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ISOLATION AND CHARACTERIZATION OF CORTISOL METABOLITES FROM LIVER OF ADRENALECTOMIZED RATS

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SUMMARY

[14-^{14}C]-cortisol was administered intraperitoneally to adrenalectomized female rats. After 15 min the rats were killed by decapitation. Liver homogenates prepared in 70% methanol were centrifuged. The supernatant was extracted with organic solvents. The radioactivity remaining in the aqueous fraction was about 14% of the total counts in the original supernatant. The metabolites in the aqueous fraction were identified as 3α,11β,17α-trihydroxy-5α-pregnan-20-one-21-yl-sulphate and 3β,11β,17α-trihydroxy-5α-pregnan-20-one-21-yl-sulphate. The ratio of the former to the latter was 7:3. Two of the U.V. absorbing materials which were eluted in the steroid fraction after gel filtration through Sephadex column were identified as inosine and hypoxanthine.

INTRODUCTION

EVIDENCE for the early conversion of corticosteroids to various metabolites in the liver was demonstrated by several investigators[1–4]. Shortly after the administration of labelled corticosteroids to rats, radioactive material accumulated in the supernatant and microsomal fractions of the liver[1–3]. When [^{14}C] cortisol was administered intraperitoneally to rats, the radioactivity was concentrated in the cytoplasm and microsomal fraction and reached a peak at 45 min while the induction of tyrosine transaminase occurred 15 min later[4]. In support of this finding other investigators[5] reported that the induction of tyrosine transaminase and tryptophan pyrrolase was observed about 60 min after the intraperitoneal administration of a single dose of cortisone acetate to adrenalectomized rats. Since the accumulation of injected corticosteroids in the liver occurred prior to any demonstrable biological action, it is conceivable that the administered steroids could have undergone transformation within a short time to an active metabolite(s). Testosterone has been demonstrated to be converted to a biologically active metabolite, dihydrotestosterone[6].

The formation of sulphate conjugates of corticosteroids was presented by Litwack and co-workers[7–9] who showed that radioactive cortisol administered intraperitoneally was converted rapidly into unknown anionic metabolites in the liver. These metabolites, presumably sulphate conjugates were bound to cytosol proteins. In a similar type of study Morris *et al.*[10] reported that the radioactive complex isolated from rat liver after injection of [4-^{14}C]-cortisol consisted of steroid, polynucleotide, and peptide with a molecular weight of 5000 daltons. An unidentified cortisol conjugate was reported to occur in the circulation of human patients[11].

In the present study [^{14}C]-cortisol was administered to adrenalectomized rats

and the early conjugated metabolites formed in the liver were identified. Adrenalectomized rats were used to avoid possible interference by endogenous corticosteroids in the analysis of the metabolites. The identification of the metabolites might contribute information on the possible mechanism of action of corticosteroids.

EXPERIMENTAL

Materials

[4-C^{14}] Cortisol (S.A. 152 μCi/mg) was purchased from New England Nuclear, Boston, U.S.A.; 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one, tetrahydrocortisol (THF: 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one); hypoxanthine, cortisol-21-phosphate and sulphatase of *Helix pomatia* from Sigma Chemical Co., St. Louis, U.S.A.; 3β,11β,17α,21-tetrahydroxy-5α-pregnan-20-one (Reichstein's substance "V") 3β,11β,17α,21-tetrahydroxy-5β-pregnan-20-one from Ikapharm, Israel; β-glucuronidase from Worthington Biochemical Corporation, Freehold, U.S.A.; [^{14}C] cortisol-21-sulphate was a generous gift of Dr. D. Fukushima. Thin layer silica gel chromatogram sheet 6060 was purchased from Eastman Kodak Co., Rochester, U.S.A.; Whatman No. 1 paper was pre-washed with water and methanol.

Methods

Radioactivity was counted in a Packard Tricarb Scintillation Spectrometer Model 3003. The radioactive chromatogram was scanned by Packard radiochromatogram scanner Model 7201. Melting points were determined in a Thomas–Hoover melting point apparatus and were uncorrected. Elemental analysis was done by the Rockefeller University microanalysis laboratory. Ultra-violet spectra were obtained with Cary 15 spectrophotometer. Infrared spectra were measured with a Perkin–Elmer infrared spectrophotometer, Model 237B using KBr pellets. Nuclear magnetic resonance spectra were taken with a Varian HR-220 N.M.R. spectrophotometer using pyridine-d_5 or deuterium oxide as solvent and tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard. High voltage paper electrophoresis was performed in a Savant electrophoresis tank using formic acid-acetic acid mixture at pH 1·9 and pyridine-acetic acid at pH 6·4.

Methylene blue test was carried out as described by Roy[12] and Crepy[13]. Rhodizonic acid test was performed according to the procedure of Burma[14] and Schneider[15]. Periodic acid–benzidine test was performed as described by Gordon *et al.*[16]. The reaction of steroid sulphate with periodic acid was performed by dissolving small quantity of steroid sulphate in 1 ml of 70% methanol, and by the addition of 10 mg of periodic acid. The reaction mixture was left at room temperature for 6 h. The solvent was removed and the reaction mixture spotted on paper and subjected to high voltage electrophoresis. Samples were treated with β-glucuronidase by incubation in 0·1 M acetate buffer, pH 5, at 37° for 10 h. Steroid sulphate was hydrolyzed with sulphatase by incubating in 0·1 M acetate buffer, pH 5, at 37°C overnight. The hydrolyzed steroid was purified by high voltage electrophoresis at 3000 V, pH 1·9, for 4 h and by paper chromatography in a system of *iso*octane-*t*-butanol–water (10:5:9 by vol).

Purification Procedures. Adrenalectomized female rats weighing about 200 g were purchased from Holtzman Co., Madison, U.S.A. and utilized 7 days after

operation. The rats were maintained on regular Purina chow and saline as drinking water *ad libitum*. [4-^{14}C]-Cortisol (5 μCi) with carrier of 1 mg of cortisol-21-phosphate was administered intraperitoneally to each rat. The rats were sacrificed 15 min later. Each experiment was conducted with 25 rats. In ten separate experiments variations in the recoveries of radioactivity in the various fractions were within 5%. Liver was perfused with saline, excised and homogenized in 70% methanol (5 ml/g) as shown in scheme I. The homogenate was centrifuged at 1200 *g* for 10 min. The supernatants were pooled and flash evaporated to remove the methanol. The concentrated solution was extracted in sequence with petroleum ether, dichloromethane and *n*-butanol. The remaining aqueous fraction was lyophilized. The lyophilized powder was extracted twice with 70% methanol. After removal of methanol by flash-evaporation, the concentrated aqueous fraction was added to acetone with constant stirring. The precipitate was removed by centrifugation. The acetone-water extract was flash-evaporated and the residue chromatographed on paper in a system of benzene–methanol–water (10:9·2:1 by vol). In this step the radioactive metabolites and U.V. absorbing materials (maxima 248 nm) were separated.

The major radioactive peak (R_F 0·75) and U.V. absorbing material (R_F 0·2) were extracted separately with 70% methanol. The radioactive and U.V. absorbing materials were subjected to high voltage paper electrophoresis at 3000 V, pH 1·9, for 4 and 10 h, respectively. Three peaks of radioactivity were observed with the former fraction: fast-moving, intermediate-moving and slow-moving peaks which were designated as steroids No. 1, No. 2 and No. 3, respectively (Fig. 1). The steroid No. 2 was the major component. This fraction was further purified by three paper chromatographic systems: *iso*-butanol–acetic acid–water (6:1:4 by vol), R_F 0·64; ethyl acetate–*iso*-butanol–3% ammonia (8:4:12 by vol), R_F 0·41; benzene–methanol–water (10:9·2:1 by vol), R_F 0·75. Figure 2 illustrates a scanning record of the radioactivity of the final purified product.

When subjected to electrophoresis, the U.V. fraction was separated into two zones with mobilities of 6·5 and 25 cm toward cathode. The slow and fast-moving peaks were designated as U.V. fraction No. 1 and No. 2. Both fractions possessed a U.V. absorption maximum of 248 nm. Each fraction was further purified by paper chromatography in a system of benzene–methanol–water (10:9·2:1 by vol). Fraction No. 1 was crystallized from water and fraction No. 2 from a water–methanol mixture. Needle-like crystals were obtained from both fractions.

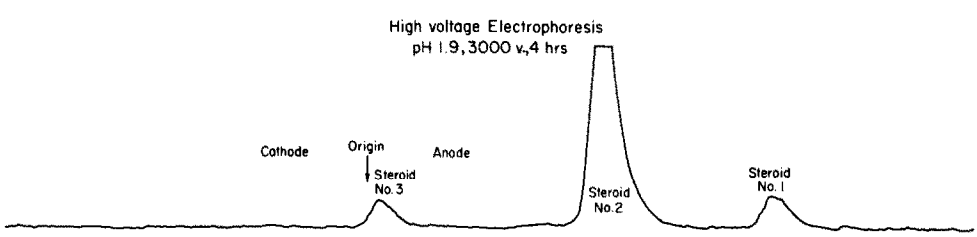
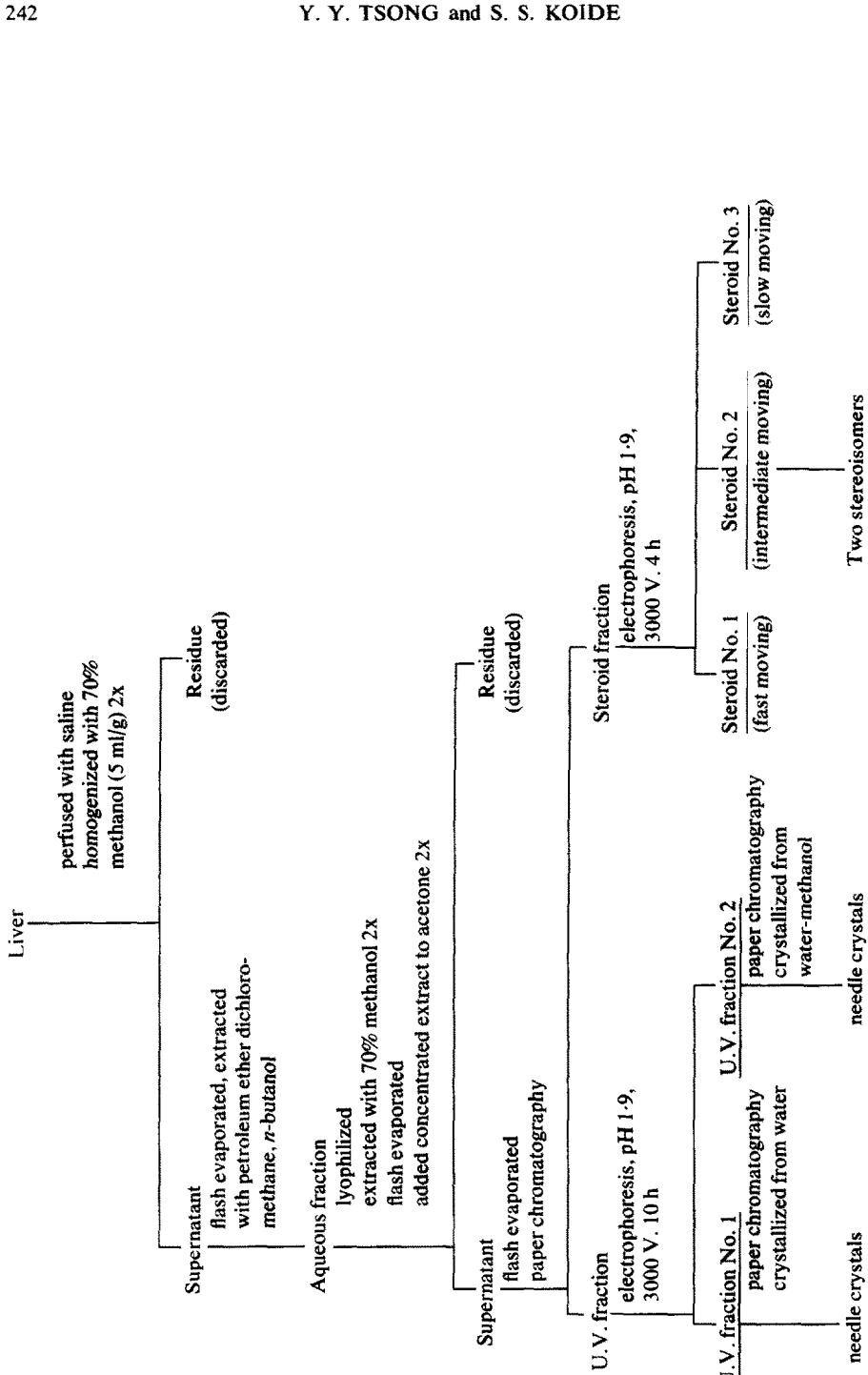


Fig. 1. Scanning record of radioactivity of cortisol metabolites on the electrophoregram. The major radioactive peak found in the first paper chromatogram of cortisol metabolites was extracted with 70% methanol and subjected to high voltage electrophoresis at 3000 volts for 4 h in formic acid–acetic buffer (pH 1·9). The steroid No. 2 was extracted and purified by paper chromatography.



Scheme 1. Purification of steroids and U.V. absorbing compounds.

Fig. 2. Scanning record of radioactivity of purified steroid No. 2 metabolite following paper chromatography. The system was developed in benzene-methanol-H_2O (10:9·2:1 by vol). Details of the purification procedures are given in Scheme 1.

RESULTS

The recoveries of radioactivity during the purification steps are shown in Table 1. Steroid No. 2 accounted for 7% of the radioactivity present in the original 70% methanol extract. It should be noted that only 2% of radioactivity was recovered in the fourth *n*-butanol extract.

Characterization of steroid No. 2

The scanning record of radioactivity of the final paper chromatogram showed a symmetrical peak demonstrating that the compound was relatively pure. We were unable to obtain a crystalline product. Purity of the compound was further checked by thin layer chromatography in 3 systems and by paper chromatography in 3 systems and by high voltage paper electrophoresis in 2 systems (Table 2). In all of these systems, the final steroid No. 2 compound exhibited a single spot. The compound gave a positive reaction with methylene blue and rhodizonic acid reagents, indicating that it was probably a sulphate ester. This notion was supported by the finding that the compound was hydrolyzed with sulphatase but not

Table 1. Recovery of radioactivity from adrenalectomized rat livers during various steps of purification

Procedures	Recovery of radioactivity (d.p.m.)	%
Initial 70% methanol extract	$1{\cdot}27 \times 10^7$	100
Petroleum ether X3	$1{\cdot}32 \times 10^6$	10
Dichloromethane X3	$2{\cdot}68 \times 10^6$	21
n-Butanol X3	$6{\cdot}80 \times 10^6$	53
n-Butanol 4th	$2{\cdot}70 \times 10^5$	2
Extracted aqueous fraction	$1{\cdot}80 \times 10^6$	14
70% methanol extract of lyophilized powder	$1{\cdot}94 \times 10^6$	15
Acetone–water extract	$1{\cdot}41 \times 10^6$	11
Paper chromatography extract	$1{\cdot}07 \times 10^6$	8
High voltage electrophoresis		
Steroid No. 1 (fast moving peak)	$1{\cdot}29 \times 10^5$	1
Steroid No. 2 (intermediate moving peak)	$8{\cdot}27 \times 10^5$	7
Steroid No. 3 (slow moving peak)	$1{\cdot}22 \times 10^5$	1

[4-^{14}C]-Cortisol ($1{\cdot}13 \times 10^8$ d.p.m. equivalent to 328 μg) was administered to 25 rats.

Table 2. Chromatographic and electrophoretic characteristics of steroid No. 2

Thin layer chromatography:	R_F	mobility (cm)
methanol–chloroform (1 : 9 v/v)	0·1	—
acetone	0·28	—
methanol–ethyl acetate (3 : 7 v/v)	0·31	—
Paper Chromatography		
Benzene–methanol–H_2O (10 : 9·2 : 1 by vol)	0·75	—
iso-Butanol–acetic acid–H_2O (6 : 1 : 4 by vol)	0·64	—
Ethyl acetate–*iso*-butanol–3% NH_4OH (2 : 1 : 3 by vol)	0·41	—
High voltage paper electrophoresis (3000 V, 4 h)		
pH 1·9 (toward anode)	—	22
pH 6·4 (toward anode)	—	23

with β-glucuronidase. The hydrolyzed product did not move when subjected to analysis with high voltage electrophoresis. When steroid No. 2 and authentic cortisol-21-sulphate were subjected to high voltage electrophoresis, the two compounds showed identical mobility, indicating that steroid No. 2 was a monosulphate ester. The compound did not exhibit any ultraviolet absorption. Its infrared spectrum showed absorption peaks at 3420, 1725, and 1200 cm^{-1} indicating the presence of hydroxyl, carbonyl and sulphate groups, respectively. These findings verify the fact that sulphate moiety exists in steroid No. 2 and indicated that the 3-keto group and the C4-5 double bond of cortisol were reduced. Moreover, the N.M.R. spectrum showed peaks at δ value of 0·88 (3H), 1·02 (3H), 3·49 (1H), 4·13 (1H) and 4·23 (2H) ppm, indicating that the metabolite is a tetrahydrocortisol sulphate. Steroid No. 2 did not react with tetrazolium blue reagent. However, after treatment with sulphatase, the product gave a positive test with tetrazolium blue reagent. These results suggest that the sulphate moiety is at C-21[17]. Steroid No. 2 was treated with periodic acid and its mobility checked by electrophoresis. The product remained near the origin, indicating that the sulphate group was eliminated and was probably located at C-21 since Calvin and Lieberman[18] showed that sulphate group at C-3 position was unaffected on treatment with periodic acid. Under identical conditions the sulphate group of authentic cortisol-21-sulphate was split off.

Steroid No. 2 was treated with sulphatase and the product purified by high voltage electrophoresis (pH 1·9, 3000 V, for 4 h) and by paper chromatography using Bush E_2B solvent system. The radioactive peak on the chromatogram was asymmetrical, suggesting that the product contained more than one stereoisomer. In our hands we were unable to separate the various stereoisomers by TLC. An alternate paper chromatographic system using petroleum ether–benzene–methanol–water (2 : 8 : 5 : 5 by vol) was tried but the stereoisomers were not separated. I.R. spectrum of the major component of the hydrolyzed product of steroid No. 2 obtained after paper chromatography showed peaks at 3420, 1172 and 1000 cm^{-1}. In an A/B trans system 1000 cm^{-1} indicates that an axial OH is present at C-3 position. Hence, it can be concluded that the major com-

ponent is most likely 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one. Because of the limited quantity of materials used, only two peaks were discerned in the N.M.R. spectrum which showed values of 1·21 and 1·38 ppm, corresponding to the C-18 and C-19 methyl protons of 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one.

The hydrolyzed steroids were recrystallized with 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one, tetrahydrocortisol, 3β,11β,17α,21-tetrahydroxy-5α-pregnan-20-one and 3β,11β,17α,21-tetrahydroxy-5β-pregnan-20-one. Constant specific radioactivity was obtained with 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one and 3β,11β,17α,21-tetrahydroxy-5α-pregnan-20-one (Tables 3 and 4). The approximate ratio of the amount of the former compound to the latter in the steroid No. 2 fraction was 7 : 3.

Table 3. Recrystallization with authentic 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one

Number of crystallization	Solvent	Radioactivity in crystals (d.p.m./mg)	Mother liquid (d.p.m./mg)
first	Methanol–ether–petroleum ether	1521	2585
second	Acetone–petroleum ether	1115	1668
third	Ethyl acetate	1129	1114
fourth	Methanol–ether–petroleum ether	1112	1121

Table 4. Recrystallization with authentic 3β,11β,17α,21-tetrahydroxy-5α-pregana-20-one

Number of crystallization	Solvent	Radioactivity in Crystals (d.p.m./mg)	Mother liquid (d.p.m./mg)
First	Methanol–ether–petroleum ether	1174	6560
Second	Methanol–acetone–petroleum ether	807	2659
Third	Methanol–ethyl acetate	816	827
Fourth	Methanol–ether–petroleum ether	813	810

Characterization of U.V. fraction No. 1 and No. 2

As an alternate procedure of purification the crude material containing the cortisol metabolites were subjected to gel filtration on Sephadex columns. Fractions possessing radioactivity were collected. These fractions showed absorption maximum at 248 nm. The U.V. absorbing compounds were identified as inosine and hypoxanthine, respectively (Tables 5 and 6).

DISCUSSION

Although sulphate metabolites of corticosteroids have been isolated and identified from various tissues and body fluids [19–24], they have been considered to be excretory products. There are reports suggesting that steroid sulphate conjugates may serve a biological function as intermediates in the biosynthesis of

Table 5. Characterization of U.V. fraction No. 1

Crystals:	needle
Purity	single compound when analyzed by thin layer, paper and gas chromatography and high voltage electrophoresis
Melting point:	212°C (decomposed)
Elemental analysis:	$C_{10}H_{12}N_4O_5$ calculated: C = 44·78 H = 4·51 N = 20·89 found: C = 44·54 H = 4·95 N = 20·87
I.R. (KBr, cm^{-1})	1700 (C=N), 1595, 1555, 1520, 1475 (C=C) superimpose with authentic inosine
N.M.R. (D_2O):	δ value (ppm) 8·16 (1H), 8·30 (1H), 6·07 (1H) 4·75 (1H), 4·43 (1H), 4·20 (1H) 3·89 (2H)
U.V. (H_2O):	pH 7·0; $\epsilon = 1{\cdot}21 \times 10^4$ Neutral and acidic pHs: $\lambda_{max.}$ 248 nm, $\lambda_{min.}$ 223 nm Alkaline pHs: $\lambda_{max.}$ 253 nm, $\lambda_{min.}$ 223 nm
Periodic acid benzidine test:	positive
Acid hydrolysis:	Hypoxanthine (I.R., N.M.R.) and D-ribose (I.R.) were obtained
Mix melting point:	unchanged with inosine

Table 6. Characterization of U.V. fraction No. 2

Crystals	needle
Melting point:	172°C (decomposed)
Period acid – benzidine test:	negative
I.R. (KBr, cm^{-1}):	1625 (C=N), 1580, 1470, 1420 (C=C) superimposed with authentic hypoxanthine
N.M.R. (D_2O):	δ value (ppm) 7·87 (1H) 8·09 (1H)
U.V. (H_2O):	pH 7; $\epsilon = 1{\cdot}04 \times 10^4$ Neutral and acidic pHs: $\lambda_{max.}$ 248 nm, $\lambda_{min.}$ 223 nm Alkaline pHs.: $\lambda_{max.}$ 258 nm, $\lambda_{min.}$ 232 nm
Mix melting point:	unchanged with hypoxanthine.

steroids, e.g. dehydroisoandrosterone sulphate in the biosynthesis of estrogen [22, 23]. Ruokenen and co-worker [24] reported that several C-19 and C-21 steroid compounds with 3β-hydroxy and double bond at C-5 were isolated in large quantities from human testicular tissues as the monosulphate derivative rather than the free steroid and suggested that these sulphate conjugates may participate in the biosynthesis of testosterone by the testis. In the present study, it was demonstrated that significant amounts of sulphate conjugates are formed within 15 min after intraperitoneal administration.

During the isolation procedure it was observed that significant amounts of radioactivity remained in the aqueous fraction after 4 *n*-butanol extractions (Table 1). Since sulphate conjugates are extracted completely with *n*-butanol, the retention of the steroid sulphate conjugates in the aqueous fraction suggest the possibility of a complex formation. Although inosine and hypoxanthine were isolated and characterized as the U.V. absorbing materials associated with the steroid

conjugates, preliminary studies indicate that they are probably not the compounds responsible for the complex formation.

The finding that both isolated conjugates were 5α-pregnane derivatives supports the report of Forchielli *et al.*[25] that in the microsomal fraction of female rat liver only 4-ene-5α reductase exists; whereas in the male rat liver both 4-ene-5α and 4-ene-5β reductases are present. Roy and Pasqualini[26] reported that following the administration of [^{3}H] corticosterone to rat foetuses, the major metabolites after 30 min were dihydro- and tetrahydrocorticosterone with 5α configuration. Furthermore, Gatehouse *et al.*[27] studied the metabolism of cortisone-21-[^{35}S] sulphate in female rats with bile duct cannulaes and found that about 70% of the radioactivity appeared in the bile as 3α-(β-D-glucopyranuroside)-17α-hydroxy-5α-pregnane-11,20-dione-21-[^{35}S] sulphate. Sulphate conjugation of [4-^{14}C] corticosterone occurred at a faster rate in the female rats rather than in the male rat[28].

Although 3β,5α metabolites of corticosterone were repeatedly identified in various organs of male rats[29–31], these metabolites are also produced by female animals since 3β,11β,21-trihydroxy-5α-pregnan-20-one was found in the faeces of female rats[32] and the 3β,20β-diol derivative was identified in the plasma of pregnant women[33] and in the urine of pregnant mares[34]. The results of the present study are in agreement with the above cited reports that 4-ene-3-keto steroids can be reduced to 3α-hydroxy and 3β-hydroxy-5α-pregnane derivatives in the female rats. In the present study, sulphate conjugates of dihydrocortisol or cortisol were not detected, suggesting that the sequence of transformation probably occurred initially by reduction of the 4-enebond by 5α reductase followed by the hydrogenation of 3-keto group by 3α- and 3β-hydroxysteroid oxidoreductases, and finally by the sulphation at C-21 by sulphate kinase.

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