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STIMULATORY EFFECT OF *N*-PHENYLBARBITAL (PHETHARBITAL) ON CORTISOL HYDROXYLATION IN MAN

R. KUNTZMAN, M. JACOBSON, W. LEVIN and A. H. CONNEY

Wellcome Research Laboratories, Burroughs Wellcome & Co., (U.S.A.) Inc., Tuckahoe, N.Y., U.S.A.

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Abstract—After the chronic administration of phetharbital (*N*-phenylbarbital) to man, a marked increase in the urinary excretion of 6 β-hydroxycortisol was observed. This increase in 6 β-hydroxycortisol excretion was not accompanied by a concomitant increase in the urinary excretion of 17-hydroxycorticosteroids (17-OHCS), suggesting that an increased adrenal output of cortisol is not responsible for the observed rise in 6 β-hydrocortisol excretion. The finding that phetharbital administration to guinea pigs increased the activity of an enzyme system in liver microsomes that hydroxylates cortisol in the 6 β-position does, however, offer an explanation for the observed increase in 6 β-hydroxycortisol excretion. The possible therapeutic uses of a nonhypnotic barbiturate such as phetharbital, which stimulates steroid metabolism, are discussed.

PREVIOUS studies have shown that the same factors control both drug and steroid hydroxylation by enzyme systems in liver microsomes, and the results of these studies suggest that the same enzyme systems are responsible for the hydroxylation of both classes of compounds.[1, 2] Treatment of rats with phenobarbital or several other stimulators of drug metabolism increases the levels of enzymes in liver microsomes that hydroxylate androgens, estrogens, progestational steroids, and glucocorticoids.[1–8] Since the chronic administration of phenobarbital and several other drugs increases drug hydroxylation in man,[9] one would expect that these drugs would similarly enhance steroid hydroxylation. The experiments presented in this report describe the effects of phetharbital (*N*-phenylbarbital) on the hydroxylation *in vivo* of cortisol in man and on the metabolism *in vitro* of several steroids by livers of rats and guinea pigs.

METHODS

6 β-Hydroxycortisol determination in human urine. The following modification of the methods of Frantz *et al.*[10] and of Werk *et al.*[11] was used to determine 6 β-hydroxycortisol levels in urine. A sample of urine equal to 3 per cent of the 24-hr urine output was buffered to pH 5·0 with 0·1 vol. of 2 M sodium acetate buffer. Twenty per cent by weight of sodium sulphate was dissolved in the urine sample by warming the urine to not more than 35°. The urine was extracted twice with 2 vol. of ethyl acetate. The combined ethyl acetate extracts were washed in separatory funnels, twice with a 1/20 vol. of 15% sodium sulphate in 1·0 N NaOH, once with a 1/20 vol. of 15% sodium sulphate in 0·5% acetic acid, and once with a 1/100 vol. of distilled water.

The washed ethyl acetate extracts were allowed to stand overnight at room temperature so that the water in the ethyl acetate would settle. The ethyl acetate extracts were taken to dryness *in vacuo* in a rotating evaporator and the residues were transferred to test tubes with 5 ml of an ethyl acetate:methanol (1:1) mixture followed by 5 ml methanol. The extracts were dried under nitrogen and the dried residues were dissolved in 5 ml methanol:methylene chloride (2:1). A 3·5-ml aliquot of this solution was transferred to another test tube. Both the 3·5-ml and the remaining 1·5-ml solutions were dried under nitrogen and dissolved in 0·1 ml ethyl acetate:methanol (1:1) and spotted adjacently on 3 MM chromatography paper that was previously washed with methanol. The four extracts from two urine samples were spotted on the same sheet ($8\frac{3}{4}$ in. wide) so that the two 1·5-ml samples were in the center, 1 in. apart, and the two corresponding 3·5-ml samples were 4 in. to the right or left of the center spots. Thirty μg 6 β-hydroxycortisol was added to water and urine and extracted for the determination of recoveries. The chromatography tanks were lined with paper and the chromatograms, including a blank sheet for paper blank determination, were equilibrated overnight with the lower phase of a system containing distilled benzene:ethylacetate:methanol:water (100:20:50:50). Descending chromatography was then run for 44 hr with the upper phase of the above system. On some chromatograms, 2 μg 6 β-hydroxycortisol (the minimum that could be detected) was placed between the two 1·5-ml extracts.

After chromatography, the center section of the chromatogram, corresponding to the two 1·5-ml extracts, was cut out and carefully immersed in a solution prepared by mixing 5 ml blue tetrazolium reagent (5 mg blue tetrazolium/ml formamide) with 500 ml of 2 N NaOH just before use. After 5 min, the solution was decanted and 500 ml of 2% acetic acid was added. The tray was rocked gently and after 5 min the acid was decanted and the chromatogram was washed in the tray with distilled H_2O. The chromatogram was then removed and dried.

The 6 β-hydroxycortisol areas (corresponding to the blue tetrazolium spots) from the 3·5-ml extracts on the remainder of the chromatogram were cut out. A similar section of the blank sheet was cut out. The 6 β-hydroxycortisol was eluted by shaking each section of filter paper with 10 ml methanol for 15 min. The methanol was then removed and the section washed with an additional 10 ml methanol. The combined methanol eluates were evaporated to dryness under nitrogen and the amount of 6 β-hydroxycortisol was determined by the Silber–Porter method[12] as follows: a solution of 32·5 mg phenylhydrazine in 50 ml of 11·2 M H_2SO_4 was freshly prepared. One ml of a mixture containing 2 parts of this phenylhydrazine reagent and one part of ethanol was added to each dried eluate. The tubes were allowed to stand overnight in the dark at room temperature, and the O.D's were measured at 370 mμ, 410 mμ, and 450 mμ. Standards of 5 μg, 10 μg, and 15 μg of 6 β-hydroxycortisol were also reacted with the Silber–Porter reagent. The O.D's of blanks prepared with or without phenylhydrazine were determined at the three wave lengths and the Allen correction was applied to all readings.[13] When applying this correction, the O.D. values obtained at 370 mμ and 450 mμ are averaged and subtracted from the value found at 410 mμ. The corrected O.D. value is then used in all further calculations. Each value was corrected for the recovery of 6 β-hydroxycortisol, which was 60 per cent.

Nonpolar 17-OHCS determination in human urine. Quantification of 17-OHCS in

urine was determined by a modification of the method of Silber and Porter.[12] A 2-ml urine sample was diluted with an equal volume of distilled H_2O. The pH of the sample was adjusted to 5·0 with 2 M sodium acetate–acetic acid buffer, and the sample was incubated for 2 hr at 37° with 2000 units of β-glucuronidase (Ketodase) per ml urine. The volume of the incubation mixture was adjusted to 10 ml with pH 5 buffer, and the mixture was shaken by hand for 15 sec after the addition of 50 ml of distilled chloroform. After centrifugation, the aqueous phase was removed and the chloroform was shaken for 10 sec after adding 2 ml of 0·1 N NaOH. The mixture was centrifuged and the NaOH was removed. The 50 ml chloroform extract was divided into two 20-ml aliquots. One ml of a mixture containing two parts phenylhydrazine reagent (32·5 mg phenylhydrazine in 50 ml of 11·2 M sulfuric acid) or 11·2 M sulfuric acid and one part of ethanol was added to the chloroform. The tubes were then shaken for 15 sec, centrifuged, and the chloroform removed. The remaining 1-ml aqueous phase was allowed to stand in the dark overnight and the O.D's were determined the next day at 370 mμ, 410 mμ, and 450 mμ. All calculations were made by using the Allen correction and tetrahydrocortisol as the standard 17-OHCS. All values were corrected for the recovery of tetrahydrocortisol, which was 80 per cent.

Hydroxylation of steroids by rat and guinea pig liver microsomes. The metabolism of estradiol, testosterone, and deoxycorticosterone to polar hydroxylated metabolites by rat liver microsomes and the metabolism of cortisol to 6 β-hydroxycortisol by guinea pig liver microsomes was studied as previously described.[1–3, 5–7]

RESULTS

Effect of phetharbital and phenobarbital on steroid metabolism by female rat liver microsomes. Phetharbital or phenobarbital (41 mg/kg, i.p., twice daily) was administered to adult female rats for 4 days and the animals were killed on the fifth day. As can be seen in Table 1, phetharbital and phenobarbital increased the metabolism of estradiol, testosterone, and deoxycorticosterone to polar metabolites by enzymes in liver microsomes.

TABLE 1. EFFECT OF PHETHARBITAL AND PHENOBARBITAL ON STEROID METABOLISM BY RAT LIVER*

Treatment	Substrate		
	Estradiol	Testosterone	Deoxycorticosterone
	(mμmole polar metabolites formed)		
Control	13·3 (12·1–14·5)	66·7 (66·3– 67·3)	88·0 (85·0– 93·0)
Phenobarbital	86·5 (76·9–96·8)	262·9 (236·9–286·9)	179·4 (170·7–191·1)
Phetharbital	65·2 (60·9–72·0)	204·6 (185·6–218·2)	139·4 (132·3–152·2)

* Adult female rats were treated for 4 days with phenobarbital or phetharbital. The rats were killed on the fifth day and liver microsomes from 330 mg liver were incubated at 37° with 700 mμmole testosterone or deoxycorticosterone for 7·5 min or with estradiol for 15 min in the presence of an NADPH-generating system as previously described.[1, 2, 5] The ascending systems used for chromatography were as follows: testosterone, cyclo hexane : MeOH : H_2O (100 : 100 : 10); estradiol, benzene : heptane : MeOH : H_2O (7 : 3 : 8 : 2); deoxycorticosterone, cyclohexane : dioxane : MeOH : H_2O (100 : 25 : 100 : 10). The values given represent the average and range obtained in 3 determinations with pooled livers from 3 rats used in each assay.

Increased activity of cortisol 6 β-hydroxylase in liver microsomes of guinea pigs given phetharbital. Phetharbital (100 mg/kg/day, i.p.) was administered in divided dosage (morning and afternoon) to guinea pigs for 10 days. Other guinea pigs served as controls. Administration of phetharbital caused more than a 100 per cent increase in the level of cortisol 6 β-hydroxylase in guinea pig liver microsomes (Fig. 1).

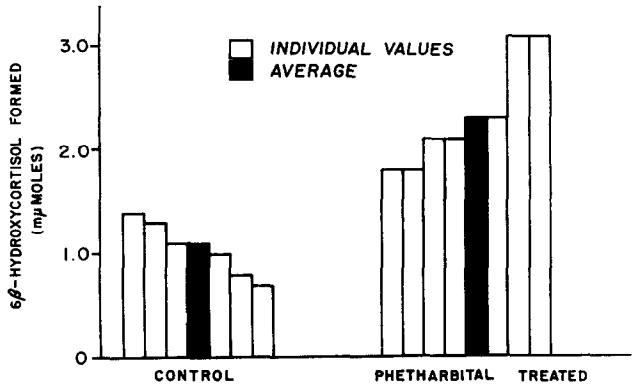

FIG. 1. The effect of phetharbital on cortisol 6 β-hydroxylase in guinea pig liver microsomes. Female guinea pigs were injected i.p. with 50 mg/kg of phetharbital twice daily for 10 days. On day 11, the guinea pigs were killed and microsomes from 666 mg liver were incubated at 37° with 100 mμmole cortisol-4-^{14}C for 30 min in the presence of an NADPH-generating system as previously described.[7] After extraction with ethyl acetate, chromatographic separation of 6 β-hydroxycortisol was accomplished as described.[7] Statistical evaluation of the data indicated an average of 1·04 ± 0·25 (S.D.) for the control, and an average of 2·31 ± 0·53 (S.D.) for the phetharbital-treated group, with a P value of < 0·01.

Increased excretion of endogenous 6 β-hydroxycortisol in human urine caused by phetharbital administration. Four human volunteers received phetharbital daily on the following dosage schedule: days 3–5, 100 mg three times a day; days 6–19, 200 mg three times a day; days 20–24, 300 mg three times a day; and days 25–36, 400 mg three times a day. No CNS stimulant or depressant effects of phetharbital were seen in any of the treated subjects. Two other volunteers received placebo tablets throughout the study. A 24-hr urine sample was collected from all six subjects on day 1 (before phetharbital treatment), day 18, and day 35, and the amount of 6 β-hydroxycortisol in the urine was measured. The results in Fig. 2 show a marked increase in the excretion of 6 β-hydroxycortisol in the urine of all four subjects treated with phetharbital (days 18 and 35), but no increase was found in the urine of the two control subjects. After 18 days of treatment with phetharbital, the urinary excretion of 6 β-hydroxycortisol increased from an average of 186 μg/day to 481 μg/day, while 35 days of phetharbital treatment increased the average urinary excretion of 6 β-hydroxycortisol to 699 μg/day. Fig. 2 also shows that the control levels (day 1) of 6 β-hydroxycortisol were always below 400 μg/24 hr, whereas on day 35, after phetharbital treatment, all four subjects had an excretion of 6 β-hydroxycortisol in excess of this amount. Three of the four subjects already excreted more than 400 μg/24 hr on day 18. The only subject who did not exceed 400 μg/24 hr on day 18 showed, at this time, a 3-fold increase in 6 β-hydroxycortisol excretion, but started from the lowest control level of only 100 μg/24 hr. Although the 6 β-hydroxycortisol level increased markedly, the chloroform-extractable

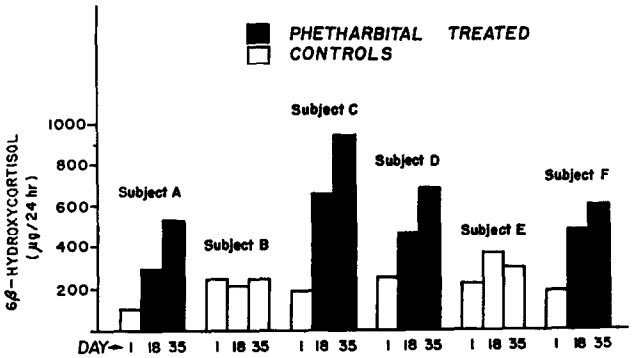

FIG. 2. The effect of phetharbital on urinary 6 β-hydroxycortisol excretion in humans. Four human volunteers received daily oral doses of phetharbital (100–400 mg, three times a day) from day 3 through day 36, according to the schedule described under Results. Two other volunteers received placebos. Twenty-four-hr urine samples were collected from all subjects on day 1, day 18, and day 35, and urinary 6 β-hydroxycortisol was measured as described under Methods.

17-OHCS (Porter–Silber reacting material) in the urine of these four subjects did not increase, indicating that the increased 6 β-hydroxycortisol levels did not come simply from an increased adrenal output of cortisol.

Effect of phetharbital on the metabolism of exogenously administered cortisol. The two control subjects and four phetharbital-treated subjects described above were given 120 mg cortisol orally on days 2 and 19, and 24-hr urine samples were collected. Phetharbital treatment for 17 days decreased by an average of 43 per cent the chloroform-extractable 17-OHCS excreted after this exogenous dose of cortisol. In contrast, the two control subjects excreted the same amount of 17-OHCS on days 2 and 19 of the study. Measurement of 6 β-hydroxycortisol in the urine after the exogenous dose of cortisol on days 2 and 19 showed small increases (14–77 per cent) in the level of this metabolite in the urine after phetharbital treatment.

DISCUSSION

Chronic administration of phetharbital causes an increased excretion of 6 β-hydroxycortisol in human urine. This increase in 6 β-hydroxycortisol excretion is not accompanied by an increase in the 17-OHCS levels in urine, indicating that an increased adrenal output of cortisol is not responsible for the increased urinary levels of 6 β-hydroxycortisol. The finding that phetharbital administration to guinea pigs increases the activity of an enzyme system in liver microsomes that hydroxylates cortisol in the 6 β-position offers an explanation for the increased urinary excretion of 6 β-hydroxycortisol in humans treated with phetharbital. Phenobarbital,[14] diphenylhydantoin,[11] phenylbutazone,[15] and *o,p'*-DDD[16–18] are examples of other drugs that stimulate the urinary excretion of 6 β-hydroxycortisol when administered to man. The first three compounds have been demonstrated to stimulate the activity of an enzyme in guinea pig liver microsomes that hydroxylates cortisol in the 6 β-position.[7, 15] The effect of *o,p'*-DDD on the liver microsomal hydroxylation of cortisol in the 6 β-position was not studied, but *o,p'*-DDD does enhance liver microsomal oxidations.[19, 20]

The increased urinary excretion of 6 β-hydroxycortisol caused by phetharbital and other drugs may offer a method for determining the induction of liver microsomal

enzymes in man. The data in this paper (Fig. 2) indicate that all individuals given phetharbital excreted more than 400 μg 6 β-hydroxycortisol/day, whereas the level of this metabolite was below 400 μg/day in the subjects treated with placebo and in the control periods before drug treatment started. In fact, the average level of 6 β-hydroxycortisol found in this study in untreated subjects was well below this arbitrary figure of 400 μg/day.

It is not clear why the level of 6 β-hydroxycortisol formed from exogenous cortisol did not increase as much after phetharbital treatment as it did when one measured the endogenous level (Fig. 2). It is possible that the higher amounts of 6 β-hydroxycortisol formed from exogenously administered cortisol can be further metabolized more rapidly than the small amounts of 6 β-hydroxycortisol formed endogenously.

When exogenous cortisol was given to phetharbital-treated subjects, a decrease in the amount of chloroform-extractable 17-OHCS was found. These results are also consistent with enhanced metabolism of cortisol via hydroxylation, which would result in a decreased urinary excretion of nonpolar 17-OHCS. The studies by Bledsoe *et al.*[18] and by Southren *et al.*[16, 17] similarly showed that *o,p'*-DDD did not decrease the urinary excretion of 17-OHCS in man by inhibiting the adrenal secretion of cortisol, but that *o,p'*-DDD caused an accelerated metabolism of cortisol to poorly extractable hydroxylated metabolites of cortisol. The stimulatory effect of *o,p'*-DDD, phetharbital, or other drugs on the extra-adrenal hydroxylation of cortisol could lead to the mistaken conclusion that a fall in easily extractable 17-OHCS in the urine reflects a decrease in adrenocortical production of cortisol.

The observations that diphenylhydantoin and *o,p'*-DDD enhance the 6 β-hydroxylation of cortisol in man and possess therapeutic value in Cushing's syndrome[11, 16–18, 21] suggest the possibility that liver microsomal enzyme inducers may have therapeutic effects in Cushing's syndrome. Southern *et al.* postulated that the increased amount of 6 β-hydroxycortisol that was found in subjects treated with *o,p'*-DDD may inhibit cortisol action and thereby alleviate the symptoms of Cushing's syndrome.[17] Phetharbital and other drugs which stimulate liver microsomal enzymes may have beneficial uses in other areas of medicine. For instance, Yaffe *et al.*[22] and Crigler and Gold[23] recently showed that chronic phenobarbital treatment markedly decreased the free bilirubin levels in the plasma of two children with congenital nonhemolytic jaundice. This effect could be explained by a stimulatory effect of barbiturates on the liver microsomal conjugation of bilirubin.[24]

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