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Effect of Diphenylhydantoin on Cortisol Metabolism in Man *

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During a study of cortisol metabolism in advanced cancer and other disease states (1), we observed in patients receiving DPH [1] therapy for convulsive seizures an abnormal pattern of excretion of cortisol metabolites in urine. This abnormal pattern was characterized by increased urinary output of the polar unconjugated metabolite 6-OHF, suggesting an interference with extra-adrenal metabolism of cortisol by DPH.

We wished to pursue this finding in more detail for two reasons: *1*) An interrelationship among convulsive seizures, the anticonvulsant action of DPH, and pituitary-adrenal function has been demonstrated by several investigators, but interpretation of this is unclear. Woodbury, Timiras, and Vernadakis have shown that cortisol and DPH are antagonistic in their effect on brain excitability in rats; treatment with cortisol enhances, whereas DPH depresses, brain excitability (2). These investigators believed that DPH administration to rats stimulates adrenocortical secretion, since *a*) adrenal hypertrophy was observed in intact rats and *b*) in adrenalectomized rats treated with DPH a more profound decrease in brain excitability was demonstrated than in intact rats. Goncharov has observed that acute administration of DPH to dogs and guinea pigs increases adrenocortical output of cortisol (3). Reports by Staple (4) and Bonnycastle and Bradley (5) have, however, indicated an inhibition of pituitary-adrenal secretion after chronic DPH treatment of mice and rats.

In humans also cortisol has been shown to enhance brain excitability (6). After chronic DPH therapy to patients, excretion of corticosteroids in urine is reported to decrease (7, 8) after an initial increase (7). These findings have led to the suggestion that the decrease in brain excitability induced by DPH might be mediated partially through adrenocortical suppression. However, levels of 17-OHCS in plasma from patients treated with DPH are usually normal (8, 9), as are responses to ACTH (8, 9) and rates of disappearance from plasma of infused cortisol (8). These observations detract from the possibility that direct adrenocortical suppression plays a significant role in therapeutic effectiveness of DPH. Recently, however, Krieger has demonstrated that control subjects treated with DPH manifest a reduced response in the methopyrapone test, which suggests an interference with ACTH release (10).

2) The increased excretion of 6-OHF produced by DPH appears to be similar to that we have observed in patients with advanced cancer and certain terminal illnesses (1) as well as to that reported after estrogen therapy (11), during pregnancy (12), and in newborn infants (13).

Thus we hoped that further investigation into the nature of this alteration in cortisol metabolism induced by DPH would contribute information bearing on the two aforementioned problems. The purpose of this paper is to describe results of

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[1] Glossary of trivial names and abbreviations: diphenylhydantoin (DPH) = 5,5'-diphenylhydantoin; 6-hydroxycortisol (6-OHF) = 6α(β),11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione; 17-OHCS = 17-hydroxycorticosteroids; ACTH = adrenocorticotrophic hormone; methopyrapone (Metopirone, Ciba Pharmaceutical Co., Summit, N. J.) = 2-methyl-1,2-di-(3-pyridyl)-1-propanone. Tetrahydro derivatives: THF (tetrahydrocortisol) = 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one; allo-THF (allotetrahydrocortisol) = 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one; THE (tetrahydrocortisone) = 3α,17α,21-trihydroxy-5β-pregnan-11,20-dione. Cortol = 3α,11β,17α,20α(β),21-pentahydroxy-5β-pregnane; cortolone = 3α,17α,20α(β),21-tetrahydroxy-5β-pregnan-11-one; 17-KS = 17-ketosteroids; 11-hydroxyetiocholanolone = 3α,11β-dihydroxy-5β-androstan-17-one; dehydroisoandrosterone = 3β-hydroxy-5-androstene-17-one; TPNH = reduced triphosphopyridine nucleotide; o,p'DDD = 2,2-bis(2-chlorophenyl,4-chlorophenyl)1,1-dichloroethane.

studies dealing with *in vivo* administration of DPH to human subjects.

Methods

Subjects studied were normal volunteers and patients in the Veterans Administration Hospital, Cincinnati, Ohio. DPH was administered as the commercially available product Dilantin,[2] given in divided doses two or three times per day. Twenty-four-hour urine collections were obtained on ice and samples frozen until subsequent chemical analysis, a period of up to several weeks.

The radioactive cortisol-4-C^{14} (approximate SA, 69 μc per mg) was obtained commercially.[3] Radio paper chromatography of this material demonstrated only one radioactive peak with a mobility comparable to cortisol. The cortisol-4-C^{14}, 0.5 μc, dissolved in 1 to 2 ml of ethanol, was diluted with 150 ml of normal saline and infused intravenously over 10 to 15 minutes, followed by a flushing of the bottle and tubing with another 150 ml of saline. The infusions were given at about 9 a.m. Twenty-four-hour urine collections were begun just before the infusion.

ACTH gel was administered intramuscularly, 40 U at 9 a.m. and 1 p.m. Urine was collected for 24 hours, 8 a.m. to 8 a.m.

Chemical methods. Analysis of 17-OHCS in urine was performed according to the general procedure of Silber and Porter (14) with certain modifications. In preparation for hydrolysis the urinary sample was diluted 50% with distilled water, adjusted to pH 4.8 with acetate buffers, and a small amount of chloroform added. β-Glucuronidase (Ketodase)[4] was added initially at a concentration of 500 U per ml of urine and later at higher concentrations as described in the Results. Butanol (0.7 ml) was added to a sample of chloroform extract (20 ml) before extraction with phenylhydrazine color reagent. Color development was allowed to proceed overnight at room temperature to minimize differences in chromogenic intensity between THF and THE (15). Optical density was read in a Beckman DU spectrophotometer at three wave lengths, 368, 408, and 448 mμ, the final calculation being made on the basis of an Allen correction factor. All samples had a peak reading at the middle wave length of 408 mμ. A sulfuric acid blank was not found to be necessary.

Determination of 6-OHF in urine was performed according to the general method of Frantz, Katz, and Jailer (16) with modifications as follows: A sample of urine equal to 5% of the 24-hour urine output was buffered with 0.2 M acetic acid and 0.2 M sodium acetate to pH 4.8. Sodium sulfate, 20% by weight, was added. Extraction with ethyl acetate and washes with alkali and acid were performed as described by the above workers. An atmosphere of nitrogen, however, was not found necessary. After evaporation of the ethyl acetate *in vacuo,* the final extract was made up to a volume of 10 ml of ethyl acetate: methanol mixture (1: 1). Samples of this were chromatographed on strips of Whatman, 3MM paper, 3 cm wide, in the solvent system, ethyl acetate: chloroform: methanol: water, 1: 3: 2: 2, for 3 to 4 hours. Quantitation was done routinely by densitometry of the strip after staining with blue tetrazolium (17) and was satisfactory between 5 and 30 μg per paper strip. If an unknown sample was found to contain 30 μg or more, a smaller amount of the extract was rechromatographed. Initially, standards of pure 6β-OHF were run simultaneously. We found, however, that standard curves were reproducible from day to day, and because of limited supply of pure 6β-OHF, standards were run only occasionally as a check. Before staining with blue tetrazolium the paper strips were viewed under ultraviolet light (UV) to check for UV absorption of 6-OHF. Rarely we noted, from urine samples with considerable pigment, that there was strong UV absorption and subsequently only minimal staining by the blue tetrazolium reaction. When such was the case, separate samples were chromatographed after further purification by solvent partition (16). We have studied in detail the precision and accuracy of this method for measuring 6-OHF. The mean difference between duplicate analyses was 6%. On comparing the densitometric method of quantitation versus elution from the paper and the phenylhydrazine-sulfuric acid color reaction, the mean difference between multiple samples was found to be 11%. Recovery of 6β-OHF added to several urine samples and quantitated by densitometry averaged 73%. Final results were corrected for this loss. Further identification of 6-OHF from urine extracts was performed in several instances by eluting unstained 6-OHF after chromatography, and examining *1*) UV absorption spectra in methanol and *2*) absorption spectra after reaction with sulfuric acid. These agreed with pure standards. Both 6α-OHF and 6β-OHF exhibit absorption maxima at 236 mμ in methanolic solution. The sulfuric acid absorption curves of these two compounds, however, were different, and the two compounds could be recognized accordingly. 6α-OHF has a peak at 280 mμ, whereas this is absent with 6β-OHF. Conversely, 6β-OHF has a peak at 335 mμ, and 6α-OHF does not. In several instances the amounts of 6α-OHF and 6β-OHF were quantitated by differential absorption curves. The amount of 6α-OHF varied from a trace to 56% without any correlation with diagnosis or treatment of the patient. Quantitating these separate isomers did not seem to be of value. Results for 6-OHF pertain to the total of 6α- and 6β-OHF. Another more polar compound was usually observed running about halfway between the origin and 6-OHF on the paper strips. This compound reacted with blue tetrazolium and absorbed UV light. The concentration of this substance correlated directly with that of 6-OHF. As demonstrated subsequently in

[2] Parke, Davis, Detroit, Mich.

[3] New England Nuclear Corp., Boston, Mass.

[4] Warner-Chilcott Div., Morris Plains, N. J.

the studies with cortisol-4-C^{14}, this polar unknown compound was a metabolite of cortisol, but its identification has not been accomplished. Incubation of several urine samples with β-glucuronidase before extraction did not increase the yield of 6-OHF, which is similar to the observation of Frantz and associates (16).

Determination of THF, allo-THF, and THE. These metabolites were analyzed from chloroform extracts of β-glucuronidase hydrolyzed urine samples (10 to 40 ml). Extracts were chromatographed on paper strips in duplicate for 24 hours in the system, toluene : methanol : water (16 : 3 : 1). THF, allo-THF, and THE were located by staining a small inner strip with blue tetrazolium. The three metabolites were eluted from the remaining paper with absolute methanol, and the phenylhydrazine-sulfuric acid color reaction was performed on the eluate. Standards of THE and THF were chromatographed on adjacent strips, and the unknown samples were quantitated according to the standards. Final calculations also took into account an average loss from extraction as determined by adding standards to various urine portions. Duplicate samples through the entire procedure agreed within 10%.

17-KS and creatinine. Levels of 17-KS were determined by the procedure of Drekter and co-workers (18). Urinary levels of creatinine were measured according to the technique of Folin and Wu (19).

Procedures with cortisol-4-C^{14}. β-Glucuronidase hydrolysis was similar to that described under the chemical analysis for 17-OHCS in urine. Acid hydrolysis was performed by acidification of the urine to pH 1 with 50% sulfuric acid and incubation for 24 hours at 37° C in the dark. When no hydrolysis was performed, urine was buffered with acetate to pH 4.8 before solvent extraction. Extraction with chloroform or ethyl acetate was performed in a manner similar to that described for the chemical method for 17-OHCS and 6-OHF, respectively. Solvents were evaporated on vacuum flash evaporators or *in vacuo* under a stream of filtered air and made up to volume with absolute methanol.

In the fractional separation by paper chromatography (Table II), ethyl acetate extracts of urine samples (5 to 7% of 24-hour output), hydrolyzed by β-glucuronidase, were chromatographed on paper in the system described under the chemical procedure for 6-OHF. The mobile phase was allowed to run to the edge of the paper, allowing separation of six major fractions as predetermined by chromatographing appropriate standards. With each run of urine extracts two adjacent strips with standards were used and stained with blue tetrazolium and phosphomolybdic acid, respectively. A 2-mm wide strip also was cut from the center of each strip containing a urine extract and stained with blue tetrazolium. As shown in Table II, six major fractions could be identified; they were divided, cut up into smaller pieces, and eluted with absolute methanol. The methanol extracts were evaporated and made up to a known volume with methanol. Samples of each fraction were taken for counting of C^{14}. After removing the sample from fraction six, the residual extract was evaporated to dryness and subsequent chromatography performed on a Florisil column to separate the 17-KS and the tetrahydro compounds with 4% and 25% concentrations of methanol in chloroform, respectively (20). The column eluates were evaporated, made up to volume with methanol, and samples taken for counting C^{14}.

Cortisol secretion rates (CSR) were determined from chloroform extracts of urine samples (10 to 50 ml) incubated with β-glucuronidase. The extracts were chromatographed on paper strips in duplicate for 24 hours in the system, toluene : methanol : water (16 : 3 : 1). THF and THE were located by staining a thin inner strip with blue tetrazolium, and the THF and THE were eluted with methanol. The phenylhydrazine-sulfuric acid color reaction was performed on one sample of the eluate and C^{14} counted on another. The final specific activities of THF and THE usually agreed within 10%. In urine samples from two of the six patients, the specific activities of THF and THE varied widely despite rechromatography. In these instances new extracts were chromatographed in the system, isoöctane : tertiary butanol : water (10 : 5 : 9), the THF and THE eluted, reduced with potassium borohydride, and oxidized with periodic acid. The final product, 11-hydroxyetiocholanolone, in each case was chromatographed on separate paper strips in the system, isoöctane : toluene : methanol : water (3 : 1 : 3 : 1) and eluted with methanol. Specific activity was determined by the Zimmerman reaction and counting on respective samples. Specific activities of the final 11-hydroxyetiocholanolone preparations, expressed as THF and THE, agreed within 10%. In four subjects, during DPH therapy, specific activities of 6-OHF were determined by the same method to isolate 6-OHF as in the chemical determination of this metabolite. Instead of densitometry, however, the 6-OHF was eluted from the paper chromatographic strip and specific activity determined by the phenylhydrazine-sulfuric acid color reaction and counting of the C^{14}. The specific activities (disintegrations per minute $\times 10^{-4}$ per micromole) of the 6-OHF in these four instances were 2.96, 0.86, 1.64, and 1.36 and compared closely to the average specific activities of the THF and THE, 2.86, 0.92, 1.66, and 1.31, respectively. CSR was calculated by dividing the average specific activities of the two or three isolated metabolites into dose of radioactivity injected.

All counting of C^{14} was performed on a Packard Tri-Carb liquid scintillation spectrometer with a usual efficiency of about 60%. Methanolic samples, 0.1 to 0.3 ml, of the various eluates were added to 10 ml of toluene scintillation solution [4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per L of toluene]. Quenching was corrected for by adding C^{14} standards to these samples, recounting, and calculating accordingly. All counting was sufficiently long to minimize error to 5% or less. Counting of radioactivity in whole urine was done according to the technique outlined by Flood and associates (21).

Results

Inhibition of in vitro β-glucuronidase hydrolysis in urine from patients receiving DPH. During the course of this investigation we noted that the amount of radioactivity extracted with ethyl acetate or chloroform from urine of patients receiving DPH and cortisol-4-C^{14} was unexpectedly low. These urine samples had been incubated with β-glucuronidase at a concentration of 500 U per ml of urine before extraction. The possibility that the poor recoveries of radioactivity were due to insufficient hydrolysis of the cortisol metabolites by β-glucuronidase was tested by adding increasing amounts of enzyme to urine samples that were subsequently extracted with chloroform or ethyl acetate.[5] As can be seen in Figure 1, maximal recovery of radioactivity was not achieved in urine from two normal subjects treated with DPH until the enzyme concentration approached 4,000 U per ml of urine, employing either chloroform or ethyl acetate for extraction, whereas in urine collected during control periods maximal hydrolysis was obtained with an enzyme concentration of 1,000 U per ml of urine.

It may also be noted in Figure 1 that when chloroform extraction was employed, recovery of radioactivity from urine during the DPH treatment period was considerably below that from urine during the control period, despite maximal enzyme concentration. This is consistent with an absolute decrease in cortisol metabolites in the chloroform-extractable fraction induced by DPH, as will be re-emphasized subsequently.

The effect of increasing concentration of β-glucuronidase during hydrolysis of urine was also evaluated by employing the routine chloroform

[5] The procedure for ethyl acetate extraction, as referred to throughout this paper, always included the addition of 20% sodium sulfate to urine before extraction.

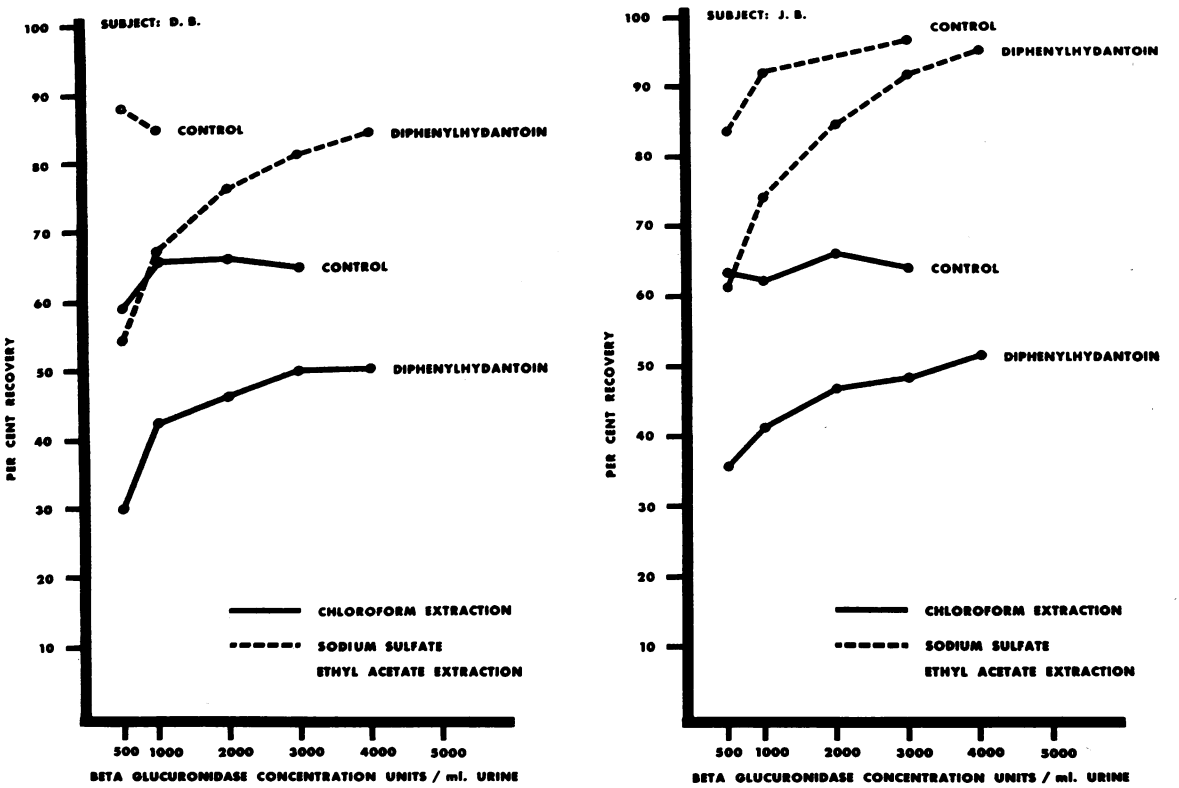

FIG. 1. EFFECT OF β-GLUCURONIDASE CONCENTRATION DURING HYDROLYSIS ON RECOVERY OF CORTISOL-4-C^{14} METABOLITES FROM URINE OF DIPHENYLHYDANTOIN (DPH)-TREATED NORMAL SUBJECTS. Cortisol-4-C^{14} was administered to each subject during a control period and again during therapy with DPH as outlined in Table IV, pertaining to cortisol secretion rates determined from the same 24-hour urine collections. The scale on the ordinate axis is expressed as percentage of recovery of radioactivity to that measured in whole urine.

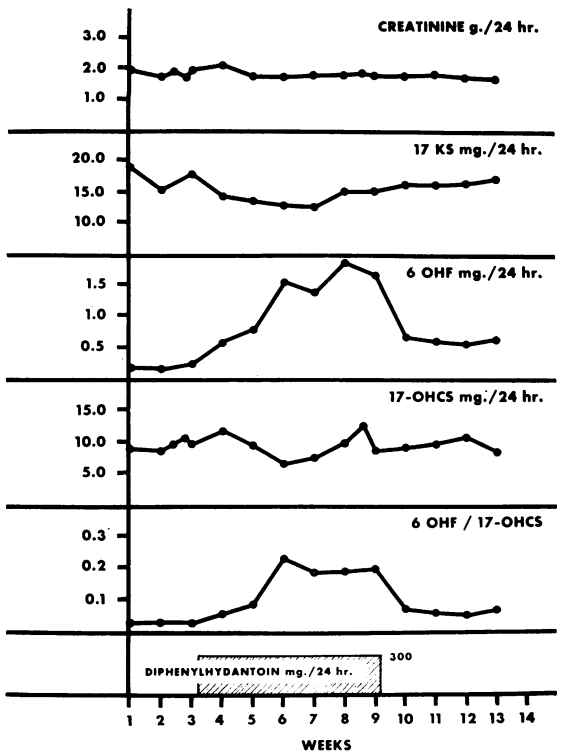

FIG. 2. EFFECT OF DPH ON STEROID EXCRETION IN URINE OF NORMAL SUBJECT D.B., 24-YEAR-OLD MALE.

extraction chemical method for 17-OHCS. The 17-OHCS values averaged 45% higher with an enzyme concentration of 4,000 U per ml than with 500 U per ml in four subjects receiving DPH. During the control period in these four subjects, the chemical values using an enzyme concentration of 1,000 U per ml of urine were slightly higher than with 500 U per ml, average increase 16%.

Thus, in all analyses pertaining to β-glucuronidase hydrolysis, concentration of enzyme employed was 1,000 U per ml of urine from control periods and 4,000 U per ml of urine from samples of subjects receiving DPH. Urine samples that had been collected early in the study and initially analyzed with a lower enzyme concentration were repeated, or in a few instances an average correction factor, based upon the previously mentioned data, was applied.

Steroid excretion. In Figure 2 weekly steroid excretion is presented for a representative normal subject given DPH, 300 mg per day for 6 weeks. The most marked effect of DPH was gradually increasing the daily output of 6-OHF from about 0.2 mg up to 1.9 mg. On the other hand, excretion of 17-OHCS and 17-KS in urine decreased slightly. The alteration in cortisol metabolism can be expressed by the ratio of 6-OHF: 17-OHCS, which increased tenfold in this subject.

In Figure 3 similar data are shown for a patient with brain trauma who had intermittent convulsive seizures. Initial urine samples were obtained when he was receiving DPH treatment. There was an abnormally large output of 6-OHF, 7.2 mg per day. After DPH had been discontinued, excretion of 6-OHF decreased to normal range, as did the ratio 6-OHF: 17-OHCS. Subsequently two other courses of DPH were given, and in each a progressive rise in excretion of 6-OHF ensued. Interestingly, output of 17-OHCS also increased up to a maximum at one point of 19.8 mg per day from the mean baseline level of 6.4 mg per day. The ratios of 6-OHF: 17-OHCS were markedly abnormal, and during one collection within the first week of discontinuing DPH the second time, daily output of 6-OHF (11.4 mg) was considerably higher than excretion of 17-OHCS (6 mg). The degree of alteration in steroid excretion demonstrated by this debilitated patient, in response to DPH therapy, was much greater than in any of the other subjects studied.

It may be noted also from Figure 3 that this patient was given a 3-week course of stilbestrol, which had been reported previously to increase

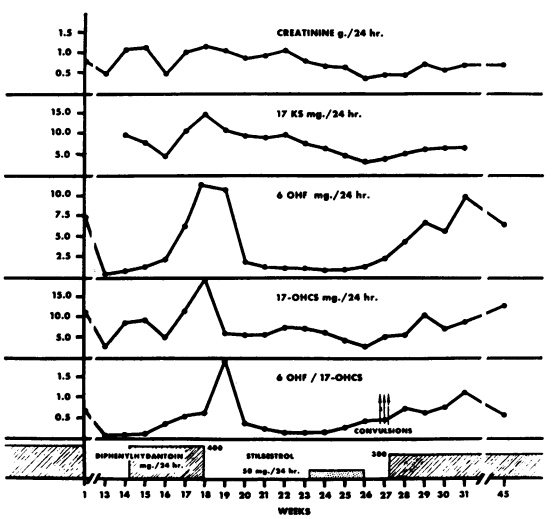

FIG. 3. EFFECT OF DPH ON STEROID EXCRETION IN URINE OF PATIENT F.J., 65-YEAR-OLD MALE. Diagnosis: old brain trauma.

TABLE I

Effect of DPH administration on steroid excretion in urine

Subject, age	Diagnosis, weight	Period of urine collections†	24-hour excretions* 6-OHF	17-OHCS	6-OHF / 17-OHCS	17-KS	Creatinine
	kg		mg	mg		mg	g
		Male subjects					
D.B. 24	Normal 66	Control (5)	0.16	9.4	0.02	17.4	1.84
		DPH, 300 mg/day, 3–6 weeks (5)	1.60	8.9	0.20	13.9	1.76
J.B. 24	Normal 100	Control (4)	0.50	13.4	0.04	22.3	2.56
		DPH, 400 mg/day, 3–7 weeks (7)	1.44	13.3	0.11	15.8	2.49
R.M. 25	Normal 82	Control (3)	0.49	10.5	0.05	20.7	2.24
		DPH, 400 mg/day, 5 weeks (3)	2.30	7.8	0.30	17.7	2.24
G.B. 25	Normal 95	Control (3)	0.43	12.8	0.03	21.6	2.65
		DPH, 400 mg/day, 5 weeks (2)	1.00	12.0	0.08	19.0	2.31
A.G. 24	Normal 93	Control (3)	0.29	10.9	0.03	16.7	2.37
		DPH, 400 mg/day, 6 weeks (3)	1.52	12.6	0.12	14.5	2.64
S.C. 74	Paraplegia 73	Control (5)	0.25	8.7	0.03	14.1	1.16
		DPH, 300 mg/day, 3–8 weeks (8)	1.22	6.3	0.21	8.8	0.92
F.J. 65	Brain trauma 50	Control (4)	0.78	6.4	0.11	8.8	0.84
		DPH, 300 mg/day, 2–4 weeks (3)	6.70	12.1	0.51	9.8	0.86
R.S.‡ 40	Tuberculosis 77	Control (4)	0.14	6.3	0.02	13.2	1.51
		DPH, 300 mg/day, 2–3 weeks (2)	1.31	6.3	0.22	11.0	1.28
L.H.§ 68	Addison's disease, tuberculosis (30 mg F/day) 64	Control (3)	0.12	9.9	0.01		1.48
		DPH, 300 mg/day, 2 weeks (2)	0.54	7.0	0.08		0.86
W.G. 32	Adrenalectomized bilaterally (30 mg F/day) 95	Control (3)	0.49	16.3	0.03	10.9	2.31
		DPH, 400 mg/day, 5 weeks (4)	1.52	13.0	0.12	11.0	2.15
		Female subject					
D.W. 24	Normal 57	Control (4)	0.24	5.4	0.04	11.2	1.34
		DPH, 300 mg/day, 2–4 weeks (3)	1.91	3.9	0.48	8.4	1.32

* Levels shown represent averages of several determinations during the period indicated; the number of determinations during each period is indicated by the number in parentheses after the period of urine collection. The first and often second week of collection values were not included in the DPH period, since the changes had not yet stabilized. In subjects R.M., G.B., A.G., L.H., and W.G. urines sample were collected only at the end of the period of DPH administration. See first footnote in text for key to abbreviations in all tables.

† Control periods varied from 1 to 3 weeks in the male subjects and extended over 4 weeks in the female to evaluate possible changes during the menstrual cycle.

‡ During the administration of DPH, patient R.S. developed a toxic hepatitis manifested by anorexia, nausea, and elevated serum enzyme levels and demonstrated by liver biopsy. Recovery was prompt after discontinuance of all drugs.

§ Patient L.H. developed severe vertigo and ataxia which probably resulted from the combination of therapy with DPH and isoniazid (22). Recovery was gradual after discontinuance of DPH. Urine collections were incomplete during the DPH period in this patient.

TABLE II

*Effect of DPH administration on fractionation of cortisol-4-C^{14} metabolites in urine by paper chromatographic separation after β-glucuronidase hydrolysis and ethyl acetate extraction**

	Subject D.B.		Subject J.B.		Subject R.M.	
	Control	DPH†	Control	DPH†	Control	DPH†
Radioactivity extracted, *dpm*	56,480	57,840	36,561	43,345	43,760	39,440
Percentage of recovery	85	87	79	82	78	73
Radioactivity applied to paper, *dpm*	55,068	57,117	36,561	43,345	43,213	38,947
Radioactivity eluted from paper, *dpm*	42,636	48,568	31,657	35,750	37,444	36,412
Percentage of recovery	77	85	87	82	87	93
Paper fraction	Percentage of radioactivity in each fraction to the total eluted from paper strip					
1) Origin to polar unknown	1.4	0.9	0.7	1.3	0.8	1.3
2) Polar unknown	1.7	5.0	1.8	6.8	2.2	6.7
3) Polar unknown to 6-OHF	0.4	0.8	3.3	3.3	0.4	1.0
4) 6-OHF	3.2	7.5	3.1	6.8	2.0	10.1
5) Cortols, cortolones	16.8	14.8	17.6	19.0	11.5	12.4
6)‡ a) THF, allo-THF, THE	73.7	66.4	69.8	59.9	73.1	59.5
b) 17-KS (11 oxy)	2.4	4.2	3.6	2.9	10.0	8.9

* All ethyl acetate extractions were performed after addition of sodium sulfate to urine sample (approximately 5 to 7% of 24-hour output), as described under Methods. The solvent system used in the paper chromatography was the same as that described in the chemical determination of 6-OHF.

† The dose and duration of therapy with DPH were the same as those shown in Table IV.

‡ Further fractionated into a) and b) by Florisil column chromatography.

6-OHF excretion (11). A slight rise in 6-OHF output occurred, which was much less than that after DPH. Moreover, excretion of 17-OHCS decreased slightly, in contrast to the rise observed after DPH.

In Table I changes in steroid excretion are summarized for 11 subjects receiving DPH for 2 to 8 weeks. A consistent elevation in output of 6-OHF, ranging from 2.5- to 10-fold, was found in all subjects. Excretion of 17-OHCS in urine decreased slightly in 7 of the 11 subjects, increased in 2, and was essentially unchanged in 2. The ratio of 6-OHF: 17-OHCS increased significantly in all subjects. Excretion of 17-KS decreased in eight of ten subjects. Of particular interest are patients L.H. and W.G., who had adrenal insufficiency and were maintained on exogenous cortisol. The usual increase in excretion of 6-OHF and in the ratio of 6-OHF: 17-OHCS was observed in both subjects. However, the level of 17-KS did not decrease in W.G.

Subject D.W. was the only female studied, and her response was similar to that of the males but was more marked, as demonstrated by the high ratio of 6-OHF: 17-OHCS, 0.48. The alterations could not be attributed to any change with the menstrual cycle.

TABLE III

The ratios of tetrahydro cortisol metabolites in urine after DPH

Subject	THF / Total	Allo-THF / Total	THE / Total	THF / THE
D.B.				
Control	0.27	0.18	0.55	0.48
DPH, 300 mg/day, 4 weeks	0.32	0.13	0.55	0.58
Percentage of change	+18	−28	0	+21
J.B.				
Control	0.22	0.16	0.62	0.36
DPH, 400 mg/day, 5 weeks	0.33	0.12	0.55	0.60
Percentage of change	+50	−25	−11	+67
R.M.				
Control	0.24	0.18	0.58	0.42
DPH, 400 mg/day, 5 weeks	0.34	0.18	0.48	0.72
Percentage of change	+42	0	−17	+71

Fractionation of cortisol-4-C^{14} metabolites in urine after administration of cortisol-4-C^{14} in vivo. In three subjects recoveries of radioactive metabolites were studied initially according to conjugation and polarity. Separate urine samples that had received *1*) no prior hydrolysis, *2*) pH 1 hydrolysis, and *3*) β-glucuronidase hydrolysis were extracted with chloroform or ethyl acetate. The main changes observed consistently after DPH treatment were as follows: *1*) a decrease, approximating 20%, in metabolites conjugated with glucuronic acid and extracted with chloroform (as

TABLE IV

Cortisol secretion rates (CSR): control and during DPH administration

Subject	Control			DPH				
	17-OHCS excretion in urine				17-OHCS excretion in urine			
	Average*	Day CSR	CSR	Dose	Average*	Day CSR	CSR	Percentage of change
	mg/24 hrs	*mg/24 hrs*	*mg/24 hrs*		*mg/24 hrs*	*mg/24 hrs*	*mg/24 hrs*	%
D.B.	9.4	9.6	18	300 mg/day, 5 weeks	8.9	12.6	32	+78
J.B.	13.4	14.4	24	400 mg/day, 6 weeks	13.3	13.0	30	+25
S.C.	8.7	8.4	18	300 mg/day, 8 weeks	6.3	5.1	27	+50
A.G.	10.9	12.1	23	400 mg/day, 6 weeks	12.6	12.9	28	+22
G.B.	12.8	14.5	23	400 mg/day, 5 weeks	12.0	12.1	21	− 9
R.M.	10.5	10.7	21	400 mg/day, 5 weeks	7.8	8.7	21	0

* Average excretion of 17-OHCS in urine for the respective period, same as shown on Table I.

may be noted from Figure 1); and *2*) an increase, about twofold, in polar unconjugated metabolites extracted by ethyl acetate. There were no significant differences noted in unconjugated chloroform-extractable fractions or acid-hydrolyzable fractions.

In Table II changes determined by a more discrete fractionation system are depicted from urine samples of the same three subjects. In these experiments extracts of urine that had been hydrolyzed with β-glucuronidase and extracted with ethyl acetate were fractionated as described in the Methods section. The principal changes after DPH therapy were the decrease in tetrahydro metabolites, THF, allo-THF, and THE, and the increase in 6-OHF and an unidentified polar unknown. No consistent differences were noted in the cortol-cortolone fractions or in the 17-KS.

Excretion of tetrahydro derivatives. In view of the changes in the tetrahydro fraction, it was considered of interest to compare excretion of individual tetrahydro metabolites. The results, Table III, are expressed in terms of ratios of individual metabolites to the sum total or to each other. The ratios of THF: total and THF: THE increased during DPH therapy, whereas the ratios allo-THF: total and THE: total tended to decrease.

CSR, Table IV. CSR were measured in six subjects, five of whom were normal, during both control and DPH periods. An increase, of relatively small degree, was found in four of the six subjects. In one subject, G.B., there was an insignificant decrease and in the remaining subject, no change.

ACTH administration, Table V. ACTH gel was given to two normal subjects and patient F.J.

TABLE V

Effect of ACTH administration on steroid excretion in urine before and during DPH ingestion*

Subject	Study period	Rise in 24-hour excretion (or ratio) above base line†			
		6-OHF	17-OHCS	$\frac{\text{6-OHF}}{\text{17-OHCS}}$	17-KS
		mg	*mg*		*mg*
D.B.	Control	7.73	66.1	0.08	21.0
	DPH, 300 mg/day, 4 weeks	20.6	49.4	0.18	12.2
J.B.	Control	3.56	45.4	0.03	14.6
	DPH, 400 mg/day, 5 weeks	20.59	67.7	0.16	15.4
F.J.	Control	2.75	28.0	0.04	3.8
	DPH, 300 mg/day, 3 weeks	27.41	34.8	0.22	2.9

* ACTH gel, 40 U im at 9 a.m. and 1 p.m.

† The base-line excretions and ratios used in the compilation of these data were the averages for the respective study periods as depicted in Table I.

TABLE VI

Steroid excretion in patients with convulsive seizures receiving long-term DPH therapy

	24-hour steroid excretions		
	6-OHF	17-OHCS	$\frac{\text{6-OHF}}{\text{17-OHCS}}$
	mg	*mg*	
Patients on DPH*			
Mean	1.57	3.2	0.65
Range	1.01–3.01	1.4–5.2	0.20–1.37
Normal†			
Mean	0.40	7.3	0.06
Range	0.15–0.84	3.0–17.2	0.02–0.14

* Six male patients: one, age 5 (Cincinnati Children's Hospital), the remainder, ages 39 to 69. DPH had been ingested from 6 months to 8 years at an average daily dosage of 100 to 400 mg. Five patients were receiving phenobarbital also.

† Fourteen males, ages 23 to 75.

The increases in output of 6-OHF after ACTH were markedly augmented while the subjects were receiving DPH. The ratios of 6-OHF: 17-OHCS also were enhanced considerably. A slightly greater increase in excretion of 17-OHCS was found in subjects J.B. and F.J. These changes, coupled with the considerably higher increase in output of 6-OHF, indicate a greater adrenal secretory responsiveness in these two subjects when receiving DPH.

Finally, steroid excretion was measured in 6 additional patients with convulsive disorders who had received long-term DPH treatment, in contrast to the previously mentioned 11 subjects. Output of 6-OHF was increased above normal (Table VI), the degree of which was comparable to that found in the subjects on short-term DPH treatment (Table I). Output of 17-OHCS, conversely, was in the low normal range. Ratios of 6-OHF : 17-OHCS were markedly abnormal, ranging from 0.20 to 1.37, the degree of which tended to be greater than in normal subjects receiving short-term DPH.

Discussion

The principal results of this investigation document an extra-adrenal alteration in cortisol metabolism induced by administration of DPH to human subjects. The alteration was characterized by an increased proportion of cortisol metabolized to polar unconjugated metabolites and a corresponding decreased proportion metabolized via A-ring reduction and glucuronic acid conjugation. The polar unconjugated metabolites consisted principally of 6-OHF, and in somewhat lesser amounts, another, as yet unidentified, polar compound. Interestingly, the alteration in tetrahydro derivatives included an increase in the proportion of THF to THE and to the total tetrahydro metabolites.

The major alterations were observed in radioactive metabolites in urine after administration of cortisol-4-C^{14} *in vivo,* indicating that the changes truly reflected metabolism of cortisol. Furthermore, the changes were found in one patient with Addison's disease and in another bilaterally adrenalectomized, indicating that the metabolic alterations occurred outside of the adrenal glands. Presumably the major site is liver, since it is well known to be the chief organ responsible for the greatest proportion of cortisol metabolism. Also the study by Lipman, Katz, and Jailer, employing human tissue incubation *in vitro,* demonstrated more 6β-hydroxylation activity in liver than in other tissues (23).

That other steroids may have been altered by DPH administration was suggested by reduction in 17-KS excretion. Since neither the radioactive 17-KS metabolites of cortisol (Table II) nor the 17-KS in adrenalectomized patient W.G. appeared to change, we suggest that the observed reduction in 17-KS in the normal subjects involved adrenal 11-deoxy 17-KS, e.g., dehydroisoandrosterone.

CSR did not change consistently after DPH administration to normal subjects. It was suggested that cortisol secretion may increase to a variable degree during 4 to 8 weeks of DPH treatment, as manifested by CSR results in four subjects and the urinary excretion steroid values in patient F.J. Responses to ACTH in two subjects, as manifested by increased excretion of 17-OHCS plus 6-OHF, also suggested adrenal hyperactivity. Previous studies of the effect of ACTH in subjects treated with DPH have suggested either normal (8, 9) or reduced (9) adrenocortical responsiveness. However, techniques employed were considerably different from ours. Specifically, 24-hour total excretion of 17-OHCS and 6-OHF in short-term treated subjects was not determined.

The disparity, in short-term DPH-treated subjects, between possible adrenal hypersecretion and

the previously reported reduced responsiveness to methopyrapone (10) cannot be explained. Further study is necessary to clarify this point.

Patients with convulsive disorders receiving prolonged DPH therapy from 6 to 8 years (Table VI) demonstrated the alteration in ratio of 6-OHF: 17-OHCS similar to that in the experimental subjects treated up to 8 weeks with DPH. Low output of 17-OHCS in this group, however, suggested a decrease in cortisol secretion despite the increase in 6-OHF excretion. This pattern of an eventual decrease in cortisol secretion after an initial increase with DPH treatment has been suggested previously by Costa, Glaser, and Bonnycastle (7).

An incidental but important finding was that urine from subjects treated with DPH requires a greater concentration of β-glucuronidase than usual to achieve maximal hydrolysis of steroid-glucuronide conjugates. This indicates an inhibition of β-glucuronidase by DPH or its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (24), which is conjugated with glucuronic acid (25). Although the mechanism of inhibition was not studied, it is conceivable that it might be a competitive one as noted with the glucuronide of acetylsalicylic acid (26). In any event it is important to know that the concentration of β-glucuronidase must be increased when analyzing for 17-OHCS in urine from DPH-treated patients. Failure to do so results in values for 17-OHCS approximately 45% too low and would partially discredit some low levels reported after DPH (8) but not others (7) where enzyme hydrolysis was not employed.

The mechanism of the increased excretion of 6-OHF is not clear. Considerable information regarding this metabolite has been contributed recently in a series of studies by Frantz, Katz, Jailer, Lipman, and Borkowski (11, 12, 16, 23, 27) and by Ulstrom, Colle, Burley, and Gunville (13). The latter group noted that 6-OHF was a major metabolite of cortisol in newborn infants. The former group initially observed an increased output of 6-OHF in pregnancy and subsequently attributed increased formation of 6-OHF to inhibition of cortisol A-ring reduction brought about by a high level of estrogen and a resultant interference with TPNH production.

The finding in the current study of a proportionate decrease in tetrahydro derivatives is consistent with the postulate of a partial block in A-ring reduction. It does not seem likely, however, that this aberration is due to an estrogenic effect, since after DPH therapy there is no increase in plasma cortisol binding, as shown by normal levels of 17-OHCS in plasma (8, 9).

Effects of other hormones, testosterone, ACTH, and thyroxine, might be considered. Testosterone depresses cortisol A-ring reduction (28–30) but does not increase 6-OHF excretion (11). ACTH has been reported to enhance 6-OHF excretion to some degree (16). In the present study, however, little change was noted in the ratio of 6-OHF: 17-OHCS after ACTH when subjects were not receiving DPH. Thyroxine is known to be displaced by DPH from thyroxine-binding globulin (31), but an excess of thyroxine would be expected to increase the proportion of THE rather than reduce it.

Thus current evidence indicates that the alteration in cortisol metabolism induced by DPH is not mediated through effects by these hormones. Perhaps DPH, or its major metabolite (24), may interfere directly with cortisol A-ring reduction or TPNH production.

Whether there is any relation between pharmacological action of DPH and this aberration in cortisol metabolism remains speculative. Levels of 17-OHCS in plasma are normal during DPH therapy (8, 9). Conceivably DPH might interfere with cortisol metabolism in brain as well as liver. Such a concept would require a reduction in cortisol activity locally, since cortisol enhances brain excitability. How this could occur in conjunction with the metabolic alteration is questionable. It is of interest, however, that other known effects of DPH are consistent with a reduction in cortisol activity, namely, lymphoid hyperplasia (32, 33), protein anabolism (34), dermal chemical response (35), and a suggestion of amelioration of Cushing's syndrome despite high levels of 17-OHCS in urine (36).

Summary

The effect of diphenylhydantoin administration on cortisol metabolism was investigated in human subjects by analyzing cortisol metabolites in urine. Diphenylhydantoin caused a net increase in ex-

cretion of 6-hydroxycortisol and an unconjugated polar metabolite concomitant with a relative decrease in conjugated tetrahydro derivatives. Similar results were found in two patients with adrenal insufficiency. Fractionation studies after administration of cortisol-4-C^{14} to subjects treated with diphenylhydantoin confirmed these changes and demonstrated them to be the major alterations in cortisol metabolism induced by diphenylhydantoin.

Administration of exogenous ACTH enhanced the alteration in cortisol metabolism in diphenylhydantoin-treated subjects.

Cortisol secretion rates increased slightly in four of six subjects treated with diphenylhydantoin for 5 to 8 weeks. In addition, one patient with brain trauma showed a marked increase in steroid excretion indicative of a true rise in cortisol secretion. In other patients receiving long-term diphenylhydantoin therapy cortisol secretion appeared to be reduced.

Output of total 17-ketosteroids decreased slightly in normal subjects receiving diphenylhydantoin but not in patients with adrenal insufficiency, which suggested an effect on the metabolism of 11-deoxy, 17- ketosteroids produced in the adrenals.

An incidental but important finding in the study was that of an *in vitro* inhibition of β-glucuronidase in urine from subjects receiving diphenylhydantoin. Thus a fourfold increase in enzyme concentration was necessary in these samples for maximal hydrolysis, compared to control urine samples.

We conclude that diphenylhydantoin alters extra-adrenal metabolism of cortisol and probably 17-ketosteroids as well. This effect may be related to the pharmacological activity of diphenylhydantoin.

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