Female Pattern of Metabolism of [4-^{14}C]Corticosterone in Male Pseudohermaphroditic Rats (37096)

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The quantitatively predominant corticosteroid in the rat is corticosterone. The metabolism of this steroid has been studied extensively and it has been demonstrated that male and female rats metabolize corticosterone along different pathways. Thus, bile from female rats contains 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one monosulfate and 3β,11β,21-trihydroxy-5α-pregnan-20-one disulphate as the predominant metabolites (1) whereas male rats do not form any 15-hydroxylated corticosterone metabolites but excrete disulfates of 3β,11β,21-trihydroxy-5α-pregnan-20-one and 5α-pregnane-3β,11β,20β,21-tetrol in bile (2). Little is known about the development of sexual differences in corticosterone metabolism in rats *in vivo* and the effects of androgens and estrogens on this development. It was therefore of interest to investigate how corticosterone is metabolized *in vivo* by the male pseudohermaphroditic rat. These rats have an inherited defect leading to lack of androgen-dependent differentiation with absence of reproductive organs other than inguinal testes (3). Bardin and co-workers have shown that the failure of the rats to androgenize is due to end-organ insensitivity to testosterone (4). However, large doses of testosterone may androgenize pseudohermaphroditic rats (4, 5) in the present study the effect of large doses of testosterone propionate on corticosterone metabolism *in vivo* in the pseudohermaphroditic rat was also studied. The pseudohermaphroditic rats can respond to estrogen treatment (6) and in another experiment the effect of estradiol benzoate treatment on corticosterone metabolism was studied.

Materials and Methods. Steroids. [(4-^{14}C)]Corticosterone (sp. act. 56.7 mCi/mmole) was purchased from the Radiochemical Centre (Amersham, England). The sources and syntheses of other steroids used in the present investigation have been described in previous publications (1, 2).

Analytical methods. Gas–liquid chromatography, radio–gas chromatography, and gas chromatography–mass spectrometry were carried out as described previously (1, 2).

Enzymes. Digestive juice from *Helix pomatia* was purchased from Industrie Biologique Francaise (Gennevilliers, France).

Animal experiments. One female, one male, and two male pseudohermaphroditic rats of the King X-Holtzman strain were used in one experiment. In another experiment one pseudohermaphroditic rat was treated daily for 2 weeks with 0.5 ml of dimethylsulfoxide intramuscularly, another pseudohermaphroditic rat with 50 mg of testosterone propionate in 0.5 ml of dimethylsulfoxide (daily for two weeks) and a third pseudohermaphroditic rat with 1.0 mg of estradiol benzoate in 0.5 ml of dimethylsulfoxide (daily for

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two weeks). The common bile duct was cannulated in all seven rats used in the two experiments. The rats were kept in restraining cages with free access to saline and food pellets. Twenty-four hours after operation all rats were injected intraperitoneally with 1 μCi of [4-^{14}C]corticosterone in a 1% (w/v) solution of serum albumin in saline. Bile and urine were collected in 24 hr portions for 3 days. The rats treated with testosterone propionate, estradiol benzoate and dimethylsulfoxide alone received daily intramuscular injections also during the bile collection period. Aliquots of the bile and urine samples were assayed for radioactivity. The samples were frozen immediately after collection and stored at —15° until analyzed.

Extraction and purification of biliary steroids. Pooled bile from 3 day collection was extracted with ten volumes of acetone-ethanol (1:1, v/v) for 24 hr at 39° (7). After filtration, the extract was evaporated to dryness under vacuum and was then dissolved in 10 ml of methanol. Aliquots of the extract were taken for radioactivity measurement and 2.5 ml was evaporated to dryness, dissolved in 4 ml of chloroform-methanol (1:1, v/v), 0.01 *M* with respect to NaCl, and applied on a 15 g Sephadex LH-20 column prepared in and eluted with the same solvent. The radioactivity was eluted in three fractions: 0–100 ml of effluent (free steroid + steroid glucuronide fraction), 100–250 ml of effluent (steroid monosulfate fraction) and 250 ml of methanol (steroid disulfate fraction). Aliquots of these fractions were taken for radioactivity measurement and 1/20 was analyzed by thin-layer chromatography using the solvent system ethyl acetate–ethanol–15 *M* ammonium hydroxide (5:5:1, by vol). The mono- and disulfate fractions contained material with the same mobilities as reference steroid mono- and disulfates, respectively. No steroids or steroid conjugates were detected in the free steroid + steroid glucuronide fraction.

The free steroid + steroid glucuronide fraction contained very small amounts of radioactivity and were not worked up further. The mono- and disulfate fractions were hydrolyzed in 17 ml of 0.5 *M* sodium acetate buffer at pH 4.5 for 48 hr by the digestive juice of *Helix pomatia* which was added at the start (0.2 ml) and after 24 hr of incubation at 37° (0.2 ml). The incubation mixture was added to a 10-g column of Amberlite XAD-2 in water which was then washed with 50 ml of water and 5 ml of hexane. The steroids were eluted with 100 ml of ethanol. The dried ethanol eluate was solvolysed for 24 hr at 37° in ethyl acetate acidified with 2 *M* H_2SO_4. The ethyl acetate phase was washed with 8.4% (w/v) $NaHCO_3$ until alkaline and with water until neutral. An aliquot of the extract was taken for radioactivity measurement. The radioactivity measurements were performed in a Packard Liquid Scintillation Counter (Model 4322) using the scintillator liquid Instagel (Packard Instrument Company, Inc., Ill. and internal quenching corrections.

Half of the hydrolyzed and solvolysed extracts from the mono- and disulfate fractions was analyzed after preparation of (trimethyl)silyl ethers using 0.3 ml of pyridine. The reaction was allowed to proceed overnight to obtain complete silylation of the 11β-hydroxyl group of the corticosterone metabolites. Quantitation of these steroids was performed by gas–liquid chromatography using cholesteryl butyrate as an internal standard. In a recent paper Cronholm reported that the response factors of a mixture of isomers of 3,11β,21-trihydroxypregnan-20-one and a mixture of isomers of pregnane-3,11β,20β,21-tetrol in relation to cholesteryl butyrate (using gas–liquid chromatography with a flame ionization detector) were 0.7 and 1.1, respectively (8). In the present investigation the response factor 0.7 was used for 3α(and 3β),11β,21-trihydroxy-5α-pregnan-20-one and 1.1 for 5α-pregnane-3α(and 3β),11β,20β,21-tetrol. The response factor for 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one could not be determined but was set to be 1.0. All samples were also analyzed by radio–gas chromatography and by gas chromatography–mass spectrometry. A steroid was considered to be identified only if it had a gas chromatographic mobilility (on SE-30 and QF-1) and a mass spectrum very similar to those

TABLE I. Relative Retention Times (Relative to 5α-Cholestane, $t_R = 1.00$) on 1% SE-30 and 3% QF-1 and Concentrations (μg/24 hr Excreted by one Rat) of Metabolites of Corticosterone in Bile from Normal, Dimethylsulfoxide, Testosterone Propionate and Estradiol Benzoate-Treated Male Pseudohermaphroditic Rats and from Normal Female and Male Rats.

Type of rat, steroid fraction		t_R SE-30	t_R QF-1	μg/24 hr
Normal male pseudohermaphroditic rats				
Monosulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.00	2.52[a]	9
	3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one	2.46	2.62[a]	121
Disulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	1.97	2.67[a]	23
	3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one	2.42	2.67[a]	28
	3β,11β,21-trihydroxy-5α-pregnan-20-one	2.70	3.79[a]	190
Dimethylsulfoxide treated male pseudohermaphroditic rat				
Monosulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.03	2.61[a]	9
	3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one	2.49	2.61[a]	70
Disulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.02	2.66	1
	3β,11β,21-trihydroxy-5α-pregnan-20-one	2.77	3.79	40
Testosterone propionate-treated male pseudohermaphroditic rat				
Monosulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.01	2.69	8
Disulfate fraction	3β,11β,21-trihydroxy-5α-pregnan-20-one	2.77	3.81	235
Estradiol benzoate-treated male pseudohermaphroditic rat				
Monosulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.01	2.65[a]	13
	3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one	2.46	2.65[a]	85
Disulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.01	2.69[a]	31
	3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one	2.48	2.69[a]	20
	3β,11β,21-trihydroxy-5α-pregnan-20-one	2.76	3.84	41
Normal female rat				
Monosulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.03	2.64[a]	16
	3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one	2.49	2.64[a]	202
Disulfate fraction	3α,11β,21-trihydroxy-5α-pregnan-20-one	2.00	2.67[a]	19
	3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one	2.45	2.67[a]	43
	3β,11β,21-trihydroxy-5α-pregnan-20-one	2.71	3.83	37
Normal male rat				
Monosulfate fraction	5α-pregnane-3β,11β,20β,21-tetrol	3.75	2.56	8
Disulfate fraction	3β,11β,21-trihydroxy-5α-pregnan-20-one	2.79	3.83	137
	5α-pregnane-3β,11β,20β,21-tetrol	3.74	2.56	270

[a] 3α,11β,21-trihydroxy-5α-pregnan-20-one and 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one did not separate on the QF-1 column.

of a reference steroid or a steroid previously identified.

Results. Excretion of [4-^{14}C]corticosterone and recovery of radioactivity. About 86% of the injected radioactivity was recovered from the bile and about 8% was recovered from the urine. No significant variations in these figures were obtained between the different groups of animals investigated. The recovery of biliary radioactivity after extraction and chromatography on Sephadex LH-20 was about 76%. After fractionation on Sephadex LH-20 all extracts showed a similar distribution of radioactivity: about 2% (1–4%) of the radioactivity recovered after the chromatography was found in the free + glucuronide fraction, about 30% (23–37%) in the monosulfate fraction and about 68% (61–76%) in the disulfate fraction. After hydrolysis and solvolysis of the monosul-

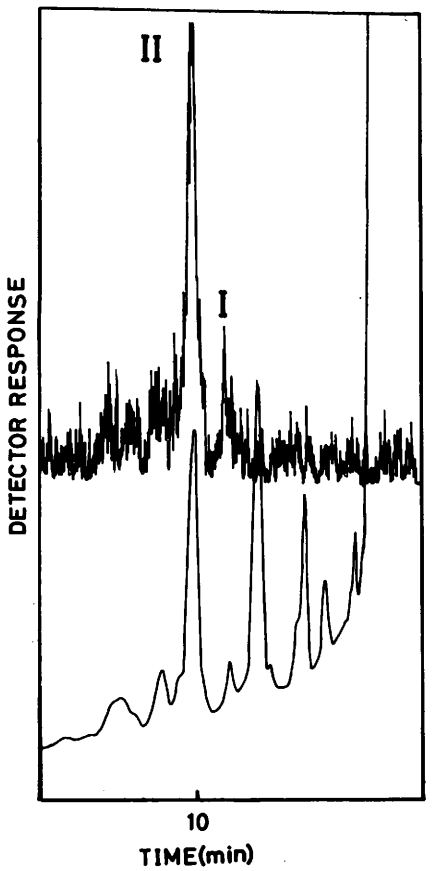

FIG. 1. Radio–gas chromatographic analysis (SE-30 column) of silyl ethers of steroids in the monosulfate fraction of bile from female rats. The lower curve represents the flame ionization detector response and the upper curve represents the recording of radioactivity. Peaks I and II were identified as 3α,11β,21-trihydroxy-5α-pregnan-20-one and 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one, respectively.

fate fraction about 84% of the radioactivity present in this fraction was recovered as free steroids. The corresponding figure for the disulfate fraction was about 82%.

Identification and quantitation of steroids. Table I summarized the identifications and quantitations of corticosterone metabolites in bile from male, female and male pseudohermaphroditic rats, and from male pseudohermaphroditic rats treated with testosterone propionate, estradiol benzoate or dimethylsulfoxide alone. Radio–gas chromatographic analysis of the silylated steroids on SE-30 (cf. Figs. 1 and 2) showed that the identified corticosterone metabolites were radioactive. The identifications were based on gas–liquid chromatography–mass spectrometry. The steroids identified have previously been described in bile from male and female rats (1, 2).

The male pseudohermaphroditic rats were shown to excrete metabolites of corticosterone that were identical to those excreted by the female rats. Thus, 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one was present in the monosulfate fraction—where it represented the quantitatively predominant steroid hormone metabolite—and in the disulfate fraction of bile from both female and male pseudohermaphroditic rats. As expected, no 15-hydroxylated corticosterone metabolites were found in bile from the male rat. The quantitatively predominant biliary steroid in this rat was 5α-pregnane-3β,11β,20β,21-tetrol disulfate which was absent from bile from both female and male pseudohermaphroditic rats.

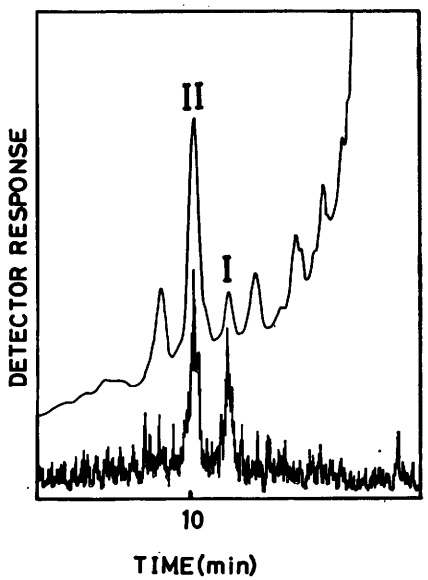

FIG. 2. Radio–gas chromatographic analysis (SE-30 column) of silyl ethers of steroids in the monosulfate fraction of bile from male pseudohermaphroditic rats. The upper curve represents the flame ionization detector response and the lower curve represents the recording of radioactivity. Peaks I and II were identified as 3α,11β,21-trihydroxy-5α-pregnan-20-one and 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one, respectively.

The control male pseudohermaphroditic rat treated with dimethylsulfoxide showed a reduction in total output of biliary steroids. No quantitative differences were seen in the pattern of biliary corticosterone metabolites in the male pseudohermaphroditic rat treated with estradiol benzoate when compared to the control rat. Treatment with testosterone propionate, on the other hand, resulted in disappearance of 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one both from the mono- and disulfate fractions of bile. No steroids were found in the monosulfate fraction following treatment with testosterone propionate.

Discussion. Several authors have reported sexual differences in the metabolism of corticosteroids by rat liver *in vitro* (see, *e.g.*, 9, 10). *In vivo* studies on the metabolism of [4-^{14}C]corticosterone in rats have revealed sexual differences that are even greater than could be expected from *in vitro* results. One of the most interesting differences is the occurrence of 15-hydroxylated corticosterone metabolites in urine, feces, and bile from female rats and the total absence of these metabolites from male rats (1, 2, 11, 12); no hydroxylated corticosterone derivatives have been identified in bile from male rats (2). These characteristics of corticosterone metabolism in male and female rats are somewhat unexpected since male rats generally hydroxylate steroids (and drugs) more efficiently than female rats (13).

The present study demonstrates that male pseudohermaphroditic rats metabolize corticosterone in a similar way to female rats. Thus, pseudohermaphroditic rats were found to excrete radioactive 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one mono- and disulfate after injection of [4-^{14}C]corticosterone. Actually the qualitative pattern of corticosterone metabolites in male pseudohermaphroditic rats was identical to that in female rats. One of the quantitatively predominant metabolites in bile of male rats, 5α-pregnane-3β,11β,20β,21-tetrol disulfate, was absent from bile from both male pseudohermaphroditic and female rats.

Previous studies with isolated perfused rat livers have demonstrated that corticosterone is 15-hydroxylated in the liver (14). The finding of 15-hydroxylated corticosterone metabolites in bile from male pseudohermaphroditic rats shows that hepatic 15-hydroxylation of corticosterone is not dependent on ovarian steroid secretion. Treatment of male pseudohermaphroditic rats with large doses of testosterone propionate abolished the excretion of 15-hydroxylated metabolites showing a suppressive effect of androgens on hepatic 15-hydroxylation of corticosterone. The finding that male pseudohermaphroditic rats respond to large doses of testosterone is not surprising since similar effects of heavy androgen load have been noted when other potentially androgen-responsive parameters have been studied in these rats (5).

Preliminary experiments have shown that castration of adult male rats does not lead to the appearance of 15-hydroxylated corticosterone metabolites in bile (15). This is probably explained by the "imprinting" effects testicular androgens have on the steroid metabolizing enzymes of the liver in the neonatal period (10, 16). Thus, neonatal testectomy results in a female type of hepatic cortisol metabolism *in vitro* in the adult rats (10). The results presented in this paper demonstrate that male pseudohermaphroditic rats metabolize corticosterone *in vivo* in a similar way to female rats. This may be due to the lack in pseudohermaphroditic rats of a postnatal testosterone imprinting on the liver (due to end-organ insensitivity to testosterone and/or deficient testicular biosynthesis of androgens) combined with hepatic insensitivity to testosterone in adult life.

Summary. The metabolites of [4-^{14}C]corticosterone appearing in bile fistulae of male pseudohermaphrodites and littermate male and female Stanley–Gumbreck rats were determined by gas–liquid chromatography, radio–gas chromatography and gas chromatography–mass spectrometry. Male pseudohermaphroditic rats excrete corticosterone metabolites identical to those in bile from female rats: 3α,11β,21-trihydroxy-5α-pregnan-20-one and 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one in the monosulfate fraction and 3α,11β,21-trihydroxy-5α-pregnan-20-one, 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one, and 3β,11β,21-trihydroxy-5α-pregnan-20-one in the

disulfate fraction. Male rats excrete 5α-pregnane-3β,11β,20β,21-tetrol in the monosulfate fraction and 3β,11β,21-trihydroxy-5α-pregnan-20-one and 5α-pregnane-3β,11β,20β,21-tetrol in the disulfate fraction. The female type of corticosterone metabolism in the male pseudohermaphroditic rat is probably due to the lack in these animals of a postnatal testosterone imprinting effect on the liver combined with hepatic insensitivity to testosterone in adult life. Large doses of testosterone propionate completely suppress the excretion of 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one in bile from a pseudohermaphroditic rat indicating a relative rather than an absolute testosterone insensitivity of the liver of these rats.

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