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IDENTIFICATION OF 5-*meta*-HYDROXYPHENYL-5-PHENYLHYDANTOIN AS A METABOLITE OF DIPHENYLHYDANTOIN*

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Abstract—5-*meta*-Hydroxyphenyl-5-phenylhydantoin has been identified in conjugated form in the urine of dogs and patients treated with diphenylhydantoin (DPH). This metabolite appears to be the major product of the hydroxylation of DPH in dogs, but it accounts for relatively little of the DPH hydroxylated by patients.

A CORRELATION between blood levels of diphenylhydantoin (DPH) and the therapeutic or toxic response of patients treated with this drug has been demonstrated in a number of studies.[1–3] These studies have emphasized also that the same dose of DPH, on a milligram per kilogram basis, produces quite different blood levels in different patients, and these findings have served to direct attention to individual differences in the metabolism of this drug. Recently, a genetically determined deficiency in the rate of formation of the *para*hydroxylated metabolite of DPH has been shown to make some patients especially prone to DPH toxicity.[4] In 1957, Butler[5] reported that this metabolite, 5-*para*-hydroxyphenyl-5-phenylhydantoin (*p*-HPPH), was the primary product of DPH metabolism in dogs and man, and since then recovery of the glucuronide conjugate of *p*-HPPH from urine has been reported to account for most of the DPH given to rats, dogs and man.[6–8]

Differences in the rate of metabolism of DPH could also result from differences in the capacity of alternate, competing pathways of metabolism of this drug. Kozelka and Hine[9] have presented evidence for the existence of metabolic pathways in which the hydantoin ring is fragmented and Woodbury[7] found that this fragmentation may account for the fate of 5·5 per cent of a dose of DPH given to rats. On the other hand, Maynert[6] gave ^{15}N-labelled DPH to dogs and recovered less than 3 per cent of the given isotope as urinary ammonia, thus indicating that complete hydrolysis of the hydantoin ring does not occur to a great extent in this species.

The existence of hydroxylation pathways, in addition to that described by Butler, has been suggested by Woodbury.[7] He has made a preliminary report that in rats, two hydroxylated DPH metabolites are formed in addition to *p*-HPPH: one with both

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phenyl rings hydroxylated in the *para* position, and the other with the *meta* and *para* positions of one ring hydroxylated to form a catechol. In the present studies 5-*meta*-hydroxyphenyl-5-phenylhydantoin (*m*-HPPH) was identified as a metabolite of DPH in dogs and in humans. This metabolite is similar to *p*-HPPH in that it is excreted in the urine in conjugated form.

METHODS

Reference compounds and sample preparation. Reference compounds were sodium diphenylhydantoin (Dilantin, Parke, Davis & Co., Detroit, Mich.), recrystallized as the free acid from ethanol–water and *m*-HPPH, *p*-HPPH and 5-(4-methyl-phenyl)-phenylhydantoin (MPPH) (kindly supplied by Dr. A. J. Glazko, Parke, Davis & Co.).

Urine samples were obtained from patients who were receiving DPH for control of seizures and from dogs. Dogs 1 to 3 were anesthetized with 30 mg/kg sodium pentobarbital, and urine was collected after the intravenous administration of 100–250 mg DPH. Dog 4 had received DPH 400 mg/day orally for 2 months and urine was collected from a metabolic cage without the dog being anesthetized (specimen kindly provided by Dr. Leon J. Sholiton, University of Cincinnati College of Medicine). The patients had been receiving DPH in a daily oral dose of 50–500 mg for several months. J. G. and E. C. were taking no other medicines. Medicines taken by the other patients were: B. W.—phenobarbital; G. P.—phenobarbital and diazepam; and R. M.—ethosuximide, methyldopa, chlorothiazide, reserpine and hydralazine.

The extraction and alkylation procedures initially used in these studies were essentially the same as those which have been described for the determination of DPH in plasma by gas–liquid chromatography (GLC).[10] One-half-ml specimens of urine were buffered with an equal volume of 0·3 M NaH_2PO_4 before extraction with 5 ml of toluene. After mixing and centrifugation, the toluene layer was removed and 50 μl of a 24 per cent solution of tetramethylammonium hydroxide in methanol (Southwestern Analytical Chemicals, Austin, Tex.) was added to this extract. The extract was then mixed and centrifuged and GLC was performed on 2 μl portions of the tetramethylammonium hydroxide layer.

Acid hydrolysis of additional 0·5-ml specimens of urine was accomplished by adding an equal volume of 12 N HCl and then placing these specimens in a boiling water bath for 1 hr. After this, these samples were neutralized with 6 N NaOH and the extraction and methylation procedure carried out as described above. This procedure was also modified by substituting tetraethylammonium hydroxide and tetrapropylammonium hydroxide (Eastman Organic Chemicals, Rochester, N.Y.) for the tetramethyl reagent.

Enzymatic hydrolysis of 0·5-ml urine specimens was carried out by adding from 250 to 20,000 Fishman units of β-glucuronidase (Ketodase, Warner-Chilcott, Morris Plains, N.J.) and 0·5 ml of 0·3 M NaH_2PO_4 buffer to the urine. Replicate specimens were then incubated for 3 and 18 hr at 37°. After incubation, the hydrolysate was extracted with toluene and the tetramethylammonium salts formed as above in preparation for GLC. Controls included urine incubated with distilled water in place of enzyme, urine hydrolyzed with acid as above and urine that was incubated with enzyme and then hydrolyzed with acid. The relative extent of hydrolysis of replicate urine samples treated in these ways was determined by comparing the GLC peak heights obtained under identical instrumental conditions. It was found that incubation

with 10,000 units of enzyme for 3 hr was sufficient to ensure maximal enzymatic hydrolysis of conjugated *p*-HPPH. However, it was consistently found that strong acid hydrolyzed more conjugated *p*-HPPH and *m*-HPPH than did β-glucuronidase.

After the qualitative studies had been completed, the initial procedures were modified to permit the quantitative measurement of *m*-HPPH and *p*-HPPH in urine samples. Five-tenths-ml urine specimens were hydrolyzed with acid as above, then neutralized with 0·5 ml of 12 N NaOH and buffered with 0·5 ml of 3·0 M NaH_2PO_4. The extraction and alkylation techniques were then carried out as above except that the initial extracting solvent was changed to 1:1 toluene–ethyl acetate. This solvent mixture contained 10 μg/ml MPPH as an internal standard for GLC analysis. After demonstrating that the acid hydrolysis step resulted in no loss of *m*-HPPH or *p*-HPPH added to control urine samples, standard solutions were prepared by adding known amounts of *m*-HPPH and *p*-HPPH to 1:1 control urine, 6·0 N NaCl. One ml of 1:1 3·0 M NaH_2PO_4–6·0 N NaCl was added to 1-ml aliquots of these standard solutions before extraction with 1:1 toluene–ethyl acetate, containing internal standard, and alkylation.

Gas–liquid chromatography (*GLC*). A Varian 1740 series gas chromatograph equipped with a hydrogen-flame ionization detector was used for analytical GLC (Varian Aerograph, Walnut Creek, Calif.). The column was a 5 ft by $\frac{1}{8}$ in. (O.D.) stainless steel coil packed with 3% SE-30 silicone on Varaport No. 30 (100–120 mesh). The flow of helium carrier gas was kept constant at 30 ml/min. The flash heater was maintained at 350 ± 10° and the detector at 275°. Column temperatures were varied from 190 to 220°, depending on the material to be chromatographed, but isothermal conditions were maintained during each run.

When urinary *m*-HPPH and *p*-HPPH concentrations were measured, the column was kept at 210°. The standard solutions were used to construct curves relating the peak height ratio of *m*-HPPH/MPPH and *p*-HPPH/MPPH to the known concentrations of these compounds. The ratios of the metabolite peaks to the MPPH internal standard peak in the urine from dogs and patients were then converted to concentrations of *m*-HPPH and *p*-HPPH using these curves.

Samples were collected for mass spectrometry by means of a Research Specialties, B-600 gas chromatograph equipped with a ^{90}Sr-Argon ionization detector (HCL Scientific, Inc., Rockford, Ill.). The column was a 4 ft by $\frac{1}{4}$ in. (O.D.) stainless steel U-tube packed with 2·5% SE-30 silicone on Chromosorb G, AW-DMCS (80–100 mesh) (Perkin–Elmer Corp., Norwalk, Conn.). The flow of argon carrier gas was maintained at 26 ml/min. Injector and detector temperatures were 350 ± 10°, and the column was kept at 220°. The collected samples were washed from collecting tubes with ethanol and then evaporated to dryness in test tubes before mass spectrometry.

Thin-layer chromatography. Thin-layer chromatography was carried out with chromAR sheet (Mallinckrodt Chemical Works, St. Louis, Mo.) which contains a phosphor. Chromatograms were developed with diethyl ether in a covered chromatography jar. Material applied at the origin was chromatographed for 10 min and then located under ultraviolet light after the chromatogram was dried. In this system *m*-HPPH moved 7·65 cm ($R_f = 0·58$) and *p*-HPPH moved 6·70 cm ($R_f = 0·51$). The area corresponding to an *m*-HPPH guide was cut from the chromatogram of acid hydrolyzed urine from a DPH-treated dog. Material from this area was eluted with 1:1 methanol–methylene chloride and evaporated to dryness before GLC and mass spectrometry.

Mass spectrometry. An Hitachi–Perkin–Elmer RMU-7E double-focusing mass spectrometer (Perkin–Elmer Corp.) was used for structure identification of the fractions collected by GLC and of the eluate from the thin-layer chromatogram. All samples were run under the same instrumental conditions. The ion source was kept at 250°, and the ionizing energy was 70 eV. DPH and the alkylated metabolites of DPH were introduced into the mass spectrometer by means of the liquid-volatile-solid sample port which was heated to 200°. However, direct ion chamber injection was necessary to obtain spectra of unalkylated *m*-HPPH and *p*-HPPH.

RESULTS AND DISCUSSION

Injection of the tetramethylammonium salt of DPH into a gas chromatograph

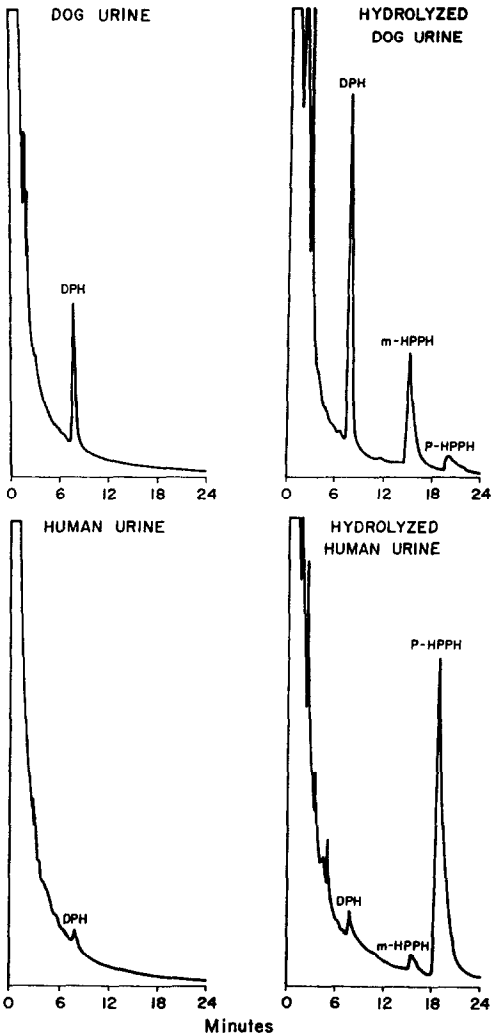

FIG. 1. Gas-chromatographic analysis of dog and human urine showing methylated metabolites of DPH. Since acid hydrolysis releases these compounds from their conjugated excretion products, a parallel analysis of an equal amount of unhydrolyzed urine containing only unconjugated DPH metabolites is shown to provide an estimate of the extent of conjugation.

injection port at high temperature results in the formation of N^1, N^3 dimethyldiphenylhydantoin.[10] This technique of flash methylation was used to identify DPH metabolites in the urine of dogs that had received an intravenous injection of this drug. GLC of this urine, extracted with toluene and methylated in this way, showed a peak corresponding in retention time to that of methylated DPH (Fig. 1). When acid hydrolysis preceded extraction of the urine with toluene, 2 additional GLC peaks were observed. The retention time of the later peak corresponded to that of methylated *p*-HPPH. The retention time of the earlier peak was the same as that of methylated *m*-HPPH. These same two peaks were identified in acid-hydrolyzed urine specimens from patients who were receiving DPH (Fig. 1).

These results suggested that the early peak might represent either *m*-HPPH or 5-*meta*-methoxyphenyl-5-phenylhydantoin and attempts were made to isolate such a metabolite of DPH in unalkylated form. Identifiable peaks were not obtained from GLC of unalkylated *m*-HPPH and *p*-HPPH, but these reference compounds could be separated by thin-layer chromatography [R_x (ratio of R_f values of 2 compounds) = 1·14]. Therefore, acid-hydrolyzed urine from a DPH-treated dog was chromatographed and an eluate was prepared from the area of the thin-layer chromatogram corresponding to the location of reference *m*-HPPH. Methylation and GLC of a portion of this eluate gave the expected peak, corresponding in retention time to methylated *m*-HPPH. The absence of a peak corresponding to methylated *p*-HPPH indicated that the eluate contained no appreciable *p*-HPPH.

The mass spectrum of this eluate, along with reference spectra of DPH, *p*-HPPH and *m*-HPPH, are shown in Fig. 2. Structures of the parent ions of these reference compounds are included in this figure, as well as structures postulated for several of the fragment ions. The mass spectra of these hydantoins are characterized by fragmentation of the hydantoin ring in preference to loss of the phenyl and phenolic substituent groups. The same mass spectral fragmentation pattern has recently been described for DPH by other investigators,[11] and is similar to the pattern of the phenyl-substituted barbiturates in which the barbiturate ring is fragmented before the phenyl substituent is lost.[12]

Although *m*-HPPH and *p*-HPPH have parent ions of identical mass ($m/e = 268$), they can be distinguished by comparing the relative intensity of these parent ions and of the fragments with m/e of 239 and 225. The intensity of these ions relative to the principal fragment ion with m/e of 196 (base peak) is shown in Table 1. Comparison

TABLE 1. RELATIVE INTENSITY* OF PARENT AND HIGH m/e FRAGMENT IONS OF *p*-HPPH, *m*-HPPH AND ELUATE

m/e	*p*-HPPH	*m*-HPPH	Eluate
	(%)	(%)	(%)
196	100	100	100
225	44	67	71
239	89	50	47
268	74	88	84

* Intensities shown are relative to that of the base peak of the *p*-HPPH and *m*-HPPH spectra ($m/e = 196$) which is assigned an intensity of 100 per cent.

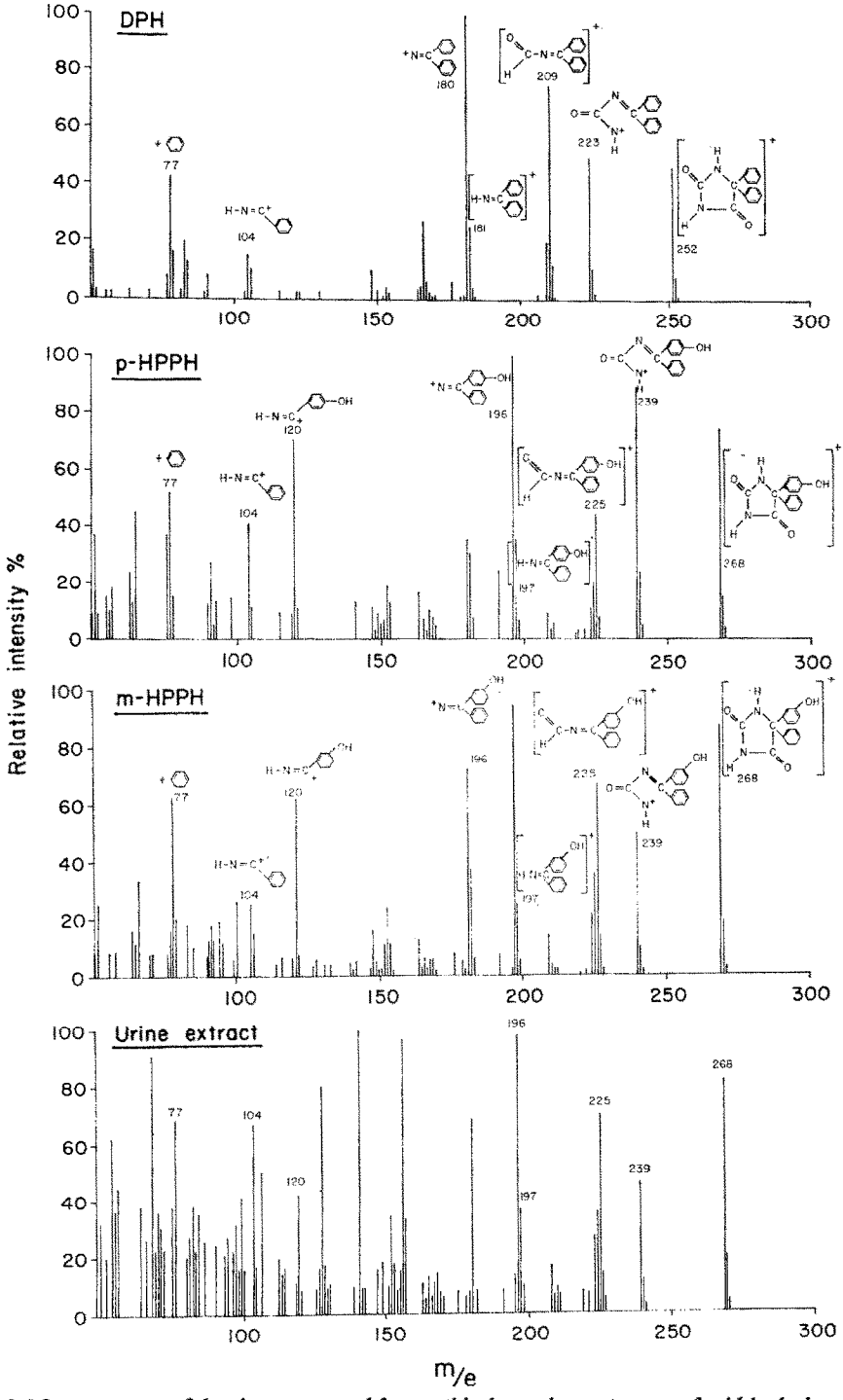

Fig. 2. Mass spectrum of the eluate prepared from a thin-layer chromatogram of acid-hydrolyzed dog urine. Comparison with reference spectra of DPH, *p*-HPPH and *m*-HPPH shows that the metabolite of DPH contained in the eluate is *m*-HPPH.

of the mass spectrum of the eluate with the spectra of these reference compounds shows that the fragmentation pattern of the eluate resembles that of *m*-HPPH and is different from that of *p*-HPPH. At the low-mass end of the spectrum of the eluate, additional ions are encountered which are not present in the mass spectrum of the reference *m*-HPPH. These ions apparently represent contaminants of the *m*-HPPH eluted from the thin-layer chromatogram.

Samples of *p*-HPPH and *m*-HPPH gave only decomposition spectra when they were introduced into the mass spectrometer through the liquid–volatile–solid sample port. However, satisfactory spectra of these compounds and of the eluate were obtained when these samples were introduced directly into the ionizing electron beam of the mass spectrometer through the direct ion-chamber inlet. This method greatly reduces the time during which a sample is exposed to high temperature before being ionized and, therefore, minimizes thermal decomposition of the sample. Presumably, sample decomposition was also the cause of our inability to isolate *p*-HPPH and *m*-HPPH by GLC. These difficulties were not encountered with the alkylated derivatives of these metabolites or with DPH itself.

Methylated, ethylated and propylated derivatives were prepared of hydrolyzed urine samples from dogs and patients treated with DPH. These derivatives produced gas-chromatographic peaks with retention times comparable to those of derivatives prepared from *m*-HPPH (Table 2). Retention times of the comparable *p*-HPPH peaks

TABLE 2. COMPARISON OF GLC RETENTION TIMES OF DERIVATIVES OF HYDROXYLATED METABOLITES OF DPH OBTAINED FROM VARIOUS SOURCES

Source	Retention times of derivatives (min)			
	Methyl	Ethyl		Propyl
		Peak I	Peak II	
m-HPPH				
Authentic compound	4·8	6·3	7·5	11·4
Dog urine	4·9*	6·3*	7·3*	11·3*
Patient urine	4·9	6·3	7·5	11·4
p-HPPH				
Authentic compound	6·0	8·4	10·3	15·4
Dog urine	6·1	8·9	10·5	15·4
Patient urine	6·0	8·3	9·4	15·2

* Structures of these derivatives were confirmed by mass spectrometry.

are also shown in Table 2. The structure of all the *m*-HPPH derivatives prepared from dog urine was confirmed by mass spectrometry. Methylation and propylation resulted primarily in alkylation of the phenol and N^1 and N^3 of the hydantoin ring. However, ethylation produced 2 gas-chromatographic peaks of almost equal size. Material in the later peak appeared to be ethylated in only the N^3 and hydroxyl positions, whereas ethylation at N^1 had also occurred to form the product composing the earlier peak. Results compatible with these have been reported by other investigators who found that alkylation of *p*-HPPH with diazomethane yields a product

methylated at N^3 and the hydroxyl position.[11] The alkylation of N^3 in preference to N^1 of the hydantoin ring is in keeping with the greater acidity of N^3.

The chromatographic patterns in Fig. 1 suggest that both hydroxylation products of DPH are excreted in the urine in conjugated form. This agrees with the report by Maynert[6] that in dogs and in patients less than 1 per cent of a dose of DPH is excreted in the urine as free hydroxylated DPH. In accordance with Maynert's findings, β-glucuronidase hydrolyzed much of the conjugated *p*-HPPH present in the dog and human urine. This enzyme also hydrolyzed most of the conjugated *m*-HPPH excreted by dogs. Incubation of human urine with β-glucuronidase for 24 hrs. but not for 3 hr, yielded part of the *m*-HPPH that could be recovered after acid hydrolysis.

Concentrations of *m*-HPPH and *p*-HPPH present in specimens of acid hydrolyzed urine from several dogs and patients treated with DPH are shown in Table 3 to provide

TABLE 3. COMPARISON OF *meta*HYDROXYLATION AND *para*HYDROXYLATION AS PATHWAYS OF DPH METABOLISM

Subject	*m*-HPPH (μg/ml)	(%)*	*p*-HPPH (μg/ml)	(%)*
Dog				
1	180·0	74·0	64·0	26·0
2	52·0	82·0	12·0	18·0
3	76·0	78·0	21·0	22·0
4	250·0	65·0	135·0	35·0
Patient				
J. G.	9·0	7·0	116·0	93·0
E. C.	3·0	6·0	51·0	94·0
B. W.	3·0	5·0	56·0	95·0
G. P.	6·0	13·0	39·0	87·0
R. M.	21·0	6·0	359·0	94·0

* Expressed as percentage of total *m*-HPPH plus *p*-HPPH.

an estimate of the relative contribution of each of these competing pathways of DPH hydroxylation. It can be seen that *para*hydroxylation is by far the more important pathway of DPH metabolism in these patients, but that *meta*hydroxylation is the major pathway in dogs. In view of this inter-species difference, it is interesting that dogs metabolize DPH three to ten times more rapidly than man.[13] If the pathway of *meta*hydroxylation is responsible for the more rapid metabolism of this drug in dogs, some of the variation in the rate of DPH metabolism in patients might be because of individual differences in the relative development of these competing pathways of *meta*hydroxylation and *para*hydroxylation.

Identification of both *m*-HPPH and *p*-HPPH as hydroxylation products of DPH metabolism in dogs and in man contrasts with the previous findings of Butler.[5] It is possible that the counter-current distribution method that he used failed to separate these two isomers and that what he regarded as a racemic mixture of *levo*rotatory (37 and 44 per cent) and *dextro*rotatory (63 and 56 per cent) *p*-HPPH in dog urine actually may have represented a mixture of *levo*rotatory *p*-HPPH, similar to the product which he isolated from patients and *dextro*rotatory *m*-HPPH. Insufficient *m*-HPPH has been isolated from dog urine to measure its optical rotation, but this

explanation of Butler's results is suggested by the findings shown in Table 3 that *para*hydroxylation predominates in man, whereas *meta*hydroxylation is favored in dogs.

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REFERENCES

1. F. Buchthal, O. Svensmark and P. J. Schiller, *Archs Neurol., Chicago* **2**, 624 (1960).
2. H. Kutt, W. Winters, R. Kokenge and F. McDowell, *Archs Neurol., Chicago* **11**, 642 (1964).
3. J. T. Bigger, D. H. Schmidt and H. Kutt, *Circulation* **38**, 363 (1968).
4. H. Kutt, M. Wolk, R. Scherman and F. McDowell, *Neurology* **14**, 542 (1964).
5. T. C. Butler, *J. Pharmac. exp. Ther.* **119**, 1 (1957).
6. E. W. Maynert, *J. Pharmac. exp. Ther.* **130**, 275 (1960).
7. D. M. Woodbury, *Epilepsia* **10**, 121 (1969).
8. A. J. Glazko, T. Chang, J. Baukema, W. A. Dill, J. R. Goulet and R. A. Buchanan, *Clin. Pharmac. Ther.* **10**, 498 (1969).
9. F. L. Kozelka and C. H. Hine, *J. Pharmac. exp. Ther.* 77, 175 (1943).
10. J. MacGee, *Analyt. Chem.*, **42**, 421 (1970).
11. G. Grimmer, J. Jacob and H. Schäfer, *Arzneimittel-Forsch* **19**, 1287 (1969).
12. H. F. Grützmacher and W. Arnold, *Tetrahedron Lett.* **13**, 1365 (1966).
13. H. H. Frey, E. Kampmann and C. K. Nielsen, *Acta pharmac. tox.* **26**, 284 (1968).

Note added in proof—The recent report that 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin is present in the urine of rats and monkeys treated with DPH [T. Chang, A. Savory and A. J. Glazko, *Biochem. biophys. Res. Commun.* **38**, 444 (1970)] and can be converted to *m*-HPPH and *p*-HPPH by acid but not by β-glucuronidase hydrolysis raises the question that some of the *m*-HPPH and *p*-HPPH that we measured could have been produced by acid hydrolysis of this glycol. In view of this, future attempts to measure *m*-HPPH and *p*-HPPH in urine should use enzymatic rather than acid hydrolysis.