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IDENTIFICATION OF THE ANDROSTENETRIOLONES AND ANDROSTENETETROLS PRESENT IN THE URINE OF INFANTS

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SUMMARY

Some of the androstenetriolones and androstenetetrols present in extracts of infancy urine have been characterized by gas chromatography–mass spectrometry. The following steroids were identified: 3β,15β,16α-trihydroxy-5-androsten-17-one, 3β,16β,18-trihydroxy-5-androsten-17-one, two 5-androstene-3β,15,16,17-tetrols and 5-androstene-3β,16β,17β,18-tetrol. Quantitatively the most important steroid of this group was 3β,16β,18-trihydroxy-5-androsten-17-one, its urinary excretion (700–2000 μg/24 h) frequently being as high as for the major 3β-hydroxy-5-ene steroids previously identified in infancy urine (*e.g.* 3β,16α-dihydroxy-5-androsten-17-one, 3β,16α-dihydroxy-5-pregnen-20-one). These androstenetriolones and androstenetetrols are excreted in urine for at least the first three months of life.

INTRODUCTION

The urinary excretion of androstenetriolones* and androstenetetrols is quantitatively very important in the first weeks of life [1–4]. Their possible structure was discussed by Chambaz and co-workers[1] and Shackleton *et al.*[2], but the lack of suitable reference steroids rendered absolute characterization impossible. In view of their possible role as precursors for oestrogen synthesis during pregnancy this investigation was undertaken to identify them positively.

EXPERIMENTAL

Urine hydrolysis, steroid extraction and liquid chromatography

The method used for the recovery of urinary steroids has been described in detail previously [5]. Steroid conjugates were extracted by passing urine through Amberlite XAD-2 resin columns after which they were recovered with ethanol and the dried extracts were dissolved in acetate buffer prior to hydrolysis by *Helix pomatia* digestive juice. The freed steroids were again extracted using XAD-2 resin. Columns containing 6g of Sephadex LH-20 were used for group separation of the steroid extracts using the system cyclohexane–ethanol 4:1 v/v [5, 6]. Androstenetriolones and androstenetetrols were recovered between elution volumes 80 and 150 ml.

Derivatization

The steroids were analysed both as silyl ethers and oxime-silyl ethers [7], internal standards of 5α-androstane-3α,17α-diol, 5β-cholane-3α-24-diol or cholesteryl butyrate having been added to the samples prior to derivatization. In order to positively identify individual fragments in the mass spectra of the steroids identified, silyl ethers were occasionally prepared using deuterium labelled silylation reagents.

Acetonide derivatives were prepared in 0·5 ml dry acetone to which was added about 200 mg of anhydrous copper sulphate. The mixture was left at 50°C for 2 h and was then centrifuged. The supernatant was dried under a stream of nitrogen and silyl ethers of the acetonide derivatives were prepared in the usual way.

17-*Carbonyl reduction*

In order to reduce the 17-carbonyl function of the androstenetriolones the steroids were dissolved in 0·2 ml methanol to which was added about 20 mg sodium borohydride. The reaction was allowed to proceed for 2 h at 4°C after which the excess reagent was destroyed by addition of 10% acetic acid (v/v) and the reduced steroids were extracted on small XAD-2 columns.

Gas chromatography and gas chromatography–mass spectrometry

Gas chromatographic analysis was usually carried out on a 25 m open-tubular column (coated with OV-101) housed in a Becker 409 gas chromatograph [5]. The samples were injected at 150°C and the temperature programmed to increase to a final temperature

* Trivial names and abbreviations used: androstenetriolones and androstenetetrols; generic names given to 5-androstenes with three hydroxyl and one carbonyl or four hydroxyl groups, respectively; silyl ether: trimethylsilyl ether; oxime-silyl ether: O-methyloxime trimethylsilyl ether; 16,18-dihydroxy *DHA*: 3β,16,18-trihydroxy-5-androsten-17-one; 15,16-dihydroxy *DHA*: 3β,15,16-trihydroxy-5-androsten-17-one.

of 240°C. A Varian MAT 731 instrument was used for gas chromatographic–mass spectrometric analysis.

Source and synthesis of reference steroids

3β,18-Dihydroxy-5-androsten-17-one and 3β,15β,-16β-trihydroxy-5-androsten-17-one were obtained from Dr. R. W. Kelly. 3β,19-Dihydroxy-5-androsten-17-one was a gift from Organon Ltd. Other reference compounds were obtained as follows: 3β,16ξ,19-trihydroxy-5-androsten-17-one and 3β,16ξ,-18-trihydroxy-5-androsten-17-one were obtained by microbial 16-hydroxylation [8, 9] of 18- and 19-hydroxy DHA respectively. The micro-organism *Streptomyces roseochromogenes** was grown for three days in 100 ml nutrient broth (Oxoid, "Lab Lemco" 8 g/l) incubated at 28°C. Progesterone was added to the medium as inducer of 16-hydroxylase followed 24 h later by the chosen precursor and the incubation was continued for 48 h. The steroidal end products were then extracted on Amberlite XAD-2 columns and purified by Sephadex LH-20 chromatography in similar fashion to urine. Microbial hydroxylation appeared to give only one principal product from the precursor steroids. Whether this hydroxyl group was 16α or 16β was not finally ascertained, but it was found during this study that if DHA was used as precursor over 70% of the hydroxylated product was 16α-hydroxy DHA, a finding which confirms reports by other workers [8, 9]. Although 16α-hydroxylation therefore seemed more likely, acetonide formation of the reduction product of microbial 16,18-dihydroxy DHA suggested the presence of a 16β-hydroxyl. This confusing problem will be discussed in a later section.

The fully reduced compounds 5-androstene-3β,16ξ,17β,19-tetrol, 5-androstene-3β,16ξ,17β,18-tetrol and 5-androstene-3β,15β,16β,17β-tetrol were obtained by sodium borohydride reduction of the androstenetriolone standards.

RESULTS

Chromatograms illustrating the separation of the silyl ethers and oxime-silyl ethers of steroids in the Sephadex LH-20 fraction containing the androstenetriolones and androstenetetrols are illustrated in Fig. 1. This pattern was consistent for all the infant urine samples analysed, the individual compounds always being in approximately the same relative proportions providing the infants were between one and twelve weeks of age. Compounds 1, 2, 4, 5 and 7 have been characterized by mass spectrometry during this study although the stereochemistry of the functional groups has not always been fully established. The androstenetetrols represented by compounds 3 and 6 are epimeric steroids but these have not been identified.

* Obtained from the National Collection of Industrial Bacteria (N.C.I.B. No. 9605), Box 31, 135 Abbey Road, Aberdeen AB9 9DG.

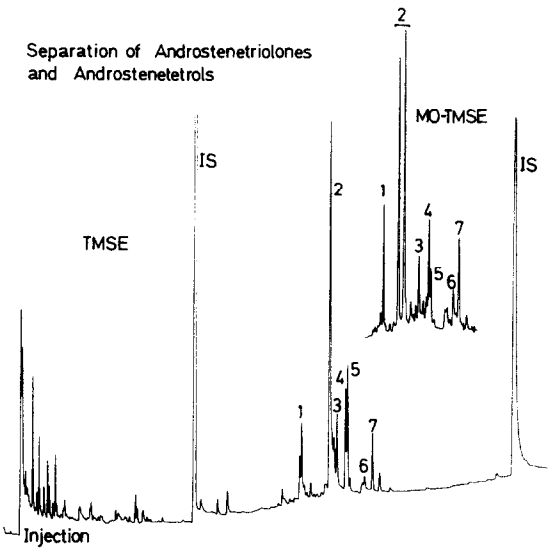

Fig. 1. Gas-chromatographic separation of the infant urinary androstenetriolones and androstenetetrols. The following steroids are indicated: compound 1, 15β,16α-dihydroxy DHA; compound 2, 16β,18-dihydroxy DHA (two peaks as MO-TMSE); compound 3, unknown androstenetetrol; compound 4, 5-androstene-3β,16β,17β,18-tetrol; compound 5, 5-androstene-3β,15β,16α,17β-tetrol; compound 6, unknown androstenetetrol (epimer of compound 3); compound 7, 5-androstene-3β,15ξ,16ξ,17β-tetrol. Peaks labelled IS are internal standards. The chromatograms representing the TMSE and MO-TMSE derivatives may not be compared quantitatively since they represent different urinary extracts.

The following sections describe the mass spectrometric identification of the individual steroids.

Identification of compound 1 (15β,16α-dihydroxy DHA)

The mass spectra of the silyl ethers of the urinary steroid and 15β,16β-dihydroxy DHA are illustrated in Fig. 2. Although these steroids have different retention times (Table 1), it is obvious from the similarity of mass spectra that they are epimeric compounds. The molecular ions (and base peaks) are at *m/e* 536. The fragment at *m/e* 405 (M-131) is formed by loss of the D-ring α-ketol silyl ether whilst the fragment at *m/e* 204 represents carbon atoms 15 and 16 with their two silylated hydroxyl substituents. This was demonstrated by the preparation of deuterated silyl ethers when the fragment at *m/e* 204 gained 18 mass units indicating the presence of two silyl groups.

The major peaks in the mass spectra of the oxime-silyl derivative of the urinary and reference steroids were as follows: *m/e* 565, M^+ (100%); *m/e* 550, M-15 (55%); *m/e* 534, M-31 (28%); *m/e* 444 (12%); *m/e* 405 (10%); *m/e* 354 (10%); *m/e* 340 (15%); *m/e* 232 (35%) and *m/e* 129 (35%).

Acetonide formation was attempted but no derivative was formed. Although the reference steroid has *cis* hydroxyl groups it also did not form an acetonide, due possibly to *cis* 15,16-hydroxyls not forming ace-

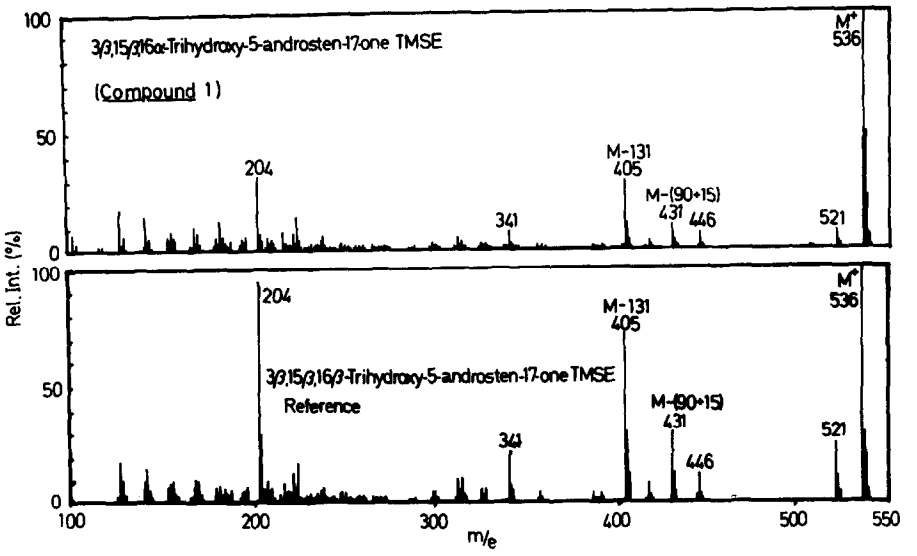

Fig. 2. A comparison of the mass spectra of compound 1 silyl ether and reference 15β,16β-dihydroxy-DHA. Although these steroids have different retention volumes, the similarity of the mass spectra shows that they are epimeric compounds.

tonides in the presence of an adjacent carbonyl function.

Sodium borohydride reduction of the reference compound produced an androstenetetrol which formed an acetonide, while reduction of compound 1 gave rise to one principal androstenetetrol which did not form an acetonide derivative. Since borohydride reduction of both 16α-hydroxy and 16β-hydroxy DHA gives rise almost exclusively to 5-androsten-3β,16α/β,17β-triol (personal observation) it seems probable that other steroids, such as compound 1, which have the same D-ring α-ketol structure would be reduced in a similar manner. Thus the failure of compound 1 to form an acetonide indicates that it has 15β and 16α hydroxyl groups; the combinations 15α,16β and 15α,16α hydroxy are both unlikely since these would form acetonides. The reduction product of compound 1 had a retention time identical to that of one of the naturally occurring androstenetetrols (compound 5).

Identification of compound 2 (16β,18-*dihydroxy DHA*)

The mass spectrum of the urinary steroid silyl ether is illustrated in Fig. 3. It seemed likely that one of the additional hydroxyl groups was at C-16 since the fragment at *m/e* 392 (M-144) is also seen in the spectra of 16α- and 16β-hydroxy DHA silyl ethers [10], and represents the loss of the D-ring. This fragment is not significant in the spectrum of 3β,17β-dihydroxy-5-androsten-16-one silyl ether, so it is unlikely that this configuration is present.

In the urinary steroid the base peak was at *m/e* 433 (M-103) and this fragmentation suggested the presence of a primary trimethylsilyl group. Only two

TABLE 1 Relative Retention Volumes in methylene units, [21] of Reference and Urinary Androstenetriolones and Androstenetetrols. This Table also indicates whether or not the steroid forms an acetonide derivative.

Urinary steroids:		Relative retention volume TMSE	MO-TMSE	Acetonide Formation
3β,15β,16α-Trihydroxy-5-androsten-17-one	(Compound 1)[1]	28.00	28.13	-
3β,16β,18-Trihydroxy-5-androsten-17-one	(Compound 2)[2]	28.93	(28.53[3] (28.70	
Unidentified androstenetetrol	(Compound 3)	29.18	-	-
5-Androstene-3β,16β,17β,18-tetrol	(Compound 4)	29.45	-	+
5-Androstene-3β,15β,16α,17β-tetrol	(Compound 5)	29.52	-	-
Unidentified androstenetetrol	(Compound 6)	29.80	-	?
5-Androstene-3β,15 ,16 ,17β-tetrol	(Compound 7)	30.35	-	+
Reference Steroids:				
3β,15β,16β-Trihydroxy-5-androsten-17-one		28.60	29.85	-
3β,16β,18-Trihydroxy-5-androsten-17-one		28.90	(28.50[3] (28.70	-
5-Androstene-3β,15β,16β,17β-tetrol		30.53	-	+
5-Androstene-3β,16β,17β,18-tetrol		29.35	-	+

Footnotes: 1. The borohydride reduction product of Compound 1 had identical retention volume to Compound 5. It did not form an acetonide derivative.

2. The borohydride reduction product of Compound 2 had identical retention volume to Compound 4. It formed an acetonide.

3. The oxime-silyl derivative of compound 2 and reference 3β,16β,18-trihydroxy-5-androsten-17-one gave two chromatographic peaks.

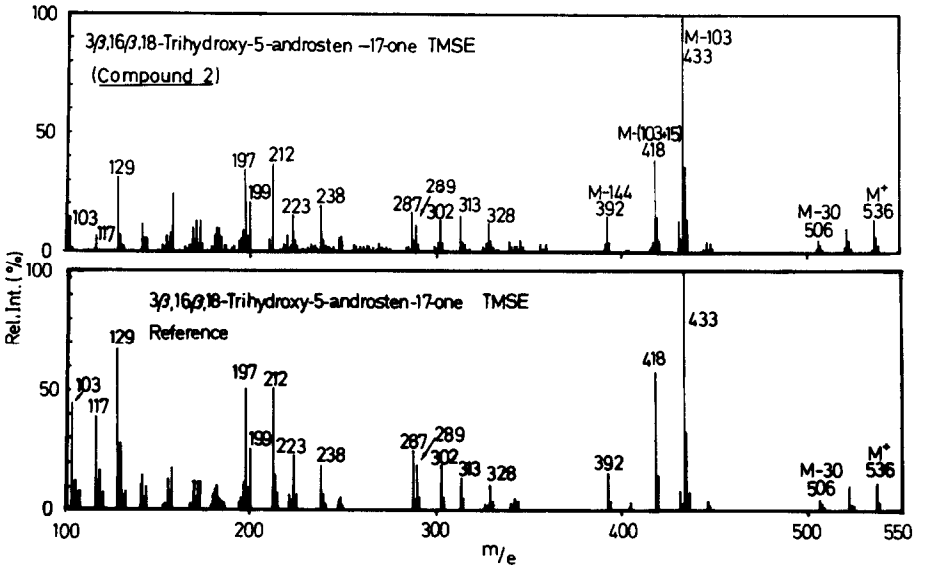

Fig. 3. Mass spectra of compound 2 silyl ether and of the compound synthesized by microbial 16-hydroxylation of 18-hydroxy DHA.

primary hydroxyl groups exist in C-19 steroids, those at C-18 and C-19, so the urinary steroid must have hydroxyl groups in one of these positions. Appropriate reference steroids were therefore synthesized by microbial 16-hydroxylation of 18- and 19-hydroxy DHA. The synthetic 16,18-dihydroxy DHA gave a mass spectrum that was almost identical to that of the urinary steroid (see Fig. 3), but no compounds present in urine gave mass spectra similar to that of synthetic 16,19-dihydroxy DHA.

The fragment at *m/e* 506 represents the loss of 30 mass units from the parent ion. This fragmentation is also important in the mass spectra of 18-hydroxy DHA silyl ether [11] and 18-hydroxyoestrone [12] and is probably formed by the migration of the 18-silyl group to the C-17 carbonyl, followed by the loss of carbon 18 as formaldehyde. This loss does not occur in the spectrum of 18-hydroxy testosterone, which confirms that a 17 carbonyl group should be present.

The oxime-silyl derivative of the urinary steroid gives rise to two peaks when analysed by gas chromatography (see Fig. 1). These peaks are probably due to *syn-* and *anti-* forms of the oxime derivatives although the presence of different epimers of the steroid which are not separated as silyl ethers cannot be excluded. Both peaks give identical mass spectra, the major fragment ions being as follows: *m/e* 565, M^+ (30%); *m/e* 550, M-15 (12%); *m/e* 534, M-31 (45%); *m/e* 444, M-(31 + 90) (25%); *m/e* 388 (75%); *m/e* 283 (40%); *m/e* 129 (85%); *m/e* 116 (80%) and *m/e* 103 (100%).

The relative retention volumes of the reference and urinary steroid were identical both as silyl ether and oxime-silyl ether derivatives (Table 1). The products of sodium borohydride reduction of the reference and urinary steroids formed acetonide derivatives although conversion did not appear to be quantitative. This at first suggested the presence of *cis*-hydroxyl groups (16β,17β) but it was also thought possible that the acetonide was being formed across the 17β,18-hydroxyl groups since these are in close proximity. This, however, does not seem likely since neither 18-hydroxyoestradiol [12] nor 5-androstene-3β,17β,18-triol [13] form this derivative although these compounds have the required 17β,18-hydroxyl groups. The possibility remains that 16α hydroxylation could change the spatial position of the 17β-hydroxyl group such that acetonide formation between 17β- and 18-hydroxyl groups can take place.

On the basis of the available evidence the conclusion reached is that the androstenetriolones (urinary and microbial) contain a 16β-hydroxy group.

Identification of compound 4 (5-androstene-3β,16β,17β,18-tetrol)

It seemed likely that one of the androstenetetrols present in infant urine would be a reduction product of the 16β,18-dihydroxy DHA described above. Sodium borohydride reduction of urinary and reference 16β,18-dihydroxy DHA gave compounds with mass spectra very similar to a urinary androstenetetrol. The upper diagram in Fig. 4 illustrates the mass spectrum of the silyl ether of the urinary tetrol and the lower diagram reference 5-androstene-3β,16β,17β,18-sterol. The molecular ions are at *m/e* 610 and base peaks *m/e* 417 M-(90 + 103), a fragmentation due to loss of the primary trimethylsilyl group and one other silyl group.

This steroid formed an acetonide derivative, a finding also reported by Chambaz and co-workers[1]. Although their compound gave an identical mass spectrum to the one illustrated in Fig. 4, no structure was assigned to it. It is concluded that both 16 and

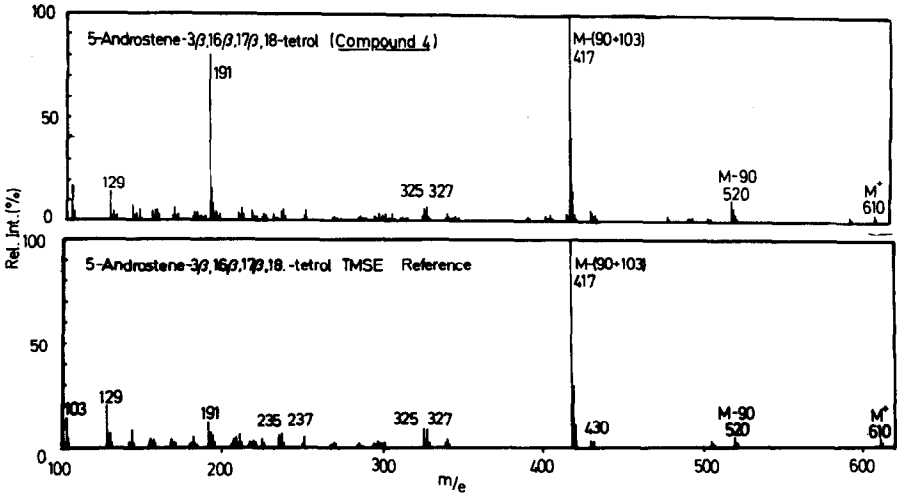

Fig. 4. A comparison of the mass spectra of compound 4 and the steroid formed by sodium borohydride reduction of reference 16β,18-dihydroxy DHA (5-androstene-3β,16β,17β,18-tetrol). The fragment at *m/e* 191 in compound 4 may be due to compound 5 contamination.

17 hydroxyls must be "β" for the reasons reported in the previous sections.

Identification of compounds 5 and 7 (two androstene-3β,15,16,17-tetrols)

The mass spectra of the silyl ether of compounds 5 and 7 are illustrated in Fig. 5; these spectra appear identical to that of reference 5-androstene-3β,15β,16β,17β-tetrol. The molecular ions are at *m/e* 610 and base peaks at *m/e* 191, an ion frequently seen in the mass spectra of C-19 steroid silyl ethers with two or more hydroxyl groups in ring D[14].

Compound 7 formed an acetonide but compound 5 did not. The stereochemical configuration of the hydroxyl group in ring D of compound 5 must therefore be "15α,16β,17α" or "15β,16α,17β", the latter structure being more likely since this steroid has the same retention time as the reduction product of urinary 15β,16α-dihydroxy DHA. It may be predicted that 6 out of a possible 8 epimeric tetrols would form acetonides, so compound 7 must be one of these. The fact that the major estetrol in pregnancy urine has 15α,16α,17β-hydroxyl groups suggests that compound 7 has the same D-ring structure.

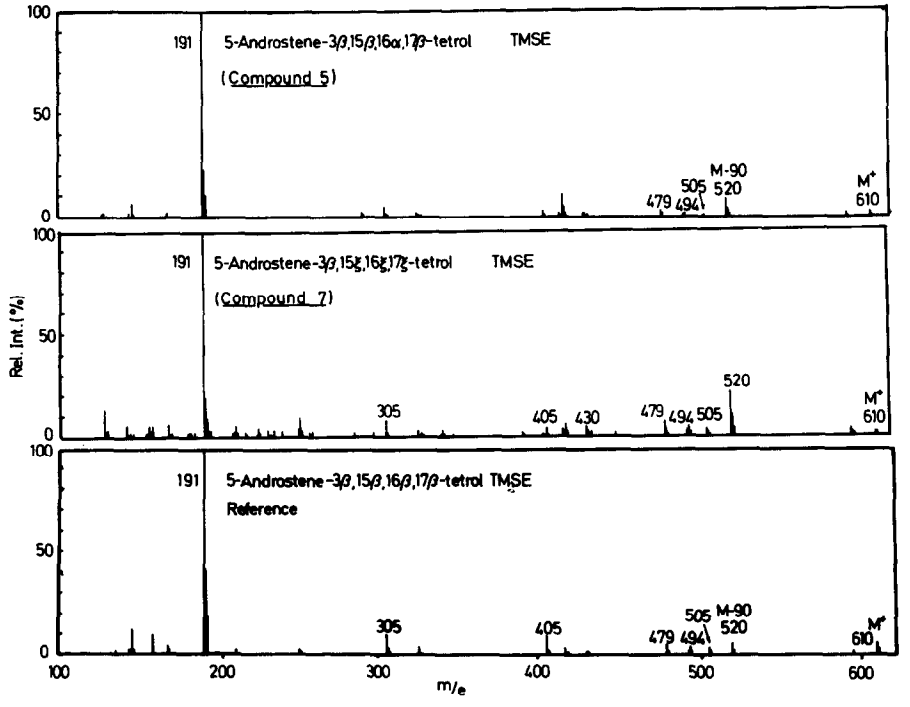

Fig. 5. Comparison of the mass spectra of compounds 5 and 7 (5-androstene-3β,15,16,17-tetrols) with reference 5-androstene-3β,15β,16β,17β-tetrol.

Table 2

Rates of excretion of urinary androstenetriolones and androstenetetrols by infants between 1 and 6 weeks of age.

Compound 1	15β,16α-dihydroxy DHA	300 - 600 μg/24 h
Compound 2	16β,18-dihydroxy DHA	700 - 2000 μg/24 h
Compound 3	Unidentified "tetrol"	300 - 600 μg/24 h
Compound 4	5-androstene-3β,16β,17β,18-tetrol	400 - 800 μg/24 h
Compound 5	5-androstene-3β,15β,16α,17β-tetrol	400 - 800 μg/24 h
Compound 6	Unidentified "tetrol"	50 - 200 μg/24 h
Compound 7	5-androstene-3β,15ξ,16ξ,17β-tetrol	300 - 600 μg/24 h

Compounds 3 and 6

These two epimeric steroids were not characterized. The mass spectra (published previously [2]) of the silyl ethers resembled those of 5-androstene-3β,16,17,18-tetrols in that the base peak was at *m/e* 417 M-(90 + 103) indicating the possible presence of a primary trimethylsilyl group. There was also an important fragment at *m/e* 191 showing the presence of at least two hydroxyl groups in ring D. However, these steroids had strong peaks at *m/e* 404 (M-206) and *m/e* 390 (M-220), fragments which are not seen in the spectra of the 5-androstene-3β,16,17,18-tetrols. It seems probable from the available evidence that these compounds are 5-androstene-3β,15,17,18-tetrols but as yet it has not been possible to synthesize the appropriate reference steroids for comparison.

Unidentified androstenetriolones

This study has described the identification and partial characterization only of the androstenetriolones and androstenetetrols found in urine collected from infants more than one week of age in order to exclude steroids which may have been synthesized during pregnancy. In the first urine specimens collected from newborn infants another important androstenetriolone is present, this steroid probably having arisen in the intact foeto-placental unit. The mass spectrum of its silyl ether has the following major fragment peaks: *m/e* 536 M^+ (5%); *m/e* 521, M-15 (45%); *m/e* 446, M-90 (12%); *m/e* 431, M-(90 + 15) (100%); *m/e* 404, M-132 (65%); *m/e* 356 (25%); *m/e* 341 (25%); *m/e* 314 (30%); *m/e* 298 (15%); *m/e* 224 (20%) and *m/e* 194 (45%). This steroid has not yet been characterized.

Quantitative importance of individual steroids

Although accurate measurement of the excretion of the individual steroids during the first weeks of life has not yet been carried out, it is possible to report the approximate range of excretion obtained for the few infants studied. These figures apply to infants between 1 week and 6 weeks of age (Table 2).

DISCUSSION

The major problem encountered in this study has been the determination of the stereochemistry of the hydroxyl groups. Much of the evidence presented was obtained by acetonide formation but the reliability of this procedure may be questioned. For example, it appears that 15β,16β-hydroxyl groups do not form an acetonide in the presence of a 17-carbonyl. It was a little surprising to find that two of the steroids identified almost certainly had 15β-hydroxyls although a 15α-hydroxyl is present in oestetrol, an important steroid of pregnancy urine [15]. Similarly, both 16,18-dihydroxy DHA isolated from urine and microbially synthesized have 16β-hydroxyls although 16α-hydroxylation usually predominates in the newborn and *Streptomyces roseochromogenes*. Although some doubt remains with regard to the stereochemistry of the hydroxyl groups of these steroids, the position of these groups is not questionable.

Recent analyses carried out in this laboratory have demonstrated that the androstenetriolones and androstenetetrols are more important constituents of infant urine than had previously been thought. This is due most probably to improvements in analytical techniques, especially Amberlite XAD-2 extraction rather than solvent extraction and group separation on Sephadex LH-20 rather than on silicic acid. These changes in methodology could be significant since 16β,18-dihydroxy DHA and possibly other compounds of the group are relatively unstable, being degraded on storage in ethanol or exposure to alkali. Accurate measurement of 16β,18-dihydroxy DHA is therefore extremely difficult but it is probable that this compound is as important quantitatively as the well-known 16α-hydroxy DHA or 16α-hydroxypregnenolone.

The 18-hydroxylation of androstenes most probably takes place in the foetal and newborn liver since it has been shown by Lisboa and Gustafsson[16] that this tissue efficiently converts testosterone to 18-hydroxytestosterone. Although small amounts of 18-hydroxy DHA are found in meconium [11] this steroid has not yet been identified in urine from infants, which suggests that 16-hydroxylation precedes 18-hydroxylation. Pregnancy urine contains 18-hydroxy oestrone [17], a steroid which can be formed from 18-hydroxyandrostenedione by placental preparations [18], so it is possible that the foetus provides 18-hydroxylated neutral precursors for the synthesis of the maternal urinary steroid. An 18-hydroxyoestriol has recently been identified in extracts of pregnancy urine [19] and it is possible that this steroid is formed from a 16,18-dihydroxy DHA sulphate precursor synthesized in the foetus.

15-Hydroxylation in the foetus probably takes place in the liver since this tissue has been shown to 15α-hydroxylate oestrone and oestradiol [20], although the 15-hydroxylation of C-19 steroids has not yet been demonstrated. One or more of the 15-hydroxylated androstenetriolones and androstenetetrols may be precursors of pregnancy urine oestetrols.

The absence of 16α,19-dihydroxy DHA and 5-androstene-3β,16α,17β,19-tetrol from infant urine was not unexpected. Although these steroids are possible

intermediates in placental oestriol synthesis, it is probable that 19-hydroxylation takes place almost exclusively in the placenta. However, these compounds may in the future be detected in amniotic fluid or umbilical cord blood.

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