METABOLIC FATE OF DRUGS: BARBITURATES AND CLOSELY RELATED COMPOUNDS[1,2]

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The literature on drug metabolism has been increasing so rapidly that it is clearly impossible in the present article to cover more than a very narrow area of the subject. There are a number of recent comprehensive papers or reviews (1–7). In order to avoid undue repetition (which is indeed difficult!) the authors have chosen to focus their attention on the barbiturates and certain closely related compounds, an area which apparently has not been brought up to date since the reviews of Williams & Parke (8) and Mark (9). The moderate number of papers which have appeared since about mid-1963 will be reviewed from two points of view: the specific chemical alterations brought about by metabolic systems, and the influence of pretreatment with the same or another drug on the rates of these enzymatic reactions. Both *in vitro* and *in vivo* studies will be considered. The many physiological factors which have been shown to affect drug metabolism (age, sex, heredity, species and strain, state of health, etc.) will not be discussed. Extensive papers on most of these subjects have recently appeared (10–13).

Since it is so important an aspect of the subject, qualitative and quantitative methodology will be discussed in relation to some of the recent improvements in techniques for the detection and estimation of barbiturates and their metabolites in biological material.

Methodology

The detection and estimation of barbiturates and closely related drugs and their metabolites continue to receive much attention, clearly because no satisfactory comprehensive systematic procedures have yet been developed. Paper, thin-layer, and now gas chromatography have become increasingly popular. The older (classical) procedures involving recovery by solvent extraction, purification by systematic multiple extractions, or countercurrent distribution (CCD), followed by identification and quantitation by ultraviolet spectrophotometry, have never been developed to their fullest potential. It would seem that coordination of the "classical" procedures with one or another of the chromatographic techniques will be necessary if a satisfactory methodology is to be worked out. Most recent authors have focused

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their efforts on a few drugs and one technique (usually chromatography). A few authors have recently applied CCD and chromatography together in a sophisticated manner to the detailed study of the metabolic fate of one or another of the barbiturates.

There have apparently been no critical reviews of this subject since the excellent one by Curry (14). An extensive bibliography has been published (15). The present senior author has discussed the general problem with special reference to barbiturates, thiobarbiturates, and metabolites (16, 17).

A number of recent papers describe the application of specialized chromatographic techniques to the detection and identification of barbiturates and some of their known metabolites in extracts of urine, blood, and tissues. Perhaps the most extensive and potentially useful study is that of Cochin & Daly (18) who successfully used thin-layer silica gel in combination with three solvent systems and two spray reagents (mercurous chloride and potassium permanganate) to separate and identify 16 common barbiturates and some of their known metabolites, and four other hypnotics of varied structure. Methylene chloride was used as the extracting solvent. It was possible to differentiate most of the barbiturates and a number of their known metabolites in various specimens of human urine. As it stands, the procedure is not suitable for quantitative investigations or the isolation and identification of unknown metabolites, but if suitably combined with systematic extraction, ultraviolet spectrophotometry, etc., it could be very helpful, as the authors suggest.

Two new studies of paper chromatographic systems have recently been published (19, 20). Both of these seem to be best suited for quick detection of barbiturate-like drugs rather than precise identification or quantitation. Ahmed et al. (21) have described several new paper and thin-layer chromatographic systems, which permitted identification of many of the common barbiturates.

The application of gas chromatography to the determination of barbiturates and related compounds has recently been reviewed briefly by Martin & Driscoll (22) who present an interesting and potentially valuable new way of handling these relatively nonvolatile drugs. The extracted crude barbiturate was treated with dimethyl sulfate to produce the relatively stable and more volatile N,N′-dimethyl derivative. Such products from several common barbiturates could then be chromatographed without the complication of decomposition.

New and superior methods of quantitation of diphenylhydantoin and primidone have been described. These drugs are commonly used together and with phenobarbital, and studies of their interaction have been hampered by lack of a method for determining them (and their metabolites) in mixtures. Wallace, Biggs & Dahl (23) reviewed the problem of analysis of phenobarbital and diphenylhydantoin in mixtures, then described a greatly improved method of determining the latter in the presence of the former (but not vice versa!). The drugs were extracted from blood with chloroform, trans-

ferred to aqueous alkali, treated with bromine and subjected to steam distillation. The distillate contained benzophenone in approximately 80 per cent yield from the diphenylhydantoin, and was measured by ultraviolet spectrophotometry. Bush & Helman (24) have described a new procedure for determining both phenobarbital and primidone in mixtures extracted from blood or urine. The two compounds were quantitatively separated by systematic fractional extraction; the primidone was then quantitatively oxidized to phenobarbital with chromic acid; both fractions of phenobarbital were then determined by an improved ultraviolet spectrophotometric procedure. Data are being accumulated to include diphenylhydantoin and the metabolites in this scheme.

Paper chromatographic separation of diphenylhydantoin and phenobarbital has been described by Cucinell et al. (25). An improved procedure for determining thiobarbiturates in biological material has been described by Bush (16).

In Vitro Metabolism

N-demethylation.—The *in vitro* demethylation of several N-methyl derivatives of barbituric acid, hydantoin, and 2,4-oxazolidinedione has recently been studied (26–29). The enzyme system(s) which catalyzes these demethylation reactions is localized in the microsomal fraction of the liver and requires NADPH and oxygen. As in other N-demethylations, the products are the corresponding demethylated analogues and formaldehyde.

Smith, Waddell & Butler (28) studied metharbital, mephobarbital, N,N′-dimethylbarbital, 5-ethyl-3-methyl-5-phenylhydantoin (mephenytoin), 5-ethyl-1-methyl-5-phenylhydantoin (N-methylnirvanol), 1,3-dimethyl-5-ethyl-5-phenylhydantoin (N,N′-dimethylnirvanol), and trimethadione as substrates for rat liver demethylases. Dimethylnirvanol was the most active substrate studied. The demethylation of metharbital, mephobarbital, and trimethadione was essentially zero, in fact, the rate of demethylation of all the substrates was much less than that commonly found for basic drugs. For example, meperidine was demethylated about ten times more rapidly than mephenytoin. The monomethyl derivative of barbital was demethylated more slowly than dimethylbarbital. A similar differential rate of removal of the methyl groups is known to occur *in vivo* (30). Pretreatment with phenobarbital and nikethamide, but not with 3-methylcholanthrene, meprobamate, or diphenylhydantoin, caused marked, but varied increases in the microsomal demethylation of all the substrates studied. Such physical properties as lipid solubility or degree of ionization were not correlated with the amount of demethylation at three hours. Data were not given to show whether or not any such correlations exist for shorter time periods. Recently, several investigators (31, 32) demonstrated that the rate of many enzymatic reactions in rat liver microsomes was constant only during the first 30 minutes or so. The time at which deviation from linearity occurred was different for each substrate studied.

McMahon (29) also studied the microsomal N-demethylation of metharbital, dimethylbarbital, and 5-ethyl-3-methyl-5-phenylhydantoin as well as hexobarbital and methohexital. Microsomes isolated from rabbit liver were more active than those isolated from rat liver, except when the substrate was 5-ethyl-3-methyl-5-phenylhydantoin. In this case, the preparations from the two species were equally active. Methohexital was demethylated by microsomal preparations of rabbit liver but not by those of rat liver. Such compounds as copper sulfate, mercuric acetate, and 2,4-dichloro-6-phenylphenoxyethylamine, which were known to inhibit microsomal oxidations, were found to be effective inhibitors of dimethylbarbital demethylation.

S-demethylation.—A new type of enzymatic metabolism, i.e., S-demethylation, has recently been demonstrated by Mazel, Henderson & Axelrod (33). Among a variety of chemical structures studied, a thiobarbiturate, 5-(S-methylthioethyl)-5-(1′-methylbutyl)-2-thiobarbituric acid (methitural), was found to be a substrate for an enzyme system of rat liver, which converted the S-methyl group to formaldehyde. This system was localized in the microsomal fraction of the liver and required NADPH and oxygen. In another study, Mazel & Henderson (34) reported that the S-demethylating system was not inhibited by SKF-525-A (β-diethylaminoethyl diphenylpropylacetate) nor was it stimulated by pretreatment with phenobarbital or mephenytoin.

Miscellaneous.—In a recent report, Toki & Tsukamoto (35) described the purification and properties of 3-hydroxy-hexobarbital dehydrogenase from a crude soluble fraction of rabbit liver. This enzyme catalyzed the conversion of 3-hydroxy-hexobarbital to the corresponding keto derivative (36). By ammonium sulfate fractionation, calcium phosphate gel treatment, and DEAE-cellulose column chromatography, a 55-fold purification of the enzyme was accomplished. The purified enzyme showed specificity for the cyclohexen-3-ol structure; 3-hydroxy-hexobarbital and 3-hydroxy-cyclobarbital were oxidized, but aliphatic alcohols, glucose, various hydroxylated steroids, and hydroxy-secobarbital were not substrates for the enzyme. The purified enzyme required NAD^+ or $NADP^+$ as cofactor, with a slight preference for NAD^+. The pH optimum was 9.5 with NAD^+ as a cofactor. Horse serum alcohol dehydrogenase failed to oxidize the cyclohexenyl derivatives.

Recent reports by Leadbeater & Davies (31) and Gram & Fouts (32) have presented some interesting and pertinent data concerning the stability of liver microsomal enzyme preparations. Gram & Fouts reported that rat liver microsomal enzymes which catalyze a variety of reactions, including the oxidation of hexobarbital, were more labile than were rabbit or mouse liver preparations. Hexobarbital metabolism by rabbit and mouse liver microsomes was linear with time up to 60 minutes, whereas deviation from linearity was observed as early as 15 minutes when rat liver microsomes were studied. No hexobarbital metabolism occurred beyond 60 minutes of incuba-

tion with rat liver microsomes. These observations emphasize the importance of incubation time when interspecies comparisons of drug metabolism are made. Also, the metabolic activities for different substrates reached a plateau at different times. Such a possibility should be considered in structure-activity studies. In apparent disagreement with Gram & Fouts, McMahon (29) found that the rate of demethylation of N,N′-dimethylbarbital by rat liver microsomes remained constant for 60 minutes.

In Vivo Metabolism

Pentobarbital.—Quantitative studies of the metabolic fate of this well-known drug in man have apparently been lacking all these years. Maynert (37) has recently presented the results of careful and detailed experiments in human subjects, of the kind he reported in dogs many years ago (38). Some further studies were done in dogs in order to get new data needed to guide the human experiments. The drug labeled with ^{15}N (about 15 per cent excess) was administered orally in single doses, 100 to 500 mg in man and 50 mg/kg or 2 mg/kg in the dog. The measurement of ^{15}N in urine extracts and in the urine could then be made with sufficient accuracy to account for about 80 per cent of the dose. In the two dogs receiving the higher dose, this amount was excreted in 24 hours, after which the isotope was too dilute to measure directly. The two men receiving the 500 mg dose excreted the isotope somewhat more slowly than the dogs, some 40 to 50 per cent in 48 hours. One of these subjects was shown to continue this excretion at a decreasing rate for another two days, for a total of 80 per cent. The determination of ether-extractable ^{15}N was much more sensitive, but the amount was less than that in the urine by 30 to 45 per cent. The rate of excretion of the small doses of pentobarbital could be followed only by this method. If we use 0.6 as a reasonable average of the ratio ether-extractable ^{15}N/total ^{15}N (obtained from the data with large doses in two dogs and one man), then the small doses were excreted somewhat more slowly and in smaller yield than the large doses, both in the dog and the human being. The unlabeled diastereoisomeric pentobarbital alcohols (38) were used in isotope dilution experiments with aliquots of the urine from patients receiving the high doses of the parent drug. In one subject, the dextrorotatory alcohol accounted for 3 per cent and the levorotatory alcohol for approximately 17 per cent of the dose of ^{15}N excreted in 48 hours (the duration of the experiment). From the other subject, the recoveries were 6.6 and 33 per cent, respectively, in 120 hours. This type of experiment could not be done with the lower doses of pentobarbital.

Thus, we now know that man (five subjects were used, four male and one female) excretes something like 40 to 50 per cent of a dose of pentobarbital as the mixture of diastereoisomeric alcohols during about four to five days. The remainder of the isotope in the urine (about 35 per cent of that administered) was not identified. Urinary urea and ammonia contained only very small amounts. The author offers no speculation, but it would seem likely

that glucuronide derivatives of the alcohols or metabolite(s) containing a carboxyl group (39) could account for much of the remainder. Products of this type would not have been extracted very well from the urine under the conditions which were used (ether, pH 6.5).

Amobarbital.—Quantitative data on the metabolic fate of this familiar drug in man have also been lacking, and Maynert (37) has reported studies with ^{15}N-labeled material. The experiments were the same in all essential respects as those with pentobarbital. The results showed that the single metabolite, amobarbital alcohol [5-ethyl-5-(3′-hydroxy-3′methylbutyl)barbituric acid], accounted for all the ether-extractable ^{15}N in one subject and for 70 per cent in the other (both received a single 500 mg dose of the labeled amobarbital). In both experiments, the 120 hour urine contained 51 per cent of the dose in the form of the metabolite. The data show that ^{15}N was excreted in appreciable though lesser amounts during the last 24 hour period. The total isotope in the urine was 100 per cent of the dose in one case, 70 per cent in the other. This left 49 and 27 per cent of the urinary isotope unidentified. Of the larger amount, almost half was ether extractable. The conditions of the ether extraction were the same as in the experiments with pentobarbital, and it is quite possible that carboxylic acid(s) or glucuronide(s) were present which were not completely extractable by ether at pH 6.5. A final accounting will doubtless require ^{14}C labeling.

Secobarbital.—A recent study by Waddell (41) is concerned with the metabolites produced by man, dog, and rat. From the urine of a young man who had taken 2 g of (commercial) secobarbital, three metabolites were isolated in pure crystalline form. These were identified as 5-(2′,3′-dihydroxypropyl)-5-(1′-methylbutyl)barbituric acid (secodiol) and two stereoisomeric forms of 5-allyl-5-(3′-hydroxy-1′-methylbutyl)barbituric acid, one of which was presumably the same as the "hydroxysecobarbital" which Tsukamoto, Yoshimura & Ide (40) isolated from rabbit urine. The carboxylic acid derivative, which the latter workers found, was not specifically searched for by Waddell. The three metabolites isolated were calculated to account for about 50 per cent of the ingested 2 g of secobarbital. About 5 per cent of the unchanged drug was in the urine of the first 48 hours. Analyses of the urines from three other patients and from dogs showed similar patterns. The rat produced only the hydroxysecobarbitals in major amounts, both *in vivo* and *in vitro*. Several other metabolites were indicated to be present in minor amounts, sporadically, in various human and dog urines. One of these was found on paper chromatograms of urine extracts from two patients. The R_F value and the ultraviolet absorption of the eluted material indicated that the substance was 5-(1′-methylbutyl)barbituric acid. This substance was presumed to be a metabolite, formed by removal of the allyl group from the secobarbital. Cochin & Daly (18) reported this as a metabolite in human urine after ingestion of secobarbital; they showed their substance to be identical (by thin-layer chromatography) with a specimen of Waddell's synthetic material. Neither Waddell nor Cochin & Daly mention the possibility that

this "metabolite" is present as an impurity in commercial USP secobarbital, which we understand is synthesized by reaction of allyl bromide with 5-(1'-methylbutyl)barbituric acid. To answer this question, a suitable analysis of a specimen of the USP drug along with a study of the metabolic fate of this impurity (if it is present) will be necessary.

The analytical methodology which Waddell worked out for this problem is noteworthy. After isolating small amounts of the metabolites from urine extracts, he measured their partition coefficients between aqueous buffer (pH 6) and six organic solvents, R_F values in three paper chromatographic systems, ionization exponents, and ultraviolet spectra. He then devised suitable countercurrent distribution systems for the separations. Final identifications and quantitations were convincingly made by suitable combinations of R_F measurements, ultraviolet and infrared spectra, melting points, and mixed melting points with synthetic material.

It may be of interest to note that while Waddell's paper was in press, Zins (42) submitted a study of diallylmelamine, in which he found both deallylation and di-hydroxylation of an allyl group to occur in rats. Niyogi (43) has recently identified secodiol as a metabolite of secobarbital in the rat.

Hexethal.—In an unusually well designed and thorough studyof side-chain oxidation *in vivo*, Maynert (44) has used 5-ethyl-5-hexylbarbituric acid (hexethal) in dogs. The ether extracts of five day collections of urines were chromatographed on columns of charcoal-Celite; the metabolites were eluted with ether and the concentrated material put through 700-transfer countercurrent distributions in a 200-tube machine. Hydrophilic fractions were combined and refractionated. In this way ten different metabolites were well separated from each other. No unchanged hexethal was found. The purity of the metabolites was demonstrated in two, three, or four paper chromatographic systems, or by further countercurrent distributions. They were all characterized by elementary analysis, ultraviolet and infrared spectra, derivatization, and suitable chemical tests. Four of the compounds were obtained in sufficient quantities to permit positive identification, two were tentatively identified, three others were shown to be alcoholic derivatives, and the remaining one was undoubtedly a carboxylic ester. In Table I we have summarized these results of Maynert's in a form which is more readily accessible than in his text. About 50 to 55 per cent of the dose of hexethal was accounted for in the form of these ten metabolites. Compounds A, B, C, and D were available in sufficient quantities to permit testing their recoveries through the initial extractions and column chromatography. They were all recovered quantitatively. A test with β-glucuronidase showed that only minute quantities of alcohols were conjugated with glucuronic acid. Ester glucuronides were not tested for. The minor metabolites were not available in sufficient quantities to test their recoveries through this method. It is quite possible that some of them were lost, in part, on the charcoal-Celite column. They should not have been lost in the ether extractions, because one can calculate that even the most polar of them (compound D, partition co-

TABLE I

STRUCTURES AND YIELDS OF THE URINARY METABOLITES OF HEXETHAL

Metabolite[a]	Fate of hexyl side-chain[b]	Yield[c]
A	CH_3–CO–CH_2–CH_2–CH_2–CH_2	9
B	CH_3–CHOH–CH_2–CH_2–CH_2–CH_2	15
C	CO_2H–CH_2–CH_2–CH_2–CH_2–CH_2	4
D	CO_2H–CH_2–CH_2–CH_2	16
E	(CO_2H)(CHOH)(C_4H_8)	2
F	CH_3–CH_2–CO–CH_2–CH_2–CH_2 or CH_3–CH_2–CH_2–CO–CH_2–CH_2	2
G	an alcohol different from B, H, and J	2
H	an alcohol different from B, G, and J	1
I	a carboxylic ester	1
J	an alcohol different from B, G, and H	1
	Total	53

[a] As designated in Maynert's text. All are barbiturates.

[b] Oxidation of the ethyl group is possible but unlikely in those metabolites not positively identified.

[c] Approximate percentage of dose; four different experiments with various dosage regimes gave essentially the same values.

efficient 0.54 between ether and pH 2 buffer) should have been extracted in 90 per cent yield or better. Possibly, there are unknown metabolites, even more polar than this one.

Maynert tested the possibility of stimulation of the metabolizing enzymes by collecting the urine from one dog from the seventh through the eleventh days while the animal was receiving 302 mg of hexethal orally every day. The pattern of metabolites and the yields of each were essentially the same as from another dog which received a single intravenous dose of the drug. It seems unlikely, to say the least, that anyone will undertake the further elucidation of the fate of hexethal unless ^{14}C-labeled material is prepared.

Thiamylal.—The metabolic fate of this 2-thio analogue of secobarbital has been investigated by Tsukamoto et al. (45) who isolated and characterized six compounds from rabbit urine after a total dosage of 30 g of the drug. Extraction was carried out with ethyl acetate, which should have avoided *in vitro* desulfuration (46). The extracted material was separated into bicarbonate soluble (5 g) and bicarbonate insoluble (6 g) fractions. These were separately chromatographed on silica gel columns. Gradient elution yielded eight fractions, two of which failed to crystallize and were not identified. Approximately 40 per cent of the weight of material put into the silica gel columns was accounted for by the sum of the weights given for the eluted fractions after crystallization. Thus, the six pure products account for about

13 per cent of the dose of thiamylal. The crystallized substances were convincingly characterized as follows (the percentage of dose follows each): unchanged thiamylal (4 per cent), 5-allyl-5-(1′-methyl-3′-hydroxybutyl)-2-thiobarbituric acid (0.2 per cent), 5-allyl-5-(1′-methyl-3′-carboxypropyl)-2-thiobarbituric acid (8 per cent); the corresponding desulfurated products: secobarbital (1 per cent), hydroxysecobarbital (0.01 per cent), and carboxysecobarbital (0.1 per cent). The principal metabolite, carboxythiamylal, was administered to a rabbit and reisolated from the urine in 64 per cent yield, unchanged, along with only a trace of the desulfurated analogue. The authors do not give any other data from which the total amount of the various products actually present in the urine might be calculated. The 64 per cent recovery of the carboxy derivative can be taken to indicate that roughly 13 per cent of the dose of thiamylal was actually excreted as this derivative. Whether or not the proportion of hydroxythiamylal excreted is actually as surprisingly low as the results indicate cannot be decided until this product is put through the isolation procedure or measured by a more accurate method.

Thiopental.—The question of whether or not this well-known thiobarbiturate is desulfurated in man was tested by Furano & Greene (47). By a gas chromatographic method which was shown to differentiate the thio and oxy analogues, no pentobarbital could be detected in a blood sample taken 15 minutes after administration of the thiopental. This is not surprising in view of the fact that this drug is known to be only slowly metabolized in man (48) as well as the likelihood that desulfuration is a very minor part of that metabolism (46).

Hexobarbital.—Toki, Toki & Tsukamoto (36) found that hydroxyhexobarbital is converted to ketohexobarbital, and vice versa, by an enzyme localized in the soluble fraction of rabbit liver. Okui & Kuroiwa (49) demonstrated that both hydroxy- and ketohexobarbital are demethylated more rapidly than hexobarbital. These papers were discussed by Williams & Parke (8) and are mentioned here in order to point out a correlation between these new findings and the results of Bush, Butler & Dickison (50). These authors concluded that in the dog hexobarbital is demethylated directly only in very small amounts, and that the relatively large amounts of ketonorhexobarbital which were isolated must have been produced by demethylation of previously oxidized hexobarbital. Bush & Parrish (51) presented results to show that norhexobarbital was not converted in detectable amounts to hexobarbital in their mice, a finding which was in disagreement with the earlier report of Deininger (52). These authors (53) have found by quantitative analyses of whole mice, that demethylation of hexobarbital is a negligible reaction in this species.

Toki & Takenouchi (54) have isolated and characterized a new metabolite of hexobarbital from rabbit urine, the glucuronide of α-3-hydroxyhexobarbital. The crude product amounted to about 14 per cent of the dose. The glucuronide was finally obtained reasonably pure, judging by the elementary analyses, as an amorphous white powder, in a yield of about 5 per cent of the

administered dose. It was convincingly characterized. The great polarity of the substance apparently has precluded the development of a method for quantitating it in the urine.

Yoshimura & Tsukamoto (55) have extended their studies of drugs containing the cyclohexenyl ring to include two hydantoins. They describe the synthesis and characterization of 5-methyl- and 5-ethyl-5-cyclohexenylhydantoin. By chromic anhydride oxidation they prepared the corresponding 3-ketocyclohexenyl derivatives, analogous to 3-ketohexobarbital. From the urine of rabbits given the parent hydantoin derivative, the corresponding ketone was isolated by extraction with ether, chromatography on alumina, and finally crystallization from ethanol. The yields from both drugs were 14 per cent. No unchanged drugs and no other metabolites could be found. It was also shown that no further excretion of the metabolite occurred after the first 24 hours.

Pharmacological activity of metabolites.—The principal metabolite of amobarbital has been reported to have hypnotic activity in mice. Irrgang (56) compared hydroxyamobarbital with amobarbital by determining their median hypnotic doses. Under his conditions the metabolite was about half as potent as the parent drug. This finding is surprising in view of the many instances in which similar metabolites have been found to be inactive. Waddell (41) showed that hydroxysecobarbital produced no hypnotic action in a mouse at a dose of 180 mg/kg, intravenously.

Chamberlin, Waddell & Butler (57) studied trimethadione and its major metabolite, 5,5-dimethyl-2,4-oxazolidinedione (DMO), in the treatment of petit mal in children. Carefully controlled experiments showed that DMO was undoubtedly responsible to a large extent for the therapeutic effectiveness of trimethadione. If this is found to be true for large numbers of patients (five were used in these studies), there might be an advantage in using DMO. Dose-effect relationships could be better quantitated, since DMO can readily be determined in blood while trimethadione cannot.

Phenobarbital and diphenylhydantoin in man.—These two drugs are so commonly used together for the treatment of epilepsy that studies of their metabolic interaction are important. Kutt et al. (58, 59) have measured blood levels of these two drugs and urinary output of the two principal metabolites, *p*-hydroxyphenobarbital and 5-phenyl-5-*p*-hydroxyphenylhydantoin. In patients under chronic treatment with daily doses of 3 to 5 mg/kg of diphenylhydantoin and 1 to 2 mg/kg of phenobarbital, there was found to be a definite correlation between impaired or abnormally low ability to hydroxylate these drugs and toxic manifestations caused by accumulation of high blood levels of the unchanged compounds. In all patients the low dosages were metabolized adequately and no signs of toxicity appeared. In several patients as doses were raised, the amounts of excreted hydroxylated derivatives rose to a plateau, at which time the blood levels of the unchanged drugs began to rise unduly and toxic signs soon appeared. In other patients there was no such plateau or ceiling, and excessive increases in blood levels

did not occur. The chemical determination of an individual's ability to metabolize these drugs and of his ability to adapt to increasing demands on the metabolizing enzymes were thus of considerable help in interpreting signs and symptoms and in regulating dosage.

Cucinell et al. have published two recent papers concerning the stimulatory effect of phenobarbital on the metabolism of diphenylhydantoin in dogs (60) and man (25). These publications will not be discussed here since they have been reviewed in an extensive paper by Burns et al. (2).

Drug Interaction

Numerous compounds with widely differing chemical structure have been shown to potentiate or inhibit the hypnotic action of various barbiturates. In this review, such effects will be considered only if they have been shown to be related to an alteration in the metabolism of the barbiturate in question, i.e., we will not consider those reports which use only altered sleeping time as a criterion of "metabolism." The majority of reports in this area are concerned with changes in the metabolism of hexobarbital. It is indeed unfortunate that in most of these studies the metabolism is determined by measuring only the disappearance of the parent compound. There are at least six known metabolites of hexobarbital. By following disappearance of the substrate, interesting possibilities such as selective stimulation or inhibition of one or more of the pathways or even appearance of a new pathway have been ignored. For the measurement of the activity of a particular enzyme system responsible for the formation of a particular metabolite, it seems clear that quantitative determination of that metabolite (as well as elucidation of its metabolic fate) is of fundamental importance. In the case of hexobarbital, for example, the activity of the N-demethylating enzyme in normal rat liver has been shown to be quite low (29). Under similar conditions the principal metabolic reaction in this species is side-chain oxidation (61) to the alcohol or ketone, or both. Smith, Waddell & Butler (28) have shown that pretreatment with phenobarbital or nikethamide enormously increases the N-demethylating activity of rat liver for several N-methyl barbituric acids. It would seem certain, therefore, that such pretreatment would greatly alter the relationship between hexobarbital demethylation and hexobarbital side-chain oxidation. Studies in which mere disappearance of hexobarbital (and probably most other drugs!) is measured therefore cannot give unequivocal information about the increased or decreased activity of a particular enzyme system after pretreatment. A tentative investigation of this nature has been recently described and will be discussed next.

Effect of barbiturates and related compounds on barbiturate metabolism.— Toki, Yamasaki & Wakiya (62) studied the effect of various phenobarbital derivatives on hexobarbital sleeping time and metabolism. Phenobarbital derivatives in which the *m*- or *p*-position of the benzene ring was substituted by a nitro, amino, dimethylamino, diethylamino, hydroxy, methoxy, chloro, or fluoro group were used. Male rats were treated with 50 mg/kg once daily

for two days with the barbiturate; the duration of hexobarbital hypnosis, its metabolism, or both were determined 24 hours after the last dose. In general, *m*-derivatives were more effective than the *p*-analogues in shortening hexobarbital sleeping time. The compounds in which a chlorine or fluorine atom was substituted in either the *m*- or *p*-position were as potent as phenobarbital. Parahydroxyphenobarbital and α-3-hydroxyhexobarbital were less effective than the parent compounds. Pretreatment with either phenobarbital or *m*-chlorophenobarbital caused about a 450 per cent increase in the *in vitro* rate of metabolism of hexobarbital. Only sleeping times were measured with the other drugs. By analogy, the authors concluded that the observed effect was caused by a stimulation of hexobarbital metabolism. In the metabolism experiments using the 9000× gravity supernatant of rat liver, it was found that the main metabolite was 3-hydroxyhexobarbital, and 3-ketohexobarbital was a very minor metabolite. The authors presented no data on the point but stated that "the production of 3-ketohexobarbital was not influenced so much by phenobarbital pretreatment." A more detailed study of this apparent selective stimulation of the hydroxylating enzyme would certainly be interesting. The authors did not consider the possibility that the N-demethylation of hexobarbital might be altered by the pretreatment conditions. A study of this relationship is certainly desirable.

Ariyoshi (63) studied the effect of barbital, phenobarbital, allobarbital, norhexobarbital, and cyclobarbital and their N-monomethyl and dimethyl derivatives on the *in vitro* metabolism of cyclobarbital. One day prior to measurement of *in vitro* metabolism, female rats were pretreated with a single dose of 100–200 mg/kg of the barbiturate derivative. The cyclobarbital-metabolizing activity of the liver was determined by measuring disappearance of the parent compound as well as appearance of 3-hydroxy- and 3-ketocyclobarbital. All the parent barbiturates stimulated the *in vitro* metabolism of cyclobarbital, that is, there was a decrease in the amount of unchanged cyclobarbital. The N-methyl barbiturates had less stimulatory activity than did the parent compounds. Pretreatment with the N,N'-dimethyl derivatives had no effect. The results of the determination of the two metabolites were so frequently out of line with the disappearance data that satisfactory correlations with specific metabolite production can not be inferred. This was undoubtedly due to the considerable difficulty of making sufficiently accurate determinations of such metabolites. The analytical determination of the remaining cyclobarbital was undoubtedly more accurate and the disappearance data can thus be accepted as more reliable. Some 30 per cent of the drug which disappeared was not accounted for by the metabolites measured.

Ariyoshi measured the duration of hypnosis produced by an arbitrary dose of 100 mg/kg of the several barbiturates and N-methylated derivatives used for pretreatment. There was no apparent correlation between these results and the effects on cyclobarbital metabolism. A possible explanation of this will be suggested in relation to the following work of Remmer.

Remmer (64) showed that the stimulating action of hexobarbital on its own metabolism was related to the frequency of the administered dose. A pretreatment regime of two doses of hexobarbital per day for several days was required to stimulate its own *in vitro* metabolism by rat and rabbit liver microsomes. These species are known to metabolize hexobarbital very rapidly. However, one dose of 30 mg/kg given to the dog, which metabolizes this barbiturate much less rapidly, was sufficient to stimulate the metabolism of subsequent doses of the drug.

Remmer's results with hexobarbital, together with his earlier demonstration that phenobarbital is a potent stimulator (65), imply that a sufficient concentration of the stimulating drug must be available to the liver *in vivo* for a sufficient length of time if significant increase in activity is to be produced. To test the question of whether or not a particular drug is inherently capable of bringing about this effect it would seem necessary to demonstrate that it does in fact remain available to the liver, viz., to determine its plasma concentrations. Ariyoshi's comparison of the duration of the hypnosis produced by an arbitrary dose of various barbiturates and their N-methylated derivatives does not shed any light on the relative plasma levels of these compounds or their metabolites. By analogy with what we know about thiopental (66), the levels of these highly lipid-soluble N-methylated derivatives (67) are undoubtedly much lower at all times than those of the parent drugs. In the absence of quantitative data on their plasma levels and of information about their metabolism in the rat, it seems unlikely that we can reconcile the results of Ariyoshi with the expectation that these compounds should have inherent stimulatory capabilities. Another aspect of the problem, the demethylation of N-methylbarbital, N-methylphenobarbital, etc., can be presumed to be of minor importance over a short period of time since this is a slow process in the normal rat. Other routes of metabolism, such as hydroxylation, are undoubtedly more important than demethylation in this species. Hydroxylation produces inactive polar derivatives rather than the active parent compounds.

Ariyoshi & Takabatake (68) have reported similar studies on the effect of various malonic acid derivatives on the *in vitro* metabolism of cyclobarbital.

Ebert, Yim & Miya (69) demonstrated that tolerance to barbital, as evidenced by a decrease in sleeping time after chronic administration of the barbiturate to rats, was not mediated by an accelerated metabolism of the drug. Barbital-^{14}C was administered to tolerant and control rats. The amount of unchanged drug and metabolite excreted in urine was determined by paper chromatography and subsequent scanning for radioactivity. During 24 hours after the administration of barbital-^{14}C, 6 per cent of the dose was metabolized in tolerant rats and 3 per cent in the controls. The only metabolite identified was 5-ethylbarbituric acid; at least two other metabolites were present in the urine of both tolerant and control rats.

Two reports have appeared in the recent literature concerning *in vitro*

competitive inhibition of reactions catalyzed by rat liver microsomal oxidative enzyme systems. Rubin, Tephly & Mannering (70) showed that a variety of compounds with widely differing chemical structures (hexobarbital, chlorpromazine, zoxazolamine, phenylbutazone, and acetanilide) competitively inhibited microsomal N-demethylation of ethylmorphine. All the compounds found to be active as inhibitors were themselves substrates for the enzyme system. Compounds such as barbital and acetazolamide, which were not oxidized by these microsomal preparations, had no effect on the metabolism of ethylmorphine. Hexobarbital and ethylmorphine were mutually inhibitory. Three possible explanations were offered for these results: all the drugs reacted with a single enzyme, two enzymes were involved and the competition was for a common intermediate, or two enzymes were involved but one drug combined with both enzymes and yielded a product with only one. A single common mechanism could not be formulated to explain all the observed inhibitions.

Butler, Waddell & Poole (71) studied the *in vitro* demethylation of ^{14}C-trimethadione and ^{14}C-metharbital by rat liver microsomes. The rate of metabolism was determined by measuring the appearance of the corresponding demethylated analogues. Trimethadione had a much lower affinity for the rat liver microsomal demethylating enzyme system than metharbital. It was demonstrated that each of the compounds had an inhibitory effect on the demethylation of the other. On the basis of these observations, it was proposed that the two substrates were competing for the same active site. However, other possibilities as suggested by Rubin, Tephly & Mannering should not be excluded. Butler's group also found that DMO, the demethylated product of trimethadione, inhibited the demethylation of both metharbital and trimethadione. Since DMO was apparently not a substrate for any microsomal enzyme system, it would be of interest to determine if the observed inhibition were competitive in nature. This is especially important in the light of the earlier results of Rubin, Tephly & Mannering (70).

Effect of analgesics on barbiturate metabolism.—Pretreatment of rats with morphine (20 mg/kg intraperitoneally daily for four days) increased the duration of hexobarbital hypnosis, but had no effect on the hypnotic action of barbital (72). Hexobarbital and morphine metabolism were studied in liver slice preparations from control and morphine pretreated rats. The *in vitro* metabolism of both these drugs was inhibited by chronic administration of the analgesic. The administration of ACTH prior to hexobarbital reduced the sleeping time of morphine pretreated rats to that of control animals. It was suggested that the action of morphine was related to a block of endocrine function. In a similar study, Jóhannesson and co-workers (73) found that chronic pretreatment with codeine inhibited the *in vitro* metabolism of hexobarbital by rat liver microsomes. A variety of other enzymatic reactions were depressed in codeine pretreated rats.

Rubin, Tephly & Mannering (74) demonstrated that ethylmorphine and codeine inhibited the *in vivo* metabolism of hexobarbital in rats. Coadminis-

tration of 52 μmoles/kg of either drug with hexobarbital slowed the rate of disappearance of the barbiturate from the blood of either normal or nephrectomized rats. Norcodeine, morphine, methorphan, meprobamate, and acetanilide had no effect on the blood levels of the barbiturate. Since morphine had no effect on hexobarbital blood levels, it was concluded that the effects observed with ethylmorphine and codeine were not related to the CNS depression produced by these agents. An alternative substrate mechanism as proposed to occur *in vitro* (Rubin, Tephly & Mannering, discussed earlier) could explain these *in vivo* effects of ethylmorphine and codeine.

Effect of chlorinated hydrocarbons on barbiturate metabolism.—The action of γ-chlordane in adult, male squirrel monkeys was studied by Cram, Jauchau & Fouts (75). This animal was used since it is phylogenetically more closely related to man than are the laboratory animals used previously (i.e., mice, rats, and rabbits). Pretreatment of monkeys with chlordane (10 mg/kg daily for seven days) resulted in an increase in the activity of hepatic microsomal enzymes which metabolized hexobarbital, benzpyrene, zoxazolamine, and *p*-nitrobenzoic acid.

Several investigators have recently studied the action of 2,2-(*o*-chlorophenyl,*p*-chlorophenyl)-1,1-dichloroethane(*o,p'*-DDD) on drug metabolism. Straw, Waters & Fregly (76) demonstrated that the administration of this substance to rats resulted in an increase in the *in vitro* metabolism of pentobarbital. The pretreatment schedule was either 100 mg/kg subcutaneously or 300 mg/kg orally, daily for three days. On the fourth day, the animals were sacrificed and the metabolism of pentobarbital in liver slices determined by measuring its disappearance. Ethionine (given in combination with *o,p'*-DDD) blocked the effect of the hydrocarbon on pentobarbital metabolism. Comparable doses of *o,p'*-DDD had no effect on the metabolism of deoxycorticosterone. In contrast, Kupfer & Peets (77) found that the administration of *o,p'*-DDD to immature and adult rats resulted in a stimulation of the *in vitro* metabolism of hexobarbital and cortisol. Male rats were pretreated with 300 mg/kg of *o,p'*-DDD daily for three days in the case of immature rats (body weight about 60 g) or nine days in case of mature animals (body weight about 200 g). The stimulation of metabolism by *o,p'*-DDD was more pronounced in immature animals than in adults. The results, using the control rat preparations, are interesting in light of the work of Kato et al. (78). Kupfer & Peets reported that preparations of "adult" control rat livers metabolized hexobarbital about three times more rapidly than did preparations of livers from "immature" animals. Unfortunately, the age of the animals was not reported. Kato and associates found that the activity of the enzyme(s) responsible for hexobarbital metabolism reached a maximum in the 30-day-old rat (body weight about 70 g), then began gradually to decrease. The enzyme activity of the 100-day-old rat (body weight about 240 g) was only about 70 per cent of that of the 30-day-old rat. In addition, Kato and co-workers found that the low metabolic activity in the adult livers was not caused by a more rapid loss of enzyme activity *in vitro* since the age

differences were observed in both early and late periods of incubation. The explanation of this discrepancy in the results of the two groups of investigators is not apparent, unless it is due to a sex difference. Kupfer & Peets used male rats; Kato and co-workers, females.

Waters, Straw & Fregly (79) recently examined the effect of *o,p'*-DDD pretreatment on the action of pentobarbital and hexobarbital in the dog. Such pretreatment (200 mg/kg daily for two days) resulted in an increase in the sleeping time due to pentobarbital. A similar pretreatment schedule had no effect on the duration of hexobarbital hypnosis. In addition, the serum half-life of the former barbiturate was increased by the pretreatment. When glucocorticoids were administered with *o,p'*-DDD, both the sleeping time and half-life of pentobarbital returned to control values. These results suggested that glucocorticoids have some regulatory action in the metabolism of pentobarbital, but not hexobarbital.

Effect of miscellaneous drugs on barbiturate metabolism.—The administration of a single, large dose of norepinephrine to rats (1 mg/kg intraperitoneally) had little effect on the hepatic microsomal metabolism of hexobarbital, aminopyrine, and aniline (80). Chronic pretreatment with the catecholamine (2, 4, or 6 doses of 1 mg/kg each 12 hours) decreased the metabolism of these substrates. In both the acute and chronic experiments, the hepatic glycogen levels were markedly depressed. The mechanism of the inhibitory effect of norepinephrine was not clear, but was possibly related to a sustained depression of glycogen levels. Mullen & Fouts (81) found that pretreatment of mice with a number of adrenergic blocking agents prolonged hexobarbital sleeping time. The effect of hydralazine, phentolamine, phenoxybenzamine, and azapetine was related to inhibition of the metabolism of the barbiturate (demonstrated *in vitro*). However, the action of yohimbine and tolazolamine was correlated with a depression of body temperature.

Kato, Vassanelli & Chiesara (82) described the properties of a potent inhibitor of microsomal drug metabolism, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA, Lilly 32391), a compound previously studied by McMahon & Mills (83). The agent was an effective inhibitor both *in vivo* and *in vitro,* and appeared to have no other pharmacological activity. At a concentration of about $1\times10^{-5}M$, DPEA effectively inhibited the *in vitro* metabolism of hexobarbital, pentobarbital, meprobamate, and carisoprodol. The enzyme responsible for the metabolism of pentobarbital was more sensitive than the hexobarbital-metabolizing enzyme; the concentrations causing 50 per cent inhibition were 6×10^{-6} M and 1×10^{-5} M, respectively. DPEA was about as potent an inhibitor as SKF-525-A (β-diethylaminoethyl diphenylpropylacetate). These same authors (84) also studied the effect of imipramine on drug metabolism in female rats. An intraperitoneal injection of 25 mg/kg of this amine 20 minutes prior to the administration of pentobarbital or meprobamate resulted in an inhibition of the *in vivo* metabolism of both of these agents. At this dose level, imipramine potentiated the pharmacological effects of pentobarbital, hexobarbital, meprobamate, and carisoprodol. Studies

with rat liver slices and microsomal preparations revealed an *in vitro* inhibitory effect on the metabolism of all four drugs. As with DPEA, the pentobarbital-metabolizing enzyme was most sensitive to imipramine. This agent was only about one fifth as potent as SKF-525-A. The *in vivo* potentiating effect of imipramine could not be explained solely on the basis of an inhibition of drug metabolism.

Recent studies have demonstrated that disulfiram (85), triparanol, and benzmalecene (86) added *in vitro* inhibited the metabolism of pentobarbital. Tolbutamide and carbutamide have been shown to increase microsomal oxidation of hexobarbital (87). Sulfanilamide and sulfaethidole were ineffective.

Novick, Stohler & Swagzdis (88) have recently demonstrated that pretreatment of male mice with 40 mg/kg of testosterone daily for four days decreased the *in vitro* metabolism of hexobarbital. The same pretreatment dosage of several 19-nortestosterone derivatives resulted in an increase in the metabolism of the barbiturate. The steroids found to be effective stimulators were 19-nortestosterone, norethandrolone, 4-chloro-19-nortestosterone, and 4-chloro-17-α-methyl-19-nortestosterone. Because of the small number of compounds tested, no strict structure-activity relationship could be formulated. Pretreatment of female mice in similar experiments produced decreases in sleeping time comparable to those in the males.

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