Journal of Steroid Biochemistry, Vol. 10, pp. 397 to 400
Pergamon Press Ltd 1979. Printed in Great Britain

STEROID 17α-HYDROXYLASE OF THE RAT ADRENAL*

DONALD C. JOHNSON
Departments of Gynecology and Obstetrics & Physiology,
Ralph L. Smith Research Center, University of Kansas Medical Center,
Kansas City, KS 66103, U.S.A.

(*Received* 10 *August* 1978)

SUMMARY

The 105,000 *g* microsomal fraction of male or female rat adrenal contains steroid 17α-hydroxylase activity, as measured by a tritium exchange assay using 17α-[^{3}H]-pregnenolone as substrate. Administration of estrogen caused a pronounced increase in enzyme activity in the adrenals of both sexes, but the increase was greater in males. Ovine prolactin also caused an increase in hydroxylase in ovariectomized females. These results indicate that hydroxylase activity may play an important role in altering production of sex steroids by the adrenal.

INTRODUCTION

The adrenal cortex of the rat produces little, if any, 17α-hydroxylated corticosteroids, presumably because of a lack of steroid 17α-hydroxylase (EC 1.14.99.9) [1–4]. However, Young and Sweat[5] reported that the rat adrenal could produce 17α-hydroxyprogesterone if the 17α-hydroxylase system could be separated from the inhibition of the 11β-hydroxylase system. Presumably the latter normally prevented the action of the 17α-hydroxylase but the interaction of a mitochondrial system with a microsomal system was not clarified.

Several recent *in vivo* and *in vitro* studies [3, 6, 7] have indicated that the rat adrenal produces androgens and some reports [8] suggest that profound increases in serum estrogens can be associated with adrenal stimulation. Production of these steroids, which play important roles in reproductive functions, implies the presence and activity of a 17α-hydroxylase system, at least under some conditions. The introduction of a sensitive and specific tritium exchange assay for 17α-hydroxylase [9] prompted a restudy of this enzyme in the rat adrenal.

MATERIALS AND METHODS

Holtzman strain rats (200–250 g) were used: They were housed in plastic cages in air-conditioned (23 ± 1°C) quarters with free access to Purina laboratory chow and tap water. Lights were on 14 h/day from 0600 to 2000 h. Gonadectomy of females was performed, under ether anesthesia, via the dorsal approach while in males the testes were removed trans-scrotally. Hypophysectomy was performed, at the same time as ovariectomy, using the parapharyngeal approach. Some groups received daily subcutaneous injections of diethylstilbestrol (DES) or estradiol benzoate (EB) (Sigma Chemical Co, St. Louis, Mo.) dissolved in sesame-seed oil. When used, ovine prolactin (NIH-P-12; 35 IU/mg) and ACTH (Acthar; Armour Pharm. Co, Phoenix, AR) was dissolved in normal saline and injected subcutaneously.

Animals were killed by decapitation. The adrenals were removed, cleaned of adhering tissue, pooled, weighed on a torsion balance and homogenized in cold 0.15M KCl (100 mg wet weight tissue/ml). The homogenate was centrifuged for 30 min at 9000 *g* and then at 105,000 *g* for 60 min. The microsomal pellet was resuspended in 0.15 M $NaKPO_4$ buffer (pH 7.4) for assay of 17α-hydroxylase. The protein concentration of the suspension was determined using the Bio-Rad kit (Bio-Rad laboratories, Richmond, CA).

Hydroxylase activity was determined by the tritium exchange method of Kremers[9]. Pregnenolone-17α-[^{3}H] (15 mCi/mmol) was prepared by Dr. P. Kremers (University of Liege, Belgium). Unlabelled pregnenolone (Sigma Chemical Co.) was used to reduce the specific activity of the label to 2.4 μCi/μmol. All other chemicals used were obtained from Sigma.

The assay was performed in 20 ml glass scintillation vials. The medium (final volume = 1 ml) contained 100 nmol pregnenolone (0.25 μCi 17α-[^{3}H]-pregnenolone), 0.5 mg tween 80 to solubilize the steroid, 5 μmol glucose-6-PO_4, 1 IU glucose-6-PO_4-dehydrogenase, 1 μmol NADP, 4 μmol $MgCl_2$, $NaKPO_4$ buffer and 0.1 to 0.4 ml of adrenal microsomal suspension. The vials were incubated at 37°C in a Dubnoff shaking water bath. The reaction was stopped by addition of 1 ml of ice cold distilled water followed quickly by a 4 mg pellet of dextran-coated charcoal (IEM Screening System Inc., North Hollywood CA). The charcoal was separated by centrifugation at 2000 *g* for 30 min. The supernantant was transferred to a 25 × 200 mm glass tube and the water distilled under reduced pres-

* Presented in part at the Endocrine Society meeting, Miami Beach, Florida 1978.

sure at 40°C. A 1 ml aliquot of the distillate was placed in a scintillation vial, 10 ml Instagel (Packard Instrument Co., Downers Grove, Ill) added and the mixture counted in a Packard scintillation spectrometer with an efficiency of 64% for tritium. The enzyme activity was expressed as nmol of pregnenolone converted per mg protein per h. In all experiments the homogenates were incubated in triplicate and in most cases at two dose levels. Statistical analyses were done using Student's *t*-test; *P* values of less than 0.05 were considered significant in this study.

RESULTS

The enzyme activity as a function of the amount of adrenal microsomes (from female rats) incubated for 40 min is shown in Fig. 1. The enzyme activity in the equivalent of 20 or 80 mg (wet weight) of adrenal tissue was used in all subsequent assays. Hydroxylase activity as a function of incubation time is shown in Fig. 2. The response appeared to be essentially linear with time after the first 10 min; in all subsequent assays a 40 min incubation was used. Variation in the substrate concentration indicated an apparent K_M of 4.3 μM pregnenolone (Fig. 3).

The weight of the female adrenal (61.7 mg/pr; average for 10 pr) was 47% larger than that of males (42.0 mg/pr); ovariectomy for one week did not change adrenal weight. The 17α-hydroxylase activity of the female adrenal was 51% ($P < 0.001$) greater than that of males and loss of ovarian function for 7 days did not change activity (Fig 4). Adrenals from androgenized, constant estrus, females (weight 57 mg/pr) was 0.25 ± 0.04 nmol × mg protein^{-1} × h^{-1}, which is only 25% of the level found in females with normal cycles.

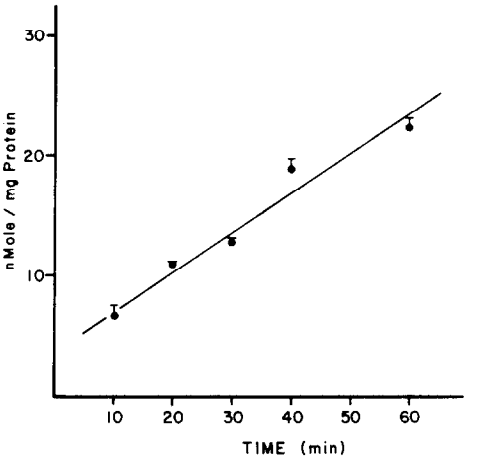

Fig. 2. Hydroxylase activity as a function of incubation time; same material assayed as in Fig. 1.

Administration of a potent estrogen (2 mg DES) for 4 days, with autopsy on the 5th day, produced a 28% increase in adrenal weight and a 45% ($P < 0.01$) increase in 17α-hydroxylase activity (Fig. 4). In males the DES treatment produced a 46% increase in adrenal weight and an increase of 153% in hydroxylase. After DES treatment males had slightly greater hydroxylase activity than did females (16%; $P < 0.05$). The stimulatory effect of DES was also evident in hypophysectomized females. While undetectable in untreated hypophysectomized rats, adrenal hydroxylase was measurable in animals given 1 IU ACTH

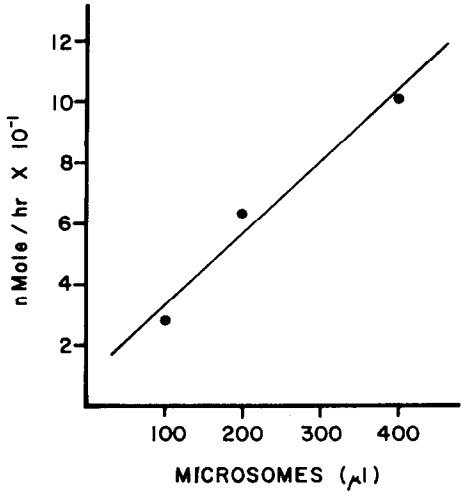

Fig. 1. Steroid 17α-hydroxylase activity as a function of the amount of rat adrenal homogenate incubated for 40 min (37°C) with 100 nmol pregnenolone. The 105,000 *g* fraction of female rat adrenals was suspended in $NaKPO_4$ (pH 7.4) buffer at a concentration of 200 mg/ml (wet weight equivalent). S.E.M. does not exceed the area covered by the points.

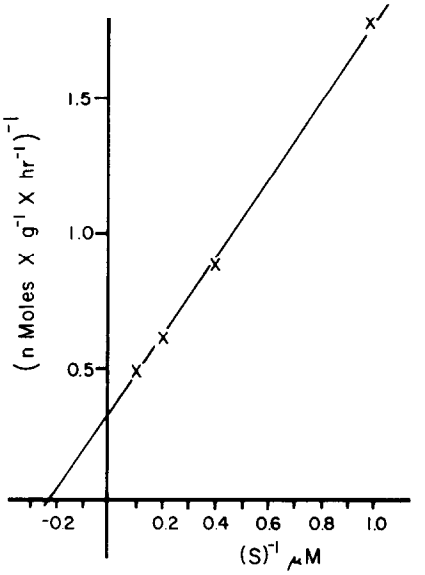

Fig. 3. The effect of varying the concentration of pregnenolone (S) on the 17α-hydroxylase activity in the microsomal fraction of the equivalent of 80 mg (wet weight) of rat adrenal; incubation time was 40 min. The apparent K_M is 4.3×10^{-6} M.

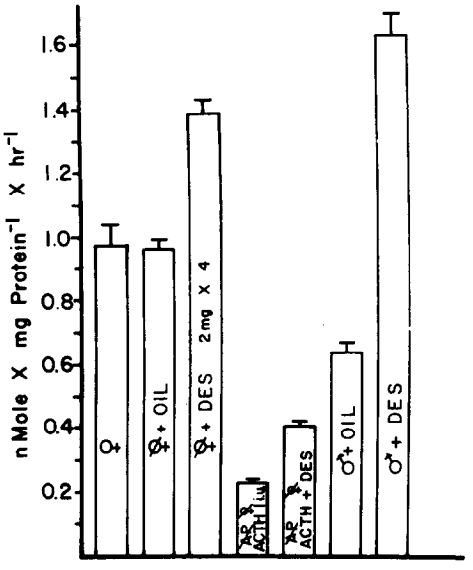

Fig. 4. 17α-hydroxylase activity in the 105,000 *g* fraction of rat adrenal homogenates. The adrenals from 10 animals on each treatment were pooled, homogenized and the microsomal fraction resuspended in $NaKPO_4$ buffer. Diethylstilbestrol (DES) was dissolved in 0.1 ml sesame seed oil and injected once daily. Ovariectomy and hypophysectomy were performed 7 days before treatment was started. Vertical lines indicate the S.E.M. for 6 determinations.

daily. Injection of 2 mg DES, as well as 1 IU ACTH, produced an enzyme level 75% larger than did ACTH alone ($P < 0.001$) (Fig. 4).

In a second study, female rats were ovariectomized 7 days prior to being randomly assigned to treatment groups. The control group received 0.2 ml of 0.15 M NaCl twice daily and 0.1 ml sesame-seed oil once daily (both subcutaneously). Injection of 5 μg estradiol benzoate (EB) in oil daily for 6 days, with autopsy on the 7th day, increased 17α-hydroxylase by 19% ($P < 0.05$); 50 μg EB/day increased activity 146% (Fig. 5). Because estrogens can cause an increase in serum prolactin levels and because the adrenal of the rat contains receptors for prolactin [10] the adrenals from animals given 500 μg (17.5 IU) of ovine prolactin twice daily for 6 days were assayed. This treatment did not alter adrenal weight but it did increase hydroxylase activity by 45% ($P < 0.01$). Included in the same assay were homogenates from female hamster and rabbit adrenals (Fig. 5). The hamster adrenals were removed from females without regard for the stage of the estrous cycle but all of the rabbit adrenals were obtained from females on the 6th day of pregnancy. The hydroxylase activity, on the basis of protein content of the microsomal fraction, was similar in the three species.

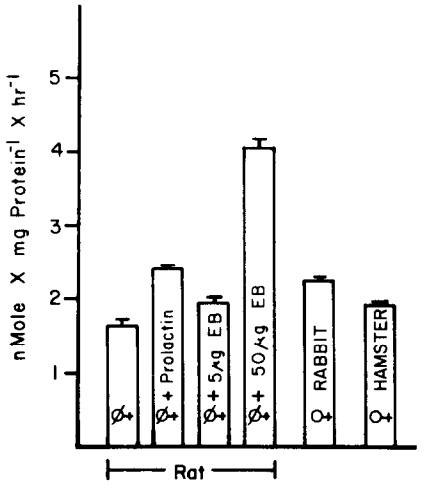

Fig. 5. Hydroxylase activity in the 105,000 *g* fraction of adrenal homogenates of ovariectomized female rats, intact female hamsters or rabbits. The adrenals were pooled prior to homogenization and 6 samples of each homogenate assayed. Ovine prolactin (500 μg, 2 ×/day) or estradiol (1 ×/day) benzoate was injected for 6 days.

DISCUSSION

The results of the present study clearly indicate that the rat adrenal has 17α-hydroxylase activity. While direct comparisons are difficult, the amount of enzyme appears to be about the same in rat, hamster or rabbit adrenals. In the latter two species the adrenals are known to readily produce and secrete 17α-hydroxy-corticosteroids whereas these compounds are not generally found in the rat [3, 4]. The reason for the apparent inactivity of the 17α-hydroxylase system in the rat, at least as far as corticosteroid production is concerned, is unknown. However, failure of several *in vitro* studies to demonstrate 17α-hydroxylase activity in adrenal homogenates may be related to the lability of the enzyme. When stored frozen in homogenates which contain 10 mg, or more, of protein per ml the enzyme activity remains constant for at least 2 months, but in protein concentrations of 1 mg, or less, the activity is lost within 48 h. In our first studies the adrenals were stored frozen in KCl for 24 h before homogenization and assay. Subsequent studies revealed that significant enzyme losses occurred with this schedule; no loss was incurred if the glands were frozen dry and assayed within 72 h.

The interactions between estrogens, ACTH and adrenal function are complex and have received considerable attention [references in 11]. Estrogen (DES) administration distinctly increased adrenal weight and 17α-hydroxylase activity in the animals of the present study. The mechanism(s) for this action is unknown but at least part of the effect was direct rather than via the pituitary and ACTH since hypophysectomized animals treated with DES + ACTH had more enzyme activity than did those getting ACTH alone. The effect of DES in this instance may have been to slow down the disappearance of enzyme already present at the time of hypophysectomy.

The increased enzyme activity found after estrogen treatment, and in females compared to males, led us

to suspect that constant estrus androgenized females would have high 17α-hydroxylase levels because they have consistently elevated levels of serum estradiol [12]; these animals also have high serum prolactin levels. However, the androgenized females had very low hydroxylase levels, actually much lower than that of males and not different from that of immature 21 day old females (data not shown). This finding suggests that the stimulatory action of estrogen, or prolactin, may be rather acute and short lived.

The stimulatory action of estrogen on adrenal 17α-hydroxylase is in distinct contrast to its inhibitory effect upon testicular [13] and ovarian (Johnson, unpublished data) hydroxylase. In the latter two organs the inhibitory action of estrogen is at least partially direct since it occurs in hypophysectomized as well as in intact animals. The nature of the inhibition is not clear but appears to involve an active process; i.e. the loss of enzyme activity is too rapid to be accounted for by half-life after removal of a trophic stimulus. We do not know as yet what is involved in the stimulatory effect of estrogen on adrenal 17α-hydroxylase.

In summary, the present study has indicated that the androgen, and subsequently the estrogen, produced in gonadectomized rats, could originate in the adrenal. The presence of 17α-hydroxylase activity and its alteration by various manipulations suggest the need for further study of the control processes for this enzyme.

Acknowledgements—Special thanks are due Dr. Pierre Kremers, of the University of Liege, for his help in setting up the assay of hydroxylase and especially for his preparation of the labelled pregnenolone. Also the excellent technical assistance of Mrs. M. Wagoner is acknowledged. This research was supported in part by a grant from the National Institute of Aging, U.S. Public Health Service.

REFERENCES

1. Brownell K. A., Lee S. L., Beck R. R. and Besch P. K.: *In vitro* 17α-hydroxylase of steroids by the adrenals of hypertensive rats. *Endocrinology* **72** (1963) 167–168.
2. Laplante C., Giroud C. J. P. and Stachenko J.: Lack of appreciable 17α-hydroxylase activity in the normal and regenerated rat adrenal cortex. *Endocrinology* **75** (1964) 825–827.
3. Koref O., Steczek K. and Feher T: Characteristics of the formation and conversion of androgenic hormones in the rat adrenal gland. *Acta endocr. Copenh.* **66** (1971) 727–736.
4. Milewich L. and Axelrod L. R.: The conversion *in vitro* of pregnenolone to C_{19} and C_{21} metabolites by the rat adrenal gland, with and without metyrapone. *J. endocr.* **54** (1972) 515–516.
5. Young R. B. and Sweat M. L.: Steroid 17α-hydroxylation in the rat adrenal gland. *Archs biochem. Biophys.* **121** (1967) 576–579.
6. Askari H. A.: Sexual differences in the biogenesis of the androgens by the adrenal cortex in rat. *Endocrinology* **87** (1970) 1377–1380.
7. Vinson G. P., Bell J. B. G. and Whitehouse B. J.: Production of testosterone and corticosteroids by the rat adrenal gland incubated *in vitro* and the effects of stimulation with ACTH, LH and FSH. *J. steroid Biochem* **1** (1976) 407–411.
8. Butcher R. L.: Changes in gonadotropins and steroids associated with unilateral ovariectomy of the rat. *Endocrinology* **101** (1977) 830–841.
9. Kremers P.: New assay for pregnenolone and progesterone 17α-hydroxylase based on the specific substitution of a tritium situated on carbon 17. *Eur. J. Biochem* **61** (1976) 481–486.
10. Posner B. I., Kelly P. A., Shiu R. P. C. and Friesen H. G.: Studies of insulin, growth hormone and prolactin binding: tissue distribution, species variation, and characterization. *Endocrinology* **95** (1974) 521–532.
11. Colby H. D. and Kitay J. I.: Relation of the thyroid gland to the effects of estradiol on adrenal corticosterone secretion in rats. *Proc. Soc. Exp. Biol. Med. (NY)* **153** (1976) 75–77.
12. Mallampati R. S. and Johnson D. C.: Gonadotropins in female rats androgenized by various treatments: Prolactin as an index to hypothalamic damage. *Neuroendocrinology* **15** (1974) 255–266.
13. Kremers P., Tixhon Ch. and Gielen J.: 17α-hydroxylase and testosterone biosynthesis in rat testes. *J. steroid Biochem.* **8** (1977) 873–877.