Metabolic Studies with Deuterated Phenobarbital[1]

JAMES M. PEREL, PETER G. DAYTON, CAROL L. TAURIELLO, LEONARD BRAND, AND LESTER C. MARK

New York University Research Service, Goldwater Memorial Hospital, Welfare Island, New York, New York, and Department of Anesthesiology, College of Physicians and Surgeons, Columbia University, New York, New York

Received November 17, 1966

Isotope effects in the metabolism of phenobarbital with dogs and rats were investigated using phenobarbital-*p-d* (II) and phenobarbital-*p-d*-ethyl-d_5 (III). The deuterated compounds were synthesized *via* benzyl-*p-d* cyanide prepared from toluene-*p-d*. This intermediate was utilized in the subsequent steps of the synthesis except that for III, ethyl-d_5 iodide was incorporated into the ethylcyanophenyl acetate precursor. Purity and extent of deuterium labeling was established by several analytical criteria. It was found that the apparent rate of plasma drug level decay for all three compounds was the same, which suggests that the rupture of the *para* C–H bond is not rate limiting in the metabolism of phenobarbital. On the other hand, a 13–26% exchange of the *p*-deuterium substituent was observed *in vivo*, which on the basis of a postulated two-step mechanism leads to a product isotope effect of 4–8. The sedative effective of the three compounds was found to be similar.

The main route of metabolism of phenobarbital (5-ethyl-5-phenylbarbituric acid) in man, dog,[2] and rat,[3] involves the hydroxylation of the *para* position of the phenyl substituent followed by appreciable formation of the phenolic β-glucuronide.

If the mechanism of action of phenobarbital (I-H) involves an equilibrium process (*e.g.*, drug–receptor interaction) the presence or absence of an isotope effect on its pharmacological activity would be evidence for or against the participation of the isotopic position. If on the other hand a rate process (*e.g.*, production of a metabolite) is involved in the mechanism, the isotope effect generally will give information regarding only the rate-determining step.[4a]

It is reasonable to expect that if scission of the aromatic *para* C–H bond is involved in the rate-controlling step of metabolism, replacement of this hydrogen by deuterium should cause a substantial decrease in the rate of metabolism. In fact, the difference in reactivity could be six- to eightfold for a primary isotope effect, whereas a secondary effect would result in a smaller decrease (1.3–1.6).[4,5]

The latter possibility may also be explored by investigating the physiological disposition of phenobarbital fully deuterated in the ethyl side chain in addition to the *para* position of its aromatic ring. Since metabolism of this drug does not directly involve the side chain, other effects which may be mainly dependent on factors such as pK_a,[4b] lipid solubility, and plasma protein binding could be observed as "secondary" isotope effects. A positive finding in this case may be construed as evidence for the involvement of this structural group in the action of this drug.

Chemistry.—The method of synthesis of phenobarbital-*p-d* (II) was essentially a modification of an earlier procedure,[6] using the precursor ethyl cyanophenyl-4-*d*-acetate prepared according to Nelson and Cretcher's[7] method. The over-all unequivocal synthesis involved the preparation of toluene-4-*d*, followed by the conversion of its cyanide *via* benzyl-4-*d* chloride.

Phenobarbital-*p-d*-ethyl-d_5 (III) was likewise synthesized in the same manner by treating the precursor ethyl cyanophenyl-4-*d*-acetate with fully deuterated ethyl iodide.

The purity of II and III was established by mixture melting point with I-H, thin layer and gas chromatography, elemental analysis, and superimposable ultraviolet spectra. The homogeneity of the deuterium labeling was established by nmr spectroscopy.

In addition, III was shown to have 82 mole % D excess in the ethyl side chain and 90 mole % D excess in the 4 position of the phenyl ring by nmr and mass spectrometric analysis.

Pharmacology.—To determine a possible isotope effect, comparisons of the rate of drug plasma level decay in dogs receiving I-H and II intravenously were made. In order to exclude the possibility of induction of drug-metabolizing enzyme systems, alternate drug administrations were kept 2–4 weeks apart. Similar studies were done in dogs using I-H and III in order to compare rates of drug plasma level decay. Concentrations of I-H, II, and III in plasma and urine were measured by a double-extraction spectrophotometric method,[8] the specificity of which was established by quantitative thin layer chromatographic techniques.

The possibility of a product isotope effect[9] in the "unchanged" II and III was examined after isolation from dog and rat urine by extraction techniques followed by purification with preparative thin layer chromatography. Suitable control studies were conducted to eliminate the possibility of artifactual exchange of deuterium in the urine.

The sedative effects of II and III were compared with that of I-H in mice, while the L-ascorbic acid excretion stimulatory effect in rats was compared for compounds I-H and II.

(1) Presented in preliminary form at the April 1966 meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J. Supported by U. S. Public Health Service Grants No. AM 4724 and GM 09069.

(2) (a) E. J. Algieri and A. J. McBay, *Science*, **123**, 183 (1956); (b) A. S. Curry, *J. Pharm. Pharmacol.*, **7**, 604 (1955); (c) T. C. Butler, *J. Pharmacol. Exptl. Therap.*, **116**, 326 (1956).

(3) B. Glasson and A. Benakis, *Helv. Physiol. Pharmacol. Acta*, **19**, 323 (1961).

(4) (a) M. J. Stern and M. Wolfsberg, *J. Pharm. Sci.*, **54**, 849 (1965); (b) K. B. Wiberg, *Chem. Rev.*, **55**, 713 (1955).

(5) H. Zollinger, *Advan. Phys. Org. Chem.*, **2**, 163 (1964).

(6) J. S. Chamberlain, J. J. Chap, J. E., Doyle, and L. B. Spaulding, *J. Am. Chem. Soc.*, **57**, 352 (1935).

(7) W. L. Nelson and L. H. Cretcher, *ibid.*, **50**, 2758 (1928).

(8) T. C. Butler, C. Mahaffee, and J. W. Waddell, *J. Pharmacol. Exptl. Therap.*, **111**, 425 (1954).

(9) The operational definition of "product isotope effect" denotes the deduction of a kinetic isotope effect by the observation of relative isotopic abundances in the product or in the chemically unchanged starting material: V. Gold and M. A. Kessick, *J. Chem. Soc.*, 6718 (1965).

Experimental Section[10]

Benzyl-4-*d* Cyanide.—Benzyl-4-*d* chloride was obtained by treating 48 g (0.5 mole) of toluene-4-*d* (prepared by an adaptation of the method of Choppin and Smith[11]) with 66 g (0.5 mole) of sulfuryl chloride.[12] The solution was thoroughly flushed with dry nitrogen for about 5 min, whereupon 0.60 g of benzoyl peroxide was rapidly added and purging with nitrogen was resumed for an additional 10-min period. The reaction was completed after refluxing for 2–3 hr. Distillation *in vacuo* gave a yield of 41 g of the chloride, bp 67–68.5° (10 mm). The total yield of benzyl-4-*d* chloride after a second pass with recovered toluene-4-*d* and fresh SO_2Cl_2 was 53 g (80%). The corresponding nitrile,[13] bp 104–105° (8 mm), was prepared by treating 30 g (0.24 mole) of benzyl-4-*d* chloride with 15 g (0.31 mole) of NaCN in aqueous ethanol solution. The yield was 25 g (95%).

Ethyl Cyanoethyl phenyl-4-*d*-acetate.—The above nitrile was treated with diethyl carbonate in the presence of sodamide, yielding colorless ethyl cyanophenyl-4-*d*-acetate,[7] bp 142–148° (8 mm), in 70% yield. Ethylation[6] of 19 g (0.1 mole) of this intermediate was effected by treating 20 g (0.13 mole) of freshly distilled ethyl iodide in 50 ml of anhydrous ether and 2.3 g (0.1 g-atom) of sodium wire. Fractional distillation *in vacuo* gave 14.5 g (66%) of a colorless oil, bp 136–140° (8 mm).

Anal. Calcd for $C_{13}H_{14}DNO_2$: C, 71.53; H, 7.39; N, 6.89. Found: C, 71.32; H, 6.99; N, 6.52.

5-Ethyl-5-phenyl-4-*d*-barbituric Acid (II).—Urea (1.7 g, 0.028 mole) was added to a stirred solution of sodium (1.11 g, 0.048 g-atom) in 22 ml of anhydrous ethanol and mixed to solution at room temperature. Then ethyl cyanoethylphenyl-4-*d*-acetate (5.5 g, 0.025 mole) was added with stirring over a 30-min period. The reaction mixture became thick and a white suspension was produced which was stirred and refluxed for 66 hr. The solvent was removed at 30 mm with heating. The residue was dissolved in 75 ml of water at room temperature and extracted with three 100-ml portions of ether. The dissolved ether in the aqueous solution was removed under a stream of nitrogen. The aqueous solution was neutralized to pH 5 with concentrated HCl and after cooling for several hours at 0–5° a white precipitate of 5-ethyl-5-phenyl-4-*d*-4-iminobarbituric acid was collected (3.4 g, 60%), mp 255–258°. The imino compound was hydrolyzed quantitatively by refluxing with 150 ml of 3.3 *N* HCl for 30 min. Upon cooling overnight at 0–5°, II separated in clusters of white crystals (3.2 g, 91%), mp 172–174°. The crude product was recrystallized from ethanol–water to give 2.2 g, mp 174–175°.

Anal. Calcd for $C_{12}H_{11}DN_2O_3$: C, 61.79; H, 5.62; N, 12.01. Found: C, 61.84; H, 5.38; N, 12.09.

Chromatography on silica gel G (Merck) using benzene–glacial acetic acid (9:1) and chloroform–acetone (9:1),[14] with visualization with $HgSO_4$ and diphenylcarbazone reagent[15] sprays, gave single spots with R_f values of 0.25 and 0.32, respectively, which were the same as those obtained with I-H standard. Gas–liquid partition chromatography (glpc) of II on a 4-ft column having 20% QF-1 (Dow Fluorosilicone FS 1265) on Chromosorb P[16] 80–100 mesh, showed the single component to be at least 99% pure. The retention times of I-H and II were the same. Mass spectrometric determination for the deuterium content in II showed the phenyl-4-*d* position to be enriched by 90 mole % D. The same enrichment was obtained by analyzing the toluene-4-*d* used as the starting compound for the synthesis. The nmr of I-H[17] showed aromatic protons at 7.37 (singlet), CH_2 hydrogens at 2.37 (center of quartet), and CH_3 hydrogens at 0.97 (center of triplet). Integration of these areas gave a relative ratio of 5:2:3, respectively. The nmr spectrum of II showed the same peaks as I-H except that integration gave a relative ratio of 4:2:3, indicating an enrichment of deuterium in the aromatic nucleus at least 90%.

Ethyl-d_5 Cyanoethylphenyl-4-*d*-acetate.—The precursors of this material were prepared from the same toluene-4-*d* batch used for the monodeuteriophenobarbital (II) synthesis. Freshly distilled ethyl cyanophenyl-4-*d*-acetate (17.5 g, 0.09 mole) was slowly added with stirring to 2.1 g (0.09 g-atom) of sodium wire in 48 ml of anhydrous ether. Ethyl-d_5 iodide, 18.5 g (0.12 mole), was added slowly and the mixture was refluxed and stirred for 11 days. The product distilled under reduced pressure had the same physical properties as the unlabeled material. The yield was 12.4 g (62%).

Anal. Calcd for $C_{15}H_{15}D_5NO_2$: C, 69.92; H, 9.48; N, 6.27. Found: C, 70.35; N, 6.19.

Ethyl-d_5-5-phenyl-4-*d*-barbituric acid (III) was prepared in the same manner as II except that the reaction mixture was stirred and refluxed for 100 hr in order to overcome isotope effects. The yield was 3.0 g (50%), mp 176–178.5°.

Anal. Calcd for $C_{12}H_6D_6N_2O_3$: C, 60.46; H, 7.56; N, 11.76. Found: C, 60.77; N, 11.54.

The purity of this compound was established in the same manner as for II. Mass spectrometric determination for the deuterium content in III indicated that the over-all average enrichment of deuterium is 41.67%. Since the *para* position of the phenyl ring has the same extent of label as its precursor toluene-4-*d* which is 90 mole % excess, the ethyl side chain is calculated to have 82 mole % excess deuterium.

The nmr spectrum of III showed only the aromatic proton peak at 7.37 (singlet) and almost a total absence of the multiplets pertaining to CH_2 and CH_3 protons found in I-H, indicating a high degree of deuterium labeling in the ethyl side chain. The pK_a of compounds I-H, II, and III, determined spectrophotometrically, were found to be 7.5 within ±0.1 units.

Animal Experiments.—II was administered intravenously to three adult dogs which ranged in weight from 6.5 to 9.1 kg. Dogs A and B received 40-mg/kg doses while C was given 50 mg/kg. The rate of decay of drug concentrations in plasma was determined by spectrophotometric measurements at 240 and 260 mμ in accordance with a previously published procedure.[5] This procedure was shown to be specific for I-H and II by quantitative thin layer chromatography on silica gel G (see below). The experiments were repeated 1 month later in dogs B and C using a dose of I-H which was the same as that of II given previously. For dog A the procedure was reversed; I-H being administered first, followed by II 2 weeks later. Urines were collected before and during drug administration.

A similar protocol was used in the intravenous administration of III and I-H to dogs E and F. The drugs were given at least 3 weeks apart at doses of 40 mg/kg. The rate of decay of drug concentrations was analogously measured and the urines were collected and pooled during the administration of compound III.

The sedative potencies of II and III were compared with that of I-H by the intraperitoneal administration of 3 mg of each drug to two separate groups of twelve mice each. The average times of onset, peak, and recovery of sedation were noted.

The effect of I-H on the increase of L-ascorbic acid excretion in rats was compared with that of II. Two groups of three male Wistar rats each were maintained on 1:1 mixture of water and evaporated milk (Carnation) for 4 days prior to and during the experiments; this diet is low in L-ascorbic acid.[18] One group of rats received I-H intraperitoneally at 90 mg/kg for the first 2 days and 135 mg/kg for the latter 2 days. The same schedule of administration was followed for the other group of rats using II. Rats were kept in a metabolism cage placed over a 10-in. polyethylene funnel equipped with an iron mesh screen. For L-ascorbic acid measurements, 24-hr urines were collected with 5 ml of 8% oxalic acid, whereas for the isolation of "unchanged" II 1 ml of toluene was added.

Isolation of Compounds II and III from Urine.—Dog urine (770 ml) collected for a 24-hr period after administration of II was initially treated by adding 100 ml of ether in order to dissolve any precipitated compound. The urine was then mixed with 55 ml of 6 *N* HCl and was extracted three times with 500 ml of ether. All extracts were combined and washed by treating

(10) Boiling points are uncorrected. Melting points were determined on a Fisher-Johns apparatus and are corrected. The C, H, and N analyses were performed by Mr. George I. Robertson, Jr., Florham Park, N. J. The mass spectrometric deuterium analysis were done by the Esspee Isotope Co., Union City, N. J., nmr spectra were obtained at a field strength of 60 Mc/sec on samples in $(CD_3)_2CO$ solution on a Varian A-60 instrument using $Si(CH_3)_4$ as internal standard and are reported in parts per million (δ) downfield sweep. Gas chromatography was carried out using an F and M Model 609 system with He carrier, hydrogen flame detection, column temperature of 185°, injection port at 300°, and detector temperature of 250°.

(11) A. R. Choppin and C. R. Smith, *J. Am. Chem. Soc.*, **70**, 577 (1948).

(12) M. S. Kharasch and H. C. Brown, *ibid.*, **61**, 2142 (1939).

(13) R. Adams and A. F. Thal, "Organic Syntheses", Coll. Vol. 1, 2nd ed, John Wily and Sons, Inc., New York, N. Y., 1944, p 107.

(14) J. Cochin and J. W. Daly, *J. Pharmacol. Exptl. Therap.*, **139**, 154 (1963).

(15) J. Kelleher and J. G. Rollason, *Clin. Chim. Acta*, **10**, 82 (1964).

(16) L. Kazyak and E. C. Knoblock, *Anal. Chem.*, **35**, 1448 (1963).

(17) G. Rucker, *Arch. Pharm.*, **299**, 688 (1966).

(18) S. S. Jacket, E. H. Mosbach, J. J. Burns, and C. G. King, *J. Biol. Chem.*, **186**, 589 (1950).

with 100 ml of 1.0 *M* phosphate buffer[8] (pH 8.0). The ether phase was shaken three times with 30-ml aliquots of 0.1 *N* NaOH. The NaOH extracts were pooled, acidified with 1 ml of concentrated HCl, and extracted with three 30-ml aliquots of ether. The ether extracts were pooled and washed with 10 ml of 1.0 *M* phosphate buffer (pH 8.0), dried (Na_2SO_4), and evaporated to dryness with nitrogen.

The residue was dissolved in 2 ml of dry ethanol and applied in a streak on a silica gel G glass plate (approximately 0.5-mm thick). A small amount of reference I-H was also applied 2 cm away from the edge of the plate. A stepwise development method was used consisting of chloroform–acetone (8:1) and then benzene–glacial acetic acid (3:1). The mobility of the isolated product was ascertained by visualization of the reference compounds. Upon drying, the relevant area of silica gel was removed from the plate by scraping and then extracted with 50 ml of dry ether. Evaporation of the ether, followed by charcoal treatment of an ethanolic solution and two recrystallizations from aqueous ethanol yielded white crystals of II, mp 173–175°.

Purity of the isolated compound was established by comparison with I-H in melting point, ultraviolet spectra, and tlc on silica gel using benzene–glacial acetic acid (9:1) and chloroform–acetone (3:1) systems.

The isolation method was also utilized to obtain II from the first 24-hr urine collected after drug administration to dog A, whereas for dog C the first 48-hr urine collection was used. Likewise, the isolation of II from pooled rat urine was accomplished, leading to white crystals melting at 176–176.5°. This material required less purification than II obtained from dog urine.

The isolation of III (pooled urines from dogs E and F) was done under the same experimental conditions as described above, yielding a compound melting at 176.5–178°. In all cases purity of the isolated material was established by the above-mentioned techniques.

Control Studies.—The *in vitro* exchange of deuterium from II in urine was examined. Fifty milligrams of II was added to 200 ml of "blank" urine of dog C and allowed to stand 24 hr at 37°. Likewise, a control experiment was done with pH 8.0 urine ($NaHCO_3$ treated). II was reisolated as described above and purity was established similarly. All of these samples of II were also analyzed for deuterium.

Urine Hydrolysis.—Urine (13.5 ml) from II-treated dog C was acidified with 2.5 ml of 3 *N* HCl and extracted twice with 10 ml of ether portions, which were then dried (Na_2SO_4). After removal of traces of ether, the aqueous phase was adjusted to pH 5.0 with sodium acetate, and the sample was divided into 6-ml aliquots. A mixture of 6.0-ml aliquot and 2.0 ml of β-glucuronidase (Ketodase, Warner-Chilcott, Morris Plains, N. J.) was incubated for 16 hr at 37°. The glucuronidase-treated sample was acidified with 1.0 ml of 3 *N* HCl and extracted with 30 ml of ether which was dried (Na_2SO_4) and evaporated to dryness with nitrogen. The residue was dissolved in a small volume of ethanol and subjected to tlc analysis on silica gel G (~0.17 mm) plates. The developing systems were benzene–glacial acetic acid (3:1) and chloroform–acetone (1:1); visualization was accomplished with the $HgSO_4$–diphenylcarbazone sprays. The R_f values, 0.25 and 0.60, were identical with those obtained with a reference of 5-ethyl-5-(4-hydroxyphenyl)barbituric acid.[19] Under the same conditions the R_f values for II were 0.52 and 0.50, respectively.

Results and Discussion

In Vivo **Rate Studies.**—The apparent rates of disappearance of I-H, II, and III from plasma are comparable (Table I), indicating the lack of a significant isotope effect. The data demonstrate that the scission of the C–H bond in the hydroxylation of phenobarbital is not rate controlling. Therefore, either bond making with oxygen in the *para* position is the slow step, or else the chemical reaction is preceded by at least one slow physical step insensitive to deuterium substitution (*e.g.*, adsorption of an enzyme–substrate complex).[20,21]

(13) Kindly provided by Dr. L. P. McCarthy of the Dow Chemical Co., Midland, Mich.

TABLE I

APPARENT FIRST-ORDER CONSTANTS FOR THE DISAPPEARANCE OF PHENOBARBITAL FROM PLASMA

Dog	Dose, mg/kg	Position of deuterium label	Rate: Half-life, hr	Rate: 10^4k^a min^{-1}	k_H/k_D
A	40	None	35	2.0	0.9
		4-Phenyl	31	2.2	
B	40	4-Phenyl	41	1.7	0.9
		None	45	1.5	
C	50	4-Phenyl	65	1.1	1.0
		None	63	1.1	
E	40	4-Phenyl, C_2D_5	65	1.1	1.1
		None	57	1.2	
F	40	None	62	1.1	1.0
		4-Phenyl, C_2D_5	61	1.1	

[a] Interpolated intercepts ranged from 50 to 69 mg/l.

On the other hand the exchange studies (Table II) indicate a loss of deuterium label ranging from 13 to 26% of the starting compound II. An over-all loss of 2.5% in deuterium label is also observed with III. However, if the exchange were to take place only in the 4-phenyl position, such a decrease becomes 14% which is comparable with the data obtained with II. The

TABLE II

DEUTERIUM ISOTOPE EXCHANGE OF PHENOBARBITAL

Species	Position of deuterium label	D, over-all mole % excess	% decrease of label
Dog A	4-Phenyl	5.50[c]	26
Dog C	4-Phenyl	6.48[a]	13
Compd II, initial	4-Phenyl	7.43[b]	
Dog C, alkaline control	4-Phenyl	7.44[a]	0
Dog C, normal control	4-Phenyl	7.21[a]	3
Rats	4-Phenyl	6.09[c]	18
Dogs E and F	4-Phenyl, C_2D_5	40.65[c]	2.5[c]
Compd III, initial	4-Phenyl, C_2D_5	41.67[b]	

[a] The over-all error in the isolation and deuterium assay is ±0.10 mole % excess. [b] The error in the deuterium assay is ±0.01 mole % excess. [c] See text.

product isotope effect corresponding to the observed exchange ranges from 4 to 8, based upon the assumption that the two-stage mechanism 1 is applicable to the aromatic hydrogen exchange.[22a]

$$\text{phenobarbital-}d + \text{HA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{phenobarbital-}h,d \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{phenobarbital-}h + \text{DA} \quad (1)$$

A = enzyme or base

The partitioning of the phenobarbital-*h*,*d* intermediate to either phenobarbital-*d* or phenobarbital-*h* is obtained from the analysis for the deuterium content of the isolated materials. Thus, the product isotope effect is equal to the ratio of mole per cent deuterium content in the original to the per cent decrease in

(20) V. J. Shiner, H. R. Mahler, R. H. Baker, Jr., and R. R. Hiatt, *Ann. N. Y. Acad. Sci.*, **84**, 583 (1960).

(21) C. Elison, H. W. Elliott, M. Look, and H. Rappoport, *J. Med. Chem.*, **6**, 237 (1963).

(22) (a) L. Melander, *Arkiv Kemi*, **2**, 211 (1950); (b) S. Olsson, *ibid.*, **14**, 85 (1959).

deuterium content of the isolated product, *e.g.*, for dog A the ratio is 4[7.43/(7.43 − 5.50)].

There seems to be no significant alteration in the metabolic pathway of deuterium-labeled phenobarbital because the analyses of urinary excretory products in dogs show that the *p*-hydroxy metabolite is present both in the free and conjugated forms in equivalent amounts, compared to I-H.

A parallelism may be observed between the data from the present study and the lack of kinetic isotope effects obtained by Melander[22a] for electrophilic aromatic substitution. Furthermore, in electrophilic hydrogen-exchange studies with toluene-4-*d* Olsson[22b] observed isotope effects ranging from 6 to 8.

While any attempt to attach mechanistic meaning to the present study would necessarily be highly speculative, the *in vivo* exchange of deuterium in phenobarbital, whether mediated by an enzyme or not, seems to involve an intermediate similar in nature to that demonstrated in the above-quoted studies.

There remains to be explored the additional possibility that the formation of *p*-hydroxyphenobarbital occurs with an intramolecular migration of the deuterium from the *para* to the *meta* position. Such a mechanism is borne out from the experiments of Guroff, *et al.*,[23] regarding the hydroxylation of *p*-deuterio- and *p*-tritiophenylalanine to tyrosine by *Pseudomonas* phenylalanine hydroxylase. Under these conditions considerable amounts of *m*-deuterio- and *m*-tritiotyrosine are produced.

It appears that biological aromatic hydroxylation is characterized by a lack of deuterium isotope effects as well as the utilization of molecular oxygen.[24] Thus, in addition to the present study, no kinetic isotope effects were observed by Soboren, *et al.*,[25] in the *in vitro* hydroxylation of 4-^{3}H-acetaniline, while studies by Corey, *et al.*,[26] also showed negative results. Moreover, Fujita, *et al.*,[27] found no isotope effect in the highly stereospecific hydroxylation of proline, and Gold and Crigler[28] reached the same conclusions while investigating the *in vivo* metabolism of cortisol-1,2-^{3}H.

On the other hand, the biological oxidations of aliphatic groups may give rise to kinetic isotope effects depending on the substrate. Thus, whereas Lemieux, *et al.*,[29] found that the rate of the *in vivo* oxidation of the methyl substituent of tolbutamide (*N*-*p*-toluenesulfonyl-*N*′-*n*-butylurea) in man was unaffected by complete deuteration, the work of Soboren and associates[25] indicate a moderate isotope effect (k_H/k_D = 2) for the *in vitro* oxidative demethylation of *o*-nitroanisole with deuterium-labeled methyl group. Furthermore, the chemical mechanism for the oxidation of α-deuterated tyrosine, phenylalanine, and tyramine[30,31] by monoamine oxidase give isotope effects ranging from 1.4 to 2.3, indicating that the removal of an α-hydrogen is rate determining.

Pharmacological Studies.—Sleep time studies with mice (Table III) did not show any significant differences in the sedative potencies among the various compounds. The urinary excretion of L-ascorbic acid in rats given II was increased to the same extent as with I-H. At the end of 4 days of drug administration the values ranged from 4.8 to 8.2 mg/24 hr. Thus, there appears to be no significant difference in the degree of stimulation of drug metabolizing enzymes. Elison, *et al.*,[2] have reported that deuteration of the *N*-methyl group of morphine weakens the analgesic and toxic effects of this drug although its duration of action appears to be unaffected. On the other hand, by using butethal (5-butyl-5-ethylbarbituric acid) with deuterium substituted in the 3′ position of the butyl side chain, Soboren, *et al.*,[25] have prolonged the sleep time of mice by a factor of 2. The same compound having the 4′ position labeled with deuterium did not show any pharmacological isotope effect. Furthermore, the implication that the 3′ position is mainly involved in the action of the drug was confirmed by the isolation of 5-ethyl-5-(3-hydroxybutyl)barbituric acid as the primary metabolite produced by the liver microsomes of mice.

TABLE III

SEDATIVE POTENCY IN MICE[a]

Drug[b]	Test group[c]	Onset of sedation	Peak[d] of action	Recovery period
Phenobarbital	A	20–38	45	151–170
Phenobarbital-*p*-*d*	B	33–65	65	136–151
Phenobarbital	C	23–38	121	211–413
Phenobarbital-*p*-*d*-ethyl-d_5	D	18–47	88	125–417

[a] Times are given in minutes. [b] The dose was 3 mg/kg ip for each animal. [c] Each test group consisted of twelve mice. Groups A and C served as controls for the simultaneous comparison with groups B and D, respectively. [d] As measured by loss of righting reflex.

The present data as well as the above-cited observations indicate that the 5-ethyl substituent participates to a minor degree in the sedative action of oxybarbiturates. Its contribution is probably due to the increase in lipid solubility that it confers to the pyrimidine nucleus of the molecule. Moreover, it would appear that alterations in the pharmacological activity of barbiturates are dependent on structural modifications involving longer or more branched 5-alkyl substituents, particularly as in the case of butyl groups. Further kinetic deuterium isotope studies would prove valuable in determining the precise pharmacological function of these molecular features.

Acknowledgment.—The authors are indebted to Dr. Samuel Blackman of The Wellcome Research Laboratories for his interpretation of the proton magnetic resonance spectra. We also thank Mr. Frank Litterio for the infrared spectral data, and Mr. Miguel A. Landrau for his able technical assistance.

(23) G. Guroff, C. A. Reifsnyder, and J. Daly, *Biochem. Biophys. Res. Commun.*, **24**, 720 (1966).

(24) H. Posner, C. Miboma, S. Rothberg, and S. Udenfriend, *Arch. Biochem. Biophys.*, **94**, 280 (1961).

(25) J. Soboren, D. M. Yasoda, M. Tanabe, and C. Miboma, *Federation Proc.*, **24**, 427 (1965).

(26) E. J. Corey, G. A. Gregorion, and D. H. Peterson, *J. Am. Chem. Soc.*, **80**, 2338 (1958).

(27) Y. Fujita, A. Gottlieb, B. Peterkofsky, S. Udenfriend, and B. Witkop, *ibid.*, **86**, 4709 (1964).

(28) N. I. Gold and J. F. Crigler, Jr., *J. Clin. Endocrinol. Metab.*, **26**, 133 (1966).

(29) R. U. Lemieux, K. F. Sporek, I. O'Reilly, and E. Nelson, *Biochem. Pharmacol.*, **7**, 31 (1961).

(30) B. Belleau and J. Moran, *Ann. N. Y. Acad. Sci.*, **107**, 822 (1963).

(31) D. J. Triggle and J. F. Moran, *Nature*, **211**, 307 (1966).