6-HYDROXYLATION, AN IMPORTANT ROUTE IN THE METABOLISM OF CORTICOSTEROIDS BY THE BABOON: THE FATE OF ADMINISTERED TETRAHYDROCORTISOL

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1. Introduction

The adult male and female baboon has been shown to excrete relatively large amounts of polar corticosteroids [1–3]. Following the administration of radiolabelled cortisol to female baboons (*Papio papio*), only 11% of the radioactivity recovered in the urine was accounted for by the metabolites tetrahydrocortisol (THF) and tetrahydrocortisone (THE), while 29% was associated with more polar metabolites [2]. Although these compounds were not identified, it was suggested that they might be hydroxylated derivatives of THF [2].

During a detailed investigation of urinary steroid conjugation by the baboon (*Papio hamadryas*), quantitatively one of the major steroids exreted was found to be more polar than THF [1]. Several milligrams of this compound were isolated from a large pool of urine and using gas chromatography-mass spectrometry (GC-MS) identified as 3α,6β,11β,17α,21-pentahydroxy-5β-pregnan-20-one (6β-hydroxy-THF) [4].

In view of the importance of 6β-hydroxylation in this animal, preliminary studies were made to investigate the pathway leading to the formation of 6β-hydroxy-THF.

2. Materials and methods

The source of chemicals and reagents have been well documented in previous communications [1,4,5]. Tetrahydrocortisol (Grade II, approx. 95% purity) was obtained from Sigma Chemical Co. (St. Louis, Mo., USA) and was used without further purification. The steroid was prepared for injection by suspending 170 mg in a 10% solution of ethanol in isotonic saline.

One adult female baboon (body wt 10 kg) of the species *Papio anubis* was injected intramuscularly with the solution of THF. Urine collections were made for two days prior to administration of the steroid and for three days thereafter. The animal was housed in a metabolism cage in a temperature and light controlled environment and the urine was immediately frozen to −70°C by collection into vessels surrounded by dry-ice.

Complete details of the analytical procedures are described elsewhere [5]. Steroids were extracted from urine using the neutral resin, Amberlite XAD-2. The steroids were then separated into their conjugate classes by anion exchange chromatography on a lipophilic ion exchange gel, diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20). Following enzymatic and combined enzymatic–solvolytic hydrolysis of the conjugate groups and preparation of the *O*-methyloxime-trimethylsilyl ether (MO-TMS) derivatives [6], the fractions were analysed by GC with open-tubular glass capillary columns and combined GC-MS.

GC was carried out on a Becker 409 gas chromatoequipped with a flame ionisation detector and housing a 25 metre open-tubular glass capillary column coated with silicone OV-101. Samples were introduced via an automatic solid injection system. Helium was the carrier gas and the flow rate through the column was approximately 2 ml/min. Both temperature programmed operation (160–260°C in increments of 2½°C/min)

and isothermal (235°C) conditions were employed.

Low resolution GC-MS was carried out using a Varian-Aerograph 2700 gas chromatograph housing a glass column (3 m X 0.4 mm) packed with 3% silicone OV-1 on Chromasorb W, and coupled to a Varian MAT-731 double-focussing mass spectrometer. The details of the GC-MS conditions have been reported previously [1].

The identification of a steroid was based upon the gas chromatography retention time relative to 5α-cholestane using isothermal conditions (t_R), the retention time relative to a homologous series of straight chain aliphatic hydrocarbons (C-20 to C-32) using temperature programmed operation (MU value), the complete mass spectrum and on selection ion current chromatograms of m/e values characteristic of MO-TMS and TMS derivatives of the steroid. The steroids were quantified as their MO-TMS and TMS derivatives by gas chromatography using 5α-androstane-3α,17α-diol and stigmasterol as internal standards [1].

3. Results

3.1. *Neutral fraction*

The GC recordings of the MO-TMS derivative of the unconjugated steroid fraction from the five daily urine collections are shown in fig.1. The major steroid with a retention time relative to 5α-cholestane of 2.2 for the MO-TMS derivative, was shown by mass spectrometry to have the structure 3α,6β,11β,17α,21-pentahydroxy-5β-pregnan-20-one (6β-hydroxy-THF). The quantitative excretion of the principal corticosteroids is listed in table 1. Following the administration of THF the excretion of 6β-hydroxy-THF in the urine increased many fold. An associated increase in the excretion of 6β-hydroxy-THE was also observed.

3.2. *Glucuronide fraction*

The GC recordings of the MO-TMS derivative of this fraction are shown in fig.1. The major steroid excreted in the urine as a glucuronide conjugate was 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one (allo-THF; t_R = 1.80 for the MO-TMS derivative) while a significant amount of the corresponding 3α,5β isomer (THF; t_R = 1.73 for the MO-TMS derivative) was also detected (table 1). Following THF administration, a relatively large amount of THF-glucuronide was excreted in the urine, particularly during the first 24 h, with a concomitant increase in the level of the 20-reduced metabolites, α-cortol and β-cortol.

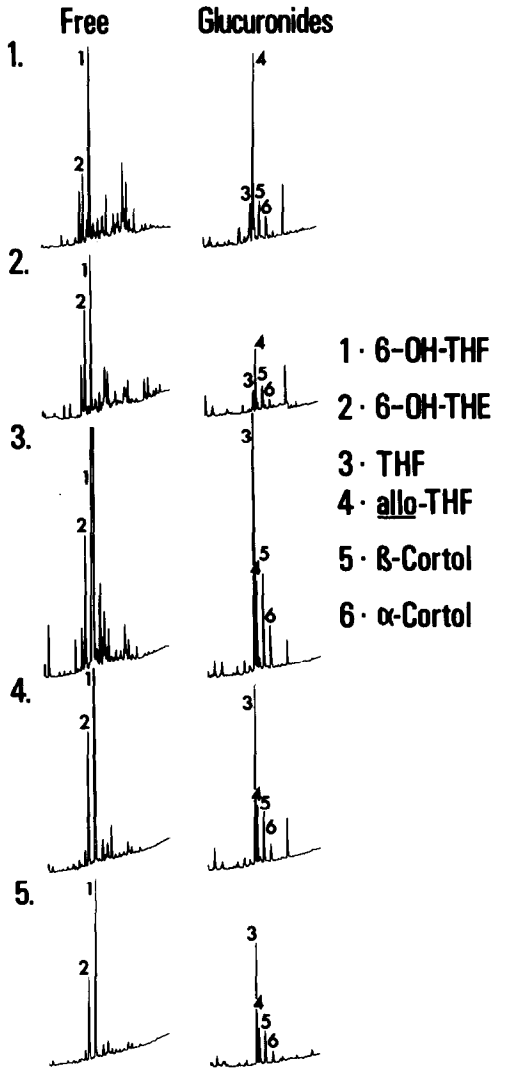

Fig.1. The gas chromatography recordings of the MO-TMS derivatives of the steroids isolated in the unconjugated steroid (free) fraction and glucuronide conjugate fractions from the urine of a baboon (*Papio anubis*). The excretion of polar steroids are shown for two days (1 and 2) prior to the injection of a dose of THF and three days thereafter (3,4 and 5). Gas chromatography was carried out on an open-tubular glass capillary column as described in the text.

Table 1
Excretion of the principal corticosteroids in baboon urine, prior to and following the administration of tetrahydrocortisol

Steroid	Excretion (μg/day)				
	Pre-THF		Post-THF		
	1	2	3	4	5
Unconjugated					
3α,6β,11β,17α,21-Pentahydroxy-5β-pregnan-20-one	948	602	6040	2884	2278
3α,6β,17α,21-Tetrahydroxy-5β-pregnane-11,20-dione	223	176	635	1061	984
Glucuronides					
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	143	53	5818	487	481
3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one	573	147	496	156	203
3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one	87	26	84	26	43
3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one / 5β-Pregnane-3α,11β,17α,20β,21-pentol	130	64	593	148	169
5β-Pregnane-3α,11β,17α,20α,21-pentol	83	23	348	70	101

3.3. *Sulphate fraction*

Only traces of corticosteroid metabolites were identified as sulphate conjugates and consequently this fraction was of little interest in this present study.

4. Discussion

Steroids extracted from urine were separated into specific conjugate groups using a lipophilic ion exchange gel, diethylaminohydroxylpropyl Sephadex LH-20, DEAP-LH-20 [5]. Ion exchange chromatography on this gel yielded fractions consisting of unconjugated steroids, glucuronide conjugates and total sulphates. The separation of steroid monosulphates and disulphates is possible using these methods [5], however, since only three steroid disulphates have been identified in baboon urine [1], in this study the sulphate conjugates were collected as a single group. The use of GC with the high resolving power of open-tubular glass capillary columns was essential to the determination of the stereochemistry of the numerous isomers of polar steroids excreted by this animal. The steroids were characterised by GC-MS as the MO-TMS and TMS derivatives.

In this study one adult female animal of the species *Papio anubis* was used and two 24-h urine collections were obtained prior to the administration of THF, to establish the normal pattern of steroid excretion.

Analysis of the urine collected on days 1 and 2 confirmed the qualitative data obtained earlier for steroid excretion by two other species of baboon, *Papio hamadryas, Papio papio*, although quantitatively the major corticosteroids were excreted in slightly greater amounts when calculated on a body weight basis (table 1).

6β-Hydroxy-THF was found to be the major corticosteroid excreted by this animal and it was shown to be mainly unconjugated in urine, although trace amounts were found as glucuronide and sulphate conjugates. These very polar steroids have previously been shown to be excreted principally unconjugated in urine [1], but this finding differed from earlier reports which showed a greater proportion of glucuronide and sulphate conjugates [2,7]. Less specific methods, which included liquid–liquid partitioning, aqueous back-washing and differential enzyme hydrolysis, were used to isolated the steroid conjugates in these earlier studies [2,7], and this probably explains the conflicting data.

Following the administration of a large amount of tetrahydrocortisol a 10-fold increase in the excretion of unconjugated 6β-hydroxy-THF was found after the first day and the excretion remained high for up

to three days thereafter. There was no evidence for 6β-hydroxy-THF being excreted in increased amounts as a glucuronide or sulphate conjugate, suggesting that this compound is either an unsuitable substrate for these conjugation systems or that its polarity is sufficient to enable transfer across the renal tubule without prior conjugation. A substantial increase in the level of 6β-hydroxy-THE was also observed after the administration of THF, indicating that to a limited extent conversion of the 11β-hydroxyl group to an 11-oxo group had occurred either prior to or following 6β-hydroxylation. This phenomenon has been demonstrated previously in studies of corticosteroid metabolism in dogs [8] and in man [9].

The principal glucuronide conjugate excreted by this animal was allo-THF, although smaller amounts of THF were also identified. After THF was administered, large amounts were excreted in the urine as the glucuronide conjugate. In spite of the large dose administered there was no appreciable excretion of THF in the unchanged form; there was however a marked increase in the excretion of the 20 reduced metabolites β-cortol and α-cortol as their glucuronide conjugates, particularly during the first day.

It is clear from the quantitative data that the yield of metabolites was low compared with the dose of THF administered. This could be due to elimination of the steroid in the faeces, however in this study faecal collections were not made. Alternatively, since the steroid was injected in a suspension it is possible that these results reflect only the metabolism of the solubilised steroid.

It is evident from this experiment that the baboon has an ability to efficiently 6β-hydroxylate tetrahydrocortisol in vivo. Furthermore, it is also possible that 6β-hydroxylation is selective towards the 3α,5β isomer (THF) thereby accounting for the greater proportion of allo-THF (3α,5α) in the urine. Whether the baboon has the ability to 6β-hydroxylate the 5α isomer (allo-THF) has not been investigated. 5β-Reduction of the 3-oxo-4-ene structure of 6β-hydroxycortisol could offer an alternative route to the formation of 6β-hydroxy-THF, but the unavailability of this steroid has made this difficult to investigate.

In spite of this it would appear that 6β-hydroxylation of THF offers an important route in corticosteroid metabolism by the baboon. 6β-Hydroxylated derivatives of 20-dihydrocortisol have been identified in urine from normal men [9] and pregnant women [10], and in view of this a search was recently made for 6β-hydroxy-THF in the urine collected at intervals from a pregnant woman. It was of interest to find that while cortisol, 6β-hydroxycortisol, THF and THE showed a slight increase during the last trimester, the excretion of 6β-hydroxy-THF was marked during this period and attained a level approximately 400 μg/day at term in this subject. In view of this, further investigations are being carried out to ascertain the significance of this steroid and other polar corticosteroids in pregnancy.

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