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STEROID EXCRETION IN THE NEO-NATAL PERIOD: A COMPARATIVE STUDY OF THE EXCRETION OF STEROIDS BY HUMAN, APE AND RHESUS MONKEY INFANTS

C. H. L. SHACKLETON

Division of Clinical Chemistry, Clinical Research Centre, Watford Road, Harrow, Middlesex, England

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SUMMARY

Analysis by gas chromatography-mass spectrometry of the steroid sulphates present in urine and faeces from a 3-week-old orang-utan indicated that, as in the human infant, 3β-hydroxy-5-ene steroids are the major urinary steroids. The production of these steroids in the neonatal period is carried out by the foetal zone of the adrenal gland which does not disappear in ape and human infants until several weeks after birth.

The major steroids identified in urine were: 3β,16α-dihydroxy-5-androsten-17-one, 3β,17β-dihydroxy-5-androsten-16-one, 5-androstene-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol, 3β,16α-dihydroxy-5-pregnen-20-one and 3β,21-dihydroxy-5-pregnen-20-one. A pregnenetriol, an androstenetetrol, an androstenetriolone, tetrahydrocortisone and cortolone were also detected.

The major steroids identified in the steroid sulphate fraction of faeces were: 3β-hydroxy-5-androsten-17-one (DHA), 3β-hydroxy-5-pregnen-20-one (pregnenolone), 5-androstene-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol, 5-pregnene-3β,16α,20α-triol, 5-pregnene-3β,20α,21-triol and 24-hydroxy cholesterol.

The total amounts of steroid sulphates excreted in urine and faeces were similar.

The urinary steroids of newborn rhesus monkeys were all "saturated", no 3β-hydroxy-5-ene-or 16-hydroxylated steroids were detected. The following were identified: androsterone, 5β-pregnane-3α,17α,20α-triol, 3α,21-dihydroxy-5β-pregnane-11,20-dione and tetrahydrocortisone. The lack of 3β-hydroxy-5-ene steroids from urine is due to the disappearance of the foetal zone prior to birth in this species.

INTRODUCTION

Human and chimpanzee infants excrete similar steroids during early infancy [1–4], and in contrast to adults 3β-hydroxy-5-ene steroid sulphates predominate. Due to active hepatic and adrenal 16-hydroxylases present during this period of life the major steroids excreted have hydroxyl groups at position 16. However, the morphology of the adrenal glands of human and chimpanzee infants is almost indistinguishable [5], so the similarities of steroid production during this period of life are not unexpected.

These studies have now been extended to include the identification of steroids in urine and faeces specimens from orang-utan and rhesus monkey infants.

MATERIALS AND METHODS

Non-standard steroid abbreviations

16-Oxo-androstenediol, 3β,17β-dihydroxy-5-androsten-16-one; tetrahydro-11-dehydrocorticosterone, 3α,21-dihydroxy-5β-pregnane-11,20-dione; cholanediol, 5β-cholane-3α,24-diol.

Reference steroids. The suppliers of the reference steroids have been detailed in previous communications [1–4].

Steroid extraction

Orang-utan urine and faeces. Urine specimens from a 3-week-old male orang-utan which was being hand-reared were collected in plastic bags attached to the animal. Faecal specimens were removed from the diapers.

The methods used for the extraction of steroids from urine and faeces have been described in detail in previous communications [1, 4, 5]. Following addition of labelled DHA–sulphate, steroids were extracted on a column of Amberlite XAD-2 resin. They were absorbed by the resin and recovered by elution with methanol. The methanol extract was dried and chromatographed on a Sephadex LH-20 column (10 g) using methanol–chloroform (1:1, made 0·01 M with respect to sodium chloride) as the eluent. Fractions of vol 2·5 ml were collected and a small portion of every third fraction was removed for measurement of

radioactivity. Labelled DHA–sulphate was present in fractions between 75 and 100 ml of eluent. Fractions between 60 and 220 ml were pooled and called the monosulphate fraction. The column was eluted with 200 ml methanol to obtain steroid disulphates.

The faecal samples were homogenized in chloroform methanol (1:1, v/v) (1). After centrifugation the solvents were removed *in vacuo* and the residue was partitioned between light petroleum (b.p. 60–70°C) and aqueous ethanol (3:7, v/v). The ethanol phase was passed through an Amberlyst-15 column in the sodium form, taken to dryness and the dried extract chromatographed on a column of Sephadex LH-20 in similar manner to the urine extract.

The monosulphate and disulphate fractions of urine and faeces were hydrolysed by the sulphatases present in the digestive juices of the snail *Helix pomatia*, and following extraction by Amberlite XAD-2 the freed steroids were purified by chromatography on small columns of silicic acid (200 mg). Three fractions were collected. 5 ml benzene–ethylacetate (19:1, v/v), 5 ml benzene–ethylacetate (65:35, v/v) and 5 ml ethylacetate. Trimethylsilyl ethers and methyloxime trimethylsilyl ethers were prepared from the second and third fractions and analysis was by gas chromatography and mass spectrometry. QF-1 and SE-30 stationary phases were used for the analyses and retention volumes were determined relative to 5α-cholestane ($r_{\text{steroid/cholestane}}$).* A semi-quantitative assay of the steroids in the urine and faecal samples has been made by relating the areas of peaks on the gas chromatogram to cholanediol used as internal standard. The recovery of [^{3}H]-DHA from [^{3}H]-DHA-sulphate was determined by measuring the radioactivity in the second fraction obtained during silicic acid chromatography. The recovery of DHA from DHA sulphate added to urine was 62% and to faeces 50%.

An LKB 9000 instrument was used for gas chromatography–mass spectrometry of the samples from the orang-utan, with the energy of bombarding electrons 22·5 eV and the ionizing current 60 μA. Mass spectra were recorded on magnetic tape using the incremental mode of operation described by Hedfjäll *et al.* [6] and the data was processed by an IBM 1802 computer.

Rhesus monkey urine. Urine was collected from five infants on the 1st and 2nd days of life. Total volume of 4 ml so obtained was divided into two portions and acetate buffer (3 ml) was added and the steroid conjugates were hydrolysed by *Helix pomatia* digestive enzymes. The freed steroids were extracted by Amberlite XAD-2 and purified on small silicic acid columns prepared in benzene. Two fractions were collected: 1, 5 ml benzene–ethylacetate (19:1, v/v) and 2, 5 ml ethylacetate. The first fraction was discarded and steroid analyses were carried out on the second fraction. Trimethylsilyl ethers and methyloxime–trimethylsilyl ethers were prepared of the steroids which were then analysed by gas chromatography and gas chromatography-mass spectrometry. The steroid extracts were chromatographed on OV-1 columns with temperature programming (200–270°C, 1·5°C/min). Mass spectrometry was carried out by using a Varian gas chromatograph on line to a Varian MAT 731 mass spectrometer (ionizing voltage, 70 eV).

RESULTS

Orang-utan infant urinary steroids

Urinary steroids were considered identified when relative retention volumes and mass spectra obtained by gas chromatographic–mass spectrometric analysis were virtually identical to the appropriate reference compounds.

Previous publications by the author and associates have described the identification of steroids in faeces [1, 2] and urine from human infants [3] and in urine from an infant chimpanzee [4]. Mass spectra of relevant steroids have been published in these communications and since the steroids detected in urine and faeces from the baby orang-utan proved to be the same as those previously identified, their mass spectra will not be given again. The compounds must also have similar retention vol. to reference compounds on the SE-30 and/or QF-1 stationary phases used for the gas chromatographic separation. These retention vol. relative to 5α-cholestane ($r_{\text{steroid/cholestane}}$) are given in Tables 1 and 2 together with semi-quantitative results.

It was impossible to collect complete 24 h samples of urine and faeces, so excretory levels are reported as μg/100 ml or μg/100 g. The urine sample from the orang-utan was collected over a period of 4 h and it seems probable that the total urinary volume would be between 50 and 100 ml. The faecal sample approximates to the total excreted during 24 h. The quantitative results have been corrected for the recovery of [^{3}H]-DHA–sulphate.

Based on a urine excretion of between 50 and 100 ml during 24 h and a faecal excretion of 10 g it can be deduced that approximately equivalent amounts of steroid were excreted in urine and faeces.

After the investigations on steroid sulphates had been completed, a further sample of urine was hydrolysed in an attempt to identify and quantitate metabo-

* The abbreviations used for relative retention volumes are those suggested in a IUPAC Information Bulletin "Appendices on tentative nomenclature, symbols, units and standards. Recommendations on nomenclatures for Chromatography" February 1972. As this bulletin was a preliminary report these abbreviations are not confirmed.

Table 1. Gas chromatographic relative retention volumes ($r_{steroid/cholestane}$) of silyl ethers of steroids identified in urine from an infant orang-utan and results of semi-quantitative estimation

	Conjugate*	SE-30 225°C Ref.†	Ur†	QF-1 225°C Ref.†	Ur	μg/100 ml‡	Other sources§
DHA	MoS	0·48	0·48	—	—	Trace	h
5-Androstene-3β,17α-diol	DiS	0·53	0·53	—	—	Trace	h, c
16α-Hydroxy DHA	MoS, DiS	0·86	0·84	1·30	1·34	265	h, c
16β-Hydroxy DHA	MoS, DiS	0·95	0·94	1·51	1·50	25	h, c
16-Oxo-androstenediol	MoS, DiS	0·95	0·94	1·69	1·64	245	h, c
5-Androstene-3β,16α,17β-triol	MoS, DiS	1·25	1·24	0·95	1·02	700	h, c
5-Androstene-3β,16β,17α-triol	MoS, DiS	1·05	1·15	0·79	0·83	25	h, c
16α-Hydroxypregnenolone	MoS	1·33	1·30	1·92	1·90	100	h, c
21-Hydroxypregnenolone	DiS	1·96	1·97	2·80	2·75	80	h, c
Androstenetriolone 433‖	MoS	—	1·54	—	—	30	h
Androstenetetrol 19¶	MoS	—	1·96	—	—	Trace	h, c

* Form of conjugation: MoS—monosulphate; DiS—disulphate.
† Relative retention time of reference steroid; Ur—relative retention time of urinary steroid.
‡ Approximate concentration in urine.
§ If steroid has been detected in urine from infant human (h) or chimpanzee (c). Shackleton and co-workers [3–5].
‖ Mass spectrum indicates mol. wt. of 536 (trimethylsilyl ether). Base peak at m/e 433.
¶ Mass spectrum indicates mol. wt. of 610 (trimethylsilyl ether). Base peak at m/e 191. Secondary peak at m/e 417. This steroid has a slightly longer retention time than a similar steroid isolated from human infant urine [3].

lites of cortisol. The method used was identical to that used in the identification of steroids in the rhesus monkey urine.

Tetrahydrocortisone and α-cortolone were identified by gas chromatography–mass spectrometry, their concentration in urine being 125 and 60 μg/100 ml respectively.

Rhesus monkey infant urinary steroids

Due to lack of material it was not possible to make such a detailed analysis of the urinary steroids from the rhesus monkey infants. Several steroids were identified and several others were detected but it was not possible to assign a structure to them. Mass spectra of the following steroids were obtained: androsterone, pregnanetriol, tetrahydro-11-dehydrocorticosterone and tetrahydrocortisone. These steroids are all "saturated" and no 3β-hydroxy-5-ene steroids were detected in the urine samples.

The mass-spectrum of the trimethylsilyl ether of tetrahydro-11-dehydrocorticosterone is illustrated in Fig. 1. The molecular ion was at m/e 492 and there is

Table 2. Gas chromatographic relative retention volumes ($r_{steroid/cholestane}$) of silyl ethers of steroids identified in faeces from an infant orang-utan and results of a semi-quantitative estimation

	Conjugate*	SE-30 225°C Ref.†	Faecal	μg/100 g‡	Other sources§
DHA	MoS	0·48	0·48	225	h
5-Androstene-3β,17β-diol	MoS	0·61	0·61	65	h
16α-Hydroxy DHA	DiS	0·86	0·86	35	h
16β-Hydroxy DHA	DiS	0·95	0·95	Trace	h
16-Oxo-androstenediol	DiS	0·95	0·95	40	h
5-Androstene-3β,16β,17α-triol	MoS, DiS	1·15	1·17	360	h
5-Androstene-3β,16α,17β-triol	MoS, DiS	1·25	1·25	380	h
Pregnenolone	MoS	0·70	0·68	1350	h
5-Pregnene-3β,20α-diol	MoS	1·20	1·18	125	h
16α-Hydroxypregnenolone	DiS	1·33	1·32	Trace	h
21-Hydroxypregnenolone	MoS, DiS	1·96	2·04	40	
5-Pregnene-3β,16α,20α-triol	MoS	1·82	1·89	35	h
5-Pregnene-3β,20α,21-triol	MoS, DiS	2·40	2·46	35	h
24-Hydroxycholesterol	MoS	5·72	5·80	545	h

* † As for Table 1.
‡ Approximate concentration in faeces.
§ If detected in faeces from human infants.

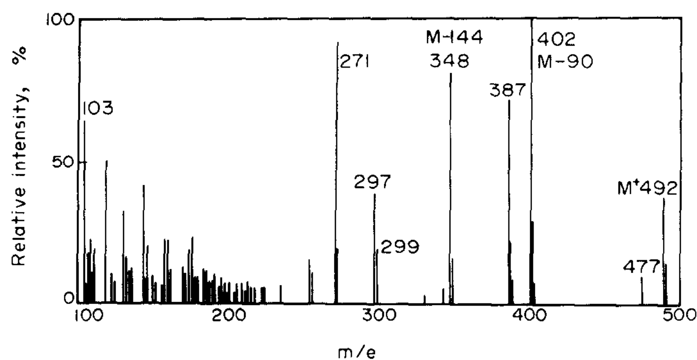

Fig. 1. Mass spectrum of the silyl ether of tetrahydro-11-dehydrocorticosterone isolated from rhesus monkey neo-natal urine.

a prominent peak at *m/e* 402, M-90. The peak at *m/e* 299 represents the loss of 103 mass units from *m/e* 402—this fragmentation indicating the presence of a "primary" trimethylsilyl group. The peak at *m/e* 348 (M-144) probably represents the loss of the side-chain and carbon 17. Unfortunately due to lack of material it was not possible to quantify the steroids excreted by these infants but the urinary concentration of the major steroids was around 200 μg/100 ml urine.

DISCUSSION

Before discussing the qualitative and quantitative excretion of steroids by infant simians it must be emphasized that only one infant orang-utan and chimpanzee were studied, and the urine collection from the infant rhesus monkeys was small and unsatisfactory. In no case was it possible to obtain accurate 24 h samples of urine. In spite of this several important similarities and differences were noted between the steroid excretion of the infant orang-utan, chimpanzee, rhesus, monkeys and human infants. These were particularly significant and were unlikely to be due to individual variation between animals.

Although the urinary 3β-hydroxy-5-ene steroids excreted by the orang-utan were identical to those previously detected in urine from human infants [3], there were apparent differences in the quantitative relationships and the overall pattern of excretion was more similar to that of a baby chimpanzee previously studied [4]. The relative quantitative importance of steroids excreted by human, chimpanzee and orang-utan infants is illustrated in Fig. 2. The most obvious difference between the species is the greater importance of 16α-hydroxypregnenolone in urine from human infants than in ape urine where androstenetriols are the predominant steroids. However, in human infants androstenetriol becomes the dominant 3β-hydroxy-5-ene steroid after the 1st month of life [7], which may indicate that the orang-utan and chimpanzee infants mature slightly earlier than human infants.

The similarities in steroid excretion between human and orang-utan infants are not restricted to urine since the present study has shown that steroid sulphate excretion in the faeces is important in the early life of the orang-utans. As in urine, 16α-hydroxypregnenolone is a minor metabolite whereas in human infant faeces it is the predominant steroid. Pregnenolone dominates the 3β-hydroxy-5-ene steroid fraction in infant orang-utans. The relative quantitative importance of the steroids present in infant humans and orang-utan faeces is illustrated in Fig. 3.

It has been reported previously [2] that approximately equivalent amounts of steroid are excreted in

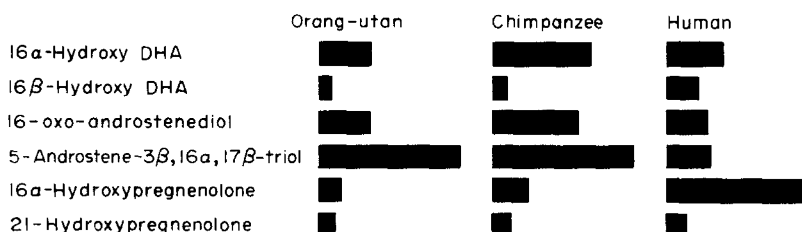

Fig. 2. The relative importance of steroids in urine from human infants, (results were compiled from the mean excretion of these steroids by eight infants 14–30 days old [15]) and an infant orang-utan and chimpanzee [4].

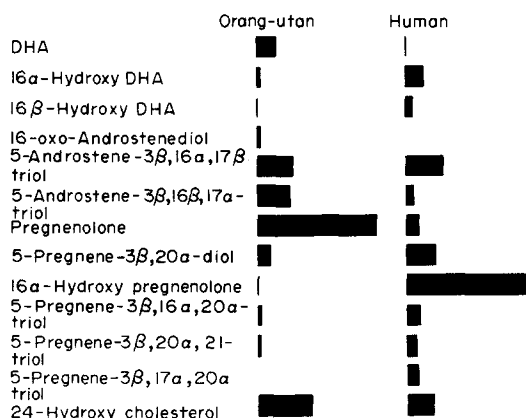

Fig. 3. The relative importance (quantitative importance of individual steroids relative to the predominant steroid) of steroids in faeces from human infants (data from Gustafsson *et al.* [1]: the results were compiled from the mean faecal excretion of these steroids by five infants 14–28 days old.

the urine and faeces of human infants during early infancy. The quantitative results reported here suggest that this is also true for the orang-utan infant studied.

The 3β-hydroxy-5-ene steroids present in urine and faeces from human infants originate in the adrenal glands although some steps of the biosynthesis (e.g. 16α-hydroxylation) also take place in the liver. This has been demonstrated by Easterling and co-workers [8] who showed that the level of 16α-hydroxy-DHA in the blood plasma of anencephalic foetuses was extremely low. In addition, Eneroth *et al.* [9] could not detect any 3β-hydroxy-5-ene steroids in urine from an anencephalic infant. Although the production of 16α-hydroxy DHA in anencephaly is extremely low the hepatic 16α-hydroxylase activity is high [10] suggesting the lack of suitable precursors.

The 3β-hydroxy-5-ene steroids must be synthesized by the foetal zone of the adrenal cortex since only this zone is absent in anencephaly. Adrenal glands of human and chimpanzee newborns are almost indistinguishable morphologically, both containing the large foetal zone distinct from the cortex [5]. It is therefore not surprising that chimpanzee and human infants excrete similar 3β-hydroxy-5-ene steroids during the peri-natal period. Other great apes, e.g. the orang-utan may also be expected to have a similar adrenal gland at birth and to excrete similar steroids.

The lack of 3β-hydroxy-5-ene steroids in urine from Rhesus monkey infants agrees well with the development of the adrenal glands during gestation. Lanman [5] has studied the adrenal gland morphology of foetal rhesus monkeys and has found that the foetal zone disappears long before birth in this species. This is reflected in the steroid excretion of those infants since no 3β-hydroxy-5-ene steroids were detected. However, in similar fashion to man, the steroid excretion of the infant rhesus monkey is different to the adult. In the adult, the major steroids excreted are 11-oxygenated androstanes and C-19 steroids represent almost all the steroidal compounds excreted [11]. In the infant, however, more C-21 steroids were found and 11-oxygenated androstanes were not identified.

Heinrichs and Colás [12] could detect only a very low activity of adrenal 16α-hydroxylase in glands from foetal and newborn rhesus monkeys, and this is reflected in the absence of 16-hydroxylated steroids from the urine of the infants.

Why the foetal gland of higher primates is so large and why it should produce such large amounts of 3β-hydroxy-5-ene steroids is still an open question. There is unlikely to be a "deficiency" of 3β-hydroxy steroid dehydrogenase* [10] in the foetal adrenal since the *in vitro* studies have shown this enzyme to be present. Bloch suggests that the placenta supplies inhibitors of 3β-hydroxysteroid dehydrogenase (e.g. progesterone) which prevent 3-oxo-4-ene steroid formation *in vivo*.

It is also possible that due to the high activity of sulphokinase in the foetal adrenal gland [11] all 3β-hydroxy-5-ene steroids are sulphurylated rendering them unsuitable substrates for 3β-hydroxysteroid dehydrogenase [10]. The foetal adrenal hypertrophy seen during primate gestation may therefore be a response to excess adrenocorticotrophin stimulation following inhibition of cortisol synthesis.

The results of this investigation have shown how useful apes would be in the study of peri-natal steroid metabolism. It is unlikely that macaque monkeys will have widespread use for this type of research as their steroid formation and excretion is so different from man and the great apes.

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*The term 3β-hydroxysteroid dehydrogenase as used here relates to 3β-hydroxysteroid oxido-reductase (EC 1.1.1.51) and to steroid Δ^{4-5} isomerases. There is evidence that there are several varieties of 3β-hydroxysteroid dehydrogenase catalysing the oxidation of different 3β-hydroxy-5-ene steroid substrates.

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