C₂₀ Steroids

THE METABOLISM OF 3-HYDROXY-19-NOR[21-¹⁴C]PREGNA-1,3,5(10)-TRIEN-20-ONE IN THE RABBIT

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1. The metabolism of 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one, a possible product of the aromatization of progesterone or pregnenolone, has been studied. 2. After oral administration of this C₂₀ steroid as the 21-¹⁴C-labelled compound to two groups of rabbits, the excretion pattern of metabolites in the urine was examined. 3. At 14 days after administration, 3.3–6.5% of the radioactivity had appeared in the urine, 71–79% in the faeces and approx. 10% remained in the gut. 4. A metabolite, isolated from urine mainly as the unconjugated steroid, was identified as 19-norpregna-1,3,5(10)-triene-3,20 α -diol and constituted 18.5–22% of the total urinary radioactivity. 5. A minor component of the urinary unconjugated steroids was identified as 19-norpregna-1,3,5(10)-triene-3,17 α ,20 α -triol. 6. A further 2–7.5% of the total urinary radioactivity, isolated only from the urinary sulphate fraction, was tentatively identified as an 18-oxygenated derivative of the administered steroid.

Biosynthesis of oestrogens by aromatization of the A ring of testosterone, androst-4-ene-3,17-dione and dehydroepiandrosterone has been demonstrated *in vitro* by using a placental enzyme system (Ryan, 1959; Gual, Morato, Hayano, Gut & Dorfman, 1962). Aromatization of these and other C₁₉ steroids also occurs during perfusion of full-term placentae *in vitro* (Cedard, Varangot & Yannotti, 1963).

The conversion of progesterone into oestrogens has been confirmed *in vivo* in a human subject (Davis & Plotz, 1958) and *in vitro* with human ovaries stimulated by follicle-stimulating hormone (Ryan & Smith, 1961). It has been suggested that progesterone may serve as the placental precursor of the C₁₉ steroids subsequently aromatized to oestrogens (Ryan, 1962). Although the conversion of progesterone into androst-4-ene-3,17-dione by human placenta *in vitro* has been reported (Warren & Cheatum, 1964) a maximum conversion of only 0.13% was obtained, suggesting that this pathway is of minor significance. A variety of techniques, including perfusion of placentae *in situ* and injection into the intact foeto-placental unit via the umbilical vein, has failed to demonstrate any production of oestrogens from labelled progesterone (Jaffe, Pion, Eriksson, Wiqvist & Diczfalusy, 1965). Moreover, the previously reported conversion of

acetate into oestrogens by perfused placentae could not be repeated (Levitz, Emerman & Dancis, 1962). Diczfalusy (1964) has reviewed the possible pathways of oestrogen biosynthesis in the light of these results. Direct aromatization of progesterone was at one time considered a possibility, but the investigations of Ryan (1959), Gual *et al.* (1962) and Jaffe *et al.* (1965) showed no conversion of progesterone or pregnenolone into 'phenolic steroids' either by the microsomal enzyme system of placenta or by the intact foeto-placental unit. It was, however, assumed in these studies that the 'phenolic steroid' produced by aromatizing progesterone or pregnenolone would behave chemically like oestrone and would be extracted from toluene by sodium hydroxide solution (Engel, Slaunwhite, Carter & Nathanson, 1950). Aromatization of the A ring of both progesterone and pregnenolone would produce 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one, a C₂₀ steroid that has been synthesized as the 21-¹⁴C-labelled compound (Schulster, Whitehead & Kellie, 1964). This 'phenolic steroid' is not significantly extracted from toluene by sodium hydroxide solution as is oestrone and oestradiol, and it might therefore have been undetected in the earlier investigations. For this reason the direct aromatization of progesterone remains a possibility.

If progesterone or pregnenolone were aromatized it is likely that further metabolism of the product, 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one,

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would occur. No work has been published on the metabolism of this type of C₂₀ steroid, which has the A ring structure of oestrone and the side-chain structure of progesterone. The present study was undertaken to establish how this type of molecule is metabolized. A preliminary account of this work has been presented (Schulster & Kellie, 1965).

MATERIALS

All solvents were distilled except ethanol (R.R. grade; James Burrough Ltd., London, S.E. 11) and acetic acid (AnalaR) and sulphur-free toluene (British Drug Houses Ltd., Poole, Dorset), which were used as supplied.

Celite 535 (Johns-Manville Ltd., London, S.E. 1) for partition chromatography was extensively washed before use with hot conc. HCl followed by water and methanol before being dried at 60°. Alumina (nearly neutral; M. Woelm, Eschwege, Germany) for adsorption chromatography had a moisture content of 4% (w/w). The following dyes were used as markers in paper chromatography, Duranol brilliant violet B.300 and Duranol violet 2R.300 (dyes F₁₁ and F₉ respectively; Neher, Meystre & Wettstein, 1959) obtained from Imperial Chemical Industries Ltd., Dyestuffs Division, London, W.C. 1, and Sudan red (Sudan IV; scarlet 04671) obtained from George T. Gurr Ltd., London, S.W. 6.

β -Glucuronidase (powder B; 5×10^5 units/g.) was prepared from *Patella vulgata* by the method of Dodgson & Spencer (1953).

3-Hydroxy-19-nor[21-¹⁴C]pregna-1,3,5(10)-trien-20-one (specific activity, 1.0 mc/m-mole) was prepared by the method of Schulster *et al.* (1964). The non-radioactive steroid was synthesized by the route of Mills, Ringold & Djerassi (1958) and was identical with the ¹⁴C-labelled compound. [1-¹⁴C]Hexadecane, [1,2-³H₂]hexadecane and [G-³H]acetic anhydride were obtained from The Radiochemical Centre, Amersham, Bucks.

METHODS

Animals: dose, collection of excreta and dissection

Rabbits were housed in cages that were cleaned daily and designed to minimize faecal contamination of the urine. A fine wire mesh below the bars of the cage floor retained the faeces. The urine fell through this on to a funnel of polythene sheeting and was filtered through fine gauze.

The doses were administered to the animals by syringe and cannula directly into the stomach.

Group I comprised two adult female rabbits (A and B), which each received a total of 35 ml. of a solution of the steroid (91 mg.; 9.5 μ C) in warm ethanolic arachis oil as four separate doses spread over 3 days. Urine and faeces were collected daily for 4 days (rabbit A) and 6 days (rabbit B) after the first dose. Both rabbits were then killed and dissected, and small portions of the gut, liver, kidney, brain, pituitary, uterus, adrenal and ovary were weighed, homogenized and freeze-dried. Group II comprised five adult male rabbits (H, I, J, K and L), which each received a single 10 ml. dose of a warm finely-divided suspension of the steroid (100.7 mg.; 1.00 μ C) in arachis oil. Urine and faeces

were collected for a maximum of 14 days after administration.

Radioactivity measurements

Samples were counted in triplicate in a Packard Tri-Carb liquid-scintillation spectrometer (model 314 EX). The settings were obtained for maximized count rates by the balance point procedure and relate accurately only to the particular machine used in this investigation.

Aqueous samples (1 ml.) of fresh urine and methanolic extracts of homogenized faeces were counted directly in dioxan scintillator fluid (15 ml.) containing 2,5-diphenyloxazole (10 g./l.), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.25 g./l.) and naphthalene (100 g./l.) (Werbin, Chaikoff & Imada, 1959). Efficiencies, measured by internal standardization, varied between 40 and 60%. All other aqueous samples were neutralized and counted in this scintillator fluid. The methanolic extracts of the faeces were also burnt in oxygen and the radioactivity was determined as ¹⁴CO₂ after absorption in methanolic ethanolamine (12%) (Kalberer & Rutschmann, 1961).

Non-aqueous samples dissolved in strong quenchers such as chloroform, ethanol etc. were, if possible, evaporated to dryness and the radioactivity was counted in 10 ml. of toluene scintillator fluid, containing 2,5-diphenyloxazole (4.0 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1 g./l.). Samples were usually dissolved in the minimum volume of benzene and counted in toluene scintillator fluid (10 ml.). Counting efficiencies were estimated by internal standardization with [1-¹⁴C]hexadecane and, when possible, by the channels-ratio method (Bush, 1963).

Paper chromatograms were analysed for radioactivity by cutting a strip of the chromatogram, 0.5–1 cm. wide, into numbered fragments and counting the radioactivity on each paper section directly in toluene scintillator fluid.

Extraction of urine

Urine was stored over chloroform at 4° for a maximum of 10 days before extraction. Daily urines from group I rabbits were combined before extraction. Daily urines from group II rabbits, collected until they contained more than 0.5 μ C (usually 1–10 days), were combined and extracted, each rabbit urine being treated individually.

Unconjugated steroids. Urine was extracted with chloroform (3 $\times$ 0.5 vol.) to obtain the unconjugated steroids.

Hydrolysed glucuronides. The extracted urine was adjusted to pH 2.0 with HCl and shaken with (NH₄)₂SO₄ (50 g./100 ml.) until the salt had dissolved. A threefold extraction with ether-ethanol (3:1, v/v; 3 $\times$ 0.5 vol.) removed conjugated steroids. The extract was evaporated to dryness and hydrolysed overnight at 37° with *Patella vulgata* β -glucuronidase (100 units/ml. of urine) in 0.5 M-sodium acetate buffer (pH 4.0; 100 ml./l. of urine) containing KH₂PO₄ (7.35 m-moles/l. of urine) as a sulphatase inhibitor. The hydrolysed steroid glucuronides were extracted with methylene dichloride (3 $\times$ 0.5 vol.). The combined methylene dichloride extract was washed with N-NaOH (3 $\times$ 0.25 vol.), then with water until neutral and finally evaporated to dryness.

Solvolysed sulphates. The extracted aqueous hydrolysate and alkali washings were combined and adjusted to pH 1.0 with H₂SO₄. Then NaCl was added (20 g./100 ml.) and the solution extracted with ethyl acetate (1 $\times$ 1 vol. and

2 × 0.5 vol.). This extract was left overnight at 37° to solvolyse the steroid sulphates (Burststein & Lieberman, 1958) and was then evaporated to one-third of the original volume. Next NaHCO₃ solution was added until effervescence ceased and the ethyl acetate was extracted with water until the washings gave no effervescence with acid. The hydrolysed steroid sulphate fraction was finally obtained by evaporating the ethyl acetate to dryness. Triplicate samples of each extract, hydrolysate and residue were counted by liquid-scintillation spectrometry.

Extraction of gut and faeces

The gut, including contents, from each group I rabbit was homogenized in ethanol in a Waring Blender. The homogenate was extracted twice with ethyl acetate and twice with chloroform. The extracts were filtered, with difficulty, through sintered glass, combined and evaporated to dryness. The gummy residue was dissolved in benzene, washed with water and centrifuged to break emulsions. The benzene layer was evaporated to dryness and purified by chromatography on alumina.

Faeces were homogenized in methanol by a Waring Blender and filtered through sintered glass under suction. This homogenization and extraction was repeated twice and the total extract was evaporated to dryness. Samples of the faecal extract were counted by liquid-scintillation spectrometry directly and after combustion (Kalberer & Rutschman, 1961).

Chromatography

Urine extracts were purified by gradient elution on 2 cm.-diam. partition columns of Celite 535 (70 g.; 0.6 ml. of stationary phase/g.). The dry extracts were dissolved in stationary phase and 1.0 g. of Celite was added/0.6 ml. of solution. This was then packed on top of the column in the minimum volume of mobile phase. A sample of every second or third fraction (10 ml., numbered to include the retention volume) was counted in the liquid-scintillation spectrometer. Fractions relating to each peak of radioactivity were combined and further purified by paper chromatography.

Paper chromatography was performed in glass tanks by downward development on Whatman no. 2 chromatographic paper (20 cm. × 56 cm.) extensively washed before use (Bush & Willoughby, 1957). Impure samples (less than 500 µg.) were chromatographed on single sheets as narrow streaks 14 cm. wide. Compounds associated with radioactive areas were cut out of the paper, concentrated by 'running-up' (Bush, 1961, p. 183) and eluted. The eluate was filtered through sintered glass to remove paper fibres. The usage of R_F , ΔR_M , ΔR_{M_s} and ΔR_{M_r} was as defined by Bush (1961, pp. xvi, xvii).

Solvent systems for chromatography and colour reactions used for steroid location. Solvent systems used for column and paper chromatography were similar to those described by Bush (1952, 1960) and had the following compositions (by vol.); light petroleum (b.p. 80–100°)–benzene–methanol–water (5:0:4:1) (L/80), (10:5:12:3) (LB21/80), (5:5:7:3) (LB11/70) and (0:2:1:1) (B/50), and light petroleum (b.p. 80–100°)–acetic acid–water (10:9:1) (L/A90).

After samples had been 'spotted' or 'streaked', papers to

be run in the L/A90 system were impregnated with stationary (lower, aqueous) phase by dipping in a trough and roller. Care was taken to avoid moving the material from the origin at this stage by dipping only to within 4 cm. of the origin. The impregnated paper was equilibrated in the chromatography tank for 1 hr. before running (Sudan red ran 30–40 cm. in 5–6 hr.) or overrunning (F₁₁ dye ran 10 cm. in 22–24 hr.). Phenolic steroids were detected on paper by dipping in 20% Folin–Ciocalteu reagent and then exposing to NH₃ vapour, or by spraying with a 5% solution of sodium cobaltinitrite in 0.2 N-acetic acid and heating at 100° (Feigl, 1955). Acetylated steroids were detected by dipping papers in 10% (w/v) phosphomolybdate in ethanol and heating at 60° for 20–30 min. The Zimmermann reaction was performed by dipping papers in ethanolic 3 N-KOH, in ethanolic *m*-dinitrobenzene (2%, w/v) and warming.

Infrared spectra

The i.r. spectra were recorded in micro-cells in carbon disulphide (3 mm.) or chloroform (1 mm.) solution on a Perkin–Elmer model 21 recording spectrophotometer with NaCl optics. Approx. 100 µg. of sample was used irrespective of cell path-length.

Micro-chemical methods

Acetylation was effected with pyridine and acetic anhydride overnight at room temperature (Zaffaroni & Burton, 1951). For approx. 30 µg. of steroid, NaBH₄ reduction was performed with 0.5 ml. of a solution, freshly made at 0°, containing NaBH₄ (25 mg.), 5 N-NaOH (0.1 ml.) and methanol (4 ml.). The steroid solution was left at room temperature for 18 hr., neutralized and extracted with chloroform. This extract was washed with water and evaporated to dryness.

Synthesis of reference steroids

19-Norpregna-1,3,5(10)-triene-3,20 α -diol. Reduction of 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one yielded 19-norpregna-1,3,5(10)-triene-3,20 α -diol and 19-norpregna-1,3,5(10)-triene-3,20 β -diol in various proportions according to the method of reduction. These two products were separated by chromatography on Celite with the LB21/80 system and recrystallized from benzene–light petroleum. After NaBH₄ reduction the 20 β -orientation was assigned to the epimer afforded in 75% yield (m.p. 232–233°) and the 20 α -orientation was assigned to the minor product (m.p. 178–179°) (Norymberski & Woods, 1955). Reduction of 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one (100 mg.) in boiling ethanol (50 ml.) by the addition of small pieces of sodium (0.5 g.) gave rather more of the 20 α -ol than the 20 β -ol (cf. Meystre & Miescher, 1946), thereby supporting the original assignment of orientation. This was confirmed by the chromatographic properties of the two epimers. The 20 α -hydroxyl group in C₂₁ steroids is usually more polar than the 20 β -hydroxyl group, but this depends on other substituents and on the solvent systems used (Neher, 1959). The ΔR_{M_r} values obtained for the change 20 α -hydroxyl to 20 β -hydroxyl (–0.15 for the LB21/80 system and –0.12 for the LB11/70 system, calculated from values given in Table 3) are similar to the ΔR_{M_r} values quoted for this

change in various C_{21} steroids (ΔR_M , between -0.13 and -0.25 ; Bush, 1961, p. 106).

19-Norpregna-1,3,5(10)-triene-3,17 α ,20 α -triol. 3,17 α -Dihydroxy-19-norpregna-1,3,5(10)-trien-20-one was synthesized from 3-benzoyloxy-17-cyano-oestra-1,3,5(10)-trien-17-ol (Schulster *et al.* 1964) by a Grignard reaction after protection of the 17-hydroxyl group as the tetrahydropyranyl ether (Ruggieri & Ferrari, 1959). Subsequent removal of the protecting groups from the 3- and 17-hydroxyl groups and crystallization from methanol afforded needles of the 3,17 α -dihydroxy-20-ketone (m.p. 229–238°) [Djerassi, Rosenkrantz, Iriarte, Berlin & Romo (1951) reported m.p. 234–240°, with m.p. 240–242° for the analytical sample inserted at 225°]. The 3-monoacetate, prepared by acetylation with pyridine and acetic anhydride overnight at room temperature, had m.p. 127–128° (literature m.p. 128–129°) and ν_{max}^{NaCl} 1764, 1707 and 1205 cm^{-1} . Reduction of the 3,17 α -dihydroxy-20-ketone with $NaBH_4$ or sodium in ethanol produced 19-norpregna-1,3,5(10)-triene-3,17 α ,20 α -triol and 19-norpregna-1,3,5(10)-triene-3,17 α ,20 β -triol in varying proportions.

RESULTS

Tissue distribution and excretion of radioactivity

The freeze-dried organs from group I rabbits were dissolved in formic acid and the radioactivity was estimated directly in the liquid-scintillation spectrometer. Most of the radioactivity was in the gut and the faeces, with small amounts in the urine, liver and kidney (Table 1). The pituitaries, brains, uteri, adrenals and ovaries each contained less than 0.1% of the administered radioactivity. The percentages of the total urinary radioactivity found in the urine fractions after extraction are given in Table 2. The radioactivity remaining in the rabbit gut 14 days after administration was approx. 10% of the original dose.

Chromatography of urinary, faecal and gut extracts

Chloroform extracts of unconjugated urinary steroids. The chloroform extract of urine from

group I rabbits, which contained a total of 0.324 μC , was purified by gradient elution from a Celite column according to the sequence of mobile phases shown in Fig. 1. Apart from the small peak eluted on the solvent front, five significant peaks of radioactivity were obtained: C¹ (0.031 μC), C² (0.118 μC), C³ (0.027 μC), C⁴ (0.014 μC) and C⁵. The last peak was eluted only after stripping the column with stationary phase. It contained little radioactivity and was not further investigated. The chloroform extract of urine from group II rabbits, which contained 0.088 μC , was chromatographed in a similar manner. The radioactive elution pattern was comparable with that of group I rabbits (Fig. 1), but the relative amount of radioactivity in each peak was different (peak C¹: 0.023 μC ; peak C²: 0.038 μC).

Hydrolysed urinary glucuronides. The extract of hydrolysed glucuronides from group II rabbits (0.013 μC) was chromatographed on a Celite column by using a sequence of mobile phases similar to that described for the extracts of unconjugated steroids. Apart from highly polar compounds, eluted by stripping the column with stationary phase, only one significant peak of radioactivity, G (0.003 μC), was found.

Solvolysed urinary sulphates. The urinary sulphate fractions obtained by ethyl acetate solvolysis were chromatographed by gradient elution from Celite columns according to the sequence of mobile phases shown in Fig. 2. The solvolysed sulphate fraction from group I rabbits contained 0.117 μC and yielded on chromatography three significant peaks of radioactivity: S¹ (0.0164 μC), S² (0.0565 μC) and S³ (about 0.005 μC). The solvolysed sulphate fraction from group II rabbits contained 0.051 μC and yielded the corresponding peaks of radioactivity (Fig. 2): S¹ (0.017 μC), S² (0.0095 μC) and S³ (0.004 μC). Peak S³ was obtained by stripping the column with stationary phase. The material that it contained was very impure and was not further investigated.

Faecal extract. This was chromatographed by gradient elution from a column of Celite (60g.; 42 cm. $\times$ 2 cm. diam.) by using the following sequence of mobile phases: L/80 system (600 ml.) followed by a gradient of LB 11/70 system (180 ml.) into L/80 system (250 ml.), then LB 11/70 system (1000 ml.). The portion of eluate containing most of the radioactivity (310–1400 ml.) was evaporated to dryness before being rechromatographed on a similar Celite column. Two peaks of radioactivity (F¹ and F²) were obtained.

Gut extract. This was obtained as a crude gum from the gut homogenate and was cleaned by preliminary chromatography on a column of alumina (160g.) with a gradient elution system of benzene-ethanol (49:1, v/v) into a recipient reservoir of benzene.

Table 1. Percentages of radioactivity excreted, and distributed in rabbit organs, 4 days (rabbit A) and 6 days (rabbit B) after oral administration of 3-hydroxy-19-nor[21-¹⁴C]pregna-1,3,5(10)-trien-20-one (91 mg.; 9.5 μC)

	% of radioactive dose	
	Rabbit A (after 4 days)	Rabbit B (after 6 days)
Gut (including contents)	66	31
Faeces	9	32
Urine	6	5
Liver	2	2
Kidney	1	0.1
Total	84	70

Table 2. *Urinary and faecal excretion of radioactivity after oral administration of 3-hydroxy-19-nor[21-¹⁴C]pregna-1,3,5(10)-trien-20-one to group I rabbits (A and B) and group II rabbits (H, I, J, K and L)*

Group I rabbits each received 91 mg. (9.5 μ C) and urine was collected for 4–6 days. Group II rabbits each received 100mg. (1.0 μ C) and urine was collected for up to 14 days.

Rabbits	A and B					
	combined	H	I	J	K	L
	% of administered radioactivity					
Faeces (1–14 days)			79	71		
Total urine	5	6.5	3.3	3.4	6.1	3.4
	% of total urinary radioactivity					
Unconjugated steroids	34.5	64	56	20	43	64
Hydrolysed glucuronides	8.5	3	1	6	23	4
Solvolysed sulphates	12.5	9	4	29	9	16
Residues and spent urine	14	15	14	36	22	24
Total accounted for after extraction	70	91	75	91	97	108

Fig. 1. Radioactivity in samples of the eluate obtained after Celite column chromatography of the chloroform extract of urine from two female rabbits (group I): 1, system L/80 (90ml.); 2, system LB11/70 (360ml.) into system L/80 (500ml.); 3, system LB11/70 (1000ml.); 4, system B/50 (300ml.) into system LB11/70 (400ml.); 5, stationary phase. The extract before chromatography contained 0.324 μ C of radioactivity, equivalent to 3.1 mg. of the administered steroid. For further details see under 'Chromatography' in the Methods section.

The radioactive material in the eluate was located (550–800ml.) and rechromatographed on another similar column. Five discrete peaks of radioactivity were obtained. The most polar peak (E) contained the most radioactivity and was the only peak to be investigated.

Fig. 2. Radioactivity in samples of the eluate obtained after Celite column chromatography of the solvolysed sulphate fraction of the combined urine from five male rabbits (group II): 1, system L/80 (150ml.); 2, system LB11/70 (450ml.) into system L/80 (600ml.); 3, system LB11/70 (1700ml.); 4, system B/50 (180ml.) into system LB11/70 (250ml.); 5, stationary phase. For further details see under 'Chromatography' in the Methods section.

Identification of metabolites

3-Hydroxy-19-norpregna-1,3,5(10)-trien-20-one, the administered steroid (metabolites C¹ and F¹). Compound C¹ was eluted as a discrete peak of radioactivity after chromatography of the chloroform extract (unconjugated steroids) of urine (Fig. 1). Compound F¹ was obtained by similar chromatography of the methanolic extract of homogenized faeces. Both were purified by paper chromatography with an overrun L/A90 system.

The R_F values of compounds C¹ and F¹ were identical with that of the title compound in three different paper-chromatographic systems (LB 21/80, LB 11/70 and L/A90). They were reduced by sodium borohydride and the products were chromatographically identical (in system LB 21/80) with 19-norpregna-1,3,5(10)-triene-3,20 α -diol and 19-norpregna-1,3,5(10)-triene-3,20 β -diol, the reduction products of 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one. The acetylated metabolites also had mobilities identical with that of acetylated 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one in the L/A90 system. The i.r. spectra of the acetylated compounds confirmed the identities of compounds C¹ and F¹ as unchanged starting material.

19-Norpregna-1,3,5(10)-triene-3,20 α -diol (metabolites C², G, S², F² and E). The rabbit urine yielded metabolites C² (unconjugated steroid fraction), G (glucuronide fraction) and S² (sulphate fraction). Metabolite F² was obtained from the faeces and metabolite E from the gut. During column chromatography of the urine and of extracts of faeces and gut, all these compounds were eluted as discrete peaks of radioactivity of similar mobility. The compounds were further purified by paper chromatography with an overrun L/A90 system. In three paper-chromatographic systems (LB 21/80, LB 11/70 and L/A90) these radioactive metabolites all had R_F values indistinguishable from that of 19-norpregna-1,3,5(10)-triene-3,20 α -diol. Further, the acetylated metabolites all had mobilities in the L/A90 system indistinguishable from that of 3,20 α -diacetoxy-19-norpregna-1,3,5(10)-triene (Table 4). The i.r. spectra of the acetylated derivatives of compounds C², S², F² and E were identical with that of 3,20 α -diacetoxy-19-norpregna-1,3,5(10)-triene and had $\nu_{\max}^{\text{CS}}$ 1765, 1733, 1243, 1205, 1056, 1012 and 933 cm.⁻¹. On the basis of these results metabolites C², S², F² and E were identified as 19-norpregna-1,3,5(10)-triene-3,20 α -diol. There was insufficient

purified metabolite G for spectroscopic studies, but the chromatographic evidence suggested that it was the same compound.

19 - Norpregna - 1,3,5(10) - triene - 3,17 α ,20 α - triol (metabolite C³). Metabolite C³ was obtained after partition column chromatography of the chloroform extract of urinary unconjugated steroids and comprised 2.9% of the total urinary radioactivity from group I rabbits. It was purified by paper chromatography in an overrun L/A90 system. The mobility of metabolite C³ in three paper-chromatographic systems (LB 11/70, B/50 and L/A90) was indistinguishable from that of synthetic 19-norpregna-1,3,5(10)-triene-3,17 α ,20 α -triol. After acetylation with pyridine and acetic anhydride overnight it had the same mobility in the L/A90 system as 3,20 α -diacetoxy-19-norpregna-1,3,5(10)-trien-17 α -ol (Table 3). The metabolite was detected on paper by Folin-Ciocalteu reagent and by liquid-scintillation counting of paper segments. Although it gave no colour with Zimmermann reagent alone, it did give the characteristic Zimmermann purple colour after oxidation, on the paper, with 0.5% (w/v) lead tetra-acetate in acetic acid (Bush, 1955). The latter method is specific for 17 α ,20 α -diols, although 17 α ,20 β -diols do react slowly. The i.r. spectrum of acetylated metabolite C³ was identical with that of 3,20 α -diacetoxy-19-norpregna-1,3,5(10)-trien-17 α -ol and had $\nu_{\max}^{\text{CS}}$ 1765, 1738, 1238 and 1206 cm.⁻¹. From these results metabolite C³ was identified as 19-norpregna-1,3,5(10)-triene-3,17 α ,20 α -triol.

Tentative identification of an 18-oxygenated derivative (metabolite S¹). This metabolite was eluted as a discrete peak of radioactivity after Celite chromatography of the solvolysed sulphate fraction of urine from group I and group II rabbits. The metabolite derived from both groups of rabbits was eluted by almost identical volumes of eluent and was purified by paper chromatography with an

Table 3. Paper-chromatographic behaviour of metabolite C³ and related steroids

Compound C³ was isolated from the urinary unconjugated steroid fraction. Solvent systems are described in the Methods section. $R_{\text{Dye F}_9}$ signifies mobility relative to dye F₉ (see the Materials section).

	Solvent system ...		LB 11/70	B/50	L/A90	L/A90 overrun
			R_F (27°)	R_F (26°)	$R_{\text{Sudan red}}$ (23°)	$R_{\text{Dye F}_9}$ (26°)
Sudan red			0.91	0.94	1.0	—
F ₁₁ dye			0.72	0.91	0.11	—
F ₉ dye			0.25	0.71	0.03	1.00
3,17 α -Dihydroxy-19-norpregna-1,3,5(10)-trien-20-one			0.39	0.82	—	—
Metabolite C ³			0.13	0.64	—	0.96
19-Norpregna-1,3,5(10)-triene-3,17 α ,20 α -triol			0.13	0.63	—	0.96
19-Norpregna-1,3,5(10)-triene-3,17 α ,20 β -triol			0.18	0.68	—	1.08
Acetate of metabolite C ³			—	—	0.31	—
3,20 α -Diacetoxy-19-norpregna-1,3,5(10)-trien-17 α -o			—	—	0.31	—
3,20 β -Diacetoxy-19-norpregna-1,3,5(10)-trien-17 α -ol			—	—	0.29	—

overrun L/A90 system. During subsequent chromatography the compound S^1 was detected by liquid-scintillation counting of paper sections (see under 'Radioactivity measurements' in the Methods section), by the Folin-Ciocalteu reagent or by sodium cobaltinitrite in acetic acid (Feigl, 1955). The metabolite was purified by overrunning for 24 hr. in the L/A90 paper-chromatographic system and acetylated. It showed $\nu_{\max}^{CH_2}$ 1765, 1733, 1365, 1242, 1196, 1071, 1056, 1018 and 949 cm^{-1} . The i.r. spectrum of the acetate was unchanged after chromatography in system L/A90. The peaks at 1765 and 1196 cm^{-1} were attributed to a phenolic 3-acetate (Jones & Herling, 1954), although a slight displacement of the latter peak was observed when the i.r. spectrum was compared with that of 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one ($\nu_{\max}^{CH_2}$ 1765 and 1205 cm^{-1}). The evidence for an intact 3-phenolic A ring was further supported by the positive Folin-Ciocalteu reaction and the positive Feigl reaction. The latter is specific for phenolic groups unsubstituted in the *ortho* positions. The intense peaks at 1733 and 1242 cm^{-1} were attributed to one or more unconjugated acetate groups.

Compound S^1 was radioactive, which indicated that the $21\text{-}^{14}\text{C}$ label was intact and that the metabolite retained the C-17 side chain.

Samples of 3-hydroxy-19-nor[$21\text{-}^{14}\text{C}$]pregna-1,3,5(10)-trien-20-one and compound S^1 were acetylated with excess of [$G\text{-}^3\text{H}$]acetic anhydride and pyridine overnight. Both were evaporated to dryness removing the excess of volatile tritium and chromatographed on the same paper with the L/A90 system. The chromatography was repeated twice and after each run the radioactive areas of the paper were eluted. The eluates were filtered through

sintered glass and duplicate fractions of each were taken for the estimation of $^3\text{H}/^{14}\text{C}$ ratios. During this purification the $^3\text{H}/^{14}\text{C}$ ratio for the monohydroxylic title compound approached the calculated value for one acetylated hydroxyl group, whereas for compound S^1 it closely approached the value for two acetylated hydroxyl groups. This is strong evidence that compound S^1 contains not more than two hydroxyl groups capable of being acetylated under the above conditions. From the i.r. data it therefore follows that the acetate of compound S^1 contains two acetate groups, one of which is a phenolic 3-acetate and the other an unconjugated acetate ($\nu_{\max}^{CH_2}$ 1733 and 1242 cm^{-1}). Paper chromatography showed that the acetate of compound S^1 was less polar than 3-acetoxy-19-norpregna-1,3,5(10)-trien-20-one (1 $\times$ acetate; 1 $\times$ ketone) and more polar than either 3,20 α - or 3,20 β -diacetoxy-19-norpregna-1,3,5(10)-triene (2 $\times$ acetate) (Table 4). This leads to the conclusion that since the acetate of compound S^1 contains two acetate groups it must also contain a polarity-increasing group that is not acetylated. That compound S^1 itself contains no acetate groups was shown by its i.r. spectrum in chloroform [$\nu_{\max}^{CHCl_3}$ 1715–1700 (weak) and 1596 cm^{-1} with no absorption in the range 1800–1720 cm^{-1}]. Compound S^1 must therefore contain a 3-phenolic group, one hydroxyl group capable of acetylation and another polar group not normally capable of acetylation.

The steroid synthesized by the method of Schulster *et al.* (1964) had a specific activity of 1.0 mc/m-mole and was diluted before administration with unlabelled material prepared by a completely different route (Mills *et al.* 1958), 33-fold for group I rabbits and 340-fold for group II rabbits.

Table 4. Paper-chromatographic behaviour of metabolite S^1 and related steroids

Compound S^1 was isolated from the urinary sulphate fraction. Solvent systems are described in the Methods section. $R_{Dye\ F_{11}}$ signifies mobility relative to dye F_{11} (see the Materials section).

	Solvent system ...	LB21/80 R_F (27°)	LB11/70 R_F (24°)	L/A90 $R_{Sudan\ red}$ (25°)	L/A90 overrun $R_{Dye\ F_{11}}$ (25°)
Sudan red		0.90	0.91	1.0	—
F_{11} dye		0.46	0.67	0.05	1.00
F_9 dye		0.09	0.21	0.02	0.19
Compound S^1 (from group I rabbits)		0.44	—	0.08	2.55
Compound S^1 (from group II rabbits)		0.43	0.67	0.09	2.45
Reduced compound S^1 (group I rabbits)		0.46	—	—	—
Reduced compound S^1 (group II rabbits)		—	0.67	—	—
3-Hydroxy-19-norpregna-1,3,5(10)-trien-20-one		0.60	0.77	0.07	1.84
19-Norpregna-1,3,5(10)-triene-3,20 α -diol		0.26	0.50	0.05	1.11
19-Norpregna-1,3,5(10)-triene-3,20 β -diol		0.33	0.57	0.05	—
Acetate of compound S^1 (group I rabbits)		—	—	0.29	—
Acetate of compound S^1 (group II rabbits)		—	—	0.29	—
3-Acetoxy-19-norpregna-1,3,5(10)-trien-20-one		0.90	0.95	0.22	—
3,20 α -Diacetoxy-19-norpregna-1,3,5(10)-triene		0.90	0.95	0.42	—
3,20 β -Diacetoxy-19-norpregna-1,3,5(10)-triene		0.90	0.95	0.45	—

The specific activity of the metabolite derived from group I rabbits based on the $^3\text{H}/^{14}\text{C}$ ratio of its [^3H]acetate was 0.025 mc/m-mole compared with the specific activity of the dose material (0.031 mc/m-mole for group I). If compound S^1 were a radioactive by-product of the chemical synthesis it would have the much higher specific activity of the undiluted compound (1.0 mc/m-mole). It was therefore concluded that compound S^1 , although representing only 0.4% of the original dose, was genuinely a product of metabolism and not a radioactive by-product of the chemical synthesis.

Compound S^1 and 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one were simultaneously reduced by sodium borohydride under identical conditions. The R_f value of reduced compound S^1 was identical with that of compound S^1 in two different systems (Table 4), although the R_f value of the concomitantly reduced 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one indicated that this had been reduced. It is possible that the 20-oxo group of the administered steroid is modified in metabolite S^1 ; either it is already reduced or it is hindered by a neighbouring group that inhibits reduction. Alternatively compound S^1 and its reduction product are different compounds with identical R_f values (cf. 18-hydroxyoestrone and 18-hydroxyoestradiol; Loke, Marrian & Watson, 1959). The possibility was considered that compound S^1 might be 19-nor-17 α -pregna-1,3,5(10)-triene-3,20 α -diol arising during the acid solvolysis procedure by inversion of the C-17 side chain (Rubin, 1963). However, when 19-norpregna-1,3,5(10)-triene-3,20 α -diol was subjected to the solvolysis procedure, subsequent paper chromatography of the products revealed only the starting compound.

In both LB21/80 and LB11/70 systems compound S^1 was more polar than the title compound ($1 \times$ hydroxyl; $1 \times$ ketone) and less polar than either 19-norpregna-1,3,5(10)-triene-3,20 α -diol or 19-norpregna-1,3,5(10)-triene-3,20 β -diol ($2 \times$ hydroxyl) (Table 4). Therefore, if compound S^1 contains a 20-hydroxyl group, it must also contain either a polarity-decreasing group or a polar group that decreases the expected polarity by influencing the 20-hydroxyl or 3-hydroxyl groups. However, the former possibility is unlikely since it has already been concluded that compound S^1 contains a 3-phenolic group, a hydroxyl group capable of being acetylated and another polar group not easily acetylated. The positive Feigl reaction of compound S^1 shows that the 3-phenolic group is unsubstituted in the *ortho* position and therefore interference with the 3-phenolic group is most unlikely. The possibility remains that compound S^1 contains a polar group interfering with the 20-hydroxyl group. If compound S^1 contains a 20-oxo group, then by virtue of its chromatographic

behaviour it must also contain either a weakly polar group of ΔR_M less than that for the change 20-hydroxyl to 20-oxo or a more polar group that decreases the expected polarity by interference with the 20-oxo group. The former alternative is not possible since the unconjugated hydroxyl group, previously concluded to be present, has a ΔR_M larger than the permissible value. The possibility remains that compound S^1 contains a polar group interfering with the 20-oxo group.

Four possibilities for compound S^1 consistent with the preceding discussion are: 3,16 β -dihydroxy-19-norpregna-1,3,5(10)-trien-20-one; 3,20 α -dihydroxy-19-norpregna-1,3,5(10)-trien-20-one; 3,18-dihydroxy-19-norpregna-1,3,5(10)-trien-20-one; 3,20-dihydroxy-19-norpregna-1,3,5(10)-trien-18-al. Intramolecular hydrogen-bonding has been found in 16 β -hydroxy-20-ketones and 16,20 α -diols (Danilewicz & Klyne, 1965) and it has been concluded that 18-hydroxy-20-ketones exist as the (18 $\rightarrow$ 20)-hemiketal (Iseli & Fukushima, 1965; Meystre *et al.* 1962).

Neher (1963) has compared the chromatographic properties of 20-oxopregnanes and 20-hydroxy-pregnanes with those of 16- and 18-substituted steroids of this series. Hydrogen-bonding in 16-oxygenated pregnanes has little or no effect on chromatographic mobility, whereas the ΔR_M for the introduction of an 18-hydroxyl group into 20-oxopregnanes is markedly less than the ΔR_M of hydroxyl groups at other positions. This observation supports the tentative identification of metabolite S^1 as an 18-oxygenated derivative of 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one.

DISCUSSION

This investigation was hampered by the poor absorption of the C_{20} steroid from the rabbit gut and the consequent low excretion of radioactivity in the urine. Attempts to increase the absorption by multiple subcutaneous injections were unsuccessful. Administration of this compound as the sodium salt was not possible because no method of preparation could be found. Consequently, to obtain sufficient material for study, five male rabbits were each dosed with 100 mg. of the title compound and the combined urinary extracts were investigated. A similar excretion pattern of urinary radioactivity was observed after administration of the C_{20} steroid to two female rabbits. Apart from the differences in the relative quantities of individual metabolites no other sex differences were noted.

19-Norpregna-1,3,5(10)-triene-3,20 α -diol, identified as the major metabolite isolated from the faeces and gut, may have arisen from bacterial action. Care was taken to avoid faecal contamination of the

urine and it is unlikely that this metabolite appeared in the urine as a result of contact with the faeces.

The administered steroid was isolated only from the free steroid fraction of the urine and not from the fractions containing conjugated steroids. 19-Norpregna-1,3,5(10)-triene-3,20 α -diol was isolated from the sulphate and possibly the glucuronide fraction of the urine. This suggests that conjugation occurred not with the 3-phenolic group but only with the 20 α -hydroxyl group of this metabolite.

The metabolic conversion of the 20-oxo group of the administered C₂₀ steroid into a 20 α -hydroxyl group may be compared with the metabolism in the rabbit of administered progesterone and 19-norprogesterone to the corresponding 20 α -hydroxy steroids (Thomas, 1962). 5 β -Pregnane-3 α ,20 α -diol has been identified in the urine as a metabolite of injected 20 β -hydroxypregn-4-en-3-one in the rabbit (Cooke, Rogers & Thomas, 1963). The existence of a 20 β -hydroxy steroid dehydrogenase possibly accounts for the absence of 19-norpregna-1,3,5(10)-triene-3,20 β -diol as a metabolite in urine in this investigation.

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