D.) is then measured at 257 mu against distilled water of constant source as reference. If $O.D._{275} \leq 0.100$, then a spectrum is recorded for 205 mu to 310 mu; the recorder is set to a full-scale sensitivity of

O.D. = 0.100, and the Δ O.D.$_{257}$ (=O.D.$_{257}$ less O.D.$_{300}$, both measured from the spectrum) is used for the calculation.

(CALCULATION)

10) An "absorbance statistic", calculated by multiplying the optical density of the distillate by its volume (or column height), is proportional to the amount of DPH recovered from the sample, up to at least 150 micrograms. Analyses of standards in drug-free human plasma (and blood) determine the exact factor to be used for conversion of this statistic to the concentration of DPH in the original sample. These standards, at concentrations of 5 to 50 micrograms per ml, are prepared from the primary DPH standard.



Diphenylhydantoin
(Acid form, HDPH*)

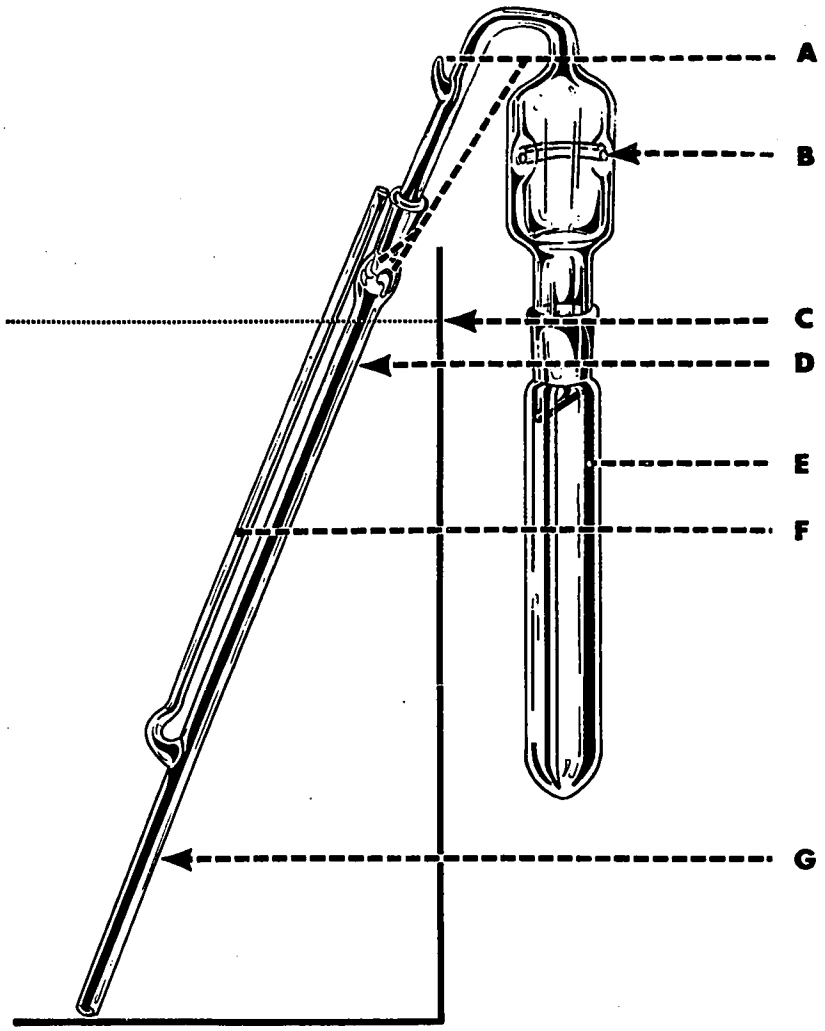
Diphenyl Ketone
(abs λ_{max} 257mμ)

FIGURE 1. REACTION CHEMISTRY.

*M_o (HDPH) = 252g, M_o (NaDPH) = 274g.

Although the correction factor for NaDPH is 92.0% to give the "acid diphenylhydantoin equivalents" of Dill et al. (1956), a pharmaceutical preparation was only 95% NaDPH (87% HDPH) when analyzed. The preferable standard for these assays is therefore the acid form and all results reported in this thesis are in terms of HDPH.

Semimicro Distillation Glassware*



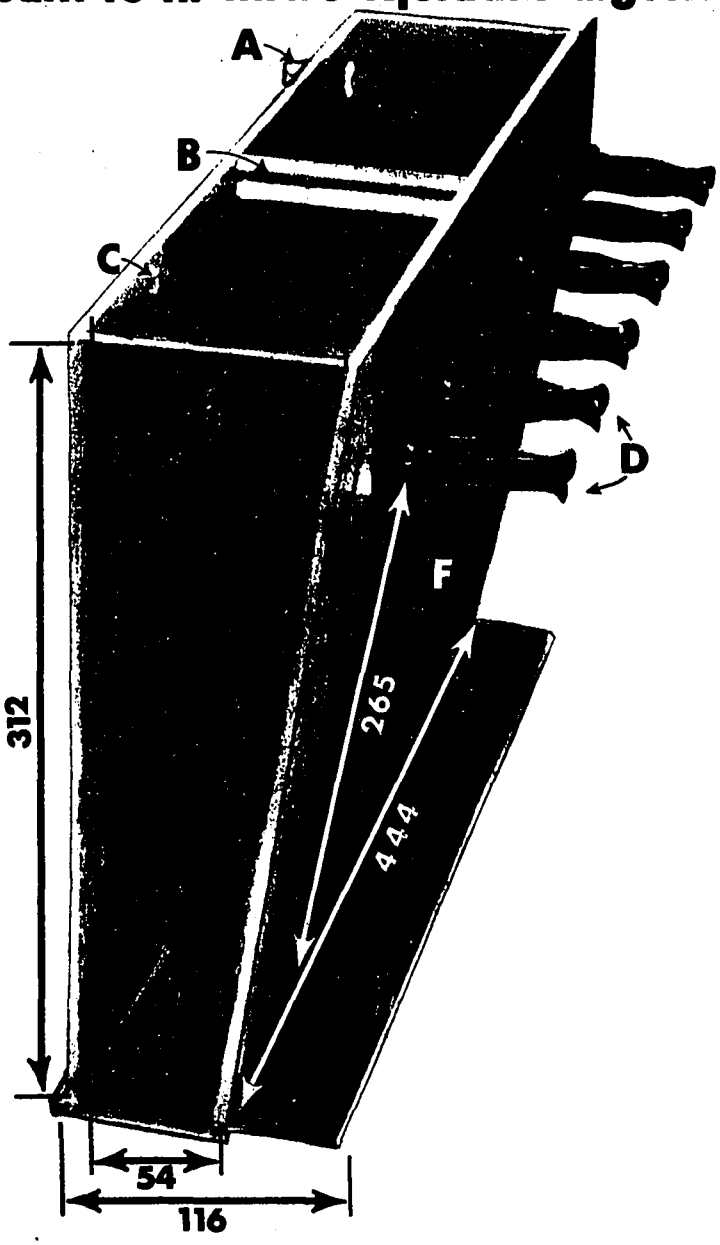
LEGEND:

A. Hooks for supporting the B10 joint between 'B' and 'D'.
B. Connecting bulb* (I.D. 30 mm, inside ht. 65 mm; total outside ht. 140 mm), with splash baffle.
C. Surface of water in bath.
D. Condensing portion of combination condensing–collection tube* (I.D. 8 mm, length 150 mm of total 260 mm length).
E. Reaction tube*, 20 mm X 150 mm, with B19 fitting. (Quickfit MF24 2 6)
F. Vent tube (I.D. 4 mm).
G. Collection portion of the combination tube (I.D. 8 mm, length 110 mm).

* This special glassware unit, as designed by the author, is now available under specification number GB 1000 from:
Canadian Laboratory Supplies, P.O. Box 2090,
Station St. Laurent, Montréal 307, P.Q., Canada.

FIGURE 2.

Waterbath to fit micro Kjeldahl digestion racks*



N.B. Bath material is Plexiglas (Lucite), 0.25 inch thick.
All dimensions labeled above are in millimeters.

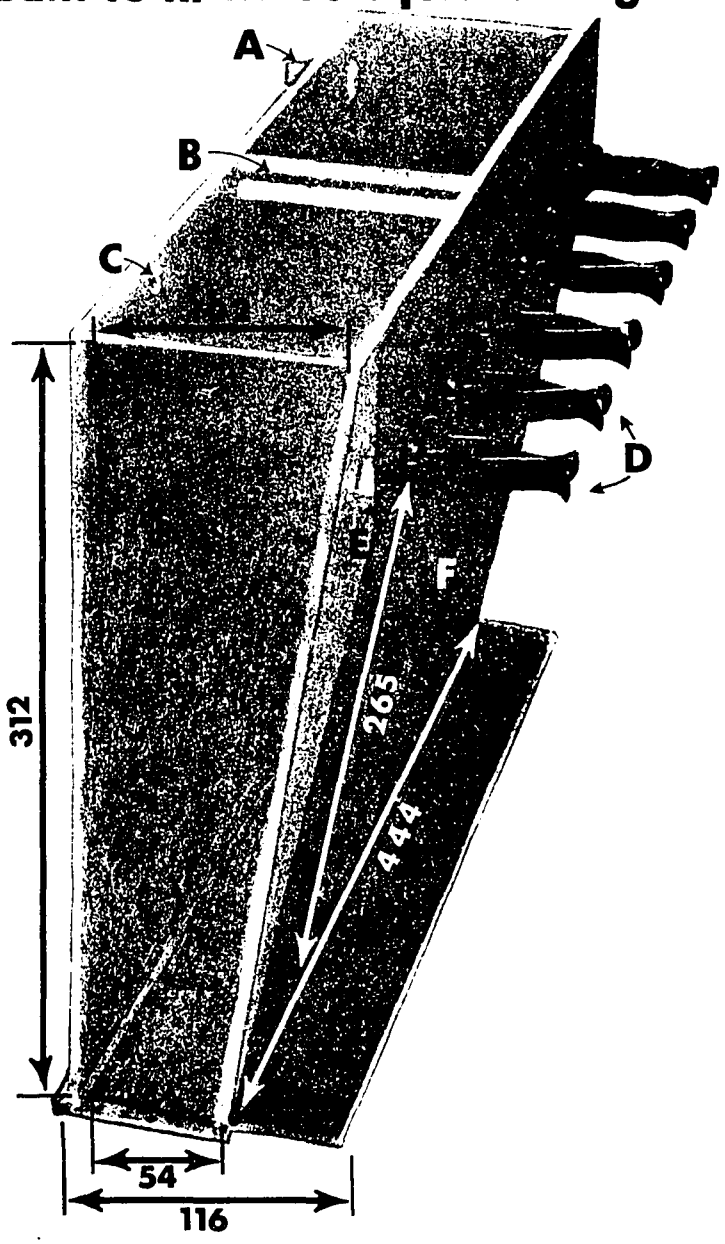
LEGEND

A. Fitting for flowing-water outlet.
B. Hollow rod, 0.75 inch O.D., to prevent warpage of bath walls.
C. Bore for flowing-water inlet tubing.
D. Tube supports; metal clips of the type used for handles of brooms and mops (available at hardware stores).
E. Plexiglas spacer (9 degree wedge), to support 'D' and to keep 0.5 inch air space between bath and sheet 'F'.
F. Aluminum sheet, 1/16 inch thick, 12.8 x 17.5 inches.

FIGURE 3.

*Rack was obtained from Precision Scientific, Chicago, Illinois, U.S.A.

Waterbath to fit micro Kjeldahl digestion racks*



N.B. Bath material is Plexiglas (Lucite), 0.25 inch thick.
All dimensions labeled above are in millimeters.

LEGEND

A. Fitting for flowing–water outlet.
B. Hollow rod, 0.75 inch O.D., to prevent warpage of bath walls.
C. Bore for flowing–water inlet tubing.
D. Tube supports; metal clips of the type used for handles of brooms and mops (available at hardware stores).
E. Plexiglas spacer (9 degree wedge), to support 'D' and to keep 0.5 inch air space between bath and sheet 'F'.
F. Aluminum sheet, 1/16 inch thick, 12.8 x 17.5 inches.

FIGURE 3.

*Rack was obtained from Precision Scientific, Chicago, Illinois, U.S.A.

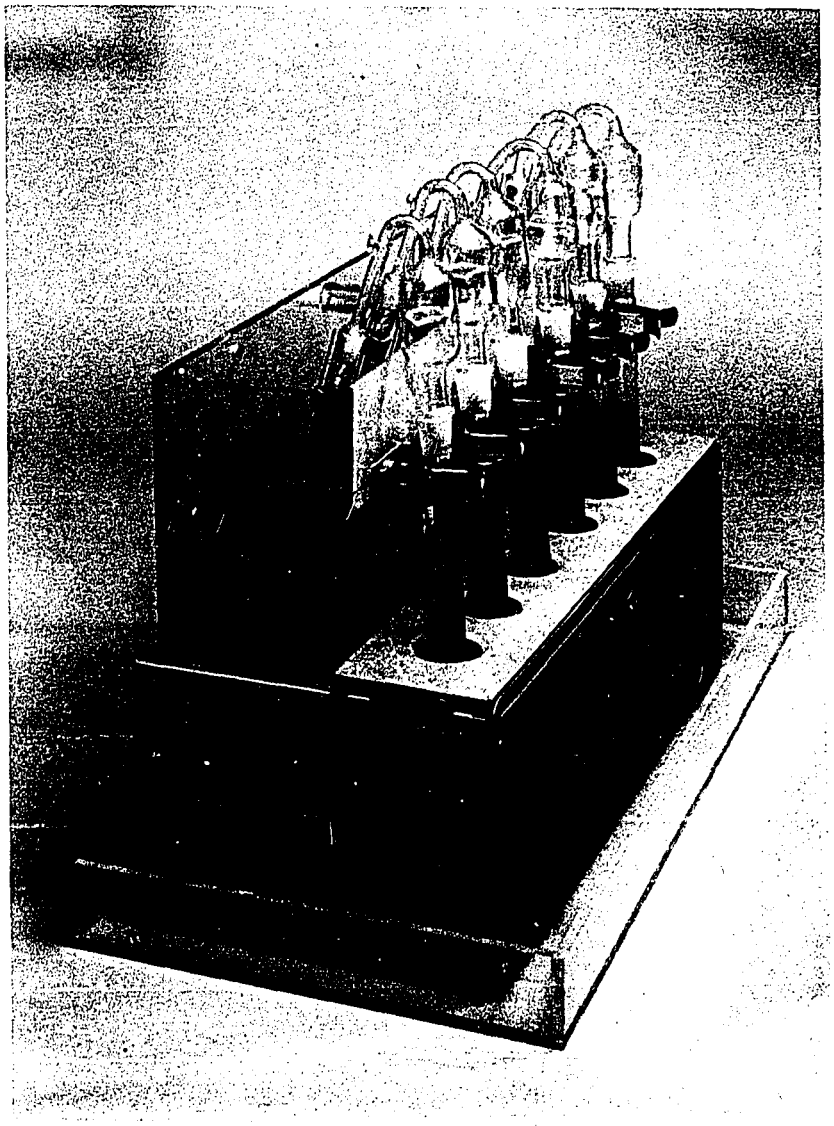


FIGURE 4. ARRANGEMENT OF APPARATUS DURING THE REACTION-DISTILLATION STEP OF THE PRESENT ASSAY METHOD FOR DIPHENYLHYDANTOIN.

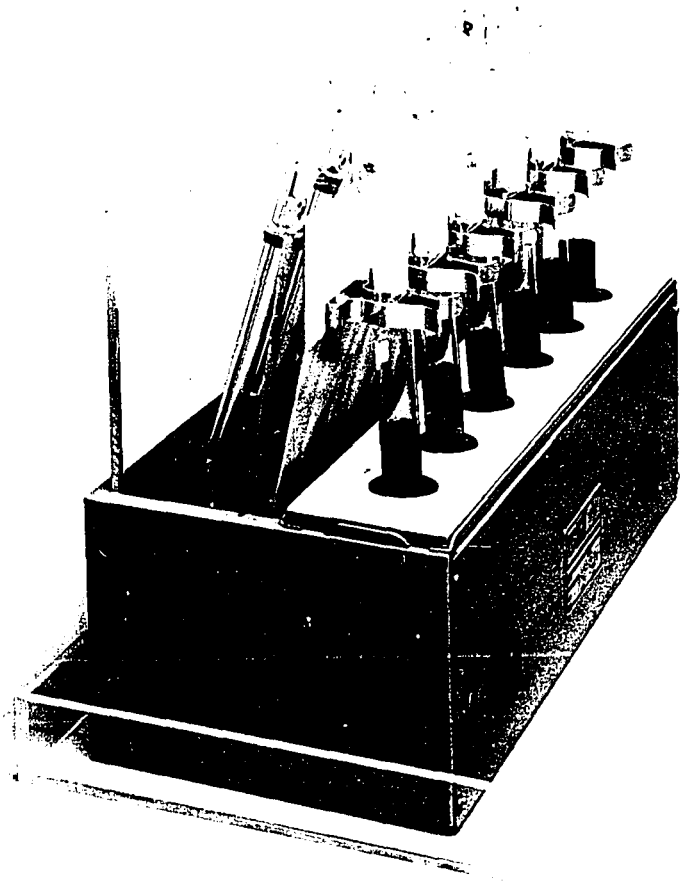


FIGURE 4. ARRANGEMENT OF APPARATUS DURING THE REACTION-DISTILLATION STEP OF THE PRESENT ASSAY METHOD FOR DIPHENYLHYDANTOIN.

TABLE X

CHARACTERISTICS OF THE ASSAY TECHNIQUE FOR DIPHENYLHYDANTOIN (DPH)

Volume of sample required per determination	1 ml
Number of purification steps for biological samples	NONE
Number of extraction steps per determination	ONE
Number of filtration steps per determination	ONE
Number of centrifugation steps per determination	NONE
Number of reactions per determination	ONE
Organic solvent employed (DPH in serum, plasma, or water)	Chloroform
Organic solvent employed (DPH in whole blood or erythrocyte lysates)	Ethylene dichloride
Volume of solvent used per extraction	10 ml
Total volume of solvent used per determination	20 ml
Test tubes employed per determination	TWO
Approximate duration of distillation step	15 min.
Volume of steam distillate collected	2 to 4 ml
Reagent blank determination, distillate at 257 mu	O.D. $\simeq$ 0.000
Plasma blank determination, distillate at 257 mu	$0.000 \leq$ O.D. ≤ 0.015 ($\simeq$0.00 to 0.75 ug DPH)
$O.D._{257}$ per ug DPH reacted (10 mm path length)	0.020-0.028
Standard deviation (S.D.) for assay of plasma	0.7 ug/ml
Threshold for assay of plasma (only $O.D._{257}$ measured) = Blank + S.D.	1.5 ug
Threshold for assay of plasma (when spectrum is recorded)	0.6 ug
Absolute limit of detection for reaction-distillation-spectrophotometry	0.2 ug
Recovery of DPH from aqueous solution*	98%
Recovery of DPH from plasma and serum*	94%
Recovery of DPH from whole blood*	82%
Recovery of DPH from erythrocyte lysates*	84%
Completeness of reaction, DPH $\longrightarrow$ diphenyl ketone	100%
Recovery of diphenyl ketone from reaction tube into distillate solution	85%
Overall efficiency of the assay from beginning to end — For aqueous DPH	83%
Overall efficiency of the assay from beginning to end — For plasma DPH	80%

*These recoveries are actual figures, uncorrected for volume losses.

TABLE XI

COMPOUNDS WHICH OFFERED NO INTERFERENCE TO THE PERMANGANATE OXIDATION TECHNIQUE FOR ASSAYING DIPHENYLHYDANTOIN (DPH)

(A) Wallace, 1966; in vitro (pure drugs):

Amobarbital	Hydantoin
Aprobarbital (Alurate)	Mephenytoin (Mesantoin)
Aspirin (ASA)	Phenacemide (Phenurone)
Barbital	Phenobarbital
Ethotoin (Peganone)	Phensuximide (Milontin)

(B) Lee and Bass, 1970; in vitro (pure drugs):

Ethosuximide (Zarontin)
Primidone (Mysoline)
Sodium warfarin (Coumadin)

(C) The present study; in vivo, i.e. from patient plasmas (drugs and their metabolites at the concentrations actually present in vivo):

Acenocoumarol (Sintrom)	Magnesium-aluminum hydroxide (Magnolax)
Amitriptyline HCl (Elavil)	Mephenytoin (Mesantoin)
Amobarbital (Amytal sodium)	Pentaerythritol tetranitrate (Peritrate)
(Angiography dye)	Phenobarbital
Chloral hydrate	Primidone (Mysoline)
Codeine	Procyclidine HCl (Kemadrin)
Diazepam (Valium)	Propoxyphene HCl (Darvon)
Ethosuximide (Zarontin)*	Secobarbital (Seconal)
Fluorescein	Trifluoperazine HCl (Stelazine)
Folic acid	Vitamin B_{12}
Haloperidol (Haldol)	

PLUS "5-parahydroxyphenyl-5-phenylhydantoin", HPPH, the main metabolite of DPH (up to 1.0 milligram reacted per analysis).

*Up to at least 195 ug/ml, as determined by gas chromatography.

B. ASSAY OF PHENOBARBITAL

Of the methods available for the spectrophotometric determination of barbiturates, those employing analysis at 260 mu are the most reliable when diphenylhydantoin (DPH) and other anticonvulsant drugs may also be present. The rationale behind these techniques was investigated using phenobarbital — absorption spectra showing the spectrophotometric behaviour of this drug are given in Figures 6 to 9.

The buffer systems and procedural variations proposed in the many reports noted previously were all tested for suitability to an anticonvulsant drug laboratory.

The buffer system chosen for the solution of pH = 10.2 was bicarbonate, approximately as proposed by Plaa and Hine (1956). The bicarbonate pK_a = 10.3 makes it far more powerful* at maintaining the proper pH than the usual borate system (pK_a = 9.2). In the present method, the final solution is stable at the desired pH (10.2) for hours after mixing; the reliability of the assay has thereby been improved. Additionally, the use of a saturated solution of potassium bicarbonate (approximately 3.3 Molar) for mixing the buffer solution entirely eliminates any need for accurately weighing reagents or for empirically adjusting the concentration of the sodium hydroxide solution regularly (e.g., Goldbaum, 1952). Overall, the time saved in this technique is substantial.

---

*A buffer is most efficient when solution pH = pK_a of the buffer (see Glasstone and Lewis, 1960).

This method serves as both a qualitative and a quantitative assay for barbiturates:

a) MATERIALS AND EQUIPMENT

1) Silicone-treated phase-separating filter paper, 11 cm diameter (Whatman No. 1 PS).

2) Glass-stoppered test tubes, 20 mm x 100 mm and 20 mm x 150 mm (Quickfit MF24/2 and MF24/2/6). Both sizes use B19-size glass stoppers.

3) Shaker, manual or electric.

4) Vortex mixer for test tubes (e.g., Fisher mini-shaker).

5) Spectrophotometer, preferably a double beam instrument, capable of measuring optical density in the ultraviolet region from 220 mu to 310 mu. Semi-micro cuvettes with a working volume of approximately 1.5 ml are employed.

b) REAGENTS

1) Chloroform, reagent grade, filtered but not necessarily redistilled (for extraction of plasma, serum, and aqueous solutions).

2) Ethylene dichloride (1,2-dichloroethane), reagent grade, filtered (for extractions from other biological materials).

3) Concentrated hydrochloric acid solution.

4) Sodium hydroxide solution, 0.50 Normal, made by appropriate dilution of a commercially available standard solution (5.00 or 10.0 Normal).

5) Potassium bicarbonate solution, saturated at 25°C, with excess salt crystals present (concentration approximately 3.3 Normal).

6) Phenobarbital in absolute ethanol, 1 to 5 mg/ml, as a primary standard solution.

c) PROCEDURE

(SAMPLE PREPARATION)

1) A 1.0 ml volume of sample plus one drop of concentrated hydrochloric acid are placed into a 20 mm x 100 mm test tube.

2) An 8 ml volume of chloroform is carefully added into each tube.

3) The tubes are then stoppered, using a few drops of distilled water to seal the ground glass joint.

4) The tubes, held in the upright position, are shaken gently by machine for five minutes. (One minute's manual shaking of each tube is as satisfactory.)

5) The organic phase of each small tube is filtered into the corresponding larger (20 mm x 150 mm) test tube; only one or two tubes should be run at a time.

   (a) Each silicone-treated filter paper cone is soaked with a small volume of pure chloroform.

   (b) The total content of each small tube is then poured into the corresponding filter.

(c) When filtration has almost ceased, the aqueous phase held back is returned to its extraction tube.

(d) This aqueous phase is then re-extracted with a 4 ml volume of fresh chloroform, by hand-shaking for ten seconds.

(e) The two phases are emptied into the same filter, and before filtration has stopped completely a rinse of the entire filter paper cone is made with a 1 ml volume of chloroform. The filter is discarded when the flow ceases.

6) A 4 ml volume of 0.50 N sodium hydroxide solution is added to the total amount of chloroform filtrate recovered from the preceding steps. The tube is then stoppered and shaken rapidly by hand for one minute.

(QUANTITATION)

7) After the tubes have set in a rack for ten minutes, to allow the phases to separate, a 1.0 ml aliquot of the aqueous phase is placed into each of two small tubes (approximately 12 mm x 100 mm). These tubes are then checked visually to assure that no chloroform has been included in either aliquot.

8) (a) A 1/3 ml volume of pure 0.50 N sodium hydroxide solution is added to one tube, maintaining the pH > 13.

(b) A 1/3 ml volume of the saturated solution of potassium bicarbonate is added to the other tube, so that the final pH = 10.2 ± 0.2 (final concentration of the carbonate ion ≃ final concentration of the bicarbonate ion ≃ 0.4 Molar).

9) The contents of each tube are mixed thoroughly on a vortex mixer.

10) Spectrophotometry is carried out with the solution at pH > 13 in the "sample" beam and the solution at pH = 10.2 in the "reference" beam.

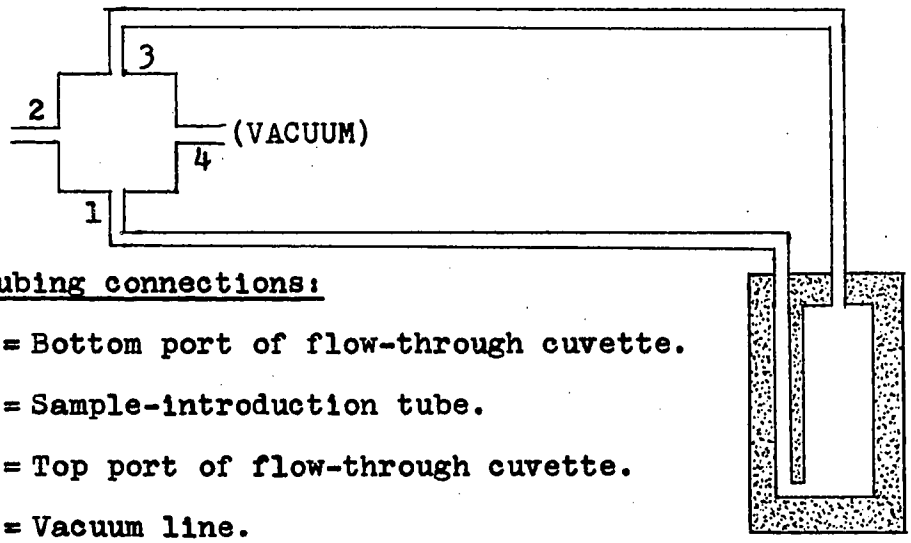
11) Interpretation of this spectrophotometric step has three aspects —

(a) Detection and quantitation of salicylates if present (as per method of Bjerre and Porter, 1965).

(b) Identification of the specific barbiturate present, by observing the spectrum of difference in optical density throughout the range 220 mu to 310 mu (see Goldbaum, 1952). The spectrum for phenobarbital is shown in Figure 6.

(c) Quantitation of phenobarbital is done from knowing the difference in optical density at 260 mu, correcting for salicylates if necessary.

A)



Tubing connections:

1 = Bottom port of flow-through cuvette.

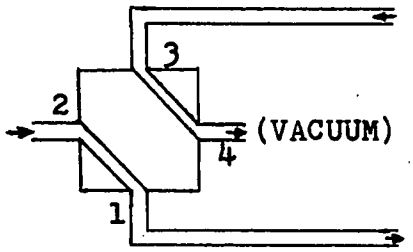
2 = Sample-introduction tube.

3 = Top port of flow-through cuvette.

4 = Vacuum line.

FLOW-THROUGH SPECTROPHOTOMETER CUVETTE

B)

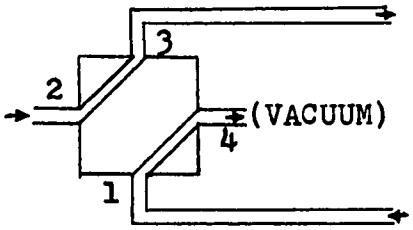


(2 = distilled water, or sample)

2 → 1
3 → 4

Water, or sample, enters the cuvette via bottom port.

C)



(2 = tube open to room air)

2 → 3
1 → 4

Contents of the cuvette pass out via bottom port into the vacuum line.

FIGURE 5. FLOW-THROUGH SPECTROPHOTOMETER CUVETTE WITH FOUR-WAY VALVE SYSTEM.
For the rapid, semi-automatic changing of samples undergoing spectrophotometric analysis:
A) Schematic diagram.
B) Filling or washing the cuvette.
C) Emptying the cuvette.

The simultaneous use of two such systems with a double-beam spectrophotometer is ideal for the present method for the determination of barbiturates; two corresponding solutions are analyzed, one in each beam.

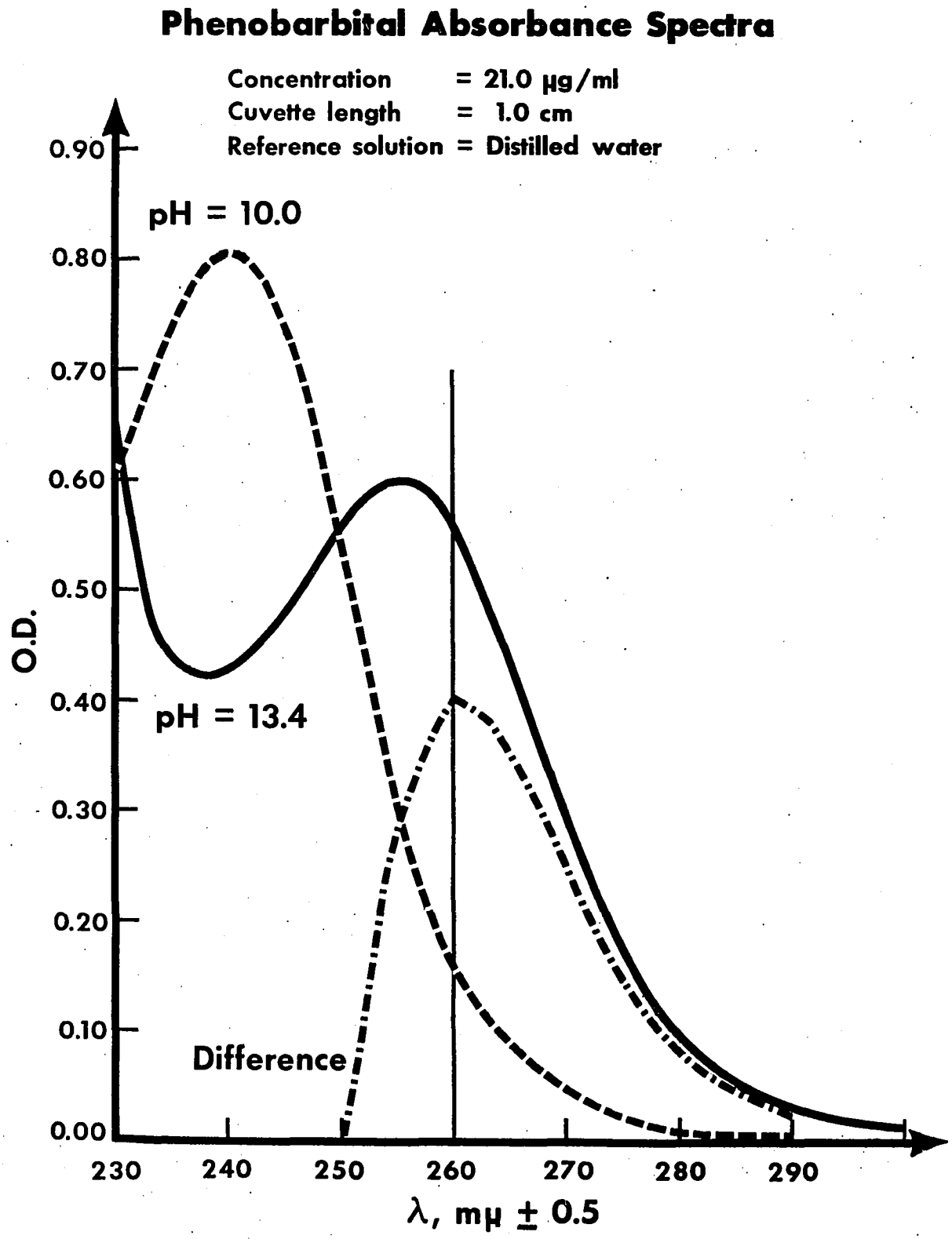


FIGURE 6. PHENOBARBITAL ABSORBANCE SPECTRA.

At 260 millimicrons (mu), the specific absorbances are:

pH ≥ 13.0, O.D. = 0.027 per ug/ml;
pH ≅ 10.0, O.D. = 0.008 per ug/ml;
Difference in O.D. = 0.019 per ug/ml.

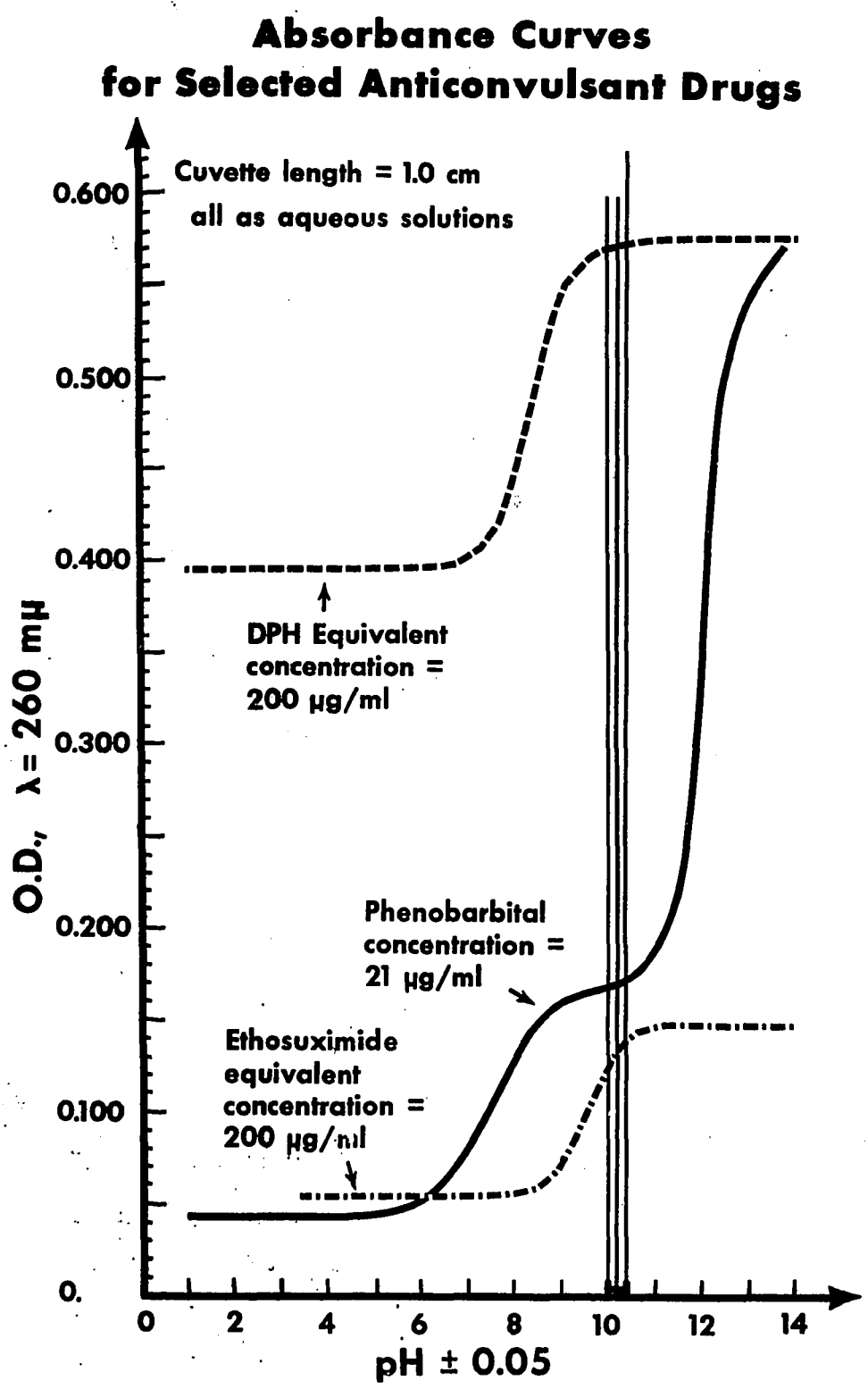


FIGURE 7. ABSORBANCE CURVES FOR SELECTED DRUGS.

For quantitative determination of phenobarbital at 260 mu, employing a solution of pH = 10.2 ± 0.2 as in the present method, common possible interferences minimized are:

400 ug DPH ——————→1 ug phenobarbital.
100 ug ethosuximide ——————→1 ug phenobarbital.

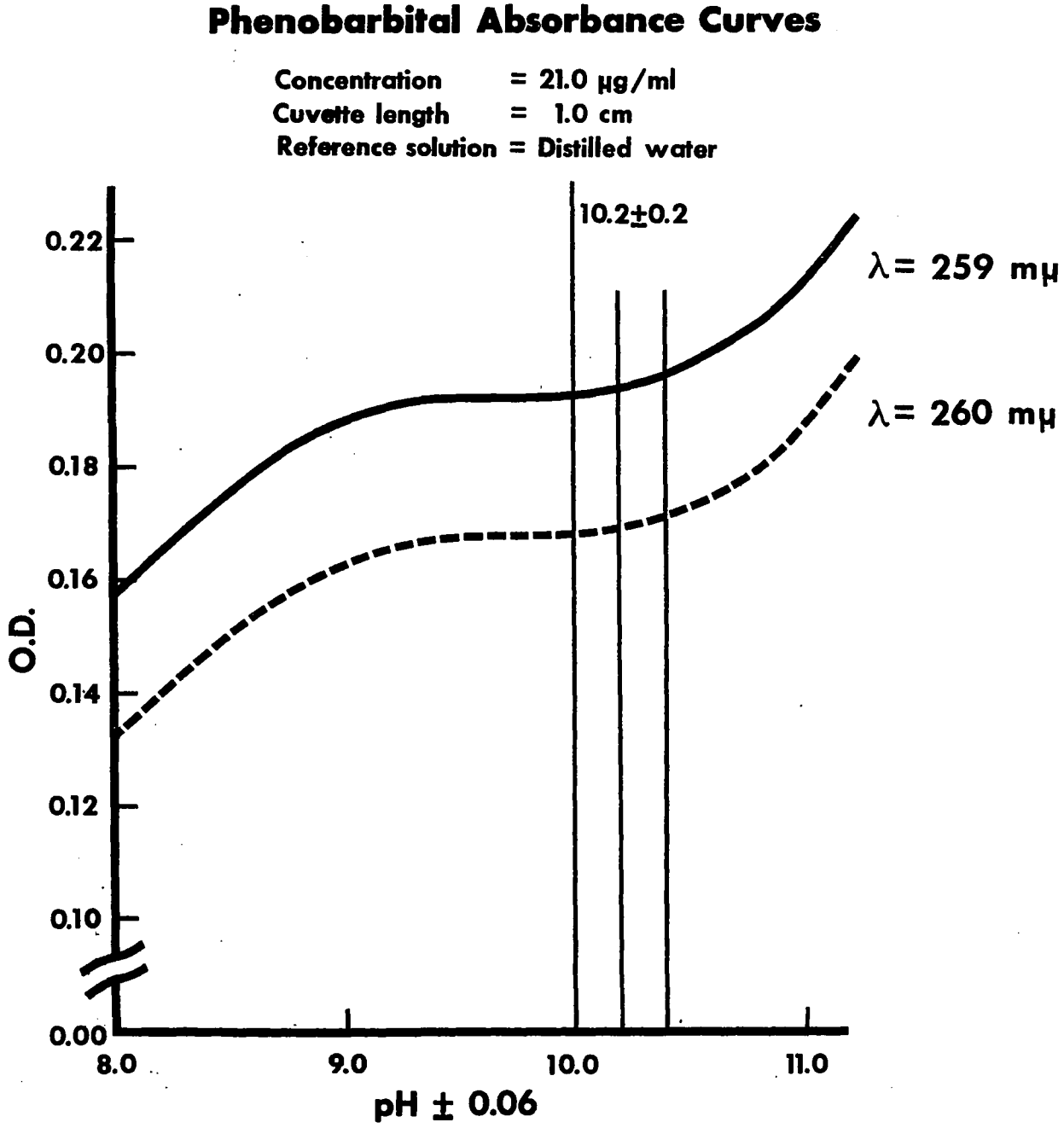


FIGURE 8. PHENOBARBITAL ABSORBANCE CURVES.

The pH region shown here corresponds to the middle portion of Figure 7, and demonstrates that the absorbance of a solution of phenobarbital is most constant around pH = 10.0. (The vertical lines indicate the limits on the pH of the buffer solution, i.e. bicarbonate, used in the present method—10.2 ± 0.2.)

Notice how important exact adjustment of the spectrophotometer's wavelength is; however, when a double-beam spectrophotometer is employed, the sharp O.D. peak shown in Figure 6 distinctly marks the proper wavelength for the analysis (260 mu).

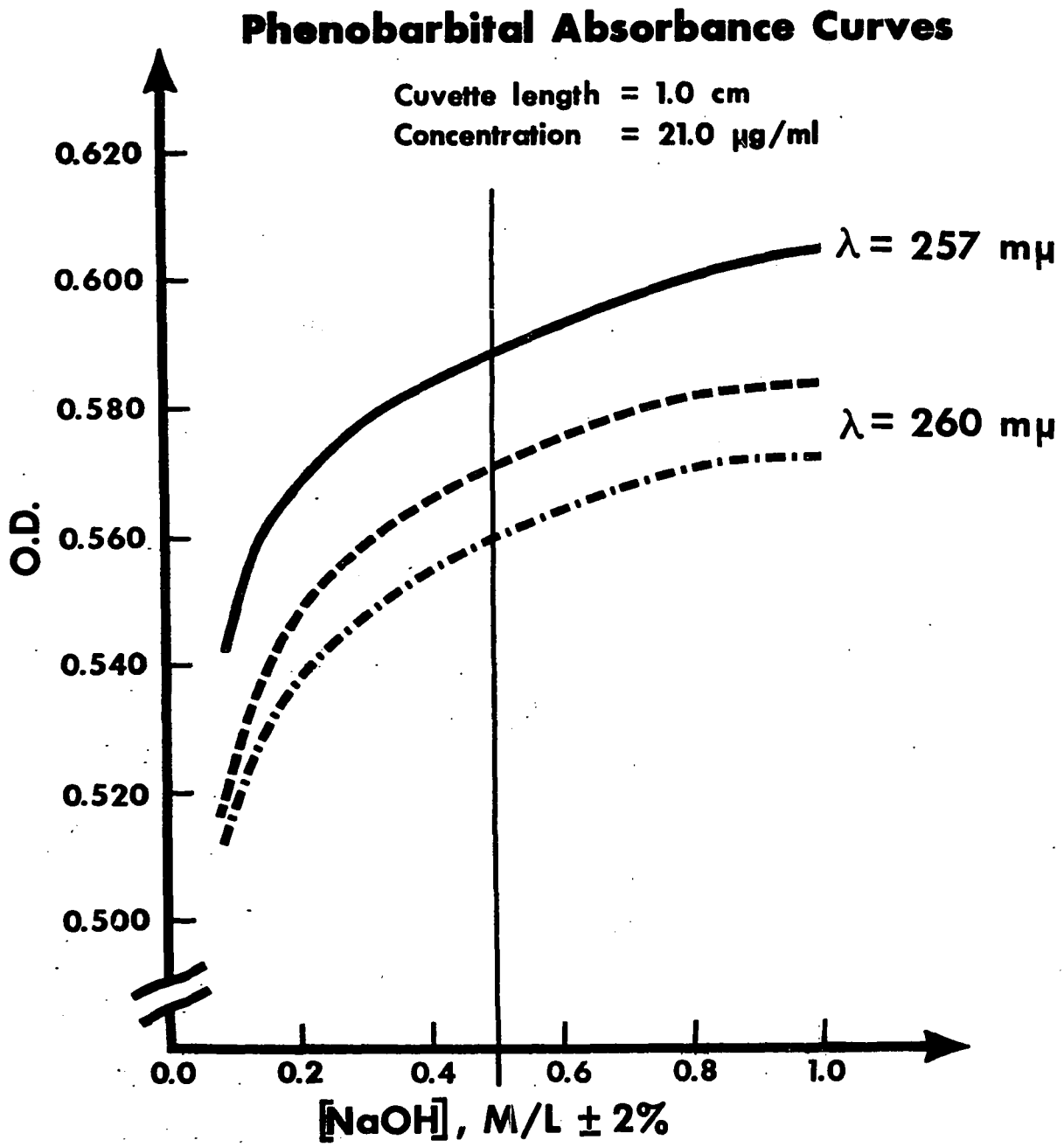


FIGURE 9. PHENOBARBITAL ABSORBANCE CURVES.

The optical density (O.D.) of a solution of phenobarbital at alkaline pH's is shown. The vertical line at 0.50 Molar sodium hydroxide indicates the concentration chosen for analysis of barbiturates by the present method.
(The two curves at wavelength 260 mu were obtained for different reference solutions: the upper was read against distilled water, the lower, against an alkaline solution of the same varying concentration but without drug.)

C. DESCRIPTION OF PATIENTS STUDIED, AND THE CRITERIA OF CLINICAL EVALUATION

PATIENTS FOR GENERAL CLINICAL CORRELATIONS

A clinical laboratory just beginning to measure anticonvulsant drugs must determine its own "therapeutic" range of concentration for each drug to be assayed; the results will be a reflection of the specific patient population and the manner of treatment emphasized by the physicians referring samples for analysis. Assays employed for this purpose by the Clinical Neuropharmacology Laboratory of the Montreal Neurological Institute, and reported here, had been requested by physicians investigating suspected abnormally high or very low levels of diphenylhydantoin (DPH). Most of the patients studied were either hospitalized in the Montreal Neurological Hospital or else were being treated in its Epilepsy Clinic. The remainder were referred as out-patients in the offices of the Hospital, while a few samples came from other hospitals in various parts of the Province of Québec.

PATIENTS IN THE SINGLE DOSE STUDY

The twenty-nine patients participating in the single vs. divided dose study of DPH resided at Foyer Savoy in Mont St. Hilaire, Québec. All had epilepsy which was being treated by the administration of DPH, usually in combination with other drugs. (This study was carried out with the permission of Dr. Bernard F. Graham, neurologist in charge at the institution.)

These patients had been receiving steady doses of DPH for at least three months prior to the beginning of the study, and the initial dosage rate was maintained in each throughout the nine month duration of the entire study. All were diagnosed as having had idiopathic epilepsy and/or generalized seizures or psychomotor epilepsy; patient ages ranged from 14 to 60 years (median = 33), and weights, from 46 to 87 kilograms (median = 71). Experimental design was a controlled cross-over study: each patient would receive his DPH as usual (in divided doses) for one full month (study "period" I), and then his total daily dosage of DPH would be given in <u>one</u> single morning dose (at 8 a.m.) each day for the following month (study "period" II). For periods (months) III to IX, the (usual) divided-dose format was resumed. For <u>all</u> months, seizure records were kept on each patient, and plasma DPH levels were monitored periodically.

Besides providing the actual comparisons of levels and clinical pictures under the two dosage regimens, this patient group was taken as being very suitable for an analysis of both the level-to-dose relationships for DPH and the variation of its level over extended periods of time: drug intake was reliable since it was supervised by the nursing staff, and the subjects were initially chosen so as to meet certain medical and neurological criteria.

These data were not included with the information derived from the general patient group described first.

CRITERIA OF CLINICAL EVALUATIONS REPORTED

All correlations of levels with clinical status were made according to the following guidelines:

1) Only those requisitions on which the referring physician had reported a specific clinical state (e.g., controlled/or uncontrolled; absence of toxicity/or specific toxic signs noted) were included when establishing ranges for any of the various clinical states considered.

2) In the case that a given patient had levels recorded at different times, only one was included in the tabulation unless a significant change in clinical status had occurred. For each patient, the levels chosen were —

the highest non-toxic level,

the lowest toxic level,

the lowest level showing adequate seizure control,

the highest level showing inadequate seizure control.

3) For practical purposes, "adequate" seizure control in epilepsy was considered to be attained when no more than a single major seizure was observed in a three month period; "complete" seizure control was taken to indicate that no major seizure had been reported in at least the past full year.

4) Finally, the tabulation of clinical status included categorization under one of three gradations of toxicity—

a) "None", for a physician's report of the absence of toxicity.

b) "Mild toxicity", when one or two of the following signs were reported: nystagmus, ataxia, drowsiness, gum hypertrophy, or hirsutism.

c) "Severe toxicity", when three (or more) of the above signs were seen, or when "severe" was specifically noted for any one sign, or when certain neuropsychiatric changes were reported.

The present study is composed of 553 determinations on 360 patients; of these, 294 were accompanied by specific statements regarding the presence or absence of toxic signs, while 263 were suitable for the correlation of level with degree of control of seizures.

D. COLLECTION AND HANDLING OF SAMPLES

Blood samples were routinely obtained by venipuncture, with ethylenediamine tetraacetic acid (EDTA) as anticoagulant; the Vacutainer system was employed (Becton, Dickinson & Company). The samples were centrifuged, stored under refrigeration, and then their plasma layers were taken for analysis in the laboratory. The levels reported herein are expressed in units of micrograms (ug) of drug per millilitre of plasma, unless otherwise noted.

As other types of blood-derived preparations were occasionally received by the laboratory, a brief study was done to compare the results of the assay of simultaneously obtained samples of plasma, whole blood, and serum. Also assessed were the effects of variation of the anticoagulant system upon the value of a determination of diphenylhydantoin (DPH) in "plasma".

For quantitative evaluation of the new assay method for DPH (Bock and Sherwin, 1971), its standard deviation for clinical purposes was estimated by analyzing successive samples in duplicate — 50 plasma samples, and a total of 100 samples of plasma, whole blood, and serum were so determined. To simulate extreme laboratory handling conditions, the effects due to sampling errors were included in this evaluation of the method — bloods were received in sealed tubes and were stored this way for several days in the refrigerator until sampling procedures were done; the technique of sampling purposely

involved no attempt to make the "plasma" layer homogeneous, but merely consisted of the gentle removal of two successive one millilitre volumes from the top of the tube for the duplicate analyses. An estimate of the "maximal" standard deviation was thereby obtained.

E. INVESTIGATION OF THE DIPHENYLHYDANTOIN CONTENT OF THE COMPARTMENTS OF WHOLE BLOOD

To investigate the effects of natural variation in the degree of protein binding of diphenylhydantoin (DPH), the erythrocyte (because of its ready availability) was to be used as a model for other cells of the body, including those of the brain. The cellular content of DPH would serve as an indication of the actual effective concentration of the drug *in vivo*.

Methods generally employed to study the drug content of erythrocytes use radioactive drugs directly (Crispell and Coleman, 1956), or involve extraction of the drug from *lysed* cells (Wall and Migeon, 1959), or both (Sandberg and Slaunwhite, 1957). Three reports investigating DPH in particular (Dill et al., 1956; Nakamura et al., 1967; Grimmer et al., 1969) gave different ratios of cellular to plasma or serum contents of the drug, but none described the exact methodology employed in reaching the figures.

Prototype procedures have produced hemolysis either by using distilled water (Crispell and Coleman, 1956; de Moor et al., 1962) or by freezing the centrifuged cells (Wall and Migeon, 1959). Drug analysis was then done by radioactive counting, or by chemical assay following extraction of the drug. In the present study, initial methodology was aimed at investigation of the drug *in vivo*, so radioactive labeling would not be used.

The procedure which was developed to study the concentration of DPH in erythrocytes involved the following scheme:

1) Unwashed erythrocytes were centrifuged twice, the plasma and buffy-coat layers being removed completely each time.

2) These "packed cells" were then stored in tightly-closed tubes in a deep freezer.

3) Just before being assayed, each sample was thawed, refrozen, and thawed again while in the same container. These steps assured complete hemolysis, as was verified microscopically.

4) The viscous lysate so produced was sampled using a pipet with a very wide bore — the 1 ml sample volumes were placed into each extraction test tube with a syringe pipettor ("Mini-Pet", Manostat Corp.). No hydrochloric acid or water was added when DPH was being extracted. The organic solvent employed for all lysates was a 10 ml volume of ethylene dichloride (1,2-dichloroethane). The actual extraction of drug was then accomplished by shaking the stoppered extraction test tube by hand for one minute.

5) The remainder of the procedure followed that used for the assay of drug in plasma samples, from filtration and re-extraction steps through the spectrophotometry. A separate standard curve for lysates was produced so that absolute concentrations of drug could be calculated from the assay results.

The related topic, protein binding, may be studied in several different ways as well. When electrophoresis is employed, a drug may unbind and move independently from the proteins (Scholtan, 1964); qualitative data can be obtained but the interpretation of this may be somewhat uncertain (Oppenheimer and Tavernetti, 1962). Gel filtration is good as a qualitative technique and is one well suited for the study of competitive binding phenomena (Desgrez and de Traverse, 1966). Ultracentrifugation produces a gradient of protein concentration and therefore partially separates protein-bound from protein-free drug in small volumes of sample (Scholtan, 1964). Ultrafiltration techniques employ centrifugal force or gas pressure to obtain protein-free "plasma water". These results are subject to errors caused by membrane phenomena (Grollman, 1926).

The methodology finally assembled for the present studies involved equilibrium dialysis, as this appeared to be the safest, most fundamental technique for investigating the quantitative aspects of protein-binding phenomena (Goldstein, 1949; Oppenheimer and Surks, 1964). Comparison studies with several different ultrafiltration techniques were also carried out. The material used for all of these studies was human plasma from individual patients, and normal plasma pooled from routine hematology tests done in the Montreal Neurological Hospital.

The exact procedure developed and the laboratory equipment chosen for binding studies were somewhat similar to those described by Scholtan (1964) and Ali and Routh (1969):

a) APPARATUS AND EQUIPMENT

1) Refractometer, for measuring protein concentrations in plasmas and in solutions of specific proteins studied.
2) Cellulose dialysis tubing, flat width equal to 1.2 inches; effective pore size is 4.8 mu, to retain substances of molecular weight greater than 12,000.
3) Cylindrical, Teflon-covered magnetic stirrer, diameter = 7 mm (0.25 inch) and height = 14 mm (0.50 inch)—by the name "Spinfin" (Bel-Art Products, Pequannock, New Jersey 07440, U.S.A.).
4) Miniature equilibrium dialysis chamber, each half-cell of volume 5 ml (Precision Cells, Inc., 221 Park Ave., Hicksville, N.Y. 11801). In order to accommodate one cylindrical stirrer upon a flat surface, the inside face of each plastic half-cell was milled (vertically 1/8 inch deep by 3/8 inch wide and horizontally at the bottom 1/8 inch deep by 3/16 inch tall by 1/2 inch wide).
5) Magnetic stirrer unit to rotate the two cylindrical stirrers in each chamber simultaneously.
6) Glass syringes with Luer-Lok fitting, 5 ml capacity. Needles, 18 gauge, 1.5-inch for introducing samples

and 3.5-inch for recovering samples and dialysates from the dialysis chamber.

b) REAGENTS

1) A concentrated "primary standard" (solution) of the drug(s) to be studied.

2) Elliott's A solution*, with disodium ethylenediamine tetraacetic acid (Na_2EDTA) added to a concentration of 1 mg/ml in order to complex the calcium present.

3) Sample—a 5 ml volume of plasma, serum, or a solution of protein to be studied.

c) PROCEDURE

(PREPARATION OF DIALYSIS CHAMBER)

1) A suitable length of dialysis tubing is washed in warm running tap water for one hour, during which time it is slit lengthwise once to create a flat sheet of cellulose. This is then rinsed thoroughly in distilled water and soaked for ten minutes in a small volume of the Elliott's solution. These steps follow the pattern proposed by Davison and Smith (1961).

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* The solution actually employed was Elliott's Solution A, but Dextrose was omitted in order to minimize the possibility of bacterial growth (Abbott Laboratories, Limited; Montréal, Québec). The composition of this commercially-available aqueous solution is, per litre:

8600. mg sodium chloride,
300. mg potassium chloride,
200. mg calcium chloride,
116. mg anhydrous magnesium chloride.

2) Once the membrane has been shaken of all free solution, one cylindrical stirrer is placed into the milled well of each half-cell, and these cells are assembled with the membrane between them. The cellulose seals each compartment and must be free from tears or folds; both magnetic stirrers must be free to rotate.

3) A 5 ml volume of the sample is introduced into one compartment through its port. The other compartment is filled with an equal volume of the Elliott's A solution prepared with EDTA. Each port is then sealed with the threaded nylon screw provided.

(DIALYSIS AND SAMPLING)

4) The chamber is placed on a magnetic stirring unit which is then set so as to rotate the cylindrical stirrers steadily at a moderate speed. Should data at other than room temperature be desired, the chamber may be placed into a glass (crystallizing) dish which is serving as a constant-temperature water bath; the entire assembly is then placed on top of the stirring unit. A combination hot plate and stirrer will of course simplify matters.

5) The dialysis system is then allowed to reach an equilibrium state — in which the concentration of unbound (free) drug is the same on both sides of the membrane. Twenty hours of continuous stirring proved satisfactory for the drugs studied in this work.

6) The actual sampling procedure is then accomplished through the port into each of the two compartments. A 5 ml glass syringe with a 3.5-inch needle is used (with care to avoid perforating the membrane).

(ANALYSES)

7) Two determinations are done on each of the two samples removed from the dialysis chamber (the plasma sample and the dialysate sample) —

(a) The water content of each solution must be determined in order to allow theoretically sound calculations; this also assures that no protein has entered the dialysate compartment. A refractometer was employed (as proposed by Kunin, 1965). The percent total solids present was determined by refractometry, and a conversion table was used to give the actual water content of each solution.

(b) The final drug concentration in each solution is determined by an appropriate assay method. In this work, the plasma was assayed in duplicate (1 ml volume each) and the dialysate was assayed using a volume of 3 ml in order to increase the accuracy of the determination.

(CALCULATIONS)

8) To assess the "true" concentration of drug in each

solution, the assay results (expressed in terms of drug per millilitre of sample) must be converted to units of drug per millilitre of water. For each sample, one may first use the formula of Breen and Freeman (1961) —

(100 mls plasma) =
(99.0-0.75 x Protein concentration in gram percent)
mls of water.

Equivalently, the ratio of assay data

$$= \left(\frac{\text{drug concentration measured in the dialysate}}{\text{drug concentration measured in the plasma}}\right)$$

may be corrected by multiplying by the factor

$$\left(\frac{\text{grams water per 100 ml of the plasma}}{\text{grams water per 100 ml of the dialysate}}\right)^{*}.$$

The results of this multiplication give directly the ratio of free drug to total drug in the plasma; the "binding fraction" = 1.00 minus this corrected ratio.

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*The "correction" factor is reported to equal approximately 0.95 (Kunin, 1965). In the present work, the numerator varied but the denominator was uniformly 0.991; the factors calculated for all experiments fell within the range 0.94 to 0.96.

IV

RESULTS AND

SPECIFIC DISCUSSION

A. ASSAY METHOD FOR DIPHENYLHYDANTOIN

The methodology developed in this work for determining levels of diphenylhydantoin (DPH) was essentially a micromodification of the technique of Wallace (1966). The standard deviations* of this new method are: (a) 7% (= the coefficient of variation* for standard solutions of the drug in plasma), and (b) 0.7 ug/ml of plasma under actual operating conditions*.

The time requirement for completion of one assay is about an hour, but the arrangement of equipment actually used (Figure 4) allowed six determinations to be done simultaneously. For best utilization of time, samples were run through the preparative procedure in groups of about twenty. Both practices were initially impossible (when the glassware and apparatus of some previous methods were employed).

In our hands, the distillates resulting from the micromethod of Lee and Bass (1970) had rather impure spectra

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*Calculations of "Standard Deviation" (S.D.):

(a) For 'n' similar determinations, $S.D. = \sqrt{\sum_n (X_i - \bar{X})^2 / (n-1)}$.

The "coefficient of variation" = the S.D. expressed as a percent of the sample mean '$\bar{X}$'.

(b) The best assessment of possible error in routine measurements is provided by the duplicate-pair method (Huisman, 1966) — for duplicate determinations on each of 'm' samples,

$$S.D. = \sqrt{\sum_m (X_1 - X_2)^2 / (2m)}.$$

(e.g., spectrum 'B' of Figure 10); and the absorbances of blank distillates were often 0.03 units and higher at the wavelength of analysis (257 mu). Frequently, when assaying biological samples, the distillate was visibly contaminated (blue or pink in color); this continued to occur even after the distillation rate was decreased to approximately one-third that recommended by Lee and Bass (1970). These problems were almost completely resolved by designing the special glassware and accessory equipment described above.

The present technique has a threshold of 0.6 ug DPH when the distillate is analyzed spectrally. Comparison of spectra 'C' and 'D' (Figure 10) shows that the assay of drug in a biological sample, as opposed to that of just pure drug, has no significant effect upon the shape of the spectrum of the distillate, even at the low concentration of DPH employed (2.5 ug/ml). Both curves have almost exactly the same shape as an aqueous solution of the pure (diphenyl ketone) reaction product (broken curves). This very pure spectrum ('C') resulted directly from an amount of DPH which, in all such previous methods (Wallace et al., 1965; Wallace, 1966; Lee and Bass, 1970), required that further purification and concentration steps be carried out prior to spectrophotometry; in fact, the 2.5 ug of DPH reacted to obtain these spectra was at or far below the thresholds stated in those previous reports (Cf. Tables I and II). In the determination of routine clinical samples, this assay results in an absorbance of about 0.1 to 1.0 optical density unit.

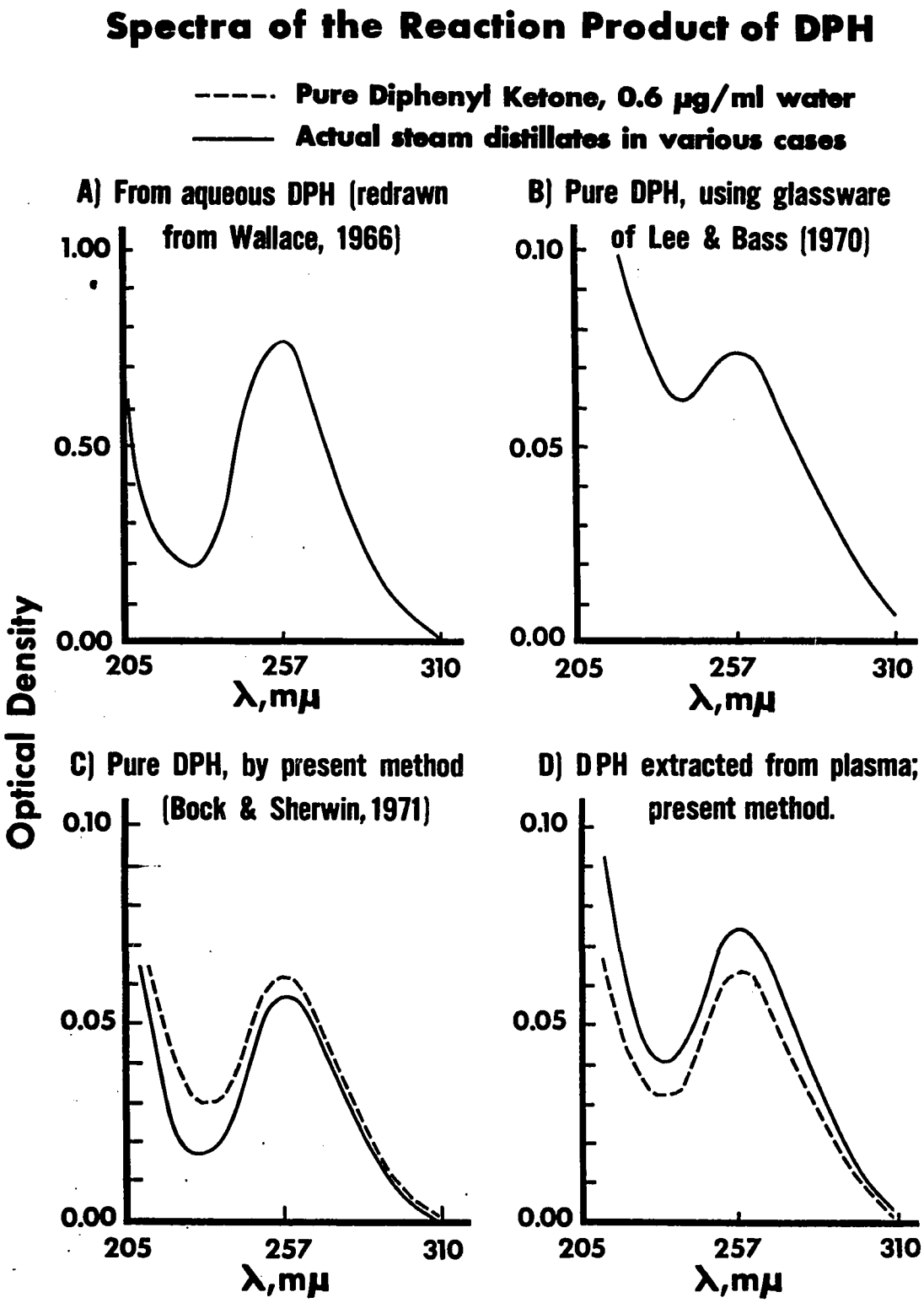
Spectra of the Reaction Product of DPH
----- Pure Diphenyl Ketone, 0.6 µg/ml water
——— Actual steam distillates in various cases
A) From aqueous DPH (redrawn from Wallace, 1966)
B) Pure DPH, using glassware of Lee & Bass (1970)
C) Pure DPH, by present method (Bock & Sherwin, 1971)
D) DPH extracted from plasma; present method.
Optical Density
1.00
0.50
0.00
0.10
0.05
0.00
205
257
310
λ,mµ
Note: 2.5 µg DPH was reacted for B and C
1.0 ml of plasma standard, 2.5 µg DPH/ml, was used for D

FIGURE 10.

An extensive list of drugs studied *in vivo* (therefore including their metabolites) has been shown to offer no interference to this method for determining DPH (see Table XI).

The usual organic solvent employed in this method for the determination of DPH in plasma and serum was chloroform. But the use of chloroform as the organic phase for whole blood or erythrocyte lysates proved to be much less satisfactory. The sample tended to congeal and a considerable amount of it remained adherent to the test tube at the termination of the extraction steps, resulting in very poor recovery of drug. Ethanol, as used by Sandberg and Slaunwhite (1957) and Wall and Migeon (1959) for lysates, not only gave poor recovery but also extracted organic contaminants which inactivated the permanganate reagent during the reaction step of the procedure. Ethylene dichloride (1,2-dichloroethane), which has been employed in a few investigations of various anticonvulsant drugs (Goldbaum and Smith, 1954; Kupferberg, 1970), proved to be the most suitable solvent for extracting DPH from such materials (whole blood or lysates). When extracting DPH from plasma, ethylene dichloride gave absolute assay results averaging 1.02 times those obtained when chloroform had been used for the same ten samples; the two solvents are therefore interchangeable for extracting *plasma*. As far as known, no such investigation or recommendation has been reported in the literature.

B. DIPHENYLHYDANTOIN LEVELS

1. VARIATIONS IN DIFFERENT TYPES OF SAMPLES*

Table XIIA presents a comparison of assay data for corresponding (and equivalent) samples of serum and plasma, plus whole blood and plasma, when the standard organic solvent (chloroform) is used during the procedure. Serum and plasma show equal amounts of extractable diphenylhydantoin (DPH). However, in the present assay methods, ethylene dichloride is the solvent recommended for samples of whole blood. Table XIIB presents data for the assay of corresponding samples of whole blood and plasma when this solvent is used.

The "plasma" referred to throughout this thesis contained EDTA as anticoagulant. Other anticoagulants are also utilized in clinical chemistry, so the potassium oxalate/sodium fluoride system was compared with EDTA: neither significantly altered the extraction of DPH from acidified water, but samples collected in oxalate/fluoride from seven clinic patients gave assay results for "plasma" averaging 15% lower than those for the corresponding samples collected in EDTA.

No warning of these preparation-dependent characteristics of DPH levels has been given previously.

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*The type of sample drawn by the physician for drug analysis is sometimes not the same as that preferred or requested by the laboratory. The present study allows interconversion of assay data to "standardize" levels within, or between, laboratories. Interpretation of levels is thusly facilitated.

TABLE XII A

Amounts of DPH Extracted by Chloroform from Various Tissue Preparations

Patients tested	Sample pairs*	DPH, mean µg/ml	Ratio (mean ± S.D.)**
30	serum, plasma	12.5, 11.5	1.08 ± 0.14
20	whole blood, plasma	7.0, 13.8	0.50 ± 0.11

TABLE XII B

Amounts of DPH Extracted by Ethylene Dichloride

Patients tested	Sample pairs*	DPH mean µg/ml	Ratio (mean ± S.D.)**
16	whole blood, plasma (uncorrected)	6.4, 10.3	0.65 ±0.09
16	whole blood, plasma (corrected)	7.7, 10.9	0.74±0.10

*Each sample pair was obtained by venipuncture from a treated patient.

**For each of the 30 (or 20 or 16) pairs of levels, a ratio relative to that specific plasma sample was calculated; the ratios were then averaged. "S.D." indicates the standard deviation of results.

The term "corrected" in Table XIIB refers to the percent recovery of drug: the raw assay results were compared directly ("uncorrected") and then after each had been corrected for its own degree of incomplete recovery. The latter therefore represents concentrations of drug.

2. PLASMA LEVELS MONITORED OVER VARIOUS PERIODS OF TIME, AND THE LEVEL-TO-DOSE RELATIONSHIP

Several other aspects of the diphenylhydantoin (DPH) level itself were investigated:

a) Blood samples stored in sealed tubes at room temperature for eight hours up to twenty-four hours prior to refrigeration (and subsequent analysis) showed no consistent or significant alteration of assay results relative to equivalent samples cooled immediately after venipuncture. Three 5 ml samples were drawn sequentially from each of eight patients and handled in the three different ways.

b) Data on twenty-one of the twenty-nine patients who volunteered for the single vs. divided dose study were suitable for assessing what effect the time of day of venipuncture had upon the outcome of DPH assay. During the same day, when the drug was being administered in the usual (divided-dose) regimen, the average level increased by 0.5 ug/ml between just before and three hours after the 100 mg morning dose was taken. This "flat" pattern may continue up to at least twelve hours later, even as the other doses are consumed (Buchanan et al., 1972). However, in the present study, individual deviations averaged ±1.4 ug/ml between the zero and third hours.

c) Over a given nine month period, corresponding levels in this same patient group were examined for constancy. The individual deviations shown between these two extremes

in time averaged ±2.5 ug/ml. Each patient had maintained a constant daily intake of DPH over this entire period.

d) In twenty-one of these patients, a series of equivalent levels (i.e., those for the same time of day relative to the dosing of DPH) were used to assess the plasma-level-to-unit-dose relationship. Dosages per unit weight and per unit surface area were both considered. Their frequency distributions approximated a Gaussian curve, so a correlation coefficient could legitimately be calculated for the best straight line through the experimental points*. The graphs and parameters of Figure 11 correspond to levels observed three hours after the 100 mg breakfast dose of DPH (during the divided-dose regimen).

At <u>all</u> times during the study, for both expressions of unit dose, the levels showed significant correlation to dosage rate ($p < 1\%$)*; i.e., level and dosage are <u>not</u> independent. The greater association was always observed when dosage rate was per unit <u>surface area</u>, the correlation coefficient* being at least 0.08 greater than that for unit weight. Therefore, all regressions with a basis of surface area (i.e., dosage rate calculated as mg/m^2) are shown together in Figure 17.

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*See Freund (1962) for statistical explanations.

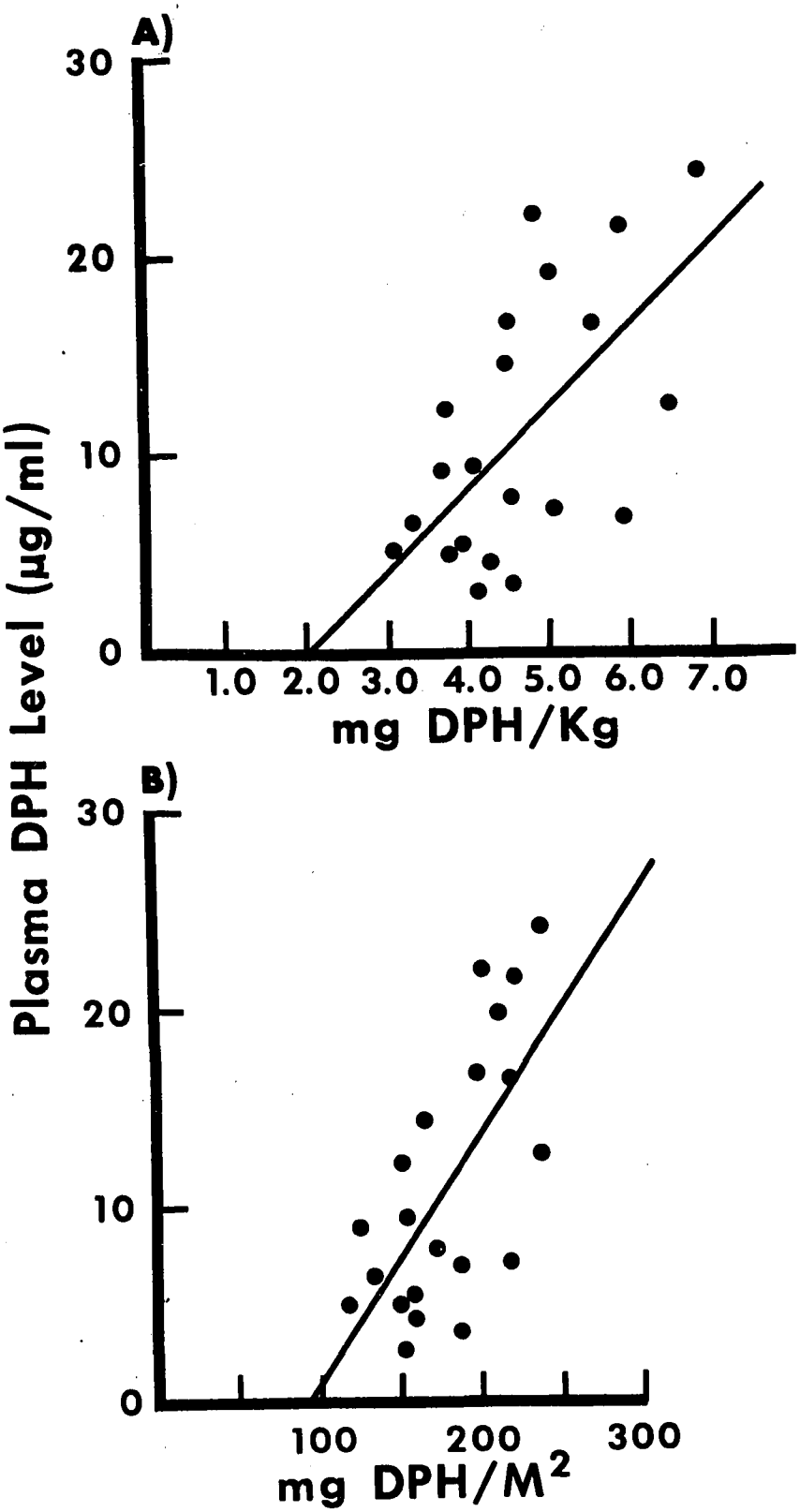

FIGURE 11. LEAST-MEAN-SQUARE LINES FOR THE DIVIDED-DOSE ADMINISTRATION OF DIPHENYLHYDANTOIN (DPH).

A) Dosage calculated per kg body weight: Level=(4.1 x dosage)-8.4; avg. weight=70 kg; correlation coefficient=0.61(actual p=0.3%).

B) Dosage calculated per m^2 surface area; Level=(0.13 x dosage)-12; avg. area=1.8m^2; correlation coefficient=0.69 (actual p=0.04%).

3. PLASMA LEVELS RELATED TO SPECIFIC CLINICAL STATES

Table XIIIA indicates that patients who were referred for laboratory testing had approximately the same levels of diphenylhydantoin (DPH) regardless of the degree of control of seizures reported. The many "low" values observed (5 to 10 ug/ml) must lead one to reject the lower limit of the drug's traditionally-stated "therapeutic" range in epilepsy (10 to 20 ug/ml). Two other recent thorough studies showed equivalent patterns (Table XIIIB).

Tables XIVA and B relate a similar situation relative to suspected toxic signs reported by the physician. The frequency distribution of the 206 "non-toxic" values of Table XIVA approximated a one-sided Gaussian curve (see Figure 12). A histogram was then drawn for each gradation of clinical toxicity (Figure 13), the plasma-level (horizontal) axes being partitioned according to the suggestion of Figure 12. The ranges and overlap of the three histograms show how misleading a statement of the average level for each clinical category actually is. The individuality of patient response to DPH has been demonstrated.

The properties of the derived Gaussian distribution (Figure 12) have implications for the interpretation of assay results on routine clinical samples. For example, of all

samples from "non-toxic" patients, 87%* had levels less than 17.0 ug/ml, and 95%*, less than 22.3 ug/ml. One can then say with less than a 13%** chance of error that a level greater than 17.0 ug/ml represents a toxic level, while only a 5%** maximum error is risked above 22.3 ug/ml. All values just given take into account the "group selective processes"*** (Buchthal et al., 1960) which will always be functioning.

When histograms were drawn for only those patients who had had multiple assays corresponding to <u>different</u> degrees of toxicity, the pattern resembled that reported by Buchthal et al. (1960) for serum levels. These are shown together in Figure 14. Considering these histograms for <u>verified</u> drug-induced toxicity, and the fact that drug assay is very frequently requested to characterize <u>suspected</u> toxic signs, it seems reasonable to choose the 17.0 ug/ml level as the beginning of toxicity when interpreting assay results. This assay is then nearly 100% sensitive (∓) to true toxicity and at least 87% specific (∓).

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*These percentages (87%,95%) are the degree of "sensitivity" (∓) of this test for detecting non-toxic levels.

**The 13% and 5% figures are the assay's <u>lack</u> of "specificity" (∓) — i.e., chance of <u>falsely</u> giving a toxic rating — when two different cut-off points are employed.

(∓)High "sensitivity" = few false-negative results.

(∓)High "specificity" = few false-positive results.

***Any given level represents the outcome of previous clinical manipulation of therapy and has reached the laboratory only for some specific clinical purpose. The process is therefore far from random.

The division between mild and severe toxicity is less clear, and the often-used 30 ug/ml level yields 90% specificity but will sacrifice approximately 20% of the test's sensitivity to true severe toxicity (Cf. Figure 14). All of these arguments assume that the assay result is the only reliable information available on the patient.

Table XV presents data on fifty-six patients who showed a large therapeutically-induced change in DPH levels and whose reported seizure frequencies were reliable*. The profile of changing toxicity serves as a good example of the arguments given above.

This procedure of alteration of dosage rate is the best way to assess the drug-induced character of any component of the clinical picture (Buchthal and Svensmark, 1971). Toxicity appears to be dose-related but the plasma level of DPH and the degree of seizure control sometimes change paradoxically (see Table XV). Therefore, should an assay result suggest that a change in dosage rate may aid the well-being of the patient, careful periodic monitoring of both the drug level and the clinical status of the patient is a must for a while thereafter. This monitoring plan maximizes the benefit to be derived from the availability of these laboratory tests.

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*For the purposes of tabulation, after each change in level had occurred: (a) a degree of seizure control noted as being "worse" meant at least a doubled frequency of seizures; (b) "better" control required that fewer than 50% as many seizures occur. Other variations are listed as "no change".

TABLE XIII A

Plasma Diphenylhydantoin Levels and Degree of Clinical Control

Clinical Control	No. Tested	Plasma Level (µg/ml)	
		Average	Range
Complete	30	10.3	1.3-42
Adequate	74	12.5	1.3-63
Complete or Adequate	104	11.9	1.3-63
Inadequate	159	10.5	1.0-46

TABLE XIII B

Plasma Diphenylhydantoin Levels and Degree of Clinical Control

Clinical Control	No. Tested	Plasma Level (µg/ml)	
		Average	Range
1. Good	118	11.3	—
Fair	80	9.1	—
Poor	84	9.4	—
2. 100%	—	6.7	0-46
50%	—	11.1	2.0-25
<50%	—	10.1	1.2-24

1. Haerer & Grace, 1969
2. Buchanan & Allen, 1971

TABLE XIV A

Plasma Diphenylhydantoin Levels and Clinical Toxicity

Clinical Toxicity	No. Tested	Plasma Level (µg/ml)	
		Average	Range
None	206	9.2	1.0 - 37.1 (87%<17 µg/ml)
Mild	48	16.4	1.3 - 41.6 (50%>17 µg/ml)
Severe	40	29.2	4.0 - 63.2 (82%>17 µg/ml)

TABLE XIV B

Plasma Diphenylhydantoin Levels and Clinical Toxicity

Nystagmus	No. Reported	Average Level (µg/ml)
1 None	220	6.3
Mild	39	18.8
Moderate	28	17.6
Mild or Moderate	67	18.3
Moderate or Marked	40	25.0
Marked	12	42.2
2 Present	—	21.8

1 Haerer & Grace, 1969
2 Buchanan & Allen, 1971

ONE-SIDED NORMAL DISTRIBUTION

The frequency distribution of the 206 "non-toxic" levels greater than 1.0 ug DPH/ml of plasma (Table XIV A) approximated a one-sided normal (Gaussian) curve. Successive refinement of the comparison produced the following Gaussian curve whose standard deviation (S.D.) was 10.6 ug/ml:

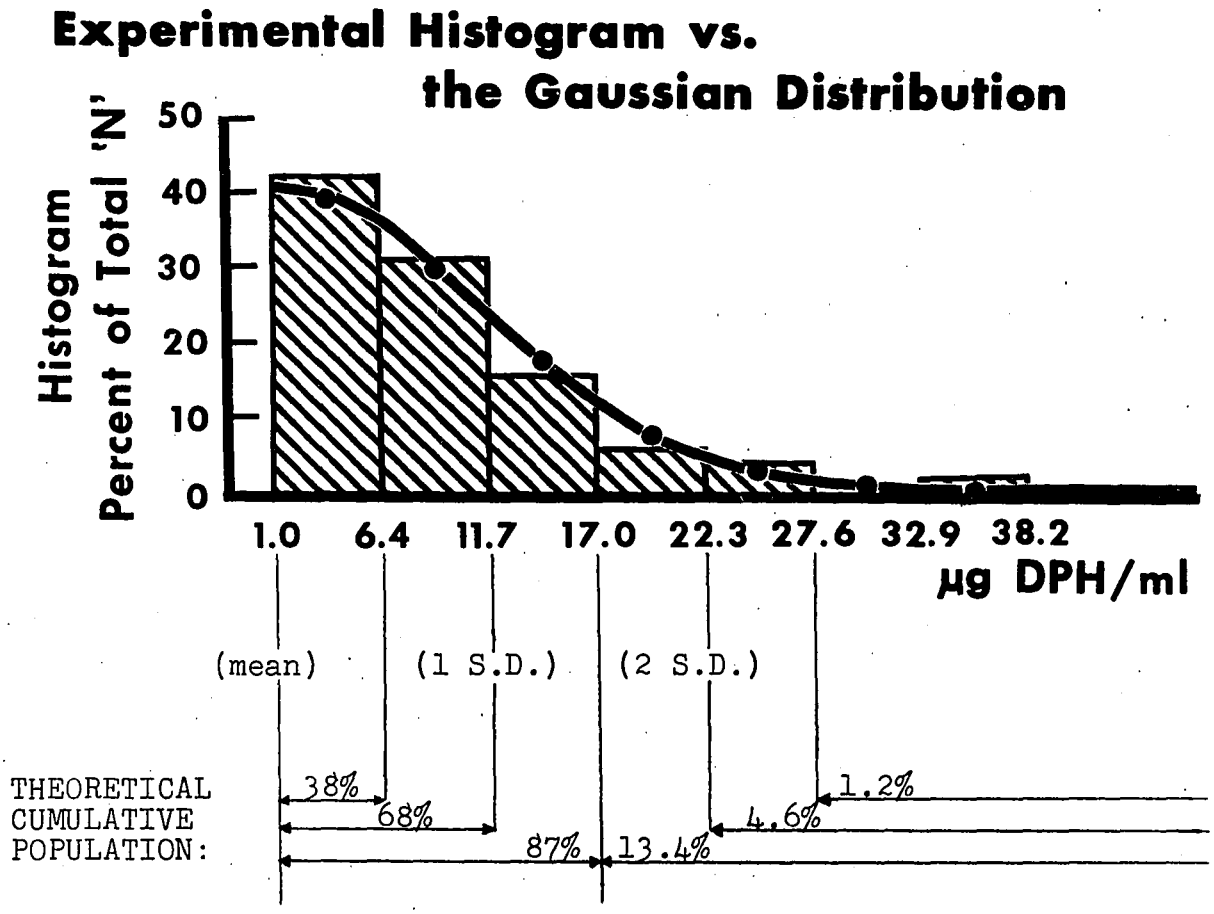

FIGURE 12. On the basis of the theoretical (Gaussian) distribution for "non-toxic" levels shown above, the 17.0 ug/ml level (at 1.5 S.D. above the "mean") was chosen to be a suitable cut-off point for clinical purposes. (The plasma level axes of all histograms are partitioned as here, and the 17.0 ug/ml level is specially marked with an arrow on each.)

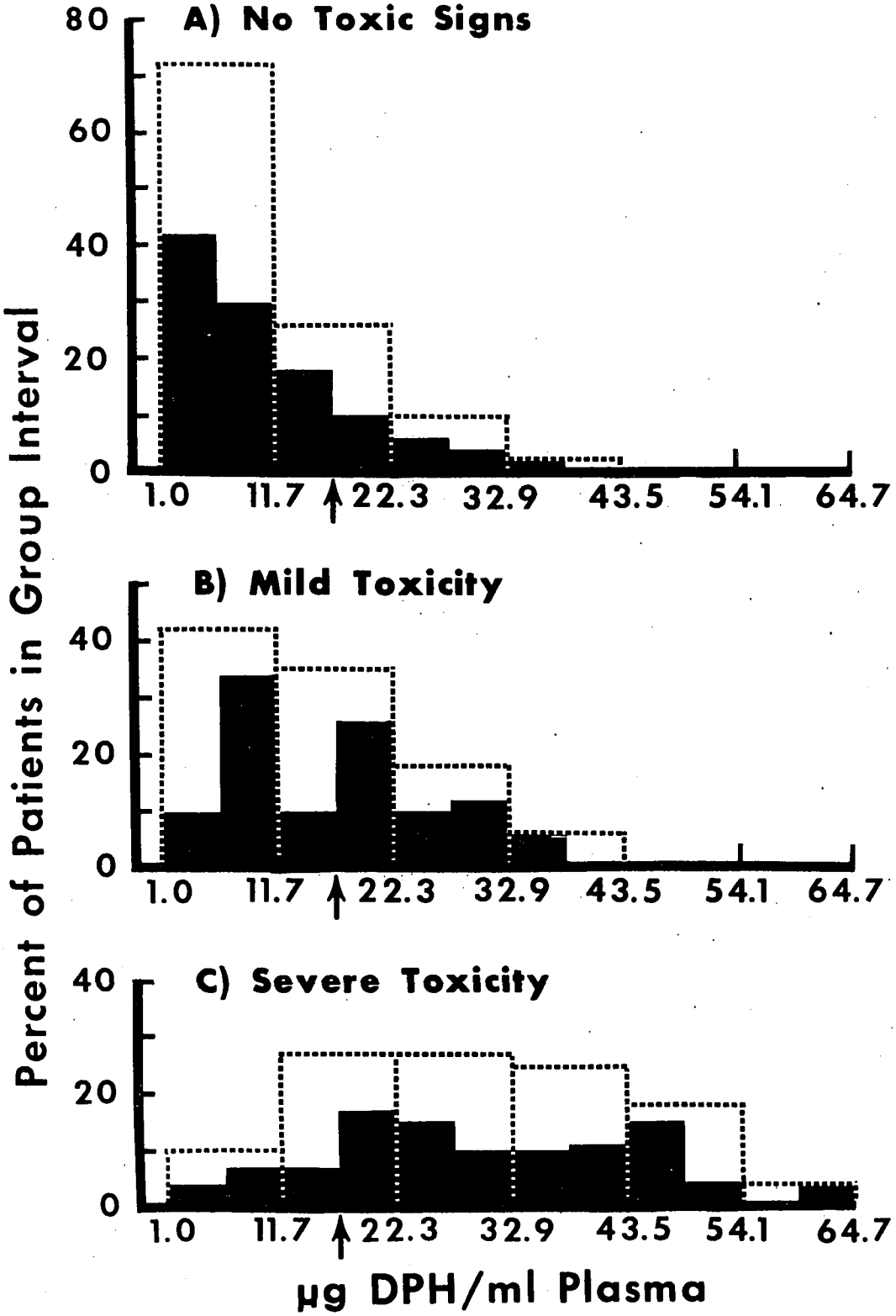

FIGURE 13. ACTUAL LEVELS OF DIPHENYLHYDANTOIN (DPH) AND THE DEGREE OF TOXICITY ESTIMATED IN THE CLINICAL IMPRESSION.

The frequency distribution for each histogram is shown for two partitioning magnitudes (5.3 and 10.6 ug/ml). The arrow on the horizontal (plasma level) axis indicates the 17.0 ug/ml level.

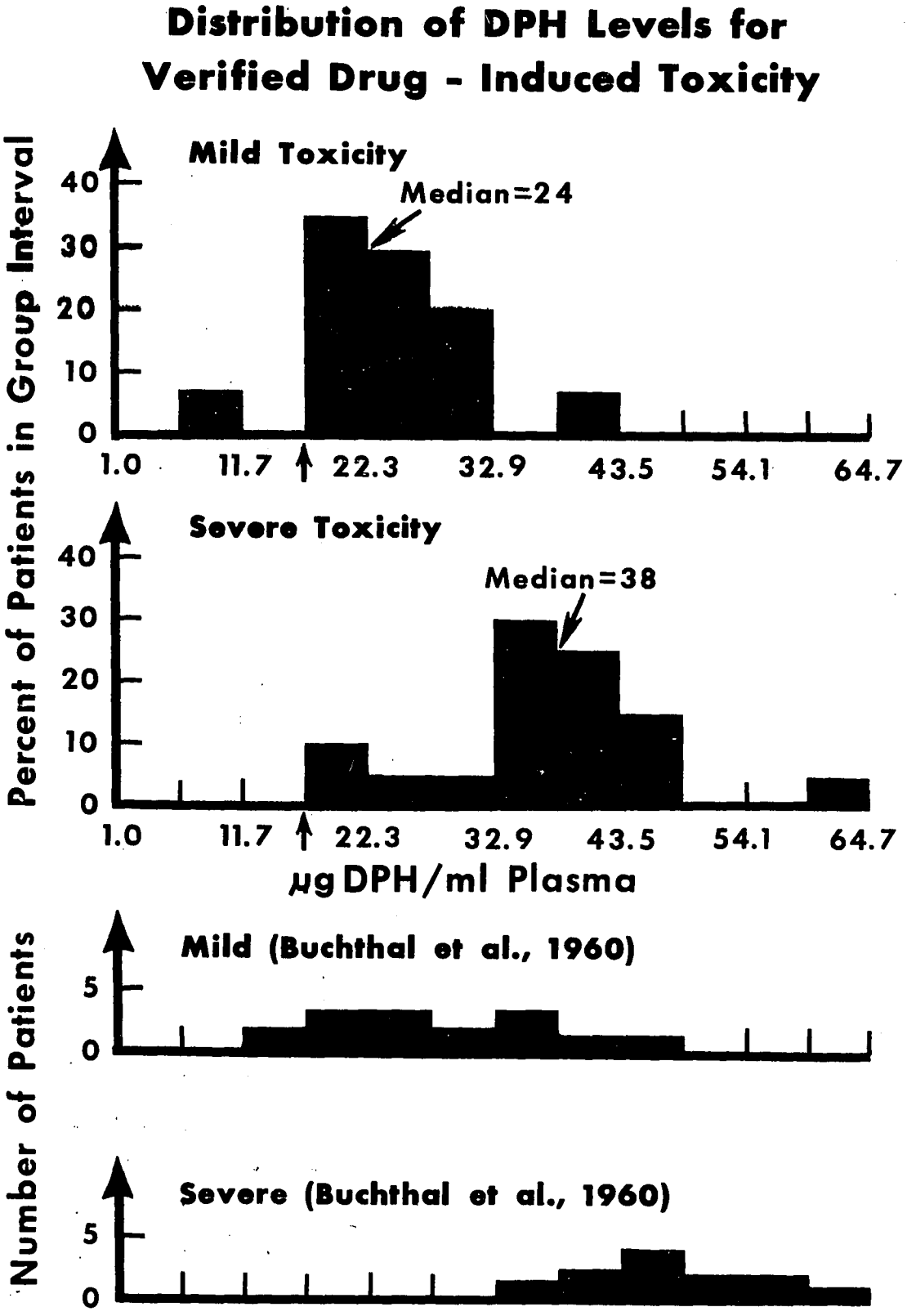

FIGURE 14. LEVELS OF DIPHENYLHYDANTOIN (DPH) AND THE DEGREE OF VERIFIED DRUG-INDUCED TOXICITY.

The frequency distributions in the upper two histograms are for toxic signs which either appeared as the measured level increased or disappeared as the measured level decreased.

Clinical Effects of Altered DPH Levels

Lines 1-7 — Patients initially uncontrolled Lines 8-9 — Patients initially controlled

Line No.	No. of Patients	DPH Level Change	Resultant Seizure Control	Average Levels µg DPH/ml Before → After	No. of Patients Toxic	
					Before	After
1	1	↑	Worse	3→6	—	—
2	7	↑	No change	7→23	0	2
3	15	↑	Better	8→17	0	2
4	11	↑	(now seizure free)	4→13	0	1
Total (1-4)	**34**				**0**	**5**
5	4	↓	Better	23→11	3	0
6	4	↓	No change	26→16	1	0
7	7	↓	Worse	23→9	1	0
Total (5-7)	15				5	0
8	4	↓	No change	36→14	3	0
9	3	↓	Worse	23→10	0	0
Total (8-9)	7				3	0
Total (5-9)	**22**				**8**	**0**

TABLE XV

N.B.--Lines 1 & 5 illustrate patients for whom the circulating level of diphenylhydantoin (DPH) and the degree of seizure control changed paradoxically (see text).

C. THE ADMINISTRATION OF DIPHENYLHYDANTOIN IN A SINGLE DAILY DOSE versus THE DIVIDED-DOSE REGIMEN

Several aspects of the investigation to determine the effects of altering the schedule of administration of diphenylhydantoin (DPH), while maintaining the dosage rate constant, have been presented separately in Tables XVI to XVIII, plus Figures 15 to 17.

Table XVII summarizes the seizure records and plasma level data for the entire group of twenty-nine patients followed for the nine month duration of the study (each month was one "period" of the study); period II was the only one during which the total daily dosage of DPH was taken in a single dose (at 8 a.m.). Comparing the periods, there was no real difference in any aspect investigated.

Table XVIII evaluates the effects of the change in administration schedule in terms of individual patient response. Considered as a whole, the data show little, if any, difference between the clinical effectiveness of the two dosage regimens. However, in many cases, the single-dose administration proved to be superior clinically and/or was preferred by the patient; in sum, the response was person-specific. With respect to side effects, some of those expected from the large single dose of DPH were observed—but all ceased spontaneously within the first week of period II. No clinical signs of even transient DPH intoxication were observed and there were no gastrointestinal complaints

at all. In several non-institutionalized patients studied separately, side effects occurred not only upon their first beginning the single-dose schedule but also upon their resuming the (usual) divided-dose schedule.

The variation of DPH level during the day was monitored for both the single- and the divided-dose periods of the study. The steadiness of DPH levels for the (usual) divided-dose schedule was described above. But during the single-dose administration of DPH, a different pattern was seen. This has been shown in Figure 15A and B for the ten patients whose levels were determined periodically from one to twelve hours after receiving the full daily dose at breakfast. A similar investigation was done up to only the sixth hour but for a full twenty-seven patient volunteers; this yielded the same temporal pattern (Figure 16). For both, the patients receiving the smallest dosage of DPH (lowest quartile* for mg/m^2 or mg/kg) showed an earlier peaking of plasma level (Figure 16), at about three hours instead of six, resembling the pattern shown in Figure 15C for an out-patient studied independently. Such comparison has verified that the shape of these curves is truly physiologic and not due to particular factors functioning in the mass study.

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*See Figure 16 for explanation of the "Quartile" partitioning scheme.

TABLE XVI

PATIENTS PARTICIPATING IN THE SINGLE DOSE v e r s u s DIVIDED DOSE STUDY*

Patient No.	& Initials	Age in Years	Weight in kg	Daily DPH Dosage mg/kg	mg/m²	3 hr. p.c. Levels† D.D.	S.D.	S.D. Levels† 0 hr.	3 hr.	6 hr.
1	JPB	44	71.8	4.18	160	9.7	10.0	8.3	10.7	11.2
2	CBr	49	80.0	3.75	154	11.3	13.7	8.5	13.1	12.2
3	PB	22	80.0	3.75	153	5.2	7.7	4.6	8.6	7.5
4	MB	38	75.8	4.62	184	4.4	7.1	2.4	-	5.6
5	HC	38	64.1	4.68	170	7.6	8.8	6.8	8.8	7.8
6	YD	23	65.0	4.62	173	8.8	11.8	7.0	10.4	11.8
7	ADu	37	79.0	3.80	155	7.7	5.8	7.0	7.2	7.6
8	MG	23	73.3	3.41	138	5.8	8.2	6.8	10.2	10.0
9	YL	25	63.2	3.16	119	5.2	5.8	2.0	7.0	5.6
10	GR	22	59.1	5.08	173	8.5	9.9	4.5	9.7	9.2
11	LSO	60	53.8	3.70	128	8.5	6.1	5.6	7.3	7.2
12	ATC	44	77.4	5.17	217	17.6	17.5	12.7	19.0	18.4
13	GM	18	75.0	5.33	219	10.3	10.7	8.2	9.1	12.5
14	NC	23	79.1	5.06	205	6.8	4.9	3.3	3.9	5.2
15	ADo	44	71.0	5.64	222	17.0	17.0	15.0	19.2	19.9
16	DL	27	61.8	6.47	238	12.9	16.8	20.7	28.7	28.9
17	DM	24	59.2	6.76	238	27.8	19.6	16.3	16.4	25.4
18	MS	22	87.2	4.59	204	12.1	15.4	12.2	15.8	22.9
19	RMo	48	76.5	5.23	225	12.0	15.8	10.9	-	17.1
20	MVa	34	70.4	4.26	164	5.7	4.7	3.9	3.6	9.1
21	MVi	45	67.7	4.43	174	5.1	10.1	7.4	11.3	9.9
22	JLa	20	71.7	4.18	160	2.7	2.9	1.7	2.0	3.2
23	LP	22	66.4	4.52	168	16.1	14.8	16.0	18.2	19.5
24	RMa	46	46.4	6.47	210	12.4	12.3	9.3	12.1	14.9
25	RP	35	77.4	3.88	160	7.1	7.4	4.3	8.0	7.6
26	AF	33	68.3	5.86	225	19.6	19.9	15.6	20.1	21.6
27	CBe	38	81.1	4.93	204	24.3	29.8	29.5	28.8	35.6
28	DV	25	60.0	6.67	233	16.5	31.6	28.1	-	-
29	JLe	28	67.4	5.93	222	10.1	10.8	6.4	9.4	10.8

*All twenty-nine volunteers were white males who had been receiving diphenylhydantoin (DPH) for many years prior to beginning the study.

† Levels are all reported in units of ug DPH/ml of plasma:

a) "3 hr. p.c. Levels" are those compared at three hours following the consumption of the breakfast dose of DPH — "D.D." for divided-dose Period I and "S.D." for the average level of the single-dose Period II. N.B. — Of the twenty-nine patients, only eight (numbers 6,16,17,18,19,21,27, and 28) showed deviations of at least ± 3.0 ug/ml between these two 3rd hour levels.

b) "S.D. Levels" correspond to concentrations of DPH observed one day at various lengths of time (0,3,6 hours) following the consumption of the single large dose at breakfast (during Period II). "-" denotes that a specific sample was not obtained.

Single vs. Divided Dose Study
(29 patients)

Study Period Month No.	Mean Plasma Level at 3rd hr.	Total Seizure Frequency/Month*			
		General	Partial	Absence	Total
I Divided	10.9	$3_{(2)}$	$40_{(12)}$	$21_{(11)}$	$64_{(16)}$
II Single	11.9	$4_{(3)}$	$44_{(15)}$	$32_{(11)}$	$80_{(16)}$
III Divided	10.1	$4_{(2)}$	20^{+}	20^{+}	44^{+}
IV-VIII Divided	—	6	24	50	80
IX Divided	10.7	$6_{(3)}$	$22_{(10)}$	$50_{(14)}$	$78_{(16)}$

TABLE XVII. SUMMARY OF SINGLE vs. DIVIDED DOSE STUDY.

*Figures = number of seizures recorded during that particular month.
Subscripts = number of patients experiencing that type of seizure during the month.

+During holiday periods, complete records were kept for only the generalized seizures.

N.B.—

A) The plasma levels noted in this table are for the same twenty-seven patients whose levels were obtained throughout the entire duration of the study; the standard error of the mean (S.E.M.) $\leq$ 1.4 ug/ml for each period ($n=27$).

B) The clinical data in this table refers to all twenty-nine patients who participated in the study.

TABLE XVIII

DIFFERENTIAL EFFECTS OF THE SINGLE-DOSE REGIMEN FOR DIPHENYLHYDANTOIN RELATIVE TO THE DIVIDED-DOSE REGIMEN

A. SEIZURE CONTROL

Seizure Type	Patients Improved	Patients Unchanged	Patients Worse
Generalized	3	23	3
Partial	4	20	5
Absence	4	22	3

B. NUMBER OF SEIZURE-FREE PATIENTS

Seizure Type	Divided Dose (Period I)	Divided Dose (Period III)	Single Dose (Period II)
Generalized	27	27	26
Partial	17	20(+)	14
Absence	18	23(+)	18

C. BENEFICIAL EFFECTS REPORTED

Elevation of mood	in 4
Better total seizure control	in 7
Better patient acceptance	in 5*

D. ADVERSE EFFECTS REPORTED OR OBSERVED

All ceased after one week of the single-dose schedule, without additional treatment required:

Lethargy	5
Dizziness	2
Confusion	1
Gastro-intestinal symptoms	0

(+) Holiday period; incomplete records kept.

* One patient refused to return to the divided-dose schedule.

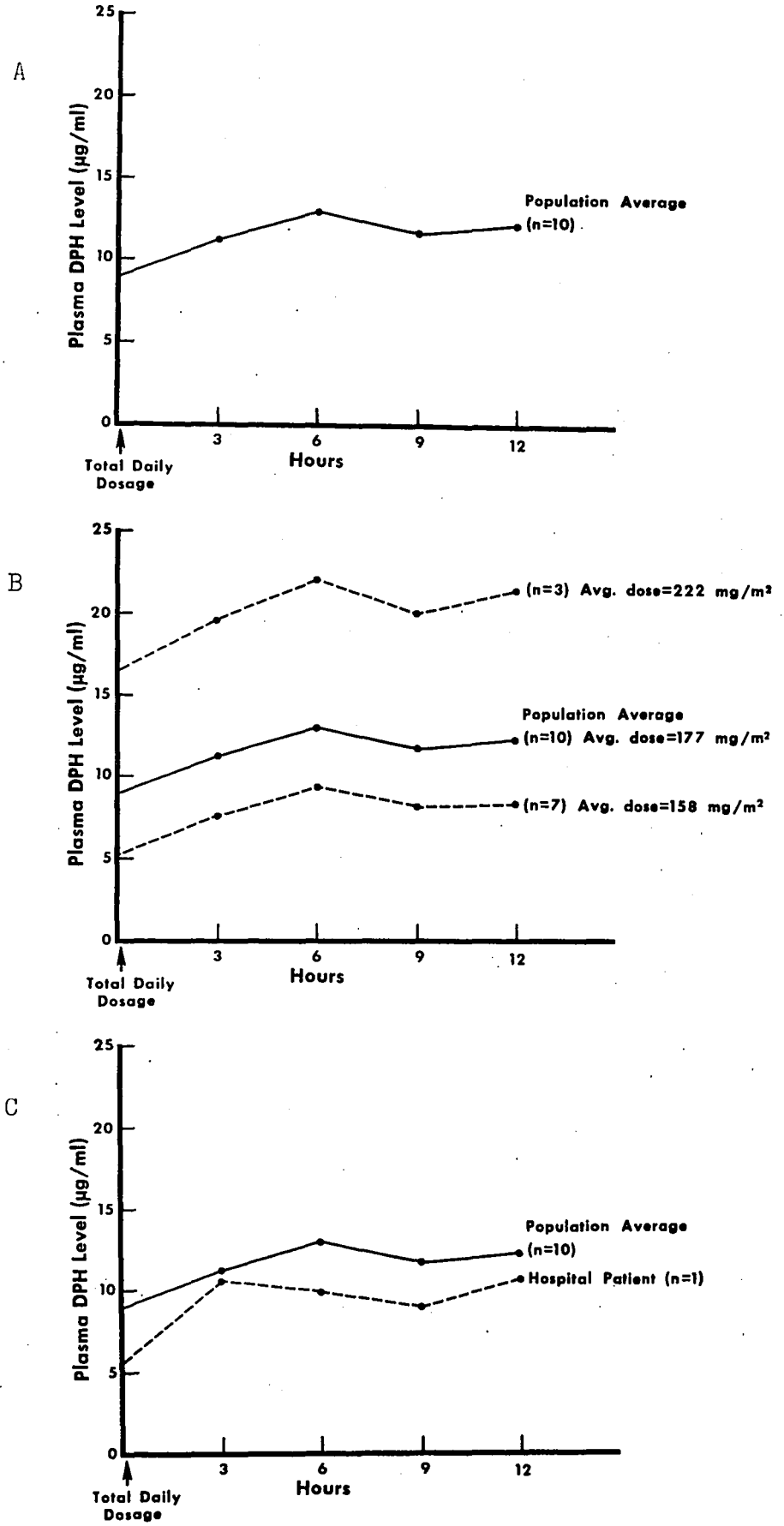

FIGURE 15. DRUG LEVELS FOLLOWING THE SINGLE-DOSE ADMINISTRATION OF DIPHENYLHYDANTOIN (DPH).

A,B: For ten patients of the institutionalized study group.
C: Comparison with an out-patient investigated separately.

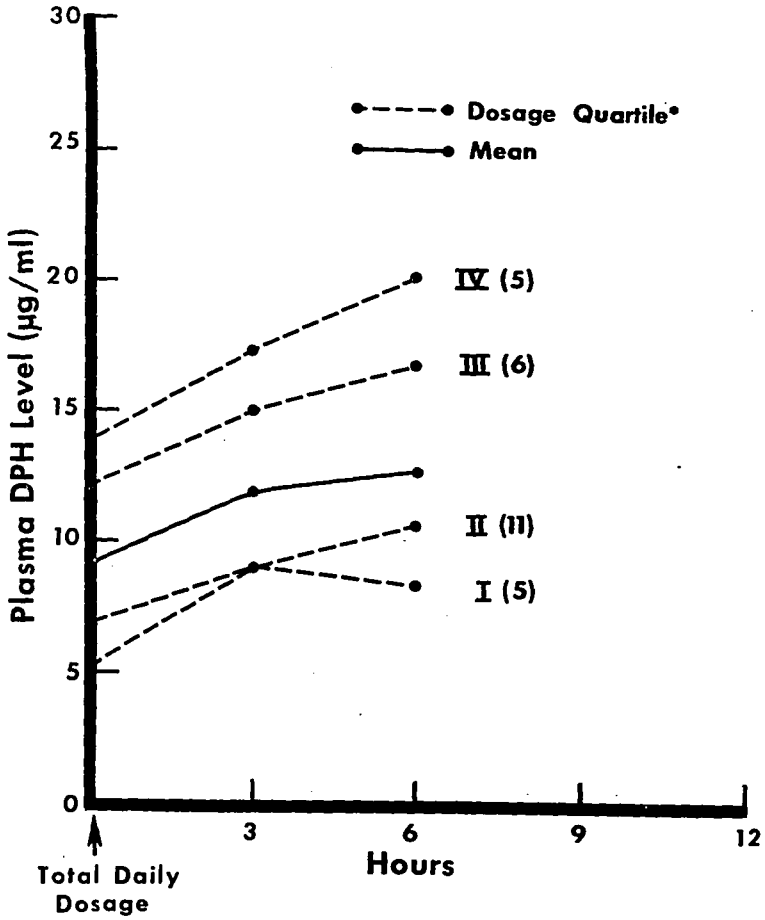

FIGURE 16. DRUG LEVELS IN TWENTY-SEVEN INSTITUTIONALIZED PATIENTS FOLLOWING THE SINGLE-DOSE ADMINISTRATION OF DIPHENYLHYDANTOIN (DPH).

For these twenty-seven patients, daily dosage rates were distributed in approximately Gaussian fashion with the mean = 4.8 mg/kg body weight, and standard deviation (S.D.) = ±1.0 mg/kg.

*For purposes of graphic presentation, the patients were grouped by statistical "dosage quartile":

I.	(mean-2 S.D.)	to (mean-1 S.D.)	= 2.8 to 3.8 mg/kg.
II.	(mean-1 S.D.)	to (mean)	= 3.8 to 4.8 mg/kg.
III.	(mean)	to (mean+1 S.D.)	= 4.8 to 5.8 mg/kg.
IV.	(mean+1 S.D.)	to (mean+2 S.D.)	= 5.8 to 6.8 mg/kg.

The average DPH level for the patients included within each quartile is plotted above (the number of patients in each being noted in parentheses beside the curve).

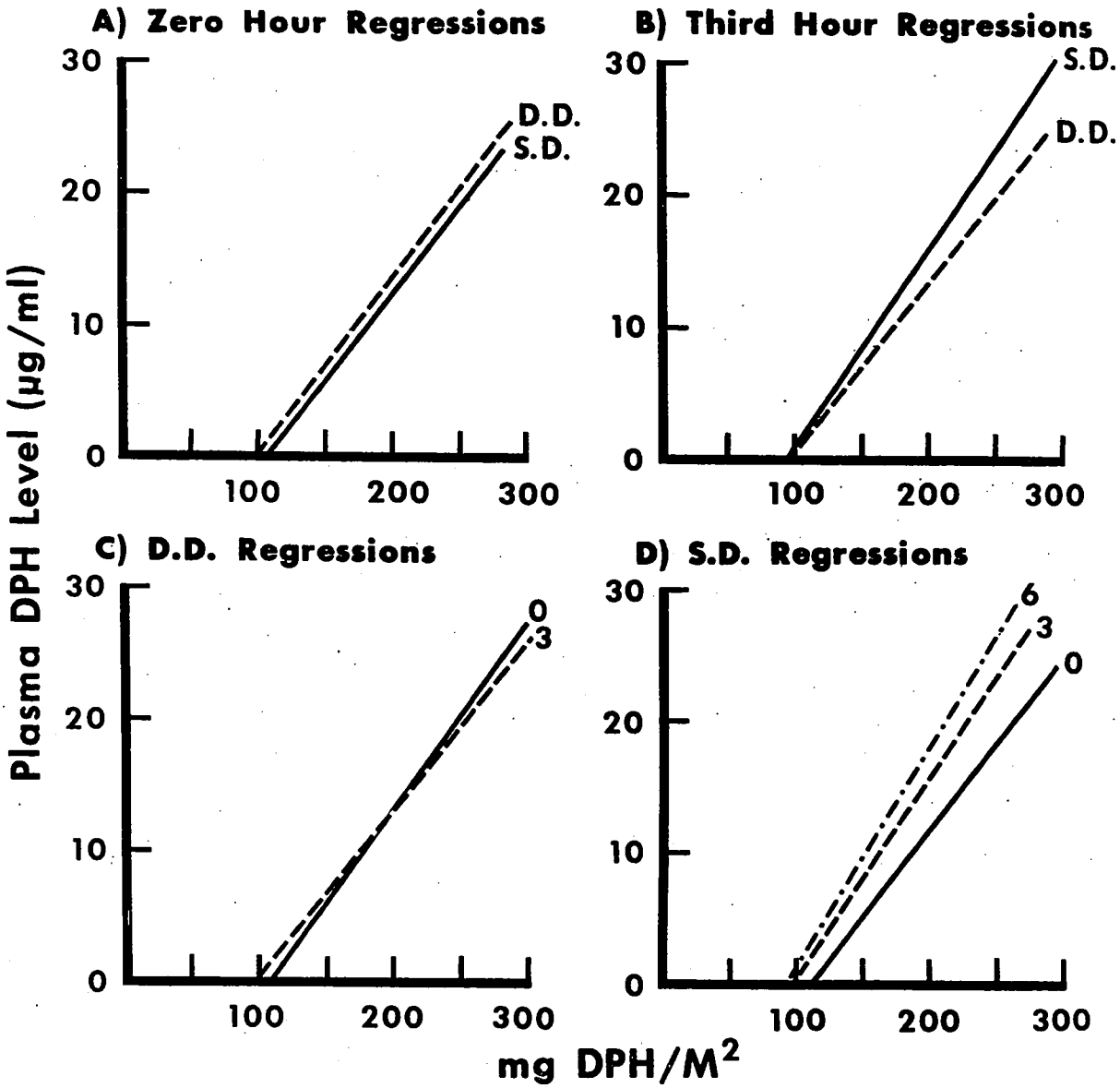

FIGURE 17. LEAST-MEAN-SQUARE LINES FOR LEVELS OF DIPHENYLHYDANTOIN (DPH) IN TWENTY-ONE INSTITUTIONALIZED PATIENTS.

"D.D." refers to the divided-dose administration schedule. "S.D." refers to the single-dose administration schedule. The hours noted (0,3,6) refer to the time elapsed between consumption of the breakfast dose of DPH and the venipuncture procedure.

Various regressions for comparable DPH levels, relative to daily dosage rate (mg/m^2), are plotted together in Figure 17. All relate plasma levels for the same twenty-one volunteers from the study group, for different hours during both the single- and divided-dose periods, without considering the question of significance of the differences shown. These demonstrate in another way the peaking of levels which occurred during period II of the present study (single-dose period).

Statistical analysis of the assay data was subsequently done—temporally-equivalent DPH levels for different periods of the study were compared, and then DPH levels for different times during the same day were also compared. Since this was a controlled cross-over study, the same group of volunteers participated throughout its entire duration, and the various sets of corresponding levels observed were <u>not</u> independent; therefore, statistical treatment had to be done in a special manner*. The results of this examination of the data are given in Table XIX.

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*See Freund (1962), page 269: These various sets of non-independent levels are best treated statistically in the form of paired data; for each <u>two</u> sets of levels to be compared, the change in level experienced by each subject is calculated and these <u>differences</u> are treated as a random sample from a normal population. For $n < 30$, as here, the "t test" is used to assess the probability that these <u>changes</u> in level observed are actually "significantly" <u>different from zero</u> (i.e., that the two <u>sets</u> of levels observed <u>differ by much more than just chance variation alone</u>). Throughout this work, the 1% error level ($p = 0.01 = 1\%$) was used as the criterion for assessing such "significance".

TABLE XIX

STATISTICAL ANALYSIS OF THE DATA ON DIPHENYLHYDANTOIN (DPH) LEVELS DURING THE SINGLE versus DIVIDED DOSE STUDY

No. of Patients	Time of study*	COMPARISON*, $Level_2 - Level_1$	AVERAGE "DEVIATION"† ug/ml	AVERAGE "CHANGE"† IN PLASMA LEVEL: ug/ml	significant?	actual probability
22	3 hr., D.D.:	III - I	±1.9	-0.5	-	>15%
		IX - I	±2.4	+0.02	-	~49%
17	3 hr., S.D.:	wk.3 - wk.1	±3.7	-0.08	-	~48%
28	3 hr.:	S.D. - D.D.	±2.7	+1.9	yes	<0.5%
21	0 hr.:	S.D. - D.D.	±3.8	-0.3	-	~40%
	D.D.:	3 hr. - 0 hr.	±1.4	+0.5	-	10%
	S.D.:	3 hr. - 0 hr.	±3.3	+3.2	yes	<0.05%
	Difference of 3 hr. - 0 hr.:	S.D. - D.D.	±2.9	+2.7	yes	<0.05%
27	S.D.:	3 hr. - 0 hr.	±3.0	+2.9	yes	<0.05%
		6 hr. - 3 hr.	±1.9	+1.4	yes	<1.0%
		6 hr. - 0 hr.	±4.3	+4.3	yes	<0.05%
10**	S.D.:	3 hr. - 0 hr.	±2.25	+2.25		
		6 hr. - 3 hr.	±2.03	+1.85		
		9 hr. - 6 hr.	±1.42	-1.26		
		12 hr. - 9 hr.	±0.96	+0.58		
		And therefore—				
		6 hr. - 0 hr.		+4.10		
		9 hr. - 0 hr.		+2.84		
		12 hr. - 0 hr.		+3.42		

*"D.D." refers to the divided-dose administration of DPH.
"S.D." refers to the single-dose administration of DPH.
"hr." refers to the number of hours after consumption of the breakfast dose of DPH.

†"DEVIATION" = The absolute value of the observed change in level (CHANGE = $Level_2 - Level_1$).
Significance was assessed at the 1% error level, p = 0.01 = 1.0%.

**The ten patients were too small a group to allow statistical analysis since the distribution of their plasma levels did not approximate a Gaussian curve.

D. DISTRIBUTION OF DIPHENYLHYDANTOIN IN THE VARIOUS COMPARTMENTS OF WHOLE BLOOD

The concentration of diphenylhydantoin (DPH) in erythrocytes averaged 52% of the corresponding plasma level with a range of ± 10% (n = 16). When these "packed red cells" were washed twice with cold Elliott's A solution (Abbott Laboratories, Limited; Montréal, Québec) prior to freezing and subsequent analysis, the "intracellular" levels averaged only 29% of the corresponding plasma values (n = 18). This washing procedure, an attempt to remove the remaining plasma from the "packed cells", was shown to leach DPH from the erythrocytes and was therefore abandoned.

The 52% figure for the "unwashed" erythrocytes agrees with the reports of Nakamura et al. (1967) and Grimmer et al. (1969). Examination of the present data relative to other results of this work reveals the following consistency:

$$\left[\begin{matrix}\text{Whole blood}\\ \text{DPH level}\end{matrix}\right] = \text{cellular DPH} + \text{plasma DPH}$$

$$= \left[\begin{matrix}\text{erythrocyte}\\ \text{DPH level}\end{matrix}\right] \times (\text{Hct}^*) + \left[\begin{matrix}\text{plasma}\\ \text{DPH level}\end{matrix}\right] \times (1-\text{Hct}^*).$$

So as a ratio of the plasma DPH level——

$$\left[\frac{\text{Whole blood level}}{\text{plasma level}}\right] = \left[\frac{\text{erythrocyte level}}{\text{plasma level}}\right] \times (\text{Hct}^*) + (1-\text{Hct}^*).$$

$$\simeq 52\% \times (\text{Hct}^*) + (1-\text{Hct}^*)$$

$$\simeq 52\% \times (0.45^*) + (0.55^*) = 0.78$$

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*"Hct" is the abbreviation for "Hematocrit". The average hematocrit in these studies was 0.451.

Experimentally, the ratio of DPH concentration in whole blood to that in plasma is 0.74 ± 0.10 (Table XIIB), which agrees with the predicted value just calculated (0.78). As far as known, no such investigation and analysis of DPH levels has been reported previously.

At a total level of 30 to 35 ug DPH per millilitre of plasma, the unbound (free) DPH represents 9.6 (±1.3*)% of the total drug when measured by equilibrium dialysis at room temperature. Table XX presents these results, which were obtained for plasma from patients who were being treated with DPH. In addition, one patient who was undergoing periodic dialysis therapy for severe renal disease, and was receiving DPH prophylactically, had 20% of the total plasma DPH unbound. This patient was the only one studied who showed a great deviation from the 9% figure. Both the 9% and 20% values agree closely with the most recent literature (Lunde et al., 1970; Reidenberg et al., 1971).

Employing the results of Lunde et al. (1970), data on the binding of DPH may be converted to other experimental conditions: The percentage of DPH free at 37°C is 1.65 times that determined at room temperature (23 to 26°C), while the percentage at a total concentration of 16 ug/ml plasma (as used by some other workers for their samples which were initially <u>without</u> DPH) is approximately 0.89 of that for the

*Maximum possible error due to the ±7% coefficient of variation of the assay procedure employed.

30 to 35 ug range of the present work. Secondly, as the binding of DPH does not change over the pH range 7.4 to 8.2, the higher-than-physiologic pH of the plasma-EDTA in the present experiments (7.6 to 8.0) would not be expected to alter the binding phenomenon*. Finally, the degree of dilution of plasma which occurs in the present dialysis technique (Cf. Figure 18) does not alter the percentage of DPH bound.

Hence, the results of the present study can be converted to predict that DPH for mid-therapeutic levels (~16 ug/ml) is 13 to 14% unbound at body temperature. This is more in line with the physiologic data which have been reported in the literature (obtained by determining the cerebrospinal-fluid-to-plasma ratio of DPH). From the data of Lunde et al. (1970), room temperature and a total DPH level of 150 ug/ml of plasma would be expected to simulate physiologic conditions (by yielding the same drug ratio as 37°C and a 16 ug/ml level). The actual outcome of these experiments is shown in Table XX.

A close examination of the methodology employed in the usual determinations of the present study was also made. Figure 18 displays the time course of equilibration between the compartments of the dialysis chamber when the contents are stirred magnetically. The routine analyses were done after twenty hours of dialysis, and therefore represent very close to equilibrium conditions. A comparison with several ultrafiltration techniques proved to be very interesting.

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*The use of a non-buffering dialyzing solution (Elliott's A with EDTA in the present work) is therefore justifiable.

TABLE XX

EQUILIBRIUM DIALYSIS OF DIPHENYLHYDANTOIN (DPH) IN HUMAN PLASMA

Sample No.*	PLASMA %T.S.**	PLASMA DPH ug/ml	DIALYSATE ug DPH/3 ml	Correction factor	Fraction of DPH unbound
1	7.3	31.0	10.5	0.955	10.8%
2	7.5	33.7	10.5	.954	9.9%
3	7.4	32.0	10.7	.955	10.6%
4	7.4	31.8	8.3	.955	8.3%
5	8.2	43.6	10.0	.949	7.2%
6	8.1	31.3	9.9	.949	10.0%
7	8.3	37.0	11.0	.948	9.4%
8	7.9	38.3	12.2	.951	10.1%
9	7.5	38.0	12.8	.954	10.7%
10	8.5	48.1	15.9	.946	10.4%
11	8.9	28.0	10.1	.942	11.3%
12	8.3	36.0	9.0	.948	7.9%
13	8.4	35.4	8.6	.947	7.7%
14	6.8	27.8	17.4	.959	20. %
15	7.6	100.	77.	.953	24. %
16	8.1	102	80.	.952	25. %
17	8.0	32.9	9.3	.953	9.0%
18	7.8	21.8	6.7	.952	9.7%
19	8.0	21.0	7.8	.950	11.8%
20	7.7	9.7	4.4	.952	14.4%
21	7.8	26.3	25.1	.952	30. %

*Nos. 1 to 7——Separate random pools of normal plasmas (pools 1-7).
No. 8——No. 8 random pool of normal human plasma, dialyzed 20 hrs.
No. 9——No. 8 random pool of normal human plasma, dialyzed 48 hrs.

No. 10—Patient showing severe toxic signs (and 62 ug DPH/ml).
No. 11—Patient with relatively low plasma level (4.4 ug DPH/ml).
Nos. 12,13——Patient showing toxic signs, a high plasma level (40. ug DPH/ml), and a long DPH half-life ($t_{\frac{1}{2}}$=50.4 hrs.):
No. 12 - Plasma dialyzed immediately ("fresh").
No. 13 - Plasma dialyzed after freezing and thawing.

No. 14——"Renal patient" undergoing periodic dialysis therapy.

Nos. 15 ff.——No. 9 random pool. Specifications: 23°C (15,16,17,18), 30°C (19), 37°C (20,21); ethanol 0.5% (18,20), 2% (19), 5% (16,17), 6% (15,21).

**"%T.S." indicates "percent total solids".

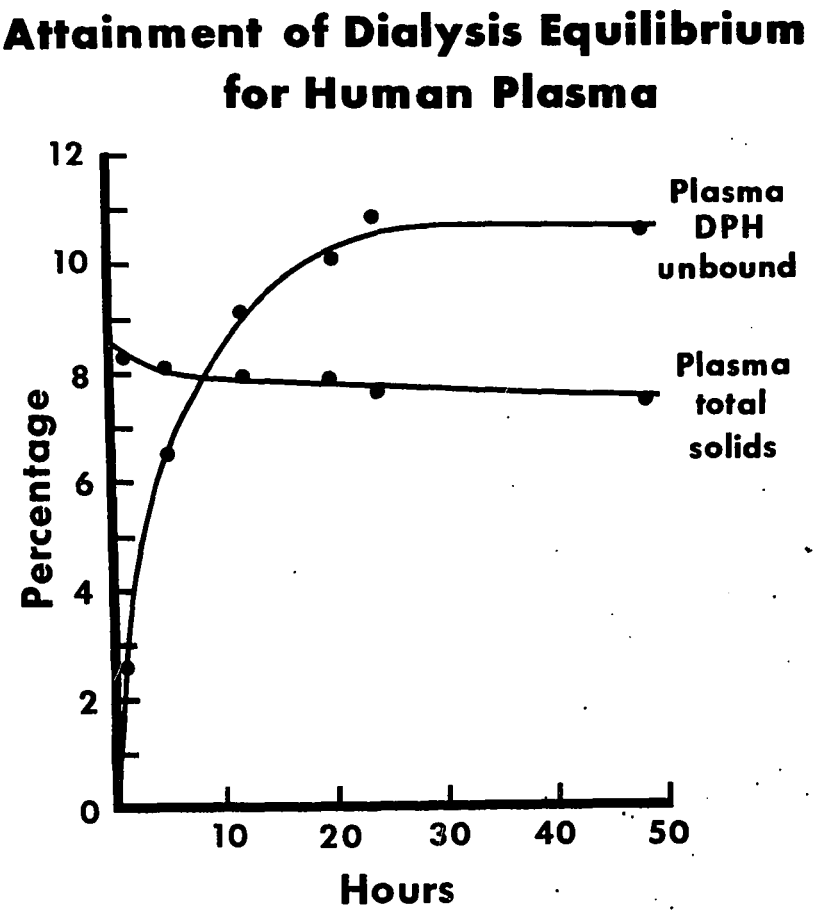

FIGURE 18. EQUILIBRIUM DIALYSIS OF DIPHENYLHYDANTOIN (DPH) AT ROOM TEMPERATURE BY THE PRESENT METHOD.

Each point represents the average result for two separate dialysis experiments. (Both solutions were stirred magnetically for the entire duration of the dialysis.)

The upper curve represents the movement of drug from the plasma sample into the dialysate, and the lower curve demonstrates the slight dilution of the plasma proteins which occurs along with the equilibration of the drug.

When the Sartorius* system was employed, the filtrate from solutions of DPH in both water and plasma contained only a trace of the drug (<0.2 ug). In fact, when a 12 ml volume of a 5.0 ug/ml aqueous solution was filtered 50% under 100 p.s.i. nitrogen, its concentration was reduced to 4.0 ug/ml, and 41. ug DPH was extracted by chloroform from the dried membrane recovered. Although this sequestration of DPH by the Sartorius membrane makes the system inapplicable to ultrafiltration studies, it does offer an easy way of indirectly determining the level of unbound drug present in a sample (since the amount sequestered is proportional to the free drug present, until the membrane begins to become saturated).

The Amicon "Diaflo" ultrafiltration membrane** was also investigated. This system gave very similar results, although less DPH was held within the membrane. The ultrafiltrate contained no DPH and the solution remaining behind the membrane increased slightly in concentration (from 5.0 to 5.2 ug DPH/ml).

When cellulose dialysis tubing (as employed in the equilibrium dialysis technique of this work) was placed in the

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*Sartorius ultrafiltration membrane No. SM 121 36 (Sartorius Membranfilter GmbH, 34 Goettingen, West Germany). Obtained through British Drug Houses (Canada) Limited.

**Amicon Corporation, Lexington, Massachusetts, U.S.A.

Amicon support, much better results were obtained than for the other membranes. Although the flow rate was very slow (4 ml/hour at 50 to 70 p.s.i. nitrogen), approximately 80% of the DPH in an aqueous solution passed through the membrane (filtrate = 4.1 ug/ml for the 5.0 ug/ml solution). This fraction increased to 90% for only 30 p.s.i. and 94% for 10 p.s.i. Therefore, the cellulose membrane does not allow DPH to be passed through as freely as water, and its characteristics are dependent upon the exact pressure applied. There was no evidence of sequestration of DPH by this membrane.

For a pool of normal human plasma (at 37 ug DPH/ml), ultrafiltration through this cellulose membrane under a pressure of 50 p.s.i. showed the drug to be 8.2% unbound. However, from the data just given, this should be corrected (+25%) to 10.2% free in order to compensate for the incomplete passage of the drug through the membrane. This corrected result agrees well with the average value of 10.4% obtained by the equilibrium dialysis technique for the same pool.

A similar correction is called for when centrifugation is employed to pass an ultrafiltrate through a membrane, although authors routinely using this method (Lunde et al., 1970; Reidenberg et al., 1971) seem to indicate that no corrections whatever are being made. No note is given as to whether such characteristics were considered or not. Although this does not invalidate their comparative results, it may explain their much lower results (approximately 6% free at a total level of 16 ug/ml) as compared with those of the present

work (8 to 9% free when corrected to the 16 ug level at room temperature), and those of the earlier reports summarized previously in this thesis (many done under physiologic conditions). The apparent unbinding of DPH (6% free increased to 9% free) caused by ethanol* at 2 (v/v)% (Lunde et al., 1970) was not observed in this work; in fact, the present data were obtained at ethanol* concentrations of approximately 3 (v/v)% and agree with a value of 9.3 (±0.7)% free (n=4) measured by these same authors using a dialysis method and much less ethanol. Perhaps the membrane phenomena affecting the non-equilibrium method (ultrafiltration) explains these differences. Additionally, the decreases in bound DPH reported to accompany increased temperature or increased drug level were of much greater proportion in the present study. The overall conclusion must be that ultrafiltration allows comparative studies of protein binding to be done relatively rapidly, but equilibrium dialysis must be relied upon to produce reliable values. Similar warnings have been issued in the past relative to filtration techniques in general (Grollman, 1926; Goldstein, 1949; Oppenheimer and Surks, 1964).

The utility and potential of the method developed in this work are being increased, and the maximum possible error decreased, by introduction of a continuous-flow half-cell for the dialysate chamber.

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*Ethanol is a solvent employed for concentrated solutions (~5 mg/ml) of many drugs including DPH.

V

GENERAL DISCUSSION

CLASSIFICATION OF THE EPILEPSIES

It has been stated that there are as many different types of epilepsy as there are persons experiencing seizures (about one-half percent of the total population). Robb (1965) suggests a classification of seizure types which emphasizes symptomatic aspects:

a) Seizures of localized onset, with or without loss of consciousness (including motor, sensory, aphasic, psychical, and autonomic seizures, plus purposeful automatisms).

b) Generalized convulsions (including tonic, clonic, and myoclonic seizures).

c) Unilateral convulsions.

d) Absence attacks (i.e., diminution of consciousness without a gross motor component).

e) Unclassified seizures.

The use of only three categories — partial, generalized, and absence — seems to be appropriate for the purposes of an anticonvulsant drug laboratory; these three are employed in this work, and are those presently used by the Clinical Neuropharmacology Laboratory of the Montreal Neurological Institute.

The system of classification proposed by McNaughton (1952 and 1971) places more emphasis on the etiology of seizure disorders:

a) Focal (or local) epilepsies — seizures of local onset, with or without generalization of the seizure pattern

(possible types include motor, sensory, aphasic, psychical, and autonomic seizures, plus purposeful automatisms). These epilepsies are associated with focal brain damage.

b) Generalized epilepsies—all seizures which are generalized from onset (possible types include absences; tonic, clonic, and myoclonic seizures; and drop attacks). This group is composed of three sub-categories—

1. "Secondary" epilepsy—due to many different causes (the identification of which can be difficult in the individual patient). Neurological or psychological abnormalities <u>are</u> commonly present.
2. "Primary" or "corticoreticular" epilepsy—generalized epilepsies of unknown cause. There are usually <u>no</u> accompanying neurological or psychological abnormalities here.
3. Undetermined generalized epilepsies (for patients with <u>apparently</u> generalized seizures which do not fit into category "a", or "b1" or "b2").

Although the antiepileptic drugs considered in this work, diphenylhydantoin and phenobarbital, have limited ranges of activity within any proposed classification scheme, their overall functions can best be understood by considering "the epilepsies" generally.

CONCEPT OF A SEIZURE THRESHOLD

Epilepsy, identified by the periodic occurrence of one or more of the above-listed types of convulsive or non-convulsive seizures, is a symptom of disturbed neural function (Penfield and Jasper, 1954; Sutherland and Tait, 1969). Figure 19 presents a unified model for neural tissue instability which is in accord both with knowledge of clinical epilepsy and with the observed actions of medications. Every brain is considered to have a "seizure threshold", designated "θ". The immediate cause of seizures would be the many stressing factors which tend to destabilize neuronal cells; these factors, which can be intrinsic or extrinsic, are listed in Figure 19. The sum of all resultant stress effects (designated "Σ STRESS") is variable over time, and its person-specific characteristics define the value of a stress parameter "α". Seizures can occur only when Σ STRESS exceeds the threshold "θ". The overall seizure frequency is related to the exact value of "α":

$\alpha \ll \theta \longrightarrow$ no seizures.

$\alpha \simeq \theta \longrightarrow$ occasional seizures.

$\alpha > \theta \longrightarrow$ frequent seizures.

In the electroconvulsive or chemical induction of experimental seizures, the difference between the non-epileptic "α" and the value of "θ" is measured.

It is the function of an anticonvulsant drug to elevate the seizure threshold (i.e., increase it from "θ" to "θ_A" or "θ_B" as in Figure 19). This decreases the frequency

of seizures. Presumably, the distance over which the seizure frequency line is moved to the right by the drug is related to its concentration within the body. Should "α" be too much greater than "θ", in any given patient, then this line may not be moved far enough to effect near total control of seizures before a toxic drug level has been reached. It therefore seems reasonable to suggest that the "therapeutic level" of a drug in any given patient is determined in major part by the exact value of his "α" parameter.

The model proposed here treats <u>all</u> epilepsies as the combined manifestation of the individual's susceptibilities to the various stressing factors to which he is exposed. The etiology of a given seizure disorder is an important consideration since suspicion of distinct pathology requires that specific investigations be done. If practical, the major treatment may be reduction of the magnitude of Σ STRESS by removing the prime causative factor (intrinsic—local or systemic—or extrinsic). Drugs are then used (often prophylactically) to elevate the seizure threshold.

CONCEPT OF A SEIZURE THRESHOLD

The magnitude of "Σ STRESS" = the summation of the destabilizing effects* on neurones due to:

Tissue pathology (including trauma, infection, neoplasm),
Emotion, Fatigue,
Endocrine changes, Fever,
Altered blood gases, Hypoglycemia,
Altered ionic balance (e.g., hypocalcemia, abnormal pH),
Vascular disturbances,
Other metabolic disturbances (local and systemic),
Immaturity of the nervous system (changing with age),
Pharmaceutical agents (all drugs),
Abnormal stimulus levels (high or low),
PLUS Genetic factors (positive and negative).

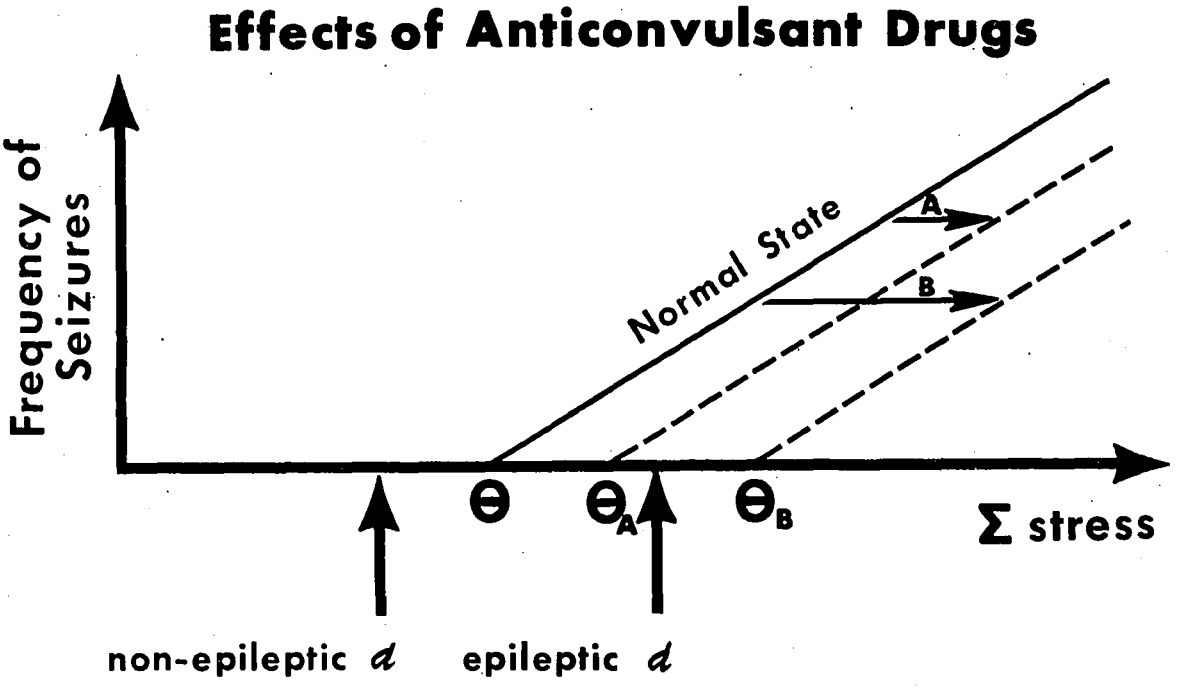

FIGURE 19. CONCEPT OF A SEIZURE THRESHOLD.
Drug "A" above is partially effective (seizure frequency > 0).
Drug "B" above is fully effective in the case demonstrated since the seizure frequency is now zero.
(N.B. — Drug "B" may merely be Drug "A" at a higher concentration.)

*The magnitude of (physiological or pathological) destabilization depends upon the strength of each factor listed above and upon the individual brain's susceptibility to it.

VALUE OF DRUG LEVELS IN CLINICAL PRACTICE

The 'need' for the assay of anticonvulsant drugs, particularly diphenylhydantoin, has been demonstrated by a number of authors. A recent monograph has reviewed the status of this field in general (Rose et al., 1971).

When the clinical situation is not in keeping with the drug dosage prescribed, Kutt et al. (1966) state that low blood levels could indicate: unreliable drug intake (most commonly), rapid metabolism (frequently), or malabsorption (rarely). The possibility of ineffectiveness of given drug therapy is indicated in the model just presented: The drug may be present in the brain in insufficient quantities to elevate the seizure threshold adequately. On the other hand, if moderate levels do exist, a value of "α" >> "θ" will significantly cut the apparent effectiveness of a given increase in the threshold; the clinical picture is deceiving. Another possibility is that the specific drug chosen may (unexpectedly) have a low potency in controlling a given case of epilepsy—so the threshold is not in fact elevated at all, but the side effects of the drug are or are about to occur nevertheless. The assay of drug levels therefore often serves as a safety measure in good therapeutics.

It is well known that increased supervision based upon knowledge of assay data leads to higher drug levels and better therapy (Gibberd et al., 1970; Dawson and Jamieson, 1971). Surprisingly though, assay data may be used in another

way to increase the (net) therapeutic effect of diphenylhydantoin (DPH)—Lascelles et al. (1970) report that ten of a total of eighteen patients whose levels were reduced from above 25 ug/ml showed such a decrease in seizures that the total seizure frequency for all eighteen equalled only 50% of the former rate. Two patients showed increased seizure frequencies. This is probably best interpreted as indicating that DPH can actually act as a (pharmaceutical) stress upon neuronal cells (Figure 19) in addition to therapeutically increasing the seizure threshold. Each effect is greater at higher concentrations of drug. The resultant individuality of response appeared in the data obtained in the present work as well: the level of DPH in plasma and the degree of seizure control sometimes changed paradoxically.

Therefore multiple assays, corresponding to different rates of dosage of a drug, allow the physician to reach a maximal therapeutic effect in each patient at his lowest possible (safest) drug concentration.

ASSAY TECHNIQUES

An assay can suffer from two possible sources of interference: 1) the biological substances contained in the samples, and then 2) the foreign materials present, e.g., drugs and their metabolites. In order that assays be selective in the determination of drugs, an extraction procedure is combined with a somewhat specific technique for the actual detection of the drug(s) of interest. In selecting a

method to be that used in a given clinical laboratory, one must consider the balance between the degree of specificity obtained and the appropriation of time and funds required in order to allow optimal utilization of the methodology. Each method has its own advantages and disadvantages. Therefore, the overall assessment of each is unique to a specific situation of clinic plus laboratory.

Gas-liquid chromatography is now considered to be the "future method of choice" for assays (Buchthal and Svensmark, 1971). It is a very specific technique, and yet the use of inadequate procedures for sample preparation, or the formation of derivatives, often defeats this advantage (Van Meter et al., 1970). The extra care required frequently lengthens the time requirement for an assay, and such techniques may then be unsuitable for screening large numbers of samples quickly (Wallace, 1969b). As well, many methods require the use of irritating or flammable reagents, a dangerous practice in general laboratories.

Present gas-liquid prototypes do represent the best technique available for the determination of small amounts of chemical agents, but their many specific requirements cause gas-liquid chromatography to fall short of its full potentiality. The high initial cost and moderate operating expenses are usually prohibitive to centers in the less-developed parts of the world and to "small" units everywhere. Such institutions should not, however, be deprived of the availability of assay data from other techniques (Rose et al., 1971).

The methods developed in the present work were designed to fill this "gap".

The assay for diphenylhydantoin employs the formation of a derivative, which is then steam distilled, the overall process allowing attainment of an outstanding degree of specificity. In the original methods using this technique (Wallace et al., 1965; Wallace, 1966), "extensive concentration of the distillate is required for accurate analysis of therapeutic levels of the drug in biological specimens" (Wallace, 1968). As well, "... the need for a constant source of steam makes it difficult to perform a number of tests simultaneously" (Wallace, 1969b). Modifications of the technique have been proposed, but these required rather expensive glassware (Lee and Bass, 1970; Morselli, 1970) or else employed flammable reagents, a dangerous practice in clinical laboratories (Wallace, 1968 and 1969a; Morselli, 1970).

In the development of the present semimicro-technique for determining diphenylhydantoin, these problems were solved by designing special glassware units and accessory apparatus which would purposely not be very expensive to obtain. The high efficiency of the connecting bulb, modeled after the Iowa State type Kjeldahl connecting bulb, reduces the contamination of distillates and so allows the use of an abbreviated procedure for the preparation of biological samples: only a single extraction process is now required, and no vacuum source is necessary for the evaporation of a volatile organic solvent, as opposed to that of the sodium hydroxide solution resulting

from the second extraction step in previous such methods (Wallace, 1966; Lee and Bass, 1970). The overall achievement is a more rapid determination of diphenylhydantoin in all types of samples.

Retention of the steam distillation technique to collect the diphenyl ketone reaction product allowed avoidance of the use of flammable reagents. Anti-bumping granules replaced an external source of steam as the means of maintaining the required equilibrium between the reaction mixture's two immiscible phases*. The instability of alkalinic permanganate solutions made by dissolving pellets of sodium hydroxide has been eliminated by the use of a commercial sodium hydroxide solution. Finally, the unique combination condensing-collection tube is ideal for a small amount of distillate; not only is it collected in toto, but a gauge of its volume (± 1%) is simply the height of the distillate column in the tube.

In the present assays of both diphenylhydantoin and phenobarbital, the use of silicone-treated filter paper at the end of the extraction process resulted in the almost total

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*A steam distillation occurs when water is distilled together with an immiscible substance — both appear in the distillate, but without the other impurities originally present; the phases must somehow be kept in equilibrium with each other in order to assure quantitative recovery (Glasstone and Lewis, 1960). The immiscible phases present in the reaction mixture of this assay are water (the alkalinic permanganate reagent) and the diphenyl ketone (reaction product); the latter is quantitatively recovered.

recovery of the drugs from samples, primarily be allowing re-extraction and 'rinsing' of the sample phase. Anhydrous sodium sulfate is not required to increase this recovery, so excessive difficulties in cleaning are avoided. Centrifugation steps can be eliminated, so time and equipment requirements are reduced, and the loss of samples due to breaking tubes is prevented. Buffer solutions are not required during either of these extraction processes because a) the recovery of diphenylhydantoin into chloroform is essentially complete for all pH $\leq$ 8.0 (Dill et al., 1971), and b) the best extraction technique for use in determining phenobarbital quantitatively employs very acid pH (Guzak and Caraway, 1963). Compared with previous methods then, the present techniques involve fewer measurements of volume and require less glassware and less time; they are relatively inexpensive.

Hence, the two rapid assay methods developed in this work are to be recommended particularly for moderately-sized and smaller institutions (for routine clinical use on their own), but also elsewhere as a supplement to other techniques such as gas-liquid chromatography, especially for emergency situations or when equipment or scheduling problems prevent the use of other apparatus. This does not preclude the adoption of some of the technology developed during this work into any of the other methods for the assay of any type of pharmaceutical agent.

In any case, regardless of the exact methodology being employed, interpretation of assay results must be done

cautiously. Analysis of a new sample is probably frequently justified, but may not correct misleading data:

> Many drugs have been shown to cause no interference in various assays for diphenylhydantoin; "This does, however, not exclude the possibility that their metabolites may interfere with the determinations" (Buchthal and Svensmark, 1960). Our own experience indicates that gas chromatographic methods can also suffer from unexpected interferences.

Careful clinical interpretation of the results from any assay technique is a must.

CLINICAL DATA

Assays for anticonvulsant drugs have been reported about equally in terms of plasma, serum, and whole blood contents. Plasma appears to be the preferred sample type on a physiological basis (Brodie and Hogben, 1957; Desgrez and de Traverse, 1966); experimentally serum and plasma proved to be equivalent with respect to content of drug and ease of laboratory cleaning operations. Duplicate assays on samples of serum or whole blood show a greater standard deviation than do those on samples of plasma.

An unexpected effect of the change of anticoagulant system upon the measured concentration of diphenylhydantoin in "plasma" was shown in the present work; use of "serum" as the sample type might therefore be expected to produce greater consistency for the routine clinical comparison of levels (except for its greater standard deviation of duplicate results as noted above). The erythrocytes remain readily available in

plasma and the morphology-preserving qualities of the anti-coagulant ethylenediamine tetraacetic acid (EDTA) prevent for a much longer time the shift of water and drugs between the intracellular and extracellular compartments which occurs in samples of whole blood in many other anticoagulants, and in samples of serum. Therefore, plasma with EDTA as anti-coagulant was requested during this work. As a clinical aid, data presented herein (Tables XIIA and B) allow one for the first time to convert an assay result for one type of sample into an equivalent level for another type. This information also allows proper interpretation of the various levels reported in the literature.

The question of the degree of level-to-dose correlation for diphenylhydantoin (Jensen and Grynderup, 1966) was studied only in a specific institutionalized group of patients, so the unreliable drug intake for the out-patient basis was eliminated. For these adult white males, there was a significant correlation; this linear association was greater when dosage was expressed in milligrams of drug per unit of body surface area (meter^2) rather than per unit of body weight (kilogram). Such a pattern is now generally accepted, and these regression lines themselves agree well with analyses given in many previous reports (e.g., Svensmark and Buchthal, 1963a; Buchanan and Allen, 1971; Buchthal and Svensmark, 1971). However, the variations in the level-to-dose ratio are rather large (note the spread of points shown in Figure 11). Regressions of level on dose must therefore serve only to estimate

the expected therapeutic daily dosage. From then on, the actual plasma levels plus the clinical picture offer the best guide for adjusting therapy.

A reasonable but arbitrary estimate of a "therapeutic level" for diphenylhydantoin (DPH) which can be made from the data of this work is 5-25 ug/ml plasma, a wider range than is usually stated (10-20). A surprising number of our patients were being controlled at very low levels of DPH (2-5 ug/ml). Buchanan and Allen (1971) suggest that earlier studies missed such levels due to the lack of specificity of their assay methods. Only one observation need be made here: the present data have emphasized the great interpersonal variation in both "therapeutic" and "toxic" levels of DPH which exists through any given patient population. So although the assay of DPH in patients treated for epilepsy is necessary in 'problem cases', the laboratory results can again only guide the physician towards the appropriate therapy for each particular patient. Clinical judgement has not been replaced.

The major remaining clinical question involves the disposition of cases showing signs which may represent drug-induced toxicity. Recent evidence indicates that there has been experimental error in the past and that "therapeutic" levels of DPH do not in fact cause any change in the CNS histology (Dam and Nielsen, 1970); however, toxic levels of DPH are accompanied by these changes — e.g., edema of brain tissue and a decrease in the density of Purkinje cells (Utterback et al., 1958; Kokenge et al., 1965). Toxic levels,

at least of DPH anyway, must therefore be reduced as soon as discovered. The difficulty is in classifying a given level as "toxic" or not.

Confirmed by the comments made above about the data of the present work, Vajda (1970) reports that clinical toxicity can occur both above and below the traditionally-noted 20 ug DPH/ml level, while much higher concentrations are well tolerated by some patients. A delicate balance occurs in many. The data of Haerer and Grace (1969) may cast doubt on the value of using nystagmus as a sign of DPH toxicity. However, it must be accepted that there is a "relatively narrow margin between full therapeutic and minimally toxic doses of the drug" (Patel and Crichton, 1968), that clinically apparent toxicity may develop slowly (Roseman, 1961), and that true toxicity in humans can lead to both temporary and permanent neurological sequelae (Kokenge et al., 1965). Nystagmus is therefore probably best considered to be a warning sign which calls for subsequent monitoring of both the clinical and drug-level situations of the patient. Should the classical progression of toxic signs then be seen (Ang et al., 1969), an immediate decrease in dosage rate is indicated while continuing to follow the patient closely.

For acute toxicity (e.g., following purposeful or accidental overdose), since the plasma level may still be increasing up to several days later (Cf. Table IV: Blair et al., 1968), less conservative measures such as peritoneal dialysis may be indicated (Andia et al., 1968; Tenckhoff et al., 1968).

Clinical improvement is usually slow (Schreiner, 1958; Blair et al., 1968). Schulte and Good (1966) suggest that measures such as dialysis "are of little value compared to metabolic detoxification of the drug", and yet the rapid reversal of DPH toxicity is to be recommended to avoid causing (further) permanent neurological damage (Theil et al., 1961; Ang et al., 1969). So, the usual treatment of clinical DPH intoxication involves only conservative and supportive measures. Even the sudden, total withdrawal of DPH from patients who did not have epilepsy has not led to the appearance of any seizures or other withdrawal symptoms (Rosemblum and Shafer, 1970; Rosenblum, 1971).

In view of the variation of DPH level in plasma which occurs throughout the day, venipuncture for comparative purposes should be done at about the same hour each time. The standard deviation of the assay employed (± 0.7 ug/ml for the method developed in this work) plus the usual physiological variation of plasma levels which has been observed to occur over several weeks' or months' time (± 2.0 to ± 2.5 ug/ml) should both be considered when interpreting a change in the reported assay results for a given patient. For the usual levels encountered, deviations of less than 2.0 or 3.0 ug/ml (approximately 25%) must still be watched, but are best considered to be equivocal unless the trend is shown to continue in the very near future.

The controlled cross-over study which compared the single-dose (q.a.m.) and divided-dose (t.i.d. or q.i.d.) schedules for administering DPH emphasized how important the exact time of day of venipuncture can be to the outcome of drug assay. The increases in level which immediately follow the ingestion of the entire daily dosage of DPH average +2.9 and +4.3 ug/ml at the third and sixth hours respectively. Deviations in level between the time of consumption of a 100 mg dose and three hours later average ± 1.4 ug/ml. On the other hand, Buchanan et al. (1972) report that the divided-dose regimen (with DPH taken at hours 0, 6, and 12) produces rather steady levels during the day, the highest level occurring at approximately hour eighteen. The peaking of DPH concentration in plasma which was observed early in the day during the single-dose period of the present study was highly significant (actual $p < 0.005$), while the divided-dose regimen produced only slight elevation ($0.1 < p < 0.2$). Hence the single- and divided-dose schedules of DPH administration are <u>not</u> pharmacologically the same. This contradicts the interpretation given by Buchanan et al. (1972) for their study done in volunteers not suffering from epilepsy. However, considering our institutionalized group of twenty-nine patients as a whole, the two schedules <u>were clinically equivalent</u>. Many individual patients nevertheless did show distinct differences between clinical behaviour during the two schedules for dosing. Again, the individuality of response to DPH has been demonstrated.

A peculiar pattern of plasma levels was observed in the ten patients who were studied from zero to twelve hours following ingestion of their single 300 mg dose of DPH —the initial increase in level was followed by a decrease and then another increase; this "dip" in level varied in characteristics from person-to-person and was reproducible. Perhaps this picture was related to meals and the individual's specific gastro-intestinal function, enterohepatic circulation, drug distribution pattern, and/or other physiological cycles. A variable effect of pH upon the rate of dissolution of DPH has been shown (Arnold et al., 1970). As well, the up/down/up pattern reported herein does mimic prominent phenomena observed over shorter periods of time following the acute oral and intravenous administration of DPH, for which similar hypotheses were advanced (Noach et al., 1958; Handley, 1970; Suzuki et al., 1970).

The non-pathological aspects of clinical picture were assessed in the twenty-nine patients through observation of their general behaviour in the institution. The "instructors" of their workshops (blindly) noticed some transient changes when the dosing schedule was altered, but could not detect any permanent effects during the one month of single-dose administration of DPH. The several hospital outpatients studied separately reported no changes in their abilities to handle their normal jobs (unsheltered). It was thereby verified that the accompanying conditions present in the institutionalized group were not in fact covering up any

side effects caused by the single-dose regimen. In both groups, the only side effects noted occurred immediately following each change in dosing schedule (at periods I to II, and then II to III). All of these adverse effects subsided spontaneously. This may indicate that some type of tolerance to the side effects of a dose of DPH actually develops with time (Cf. Buchthal et al., 1960).

This study indicates that the once-daily administration of DPH is satisfactory in patients with epilepsy. Several reports have already advocated this dosing schedule (Roseman, 1961; Merritt, 1963; Haerer and Grace, 1969; Buchanan et al., 1972). More traditional authors (e.g. Buchthal and Svensmark, 1971) seem to feel that "condensing" the administration to two doses per day is the tolerable limit. However, the data of Buchanan et al. (1972) surprisingly indicate that the peak-to-minimum differences in DPH level for its once- and thrice-daily administration (2.43 and 2.12 ug/ml respectively) are essentially the same at a daily dosage of 300 mg; it is only the timing and shape of the peaking of plasma level which differ. The present data showed that the plasma level at a full twenty-four hours after consumption of the single large dose of DPH was <u>not</u> significantly different from that observed when the total dosage had been taken in divided doses during the previous day. The <u>clinical</u> data verified that in fact no consistent alteration in the degree of therapeutic effect accompanied the change in schedule of dosing. In patients whose seizures are related to a specific precipitating

factor, e.g. hypoglycemia or the effects of physiological rhythms (Cf. Figure 19), coordination of the peak level of DPH with the highest values of ΣSTRESS during the day could be beneficial: the medication would become more efficient. The empirical adjustment of individual dosing schedule, perhaps to a non-traditional or non-stereotyped format, can be done without risking the dangers which were once predicted to accompany "condensed" therapy. In addition to this possibility for achieving better therapeutic effect from the drug when control of seizures is incomplete, the reports of Haerer and Grace (1969) and Buchanan et al. (1972) have suggested that the once-daily administration of a drug is simpler, and so leads to better motivation of the out-patient to take his medication as prescribed. In sum, there are no specific contraindications to considering the preference of the individual patient when setting the schedule for his daily intake of diphenylhydantoin.

These same factors—frequency and reliability of drug intake—may be the major causes of some of the clinical cases of supposed "drug interactions": multiple drug therapy ("polypharmacology") often leads to more frequent missing of doses and hence to lower plasma levels of the drugs involved (Vajda and Prineas, 1970). Nevertheless, it is <u>fact</u> that phenobarbital can cause both inhibition of the metabolism of DPH (apparently at the microsomes) and also induction of the enzymes involved in the para-hydroxylation of DPH (Cucinell et al., 1965; Kutt et al., 1969; Woodbury, 1969). Scores of

papers have emphasized one effect or the other and will not be mentioned here. The most important aspect clinically is the high degree of individuality of response which recent studies have noted (Kutt et al., 1965; Frey et al., 1967; Garrettson and Dayton, 1970). Our overall impression is in accord with additional clinical reports — the concomitant, chronic use of phenobarbital usually has no great effect upon either the levels of DPH or its therapeutic result (Diamond and Buchanan, 1970; Booker et al., 1971; Buchanan and Allen, 1971). But there are exceptions (e.g.: Kokenge et al., 1965; Kristensen et al., 1969; Kutt et al., 1969), so "a laboratory control of the serum concentrations is necessary if toxic actions and insufficient control of the disease are to be avoided" (Frey et al., 1967).

This entire story applies to other pharmaceutical agents as well. Besides INH and PAS (Kutt et al., 1968; Brennan et al., 1970), "other drugs have been noted to impair diphenylhydantoin metabolism. These include chlorpromazine, prochlorperazine, chlordiazepoxide hydrochloride, and estrogens" (Kutt and McDowell, 1968). Chloramphenicol (Christensen and Skovsted, 1969), dicoumarol (Hansen et al., 1966), disulfiram (Olesen, 1967b), and phenyramidol (Solomon and Schrogie, 1967) can also lead to elevation of DPH levels in the body. Monitoring of the plasma levels of anticonvulsant drugs is therefore very important when (practically) any other medication is added to or removed from a patient's <u>total</u> therapy.

One further example will illustrate the case well. In recent years, there has been much concern about the low folate levels observed in patients being treated for epilepsy. In some cases, such levels may be accompanied by recently developed anemias (Klipstein, 1964; Reynolds, 1968), neuropathies (Hansen et al., 1964), and even symptoms such as "retardation", "dementia", and "severe neuropsychiatric sequelae" (Reynolds et al., 1968; Wells, 1969). Both treatment and prophylaxis by administration of supplemental folic acid have been recommended by many of these authors, although such therapy may cause a fall in DPH levels* (Jensen and Olesen, 1970; Baylis et al., 1971). On the other hand, many authors have considered that this attempt to correct a supposed deficiency is ineffective and not really justified (Horwitz et al., 1968; Lovelace and Horwitz, 1968; Grant and Stores, 1970; Norris, 1970). Whatever course of action is chosen, care is necessary (Reynolds et al., 1968).

Drug assays are therefore to be recommended so that the physician may be forewarned of the development of any possible therapeutic problems arising secondarily to changes in the intake of any pharmaceutical agent.

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*Amazingly, this fall in the levels of DPH results from a disturbance in the metabolism of the drug which decreases the output of its main metabolite parahydroxyphenyl-phenylhydantoin, "HPPH" (Olesen and Jensen, 1970).

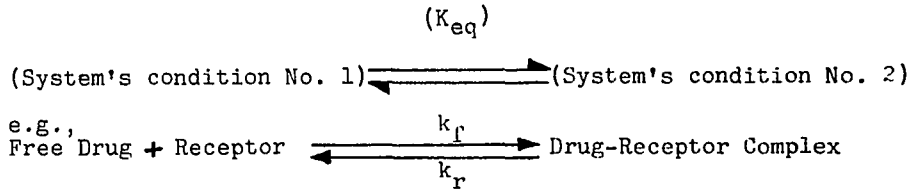
PROTEIN-BINDING PHENOMENA AND PHARMACODYNAMICS

The clinical implications of the binding of drugs by proteins in plasma and in peripheral tissues have been outlined previously. Figure 20 attempts to integrate these aspects of transport phenomena with their known relationships to drug activity at receptor sites and also to the metabolic processes occurring.

The basic concept is that of a rate of reaction: any dynamic system can be considered (at least in part) to move from one condition towards another at a rate which is equal to the product of the concentration of the reactant ("substrate") times a specific rate constant "k". As reversible processes involve two opposing changes, there is a rate constant for each direction: forward ("k_f") and reverse ("k_r"). Although changes are occurring in both directions simultaneously at all times, the overall system is well described by its "equilibrium constant" ("K_{eq}" of Figure 20A). It must be noted here that the substrate being considered in this work is free, unbound drug.

Figure 20B describes equilibrium between any two compartments of the body (e.g., intracellular and extracellular in the specific example chosen). The system is brought towards the equilibrium state by movement of free drug across membranes. These movements can be labeled as "absorption" and "excretion" relative to a single given compartment (Figure 20C).

A) BASIC RELATIONSHIP (A "REACTION")

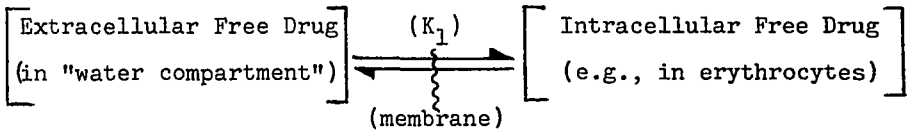


where k_f, k_r = "rate constants"

=functions of receptor activity, affinity constants, allosteric behaviour, pH.

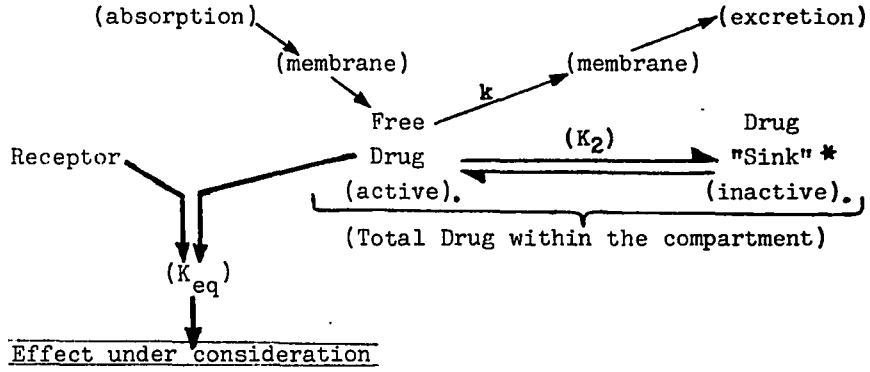
The "Equilibrium Constant" for such a reversible relationship is the ratio of the rate constants: $K_{eq} = (k_f / k_r)$.

B) INTER-COMPARTMENTAL EQUILIBRIUM



"K_1" is a function of membrane structure and potential; active or facilitated transport mechanisms; the Donnan equilibrium; plus the charge on drug molecules, their pK_a, and the pH of the compartments.

C) EQUILIBRIA WITHIN EACH COMPARTMENT



D) ONE TYPE OF RECEPTOR—THE METABOLIZING ENZYMES

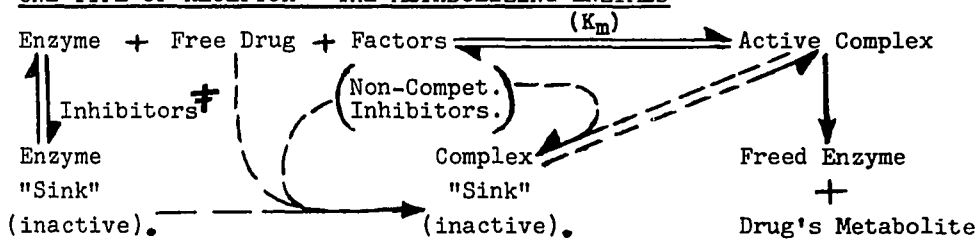


FIGURE 20. A MODEL OF DRUG DISTRIBUTION.

(Cf. particularly: Glasstone and Lewis, 1960; Martin, 1965a; Goldstein et al., 1968; Goodman and Gilman, 1970.)

*The Drug "Sink" includes drug attached to the binding proteins and is therefore inactive biologically but still available to become unbound and to replace any free drug lost by the compartment by excretion.

‡Inhibitors acting directly upon the free enzyme may be either Competitive or Non-Competitive ("Non-Compet."). These may include hormones, other drugs, and any metabolites present.

On the other hand, within this compartment, the free drug may: remain unbound; combine with specific receptors, according to constant "K_{eq}", giving rise to the effects of the drug (therapeutic and/or toxic); or else combine non-specifically (e.g., with the "binding proteins"), according to constant "K_2", forming a "drug sink" which is complexed drug, biologically inactive for the moment yet available to replace any free drug lost by the compartment (Brodie and Hogben, 1957). All of these relationships are dynamic, not static, even at equilibrium.

Therefore, although the usual emphasis is on total drug levels (the sum of free drug plus that in the "sink"), it is clearly the status of the free drug only which expresses the underlying pharmacological situations:

1) The (net) movement of drug towards a receptor site occurs at a rate proportional to the (differential) concentration of free drug. This can be evaluated by assessing the total amount of drug being stored in the erythrocyte, although variation in the affinity of cellular constituents for a drug may occur and disturb the linearity of the technique.

2) A drug effect results when the specific receptor is activated by the presence of free drug*.

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*This activation of receptor by the drug may be a function of the actual process of combination of the two to form a complex (therefore a transient effect) or else may be a function of the duration of existence of the complex itself (Paton, 1961). In either case, the magnitude of effect would be related to the concentration of free drug around the receptor.

3) A drug is (usually) metabolized at a rate which is related to the concentration of free drug made available to the enzyme(s) involved*.

To assess the status of free drug, consideration is made of its concentration in the "water compartment" since this drug is (approximately) at equilibrium throughout the body; the usual sample of this is "plasma water" (Goldstein et al., 1968). Ultrafiltration techniques attempt to obtain this sample, in very small volumes, directly from whole plasma. In the present work, the ultrafiltration membranes employed either sequestered diphenylhydantoin and allowed none into the filtrate, or else held back 5% to 20% of the free drug (depending upon the exact pressure applied). The preferences for employing an equilibrium system (i.e. equilibrium dialysis) for such determinations are many**. The method developed in the present work is also very portable and convenient because the compact dialysis chamber allows for easy temperature regulation of the sealed system, without requiring a temperature-controlled centrifuge or constant temperature room;

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*A complicating factor may be the protection of some enzymes by an additional "lipoid barrier" (Brodie and Hogben, 1957).

**See: Grollman, 1926; Goldstein, 1949; Lous, 1954a; Oppenheimer and Surks, 1964; Scholtan, 1964.

the preparation is uncomplicated. Although not involving additional labor, the requirement of equilibrium does lengthen the elapsed time for the procedure; the increased chance of bacterial growth is seldom a problem if the plasma is relatively fresh. Regardless of exactly how the free drug levels are measured, the results may explain many of the pharmacological variations which have been observed for drugs such as diphenylhydantoin (DPH).

For example, in the few patients with renal failure studied in this work who were receiving DPH at the usual dosage rate, the plasma levels of total drug were abnormally low (≤ 4 ug/ml); the binding of DPH in the plasma of this patient group was also much lower than normally measured. Both observations are confirmed by the work of others (Cf. Letteri et al., 1971; Reidenberg et al., 1971). These results probably serve to exemplify the model presented here (Figures 20 to 23): The metabolizing enzymes maintain a certain level of free drug in the water compartment of the body, regardless of the degree of protein binding of the drug in plasma. Once this concentration of free drug has been "set" (so that the rate of drug elimination = dosage rate), the drug complexes itself appropriately throughout the body (entering "drug sinks" in proportion to the affinities presented at the time). Hence, in the example above, the <u>specific</u> receptor sites may respond normally but the total level of drug in the circulation (=free plus bound drug) is much lower

than expected due to decreased binding*. The assay of total drug levels may occasionally be misleading: as here, a low level of a substantially-bound drug such as DPH does not necessarily imply that the drug therapy is inadequate.

Figures 21 to 23 mathematically examine binding and metabolic phenomena, first separately in the pattern established by Figure 20, and then together as interrelated in the organism (Figure 23). This is appropriate since most of the so-called "metabolic" problems encountered are really protein-binding phenomena — they are either related to changes in the fraction of drug unbound in the plasma or else related to the affinities shown towards various substances by the enzymes, which themselves are of course proteins.

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*In the terminology of Figure 21 (with D_f = the concentration of unbound drug, and β = the fraction of drug bound in the plasma),

$(1-\beta)$ = the fraction of drug unbound, and so

D_f = total drug x $(1-\beta)$.

Therefore, total drug = $D_f / (1-\beta)$.

The assumption made in the text above is that D_f is the same in "normal" and "renal" patients. In the present work,

$\beta_{(normal)} = 0.90$, and $\beta_{(renal)} = 0.80$.

Therefore, total $drug_{(normal)} = D_f / 0.10 = 10 \times D_f$, and

total $drug_{(renal)} = D_f / 0.20 = 5 \times D_f$.

Interpretation — the total plasma levels in "renal" patients should be half as great as normal for the same rate of long-term therapy and the same enzyme function. This fits the present data, and the data of others as mentioned above.

Let: B = Binding site

D = Drug

Reaction: $B + D \underset{}{\overset{K_{assoc}}{\rightleftharpoons}} BD$

Let: D_f = Concentration of free (unbound) drug

(B) = Concentration of uncomplexed receptor sites

(BD) = Concentration of complexed receptor sites

= Concentration of bound drug.

Then: $K_{assoc} = \frac{(BD)}{(B) \times D_f}$

and fraction of drug bound = β

$$= \frac{(BD)}{(BD) + D_f} .$$

Define "V_d" = apparent distribution space or volume*.

$$= \frac{A}{D_f}$$

where: A = Total amount of drug accumulated in the body

FIGURE 21. THE PROTEIN-BINDING REACTION, AND A DEFINITION OF "DISTRIBUTION SPACE" FOR A DRUG.

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*Cf. Goldstein et al., 1968. This volume is best assessed by measuring the level-to-dose ratio for single acute administration of the drug (Lous, 1954b).

Reaction:

$$\text{Drug} \xrightarrow{\gamma} \text{METABOLITE}$$

where: γ = a net enzyme activity*

Then rate of first-order reaction** =

$= \gamma$ x active drug concentration

$\gamma \times D_f$

This rate = $(\frac{-dA}{dt})$.

At biological equilibrium, D_f is set by "γ" such that:

$$\text{rate of drug intake} = \frac{-dA}{dt}$$

N.B. — Metabolic "inhibitors" decrease γ.

Metabolic "inducers" increase γ.

Examining the elimination half-life ($t_{\frac{1}{2}}$):

$$\frac{-dA}{dt} = \gamma \times D_f, \quad \text{but} \quad D_f = \frac{A}{V_d} \; ;$$

So $\frac{-dA}{dt} = \gamma \times \frac{A}{V_d}$, and $\frac{-dA}{A} = \frac{\gamma \times dt}{V_d}$.

FIGURE 22. METABOLIC ELIMINATION TREATED AS A REACTION.

*"γ" is related to both the activity and amount of the individual enzymes present. This can best be assessed by measuring the level-to-dose ratio for long-term therapy (Lous, 1954b).

**The assumption of first-order kinetics is justified at least for DPH at therapeutic levels (Arnold and Gerber, 1970; Gerber and Arnold, 1970).

Integration of the result of Figure 22:

$$-\log_e A = \frac{\gamma \times t}{Vd} + \text{constant}$$

$$+\log_e \frac{A1}{A2} = \frac{\gamma}{Vd} \times (t_2 - t_1)$$

But for $t_{1/2}$, $A_2 = 1/2 \times A_1$,

and $(t_2 - t_1) = t_{1/2}$

Therefore $\log_e 2 = \frac{\gamma t_{1/2}}{Vd}$

$$\therefore t_{1/2} = \frac{Vd}{\gamma} \cdot \log_e 2 .$$

CONCLUSION: Biological elimination half-life depends not only upon liver function (enzyme activity "γ") but also upon the distribution space of the drug — i.e., how much has been "stored up" in the body.

Also, $A = D_f$ multiplied by:

$$\left(V_{plasma} + V_{isf} + \frac{\beta V_{plasma}}{1-\beta} + \delta\right)$$

where δ = a factor to calculate peripheral binding of the drug.

Therefore <u>any</u> factor listed can vary from person-to-person, or over time, and change "A", and therefore alter the measured $t_{1/2}$.

<u>FIGURE 23.</u> RELATIONSHIP OF PROTEIN BINDING PHENOMENA TO DRUG ELIMINATION.

Firstly, the effects of interpersonal variation of the degree of protein binding have already been discussed. However, acute changes may also occur, as in competitive protein-binding phenomena; one substance may cause another to become unbound (by replacing it in the "sink") and thereby to temporarily flood the body in excessive quantities. But because the enzymes regulate the level of free drug, the normal free level is soon re-established with the <u>total</u> concentration at a new (lower) value*.

The manner in which protein binding may affect metabolic patterns is exposed in the mathematical treatment offered in Figure 23. Martin (1965a and 1965b) has verbalized the implications: for drugs which are highly protein bound (such as DPH), small variations in the degree of binding can cause a measured half-life to misrepresent the capabilities of the body for eliminating the drug. However, his additional remarks overstate the situation clinically. When a falling level is purposely induced (e.g., for obtaining proper responses on an electroencephalogram and during surgery), it is only the overall "elimination half-life" ($t_{\frac{1}{2}}$) which is of importance and <u>not</u> any one of the many contributing factors per se.

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*This pattern has been shown experimentally to occur for the hormone thyroxine: DPH decreases the percentage of thyroxine which is protein bound in the circulation, decreases the value of serum protein-bound iodine, and yet does not induce clinical hypothyroidism (Oppenheimer and Tavernetti, 1962). Evidently the prediction made from the model presented above is occurring in this case.

An abnormal half-life study alone—obtained when the system is entirely out of equilibrium—may suggest enzymatic changes, but many of the other factors may also cause this without affecting the clinically important equilibrium situation.

A second "metabolic" phenomenon is the alteration in receptor affinity which may occur on the basis of heredity, developing pathology, or the effects hormones, metabolites, or other drugs (Kane and Smith, 1959). These specific receptors may be either at the site of action of the drug (therefore leading to alterations in the therapeutic or toxic effects of the drug) or else at the enzymatic level. Tedesco and Mellman (1967) have reported an enzymatic defect due to abnormal affinity (therefore an alteration in the mechanism of protein binding); there was not a relative absence of the enzyme in this example. Such a situation may be the basis of some of the cases of unusually high DPH levels reported in the literature (e.g., Kutt et al., 1964a and 1964b), and on a minor scale, the basis of the usual variation in level-to-dose ratio observed in most patient populations.

Besides such hereditary trends, the drug-metabolizing enzymes are also susceptible to various inhibitory influences (Cf. Figures 20D and 22). Of the many drugs considered clinically due to their production of DPH toxicity, thorough studies have been done for only INH and PAS—these two drugs inhibit the metabolism of DPH in a non-competitive manner (Kutt et al., 1968). Dayton et al. (1970) have reported a dose-dependent variation in rate of decline of DPH levels in

plasma. The occurrence of a metabolic "self-inhibition" by DPH was theorized.

All of these "protein-binding-mediated" situations in metabolism operate by the same mechanism. As indicated in Figure 22, the metabolic rate of a drug handled via first-order kinetics is proportional to the product of free-drug concentration times enzyme activity. At equilibrium, the total elimination rate of a drug (metabolism plus excretion) must equal its dosage so that the body content of drug remains quite constant. However, when the system is perturbed by an alteration in drug intake, binding, or elimination, the level of free drug is not that which will keep the body in biological equilibrium—so a net accumulation or net loss of drug must then occur until drug elimination and drug intake are once again equal*. In the case of slowed elimination of the drug from the body, the concentration of free drug increases; clinical toxicity may become apparent.

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*The hypothesis that the enzymes are reflexly altered by the body in order to create equilibrium states (Kutt et al., 1964a) is certainly an unnecessary complication. Some induction of enzymes occurs in some specific cases, but frequently not (Arnold and Gerber, 1970). The major adjustments in pharmacokinetics are "automatic", as stated above, and are those demonstrated by plateau times and elimination half-lives.

On the other hand, a similar situation occurs when an inhibitory influence is removed; elimination of the drug increases, an initially effective drug level falls, and this may then present clinically by the appearance of signs of inadequate therapy. To re-establish an equilibrium at a therapeutic level, adjustment of dosage rate of the drug is indicated in each case.

However, above presently-unpredictable and individual-specific levels of DPH, the rate of biotransformation of the drug is no longer proportional to its level* (Gerber and Arnold, 1969; Arnold and Gerber, 1970). So, the re-establishment of equilibrium just described may be unable to occur and a steady accumulation of the drug results. Toxicity would then be an inescapable consequence, and hopefully a recognizable signal to the physician.

The best conclusion appears to be that there is enzymatic heterogeneity with respect to drug metabolism in man—for a given drug, several enzyme systems may exist with the affinities, cofactors, and other characteristics differing among them. The data of Dayton et al. (1967) would seem to

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*Pharmacokinetic studies demonstrate that the metabolism of DPH can change from the normal "first-order" process (as shown in the models proposed in this thesis) towards a "zero-order" process (for which the metabolic rate is constant and independent of the drug level presented to the enzymes). As stated above, this effect is variable and may help to explain the person-specific character of the development of toxic reactions which was referred to previously in this thesis.

to support this hypothesis since apparently both zero-order and first-order metabolic processes function simultaneously on DPH.

As in so many situations discussed previously, the reasonable recommendation is that anticonvulsant drugs be assayed periodically (especially DPH since it is very widely used and may be more frequently subject to the pharmacodynamic extremes). The detection of certain patterns in total drug content of the plasma over time, or of an unexpected pairing of clinical picture with a single assay result, will indicate when some of the further laboratory studies described above would be helpful.

PERSPECTIVES ON HEALTH CARE

The balance has been set. In order to justify institution of a given laboratory program, limits must be followed. The economic aspects of any system for delivery of health care demand that the "cost-effectiveness" of any measure be considered before use. The overall "best" utilization of a particular laboratory service (even at its inception) does not involve test data from all potential patients: the laboratory test should be requested when a specific clinical situation or question arises, and not used as a "shot in the dark" while hoping to find something. Once indicated, however, any such laboratory "consult" is worth carrying out thoroughly and also following up properly. A general utilizational scheme suggested by the present work is charted in Figure 24. The ideas presented apply to most clinical laboratory services.

With respect to anticonvulsant drugs specifically, a list of indications for having the level of drug assayed in plasma can be deduced from the results of the present work:

1) The apparent therapeutic ineffectiveness of a "normal" dosage rate of the drug.
    a) Unreliable intake of the drug.
    b) Poor absorption of the drug.
    c) Rapid elimination of the drug.
    d) Low drug potency or low drug efficacy in a particular case of epilepsy.

2) An alteration in the <u>total</u> therapy prescribed to the patient. (Potentially any drug at all may have effects.)
    a) Changing reliability in intake of the drug.
    b) Interference with absorption of the drug.
    c) Interference with metabolism of the drug (protein binding - of drug to enzyme), and induction of enzyme activity.
    d) Interference at specific receptors for the drug.
    e) Interference at non-specific receptors for the drug (protein binding in plasma and tissues).
3) The occurrence of unexpected changes in response to long-term therapy at a constant rate of dosage.
4) The presence of conditions which make correct evaluation of the patient's clinical status difficult (e.g., mental retardation, paraplegia, or brain pathology*).
5) The intention to alter dosage rate of an anticonvulsant medication in order to increase, or assess, the truly drug-induced effects. This procedure allows a safe, controlled "titration" of the patient with the drug.

The aim of all is to individualize therapy, i.e. to maximize the benefit to the patient while maintaining an adequate margin of safety for him. "Normal" in therapeutics is a variable and unique characteristic.

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*For diphenylhydantoin, brain damage may make some patients "unduly sensitive to even relatively low (therapeutic) levels of the drug" (Lascelles et al., 1970).

Since diphenylhydantoin (DPH) is a very effective drug and also relatively inexpensive, it is widely used throughout the world and is particularly prevalent in the less-developed regions. It is, however, the most frequent cause of drug toxicity in neurology and neurosurgery. Toxicity due to DPH is usually incorrectly diagnosed (Patel and Crichton, 1968) and the usual "toxic signs" may even be absent (Logan and Freeman, 1969). In practice therefore, the ability to assay DPH in plasma is of considerable importance and the cost-effectiveness ratio of this laboratory service is quite attractive. As gas-liquid chromatographic facilities are not that widely available (and even fewer are for immediate use), the inexpensive yet accurate and sensitive method developed in this work to determine DPH should prove to be quite valuable. It is outstanding for emergency use and is presently being employed in several other centers without difficulty. The methodology for assay of phenobarbital is similarly aimed to be inexpensive and uncomplicated, but especially suited for an anticonvulsant drug laboratory. These improvements in the attractiveness of laboratory technology should lead to increased availability of such services which can be considered as being necessary for safe therapeutics.

With regards to the operation of the laboratory itself, Figure 25 presents the procedure assembled during the development of the Clinical Neuropharmacology Laboratory of

the Montreal Neurological Institute. Assignment of reference numbers to samples received (with requisitions) and careful keeping of permanent records proved to be important aspects of the everyday functioning of such a laboratory. The aim of this laboratory is to benefit the patient by allowing more rational adjustment of therapy, particularly when his clinical status suggests that this may be required. One is now able to detect from controlled patients those who are either overmedicated or else are slowly and occultly building up the drug content towards a toxic level, and to identify the relatively uncontrolled patients for whom an increased dosage rate would not be advisable (high drug levels accompanying the continued occurrence of seizures). Additionally, better patient motivation to take the medications as prescribed frequently results. As mentioned previously, the assay of drug levels in plasma is the first step in this program, to be followed by other investigations whenever the initial findings so dictate.

Finally, a laboratory service relies upon careful clinical judgment for its greatest effectiveness. The physician must always avoid missing the significance of the forest while observing all the trees—laboratory data are often of utmost importance, and yet it is not these but the <u>patient</u> who should be treated. The final decision must be a clinical one. Numerous types of "normal" laboratory values and ranges are not remembered accurately by many in the medical

and para-medical professions, so the laboratory must help: a complete laboratory report certainly includes a thorough interpretation and opinion. But proper interpretation of raw results requires that certain relevant clinical information on each referred patient reach the laboratory, as demanding as this may seem. Only by a better organized interaction between clinic and laboratory can efficient use be made of these services at reasonable costs.

Anticonvulsant Drug Laboratory
General Operational Scheme

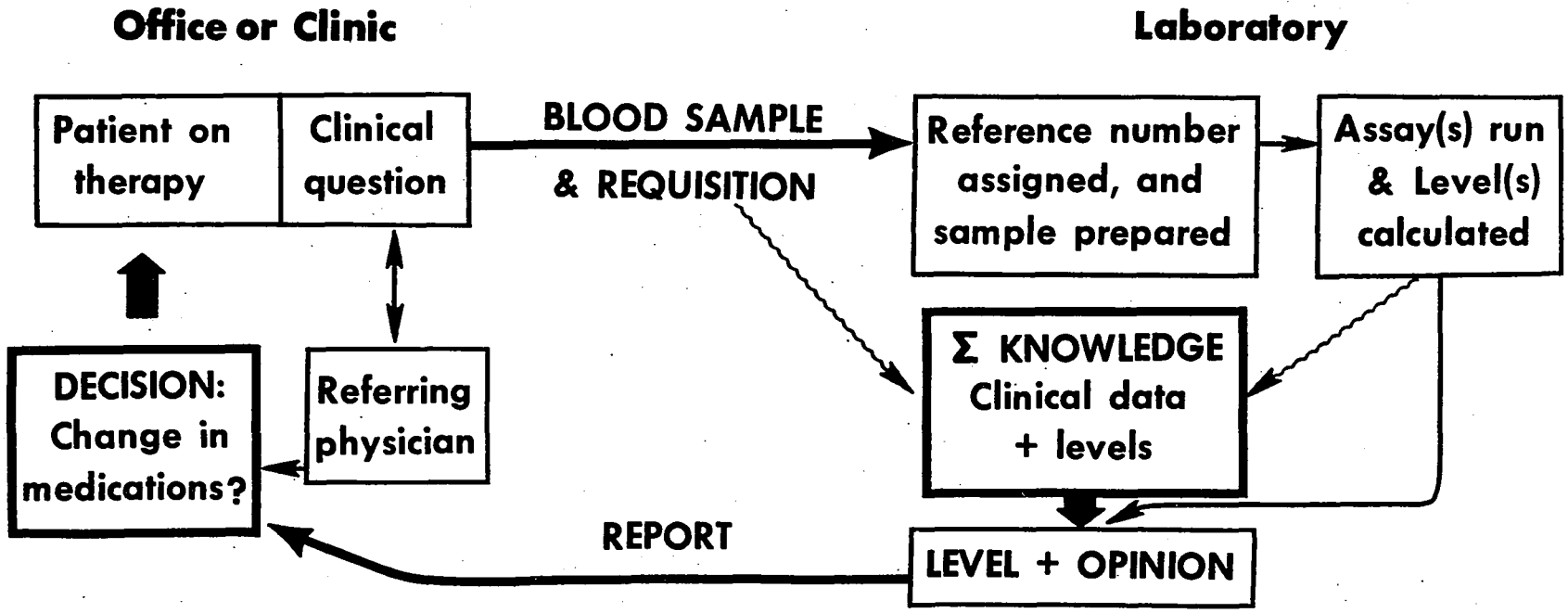

FIGURE 24

Anticonvulsant Drug Laboratory
Laboratory Procedures

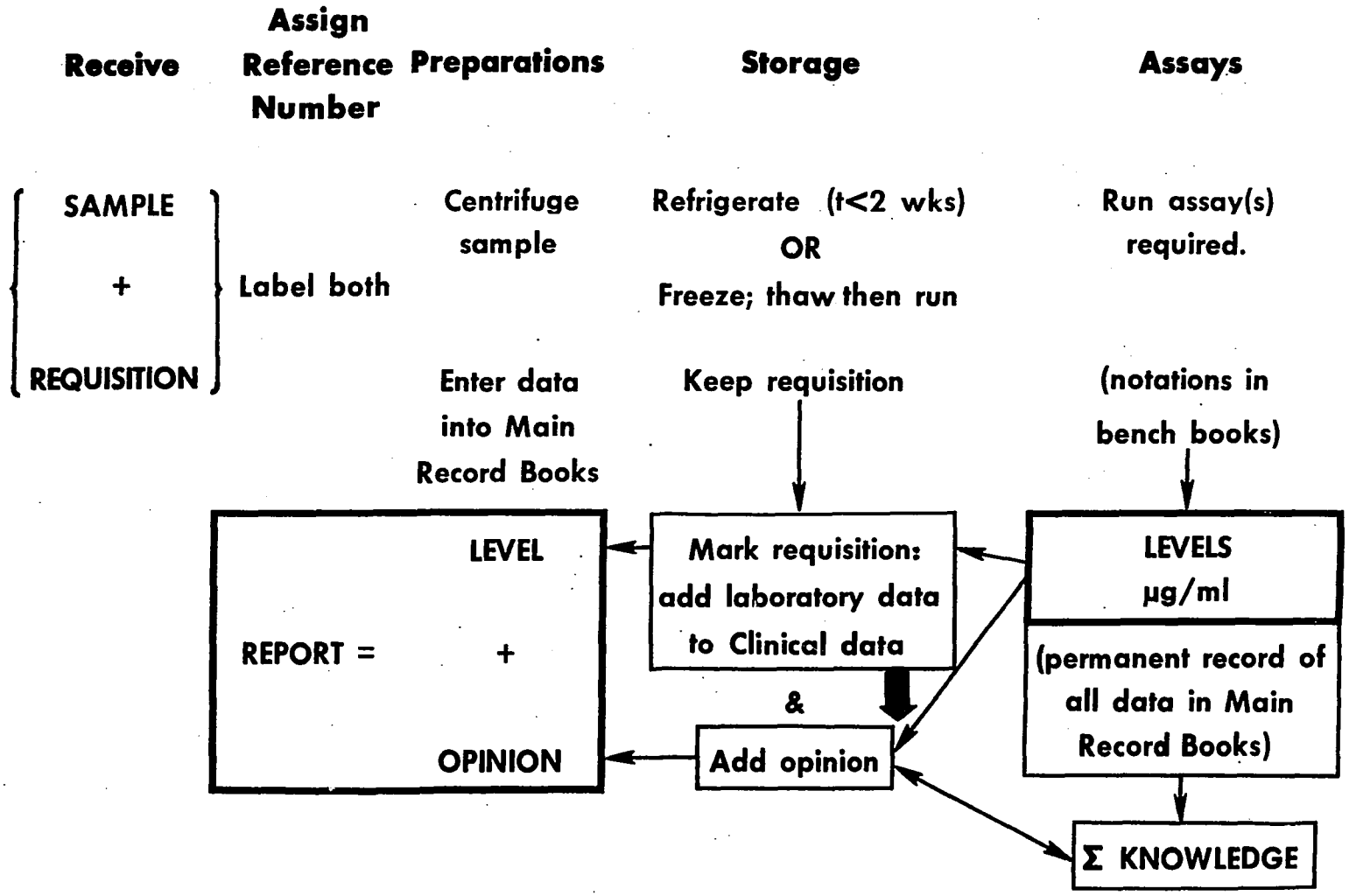

FIGURE 25

VI

ABSTRACT:

SUMMARY AND RESUME

SUMMARY

The present work investigated diphenylhydantoin (DPH) and phenobarbital, pharmaceutical agents widely used in management of "the epilepsies". Both clinical and laboratory research were done.

1) The need for studying the pharmacology of these drugs was outlined.
2) The literature concerning assay methods and drug concentrations attained clinically was reviewed.
3) Detailed descriptions were given for several practical, inexpensive laboratory techniques and apparatus all developed in this work.
4) For 360 patients, 553 determinations of DPH levels were related to specific clinical aspects.
5) DPH concentrations in whole blood, plasma, serum, and erythrocytes were compared.
6) Various clinical and laboratory data on twenty-nine institutionalized patients taking DPH in only a single dose daily were compared with those obtained when the (traditional) divided-dose administration schedule was being followed.
7) The protein binding of DPH in human plasma was studied.
8) All results were discussed relative to previous literature, to models proposed concerning seizures and pharmacodynamics, and, generally, to delivery of health care.

RESUME

Le present travail étudie la diphenylhydantoine (DPH) et phenobarbitale, ingrédients pharmaceutiques grandement en usage dans le traitement de l'épilepsie. Des recherches furent effectuées tant en clinique qu'en laboratoire.

1) Le besoin d'étudier la pharmacologie de ces deux drogues y fut souligné.
2) La documentation portant sur des methodes d'analyse et de concentration des drogues obtenues en clinique fut revisée.
3) La description détaillée de plusieurs techniques et appareillages de laboratoire pratiques et peu dispendieux y est fournie. Le tout a été developpé dans ce travail.
4) La concentration de DPH fut reliée à des aspects cliniques spécifiques (553 déterminations sur 360 patients).
5) Les concentrations de DPH furent comparées dans tout le sang, le plasma, le sérum, et l'érythrocytes.
6) Les différentes données sur vingt-neuf patients internés prenant du DPH à raison d'une seule fois par jour furent comparées aux résultats des dosages de DPH administrés à la manière traditionnelle, soit trois ou quatre fois par jour.
7) Les liens de protéines du DPH dans le plasma humain furent étudiés.
8) Tous les résultats furent discutés en relation avec la documentation antérieure, et aux models proposés sur la crise épileptique et la pharmacodynamique, et en général aux soins médicaux.

(Traduit par M. Jean-Marc Pellerin.)

VII

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*The abbreviations employed here for titles of periodicals are those of "Index Medicus" (U.S. Department of Health, Education and Welfare), with the following journals listed as per "ACCESS" of "Chemical Abstracts" (The Chemical Abstracts Service, American Chemical Society):

Acta Pharmacol. Toxicol.——Acta Pharmacologica et Toxicologica (Kobenhavn).
Can. J. Pharm. Sci.——Canadian Journal of Pharmaceutical Sciences.
Clin. Res.——Clinical Research.
Epilepsia——Epilepsia (Amsterdam).
Neurology——Neurology (Minneapolis).

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