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THE IDENTIFICATION OF 3α,6β,11β,17,21-PENTAHYDROXY-5β-PREGNAN-20-ONE (6β-HYDROXY-THF)—THE MAJOR URINARY STEROID OF THE BABOON (*PAPIO PAPIO*)

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SUMMARY

The identification of 3α,6β,11β,17,21-pentahydroxy-5β-pregnan-20-one (6β-hydroxy-THF) from the urine of the baboon (*Papio papio*) is described. Steroids were extracted from urine with Amberlite XAD-2 resin and the polar steroid fraction containing 6β-hydroxy-THF isolated by chromatography on Sephadex LH-20. The reaction products resulting from borohydride reduction, periodate oxidation and chromate oxidation were analysed by gas chromatography and gas chromatography–mass spectrometry.

INTRODUCTION

Steroid excretion by the baboon has been the subject of several recent publications [1–5]. In two studies of ^{14}C-cortisol metabolism in the baboon species *Papio papio*, a relatively large proportion of the injected dose which was recovered in the urine was associated with very polar metabolites which were not identified [3, 4]. In a comprehensive investigation of steroid excretion by the baboon (*Papio hamadryas*) several previously unidentified steroids were found [6]. These compounds were very polar corticosteroids which were quantitatively major excretory products. This paper describes their identification using gas chromatography–mass spectrometry.

EXPERIMENTAL

Chemicals and reagents. All solvents were of reagent grade and were redistilled before use. Hexamethyldisilazane and trimethylchlorosilane (Applied Science Laboratories, State College, Pa., U.S.A.) were redistilled. Methoxyamine hydrochloride was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.) and trimethylsilylimidazole from the Pierce Chemical Co. (Rockford, Ill., U.S.A.). Amberlite XAD-2 was obtained from Rohm & Hass (Pa., U.S.A.) and was washed in a large column with water, ethanol, acetone and water. This was stored in water until required. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and Lipidex 5000 from Packard–Becker Chemicals (Groningen, Holland). Diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20) was prepared as described previously [7–9] and used in the acetate form. *Helix pomatia* digestive juice was obtained from L'Industrie Biologique Francaise, Gennevilliers, France.

Collection of urine. Daily urine collections were made from two adult female baboons (body weight 5–7 kg) of the species *Papio papio* over a period of 7 days. The urines were stored at −4°C until analysed. The animals were housed in individual metabolism cages in a temperature and light controlled environment.

Isolation of polar steroids. The general scheme of analysis is outlined in Fig. 1. The urine collections were pooled, and then passed through a large column of Amberlite XAD-2 (bed size 35 × 7 cm.). The column was then washed with distilled water (1 litre) and the washings were discarded. Steroids were recovered by elution with ethanol (3 litres), and the ethanol extract was taken to dryness on a rotary evaporator. The residue was dissolved in 30 ml of sodium acetate buffer (pH 4.5; 0.5 M) *Helix pomatia* digestive juice (2 ml) was added, and the sample was incubated at 37°C for 24 h. Following incubation the steroids were re-extracted on a column of Amberlite XAD-2 (bed size 20 × 2 cm.) in a similar manner as before. The ethanol extract was evaporated to dryness, and the residue dissolved in 72% ethanol. The sample was

The following abbreviations and trivial names are used in this paper: MO, O-methyloxime; TMS, trimethylsilyl ether; MO-TMS, O-methyloximetrimethylsilyl ether; a.m.u., atomic mass unit; t_R, retention time relative to 5α-cholestane; MU, methylene unit; FIC, fragment ion current; Tetrahydrocortisol (THF), 3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one; allo-Tetrahydrocortisol (allo-THF), 3α,11β,17,21-Tetrahydroxy-5α-pregnan-20-one; Tetrahydrocortisone (THE), 3α,17,21-Trihydroxy-5β-pregnane-11,20-dione; 6β-Hydroxy-cortisol, 6β,11β,17,21-Tetrahydroxy-4-pregnene-3,20-dione. The terms pregnane-pentol-one, pregnane-hexol etc. are intended to indicate general steroid structures and do not imply any specific configuration.

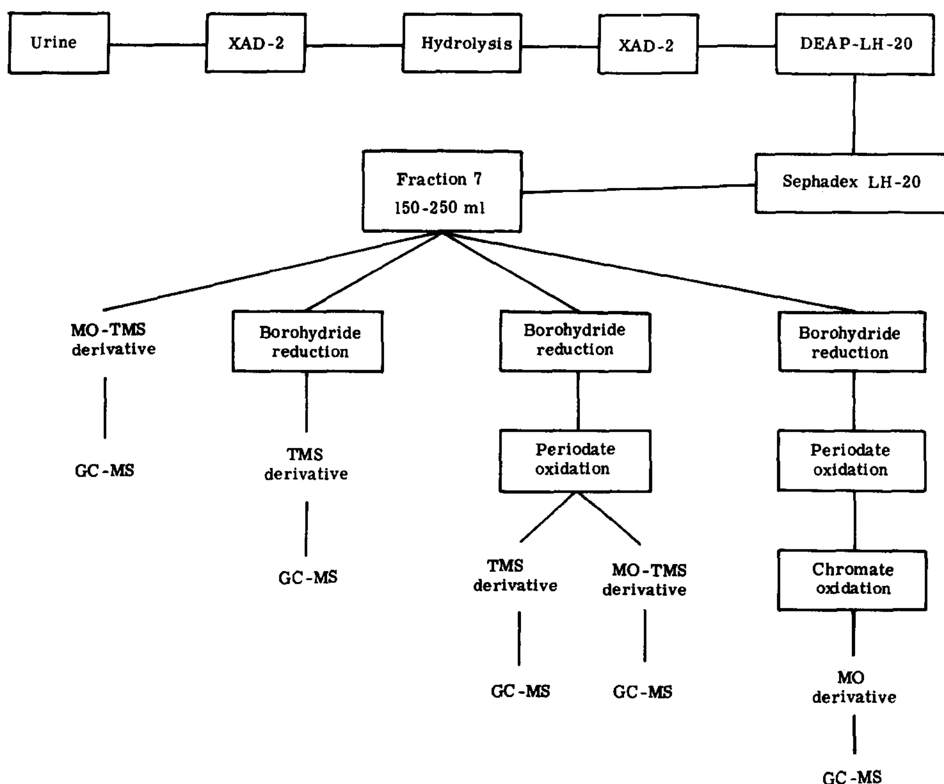

Fig. 1. General scheme of analysis outlining the extraction and isolation procedure and the chemical reactions carried out on the polar steroid fraction.

then passed through a column of diethylaminohydroxypropyl Sephadex LH-20 (6 g) suspended in 72% ethanol and prepared in the acetate form [9]. The elutent was collected and taken to dryness on a rotary evaporator. The residue was then dissolved in 3×1 ml aliquots of the solvent cyclohexane:ethanol (80:20, v/v), transferred to a column of Sephadex LH-20 (6 g) suspended in this solvent and chromatographed as described in a previous publication [10]. The fraction eluted between 150–250 ml (referred to as fraction 7) which contained the polar corticosteroids was collected, taken to dryness and redissolved in ethanol (5 ml). Aliquots of this fraction were then taken for further analysis.

Borohydride reduction. An aliquot (1/10) of fraction 7 was taken to dryness and the residue dissolved in methanol (0.5 ml). The sample was cooled in an ice bath for 15 min, and powdered sodium borohydride (5 mg) was added. The sample tube was stoppered and left in the ice bath to react for 2 h. The reaction was then stopped by addition of 0.25 M acetic acid (1 ml) and the sample diluted with distilled water (50 ml). The products were then extracted on small columns of Amberlite XAD-2 (bed size 10×0.5 cm.) and recovered by elution with ethanol (100 ml). This sample was then taken to dryness, a methyloxime-trimethylsilyl ether derivative was prepared and the sample was analysed by gas chromatography–mass spectrometry.

Periodate oxidation. An aliquot (1/10) of the sample was first reduced with sodium borohydride and the products were extracted on Amberlite XAD-2 as described above. This extract was then dissolved in dioxan (0.5 ml) and periodic acid (0.5 ml) was added. The sample was left to react in the dark for 2 h, distilled water (50 ml) was then added and the reaction products were extracted on Amberlite XAD-2 resin as described previously. The extract was divided and a methyloxime-trimethylsilyl ether derivative and a trimethylsilyl ether derivative prepared. The two derivatised samples were then analysed by gas chromatography–mass spectrometry.

Chromate oxidation. An aliquot (1/4) of fraction 7 was reduced with sodium borohydride, oxidised with periodic acid and the products were recovered from Amberlite XAD-2 as described above. The dried extract was then dissolved in acetone (0.5 ml) and 3 drops of *Jones* reagent (prepared by dissolving 26.7 g of chromium trioxide in 23 ml of concentrated sulphuric acid and diluting to 100 ml with distilled water) added [11]. The sample was shaken for 2 h at room temperature and then diluted with distilled water (100 ml). The reaction products were extracted on small columns of Amberlite XAD-2 resin, and a methyloxime derivative was prepared prior to gas chromatography–mass spectrometry.

Preparation of derivatives. (i) *O-Methyloxime-trimethylsilyl ethers, MO-TMS (persilylated).* The

method was essentially as described by Thenot and Horning [12]. Methoxyamine hydrochloride (5 mg) was added to the dried steroid extract, followed by 50 μl of dry pyridine. This was then incubated at 60°C for 15 min. The sample was then dried over a stream of nitrogen, trimethylsilylimidazole (50 μl) was added and the sample was left to react for 2 h at 110°C. Small columns of Lipidex 5000 (250 mg) were prepared in the solvent system cyclohexane–pyridine–hexamethyldisilazane (98:1:1, by vol.). After reaction, the sample was transferred to the Lipidex 5000 column with 4 × 0.5 ml of this solvent [13]. The elutent (2.0 ml) was collected. Prior to gas chromatography the solvent was evaporated to dryness and the derivatised sample redissolved in cyclohexane (50 μl).

(ii) *Trimethylsilyl ethers* (*TMS*). The dried steroid extract was dissolved in redistilled dry pyridine (250 μl). Hexamethyldisilazane (200 μl) and trimethylchlorosilane (5 μl) were added, and the tube stoppered and left to react for 4 h at room temperature [14]. The sample was then taken to dryness over nitrogen and the steroid derivatives extracted into cyclohexane (0.5 ml). By this technique usually all but tertiary hydroxyl groups become silylated.

(iii) *Methyloximes* (*MO*). To an aliquot (1/10) of the dried steroid extract was added 200 μl of a 1% (w/v) solution of methoxyamine hydrochloride in pyridine [15]. The sample tube was stoppered and left to react for 30 min at 60°C. The derivatised sample was taken to dryness and extracted into cyclohexane.

Gas chromatography. Gas chromatography was carried out using a Becker 409 gas chromatograph equipped with flame ionisation detection and housing a 20 meter open-tubular glass capillary column coated with silicone OV-101. The samples were introduced through an automatic solid injection system. Both temperature programmed operation, 160–250°C, in increments of 1°C/min, and isothermal operation at 230°C were employed and helium was the carrier gas at a flow rate of 1 ml/min.

Partial identification of a compound was based upon the retention time compared with 5α-cholestane using isothermal conditions and/or compared with a mixture of n-alkanes (C20–C32) which were added to the sample prior to gas chromatography.

Gas chromatography–mass spectrometry. Low resolution gas chromatography–mass spectrometry was carried out using a Varian-Aerograph 2700 gas chromatograph coupled to a Varian MAT-731 double-focussing mass spectrometer and the following conditions were employed; ionisation voltage 70 eV, accelerating voltage 8 KV, separator temperature 250°C, temperature of ion source 250°C, ionising current 800 μA. Repetitive magnetic scanning was carried out over the mass range 100–1000 a.m.u.

Computerized gas chromatography–mass spectrometry. Low resolution gas chromatography–mass spectrometry was carried out using an LKB 9000 combined gas chromatograph–mass spectrometer, housing an open-tubular glass capillary column coated with silicone OV-101. Modifications of the instrument and the methods for computerised evaluation of the mass spectral data have been described previously [16, 17]. Operating conditions were as follows: temperature of column 230°C isothermal, separator temperature 250°C, temperature of ion source 280°C, energy of bombarding electrons 22.5 eV, ionising current 60 μA, accelerating voltage 3.5 KV. Repetitive magnetic scanning was carried out over the mass range 0–800 a.m.u. and a delay of between 3–5 min allowed after injection of the sample and prior to the start of the scanning. The final detection of a steroid was based upon the retention time, the mass spectrum and the fragment ion current chromatograms (FIC) constructed of the characteristic ions given by the derivative of the steroid.

RESULTS AND DISCUSSION

The total volume of urine excreted by the two animals over a period of 7 days was 1300 ml. The results obtained after gas chromatography and gas chromatography–mass spectrometry of the major steroid isolated in the polar steroid fraction, and the reaction products yielded from borohydride reduction, periodate oxidation and chromate oxidation are summarised in Fig. 2.

Analysis of Sephadex LH-20 fraction 7. The major compound in this fraction had a retention time relative to 5α-cholestane of 1.78. After GC–MS the mass spectrum (Fig. 3) of this compound showed the molecular ion at *m/e* 771, which indicated a pregnane-pentol-one structure. The presence of a derivatised carbonyl group was indicated by the ion at *m/e* 740, the base peak in the spectrum, and formed by the loss of 31 a.m.u. The presence of ions at *m/e* 650, 560, 470, 380 and 290, formed by the consecutive losses of 90 a.m.u. from the base peak are indicative of five derivatised hydroxyl groups. Fragment ion current (FIC) chromatograms were constructed of the above ions and the recordings (Fig. 3) clearly indicated the presence of two isomers of the compound. Identification was not possible from this data alone, however, the prominent ions at *m/e* 244, 246 and 276 are typical of tetrahydrocorticosteroids with a dihydroxyacetone side-chain. The complete mass spectrum was similar to the spectrum of the MO-TMS derivative of tetrahydrocortisol except that the ions were 88 a.m.u. greater in magnitude due to an additional derivatised hydroxyl group. Further chemical analysis was necessary to yield more structural information.

Borohydride reduction. Reduction of carbonyl groups in positions 3, 17 and 20 will readily occur with sodium borohydride [18–20]. Reduction of this compound with sodium borohydride produced a change in its retention time ($t_R = 2.02$ for the TMS derivative) and mass spectrometry showed this product to have the structure of a pregnane-hexol. The mass spectrum (Fig. 4) showed the molecular ion at *m/e*

SAMPLE	STRUCTURE	DERIVATIVE	RETENTION TIME t_R	RETENTION TIME MU	MASS SPECTRUM M^+	MASS SPECTRUM Base peak	MASS SPECTRUM Important ions
SEPHADEX LH-20 FRACTION 7		MO-TMS_5	1.78	31.04	771	740	650,560,470,380,290,276,246,244
BOROHYDRIDE REDUCTION		TMS_6	2.02	31.55	816	243	726,713,636,623,611,546,533,521 443,431,353,341,251
PERIODATE OXIDATION		MO-TMS_3	0.94	28.32	567	536	477,446,387,356,266,213
		TMS_3	0.98	28.36	538	156	472,448,358,268
CHROMATE OXIDATION		MO_3	1.38	28.45	403	138	372,357,305,108,106

Fig. 2. A summary of the results obtained from the gas chromatographic and gas chromatography–mass spectrometric analysis of the polar steroid fraction and the major products yielded from borohydride reduction, periodate oxidation and chromate oxidation.

816. The presence of six derivatised hydroxyl groups was indicated by the ions at *m/e* 726 (M-90), 636 (M-[2 × 90]), 546 (M-[3 × 90]), 456 (M-[4 × 90]), 366 (M-[5 × 90]) and 276 (M-[6 × 90]), in addition to the ions at *m/e* 713, formed by the loss of 103 a.m.u. (due to fragmentation of a primary silylated hydroxyl group) and *m/e* 623, 533, 443, 353 and 263, formed by consecutive losses of 90 a.m.u. from this ion. The loss of 205 a.m.u. from the molecular ion giving rise to the ion at *m/e* 611 is a typical fragmentation of

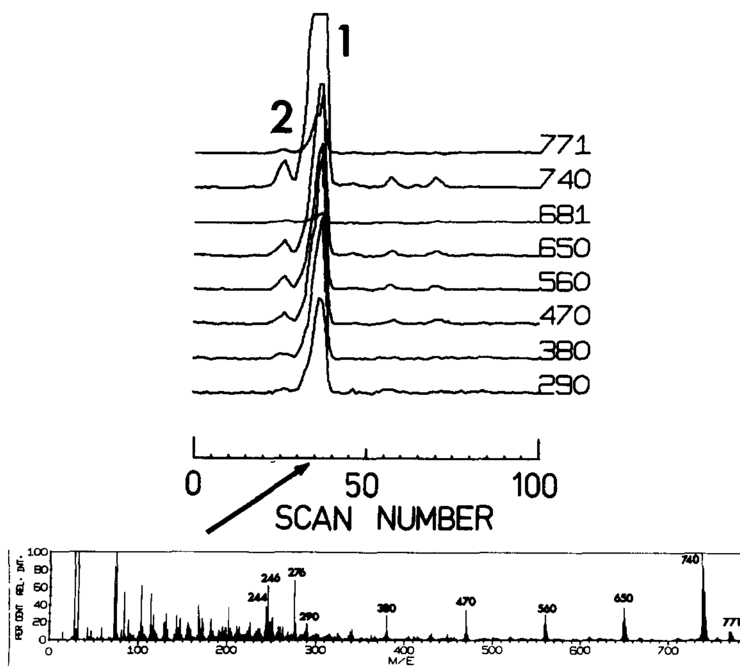

Fig. 3. Molecular ion and fragment ion current chromatograms of the ions characteristic of an MO-TMS derivative of pregnane-pentol-one. Two isomers of this steroid are indicated and the complete mass spectrum of the major steroid is illustrated.

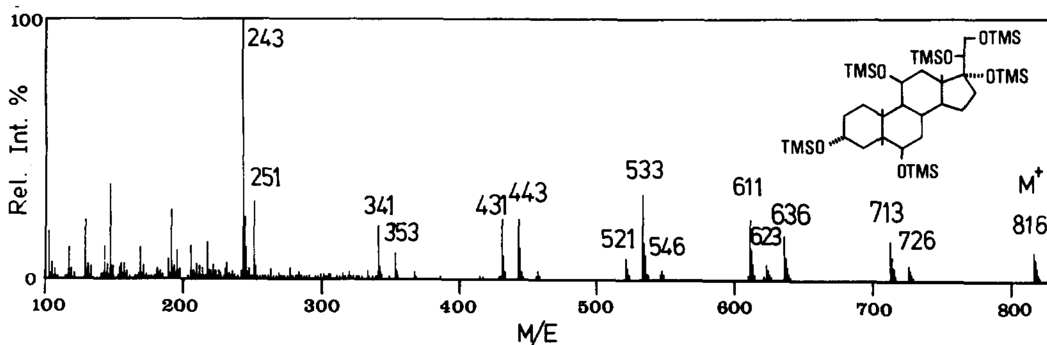

Fig. 4. The mass spectrum of the TMS derivative of 5β-pregnane-3α,6β,11β,17α,20β,21-hexol, the major product formed following borohydride reduction of the polar steroid fraction.

all corticosteroids possessing a glycerol side-chain and arises from cleavage between C-17 and 20, the loss of carbon atoms C-20 and C-21 together with two silylated hydroxyl groups. The base peak at *m/e* 243 is formed by cleavage across the *D*-ring and elimination of the side-chain together with carbon atoms 15, 16 and 17 and two silylated hydroxyl groups. This ion is also the base peak in the mass spectrum of the persilylated derivative of cortol. This spectrum showed remarkable similarities to that of the persilylated TMS derivative of cortol; however the molecular ion and fragment ions are 88 a.m.u. greater in magnitude, this being due to the additional silylated hydroxy group. This data clearly indicated that reduction of the C-20 carbonyl group had occurred with sodium borohydride, and demonstrated unequivocally a dihydroxyacetone side-chain in the original compound.

Periodate oxidation. Oxidation of a glycol side-chain in corticosteroids readily occurs with periodic acid, yielding a 17-oxosteroid [20, 21]. The sample was therefore first reduced with sodium borohydride to yield the trihydroxy side-chain and then reacted with periodic acid. The mass spectrum of the MO-TMS derivative of the major product (t_R = 0.94; Figure 5, upper panel) showed the molecular ion at *m/e* 567 and indicated a compound with an androstane-triol-one structure, thereby confirming side-chain cleavage. The presence of the derivatised carbonyl group in the C-17 position gave rise to the base peak in the spectrum at *m/e* 536 (M-31). The ions at *m/e* 477 (M-90), 387 (M-[2 × 90]), 297 (M-[3 × 90]) and

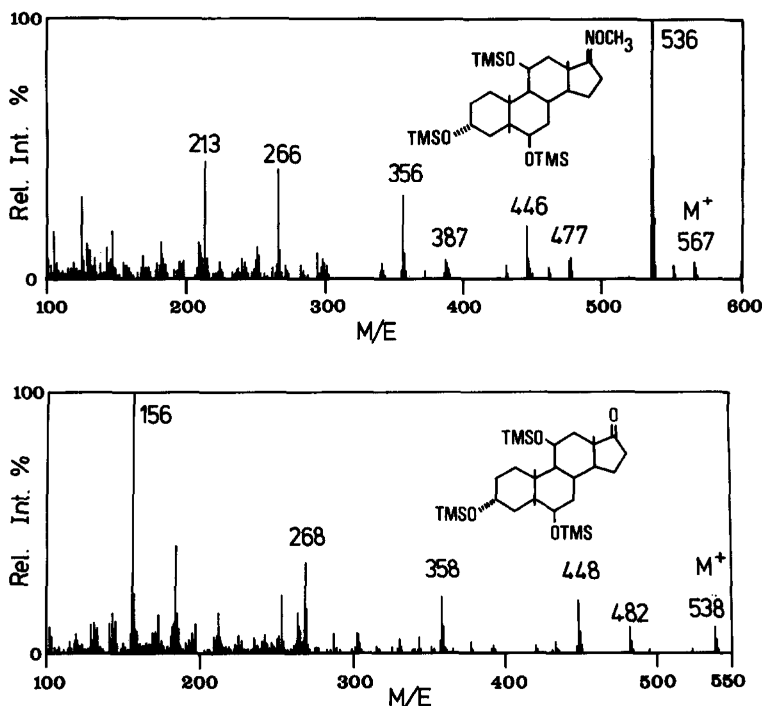

Fig. 5. The mass spectra of the 17-oxosteroids formed from borohydride reduction and periodate oxidation of the polar steroid fraction. The MO-TMS derivative (upper panel) and TMS derivative (lower panel) are illustrated.

m/e 446 (M-[31 + 90]), 356 (M-[31 + (2 × 90)]), 266 (M-[31 + (3 × 90)]) were formed by loss of the three derivatised hydroxyl groups. The prominent ion at *m/e* 213 is most probably formed by the *A*, *B* and *C* rings after loss of the silylated hydroxyl groups and cleavage across the *D*-ring.

The TMS derivative of the major product of periodate oxidation had a retention time relative to 5α-cholestane of 0.98 and the mass spectrum (Fig. 5, lower panel) also confirmed the structure to be an androstane-triol-one). The molecular ion was at *m/e* 538 and the loss of 56 a.m.u. giving rise to the ion at *m/e* 482 indicated an underivatised carbonyl group in the C-17 position and is formed by cleavage of the *D*-ring between carbon bonds 13:17 and 14:15. The three derivatised hydroxyl groups were evident from the ions at *m/e* 448 (M-90), 358 (M-[2 × 90]) and 268 (M-[3 × 90]), while the base peak at *m/e* 156 is typical of all TMS derivatives of 3,11β-dihydroxy-17-oxosteroids.

From the evidence of the above spectra the major polar steroid isolated in Sephadex LH-20 fraction 7 was shown to have the general structure of a pregnane-pentol-one. The presence of a dihydroxyacetone side-chain and hydroxyl groups in the C-3 and C-11 positions was confirmed. The position of the fifth hydroxyl group remained to be elucidated, however there was no evidence from the above spectra for it being in the *A*, *C* or *D* rings, and it was concluded that the most probable position was either C-6 or C-7. On the basis of extensive 6-hydroxylation occurring in the baboon, and the identification of other 6-hydroxylated steroids, in particular 6β-hydroxy-cortisol [6], this position seemed the most obvious choice. Confirmation of this was made following oxidation of all hydroxyl groups with chromic acid and GC–MS analysis of the methyloxime derivatives of the products. In addition to confirming a 3,6-dioxy-steroid the mass spectra also enabled the stereochemistry at the C-5 position to be elucidated [22]. The 17-oxosteroid was first formed in two stages; borohydride reduction of the C-20 carbonyl followed by side-chain cleavage with periodic acid. The product was then oxidised with chromic acid to give rise to an androstane-tetrone structure. The methyloxime derivative was then prepared and analysed by gas chromatography–mass spectrometry.

The gas chromatogram showed the presence of several products. The major component was eluted as a single broad peak ($t_R = 1.38$), a feature which is characteristic of steroids with methyloxime groups at C-3 and C-6 and a 5β-configuration [22]. Two components of lesser intensity were eluted at t_R 2.02 and 2.13, giving the appearance of a doublet, and indicating an analogous steroid with a 5α-configuration [22]. The formation of these elution patterns arises from the partial resolution of the syn- and anti-isomers of the O-methyloxime derivative of the carbonyl group in the C-3 position [23, 24].

The mass spectrum of the single broad peak

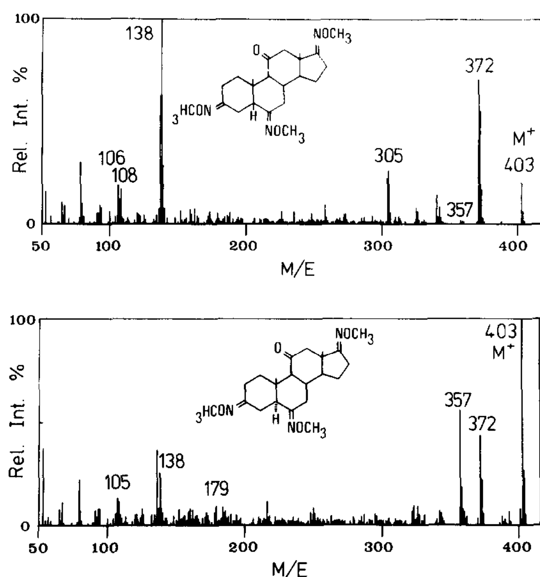

Fig. 6. The mass spectra of the methyloxime derivatives of the products formed following borohydride reduction, periodate oxidation and chromate oxidation of the polar steroid fraction. The major product gave the mass spectrum shown in the upper panel, which was identified as a 5β-steroid, while the minor product gave a mass spectrum (lower panel) typical of the 5α-isomer.

(5β-series) and one of the doublets (5α-series) is shown in Fig. 6. The molecular ion in both compounds was at *m/e* 403 confirming an MO derivative of an androstane-tetrone with an underivatised 11-oxo-group. Striking differences in the principal ions in each spectra were apparent and this was used to confirm the stereochemistry of the hydrogen at the C-5 position in both instances.

The base peak in the major compound (Fig. 6, upper panel) was at *m/e* 138, and this has been demonstrated to be the base peak in all spectra of the 5β-series of androstanes, pregnanes and cholestanes with a methyloxime group at positions C-3 and C-6, investigated by Allen [22]. The origin of this ion has not been completely defined, however it was suggested to arise after cleavage of the C-5:6 bond, transfer of a hydrogen atom from ring *A* to ring *B* and homolytic fission of the C-1:10 and C-4:5 bonds producing a fragment containing the O-methyloxime at the C-3 position.

The ion at *m/e* 372 (M-31) is formed by loss of a methoxyl group, while the ion at *m/e* 357 (M-46) is formed by the loss of a methoxyl and a methyl group. The intensity of these ions is governed by the stereochemistry at the C-5 position. In the 5β-series the order of relative intensities has been shown to be (M-31) > M > (M-46), while in the 5α series the order is M > (M-46) > (M-31) [22]. The above data clearly indicates the major oxidation product to be a 3,6-dioxy-steroid with a 5β-hydrogen.

The mass spectrum of the minor oxidation product (Fig. 6, lower panel) clearly indicated a 5α-configu-

ration. The base peak which was also the molecular ion was at *m/e* 403 and the order of relative intensity of the ions M, (M-31) and (M-46) clearly indicated the compound to have a 5α-stereochemistry. This was further supported by the ion at *m/e* 138 which is much less prominent than in compounds with the 5β-configuration.

The above data confirmed the structure of the major steroid isolated in Sephadex LH-20 fraction 7 to be 3α,6β,11β,17α,21-pentahydroxy-5β-pregnan-20-one, while trace amounts of the 5α-isomer were also present.

Although the baboon has been shown to exhibit efficient 5β-reduction, particularly of administered synthetic steroids [25], the above finding is in contrast to the relationship of THF (5β) to *allo*-THF (5α) in which the latter stereoisomer has been found to be quantitatively the predominant steroid excreted as a glucuronide conjugate by the baboon [6]. This may be explained by either selective 6β-hydroxylation of 5β-corticosteroid metabolites, which would then give rise to an increased ratio of 5α:5β corticosteroids which are not 6β-hydroxylated, or selective 5β-reduction of the 3-oxo-4-ene group of 6β-hydroxy-cortisol. The metabolic pathway leading to the formation of this steroid is at present under investigation and this will be the subject of a separate communication.

The stereochemistry of the hydroxyl group at the C-3 position is assumed to be 3α on the basis of retention data. 3β,5α-corticosteroids have been demonstrated to occur in the urine from species of macaque monkey (*Macaca fascicularis*) but have considerably longer gas chromatographic retention times [26], and although 3β,5β-corticosteroids would have similar retention times, it would appear unlikely that this is the configuration of 6β-THF because all of the principal corticosteroids identified in the urine of the baboon have been shown to have a 3α-hydroxyl [6]. The stereochemistry of the C-11 hydroxyl group is assumed to be 11β on the basis of there being no reports of naturally occurring 11α-hydroxy-steroids in mammals. While the configuration of the C-6 hydroxyl group has been assigned as 6β since reference steroids were not available to enable confirmation, this is a tentative proposal based on the evidence of the presence of other 6β-hydroxy-corticosteroids in the urine from this species, in particular 6β-hydroxycortisol [6].

Quantitatively 3α,6β,11β,17α,21-pentahydroxy-5β-pregnan-20-one has been shown to be the major steroid excreted in the urine of male baboons of the species *Papio hamadryas*. The daily level of excretion was 13–17 μg/24 h/kg body weight [6]. In a comprehensive investigation of steroid excretion and conjugation by the baboon, this steroid was found to be excreted as a neutral compound [6] and by virtue of the high polarity it is most probably unconjugated in urine.

Other polar corticosteroids. The gas chromatogram of the MO-TMS derivative of Sephadex LH-20 fraction 7 also indicated a compound with retention time relative to 5α-cholestane of 1.47. The mass spectrum of this compound is shown in Fig. 7. The molecular ion was at *m/e* 697 which indicated a pregnane-tetrol-dione structure (with one underivatised carbonyl group). The presence of a derivatised carbonyl group was indicated by the ion at *m/e* 666 (M-31) which was also the base peak in the spectrum. The ions at *m/e* 244, 246 and 276 are characteristic of corticosteroids with a dihydroxyacetone side-chain, therefore confirming the derivatised carbonyl group to be in the C-20 position. The ion at *m/e* 594 (M-103) is formed by loss of the primary hydroxyl group in the C-21 position. Ions at *m/e* 576, 486, 396 and 306 are formed by the consecutive loss of 90 a.m.u. from the base peak due to the presence of four derivatised hydroxyl groups. The underivatised carbonyl group must be in the C-11 position since this is the only position which will not form a methyloxime derivative [24]. The fragmentation pattern of this spectrum is strikingly similar to that of the MO-TMS derivative of tetrahydrocortisone, however, the molecular ion and fragment ions were 88 a.m.u. greater in magnitude. From this evidence and in view of the identification of a 6β-hydroxylated derivative of tetrahydrocortisol, it is extremely likely that this steroid is the 6β-hydroxy-derivative of tetrahydrocortisone (3α,6β,17α,21-tetrahydroxy-5ξ-pregnane-11,20-dione.)

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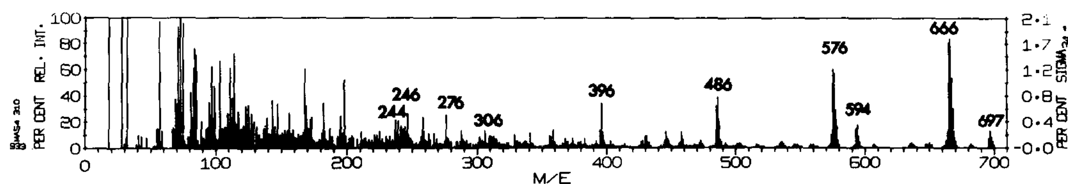

Fig. 7. The mass spectrum of the MO-TMS derivative of the compound eluted at $t_R = 1.47$ in the polar steroid fraction from Sephadex LH-20.

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