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IDENTIFICATION OF 7-HYDROXYLATED C_{19} STEROID SULPHATES IN HUMAN URINE

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SUMMARY

Gas–liquid chromatography and gas chromatography–mass spectrometry have been used in the identification of six 7-hydroxylated C_{19} steroids from human urine. The compounds characterised were: 7α-hydroxyandrosterone, 7α-hydroxyepiandrosterone, 7α-hydroxydehydroepiandrosterone, 7β-hydroxyandrosterone, 7β-hydroxyepiandosterone, and 7β-hydroxydehydroepiandrosterone. The Δ^5-unsaturated steroids were the most abundant of these compounds and the ratio between the 7α- and 7β-hydroxylated derivatives of dehydroepiandrosterone was 2–3 : 1 in the urine samples analysed.

7-Hydroxylated C_{19} steroids were present in urine as monosulphates, as evidenced by their chromatographic behaviour on Sephadex LH-20.

INTRODUCTION

THE FORMATION of 7α- and 7β-hydroxydehydroepiandrosterone* has been demonstrated to occur when dehydroepiandrosterone is incubated with homogenates of foetal liver, testes and adrenals [1, 2] and with rat liver microsomes [3]. 7α-Hydroxydehydroepiandrosterone sulphate has been identified in normal human urine [4] and 3β, 7α, 16α-trihydroxy-5-androsten-17-one has been isolated from the urine of a patient with adrenal carcinoma [5].

Previously, identifications of certain neutral steroid mono- and disulphates in normal female and male urine and in the urine of females in different trimesters of pregnancy have been published from this laboratory [6–9]. The present work is a continuation of these studies, and describes gas–liquid chromatographic and gas chromatographic-mass spectrometric identification of six 7-hydroxylated C_{19} steroid monosulphates in normal human urine. A preliminary report of part of this work has already been presented [10].

MATERIALS AND METHODS

Urine

Urine was obtained from healthy females and males 20–30 years of age. The samples were stored at −20°C until analysed.

**Trivial and systematic nomenclature of steroids.* Androsterone, 3α-hydroxy-5α-androstan-17-one; epiandrosterone, 3β-hydroxy-5α-androstan-17-one; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 7α-hydroxyandrosterone, 3α,7α-dihydroxy-5α-androstan-17-one; 7α-hydroxyepiandrosterone, 3β,7α-dihydroxy-5α-androstan-17-one; 7β-hydroxyandrosterone, 3α,7β-dihydroxy-5α-androstan-17-one; 7β-hydroxyepiandrosterone, 3β,7β-dihydroxy-5α-androstan-17-one; 7β-hydroxyetiocholanolone, 3α,7β-dihydroxy-5β-androstan-17-one; testosterone, 17β-hydroxy-4-androsten-3-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 7α-hydroxydehydroepiandrosterone, 3β,7α-dihydroxy-5-androsten-17-one; 7β-hydroxydehydroepiandrosterone, 3β,7β-dihydroxy-5-androsten-17-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one.

Reagents and reference compounds

All solvents were of reagent grade and were redistilled twice before use.

Reference steroids were kindly supplied by Dr. D. K. Fukushima, New York, Dr. J. Sjövall, Stockholm, Sweden, and Dr. L. Stárka, Prague, Czechoslovakia.

Isolation of steroid sulphates and solvolysis

The method used for the isolation of steroid mono- and disulphates has been published previously [6–9]. Briefly, the procedure is as follows: (1) Extraction of 10–20 ml of urine with 10 vol. of acetone–ethanol (1/1, v/v). (2) Evaporation of the extract to dryness. (3) Desalting of the sample by chromatography on Sephadex G-25, fine. (4) Isolation of the steroid mono- and disulphate fractions, using Sephadex LH-20 chromatography, and cleavage of the conjugates by solvolysis in ethyl acetate acidified with sulphuric acid [11–12].

Isolation of 7-hydroxylated C_{19} steroids

After solvolysis, the free steroids were fractionated on silicic acid [8, 9]. A polar fraction eluted with 20 ml of methanol contained 7-hydroxylated C_{19} steroids, and this fraction was subjected to thin-layer chromatography.

Thin-layer chromatography (TLC) of steroids was carried out with 20×20 cm precoated abrasion-resistant Silica Gel F_{254}-layers (Merck AG, No. 5715, 0·25 mm) and the solvent system chloroform/abs. ethanol, 9/1 (v/v, two developments). The zone 7·5–9·0 cm from the starting line contained 7-hydroxylated C_{19} steroids and was scraped off, eluted with methanol and analysed by gas–liquid chromatography and gas chromatography–mass spectrometry.

To permit identification of some steroids, several analyses were pooled to form samples corresponding to about 100 ml of urine.

Gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS)

During GLC and GC-MS trimethyl silyl (TMS) ether derivatives [13] of steroids were used. GLC and GC-MS were performed on 3% QF-1 and 2·2% SE-30 liquid phases, as described previously [12].

Chemical and enzymatic reactions

Reduction of steroids was carried out overnight with sodium borohydride in ethanol.

Oxidation of steroids was performed, using chromium trioxide in acetone [14].

Enzymatic oxidation of steroids with 3α-hydroxysteroid dehydrogenase was performed as described by Berséus [15]. The enzyme has been purified from rat liver and was kindly supplied to us by Dr. I. Björkhem, Stockholm, Sweden.

RESULTS

The 7-hydroxylated C_{19} steroids were eluted in a polar silicic acid fraction. In the same fraction of urinary steroids some C_{19} steroid triols were also included and TLC was used to separate 7-hydroxylated C_{19} steroids from the other compounds present using 7α- and 7β-hydroxydehydroepiandrosterone as tracers.

Figure 1 shows the GLC analysis of the TMS ethers of 7-hydroxylated C_{19} steroids in human urine. The relative retention times (RRT, relative to 5α-cholestane) of the urinary steroids identified and those of reference compounds are given in Table 1.

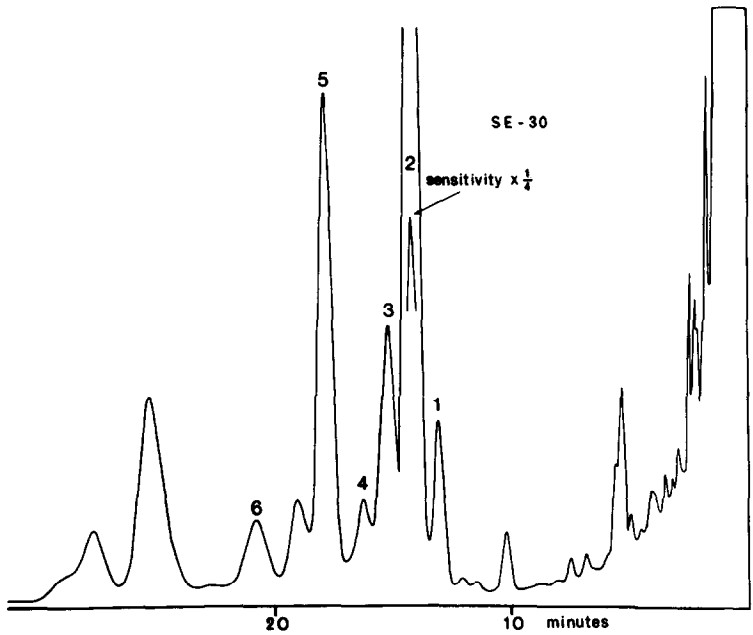

Fig. 1. Gas chromatographic analysis of the TMS ethers of 7-hydroxylated C_{19} steroids in the monosulphate fraction of urinary steroids. For identification of the compounds see Table 1 and text. Column and conditions: 2·2% SE-30, 2 m × 3·5 mm, 225°C. The amount injected corresponds to about 10 ml of urine.

After oxidation of the fraction containing 7-hydroxylated C_{19} steroids with chromium trioxide, only one product was obtained. This compound had GLC and GC-MS properties identical with 5α-androstane-3,7,17-trione[16]. Under the conditions employed, compounds with a 3β-hydroxy-Δ^5 structure were destroyed by the excess of oxidising reagent used. Therefore it was concluded that all the saturated 7-hydroxylated C_{19} steroids identified in the present work were of the 5α-androstane series.

The following six 7-hydroxylated C_{19} steroids (numbered 1–6) were identified from the monosulphate fraction of urinary steroids:

Compound 1. The TMS ether derivative of this compound gave a mass spectrum with a molecular ion at m/e 450 and a base peak at m/e 270 (M-2 × 90). Other intense ions were seen at m/e 360 (M-90), 345 (M-(90 + 15)), 305, 255 (M-(2 × 90 + 15)), 253, 242 and 231. The spectrum was very similar to that of the TMS ether of 7α-hydroxyandrosterone[16]. The steroid was oxidisable with 3α-hydroxysteroid dehydrogenase, which confirmed the 3α-hydroxy structure of the compound. After reduction with sodium borohydride, the TMS ether derivative of the reduction product gave a mass spectrum with a molecular ion at m/e 524 and an intense ion at m/e 393 (M-131). The cleavage of a fragment of 131 mass units seems to be typical of the TMS ethers of steroid triols with hydroxyl groups at carbons 3, 7 and 17[16, 17]. The RRT values of the TMS ethers of compound 1 and of its reduction product were the same as those given earlier[16] for the TMS ethers of 7α-hydroxyandrosterone and 5α-androstane-3α, 7α, 17β-triol, respectively (Table 1). In spite of the fact that reference 7α-hydroxy-

Table 1. The RRT values of the TMS ether derivatives of the 7-hydroxylated C_{19} steroids identified in the monosulphate fraction of urinary steroids and of relevant reference compounds (5α-cholestane = 1·00). 5α-Cholestane time: QF-1, = 9–11 min; SE-30, = 26–29 min

Compound	Identification	QF-1 Urinary steroid	QF-1 Reference steroid	SE-30 Urinary steroid	SE-30 Reference steroid
1	7α-Hydroxyandrosterone	1·30	—	0·53	0·53‡
Red* 1		0·55†	0·55‡	0·62†	0·62‡
2	7α-Hydroxydehydro-epiandrosterone	1·55	1·59	0·58	0·58
Red 2		0·55†	0·56	0·62†	0·62
3	7β-Hydroxyandrosterone	1·76	—	0·62	—
Red 3		0·66	—	0·78	0·79‡
4	7α-Hydroxyepiandro-sterone	1·86†	—	0·66	—
Red 4		0·64	0·63§	0·68	0·67§
5	7β-Hydroxydehydro-epiandrosterone	1·86†	1·89	0·73	0·73
Red 5		0·78	0·79	0·92	0·90
6	7β-Hydroxyepiandro-sterone	2·32	2·35	0·85	0·85
Red 6		0·89	—	1·03	1·00‡

*The reduced compound.
†Mixture compounds.
‡From ref. [16].
§From ref. [17].
Columns and conditions: 3% QF-1, 2 m × 3·5 mm, 215°C; 2·2% SE-30, 2 m × 3·5 mm, 225°C.

androsterone was not available to us, it can be concluded that compound 1 is 7α-hydroxyandrosterone.

Compound 2. The TMS ether derivative of this steroid had a mass spectrum (Fig. 2) with a molecular ion at m/e 448 and a very prominent base peak at m/e 358 (M-90). The mass spectrum of the TMS ether derivative of reduced compound 2 gave a molecular ion at m/e 522 and an intense base peak at m/e 432 (M-90). It is of interest to observe that no intense ion at m/e M-131 (391) was present in the mass spectrum of the TMS ether derivative of 5-androstene-3β,7α,17β-triol, in

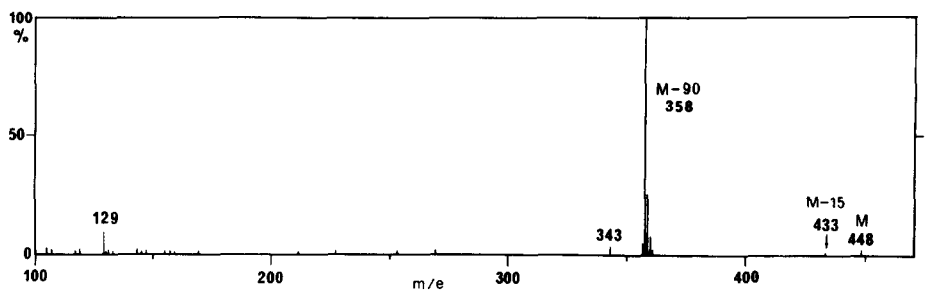

Fig. 2. Mass spectrum of the TMS ether derivative of compound 2 (7α-hydroxydehydro-epiandrosterone).

contrast to the prominent intensity of the corresponding ion in the mass spectra of the TMS ethers of androstane-3,7,17-triols[16, 17]. The RRT values and mass spectra of the TMS ether derivatives of compound 2 and its reduction product were identical with those of the corresponding derivatives of reference 7α-hydroxydehydroepiandrosterone (Table 1). These data show that compound 2 is 7α-hydroxydehydroepiandrosterone.

Compound 3. The TMS ether derivative of this compound gave a mass spectrum (Fig. 3) with a molecular ion at m/e 450 and two peaks with relative intensities of 100 per cent, namely those at m/e 360 (M-90) and 270 (M-2 × 90). Other intense ions are seen at m/e 435 (M-15), 345 (M-(90 + 15)), 332, 255 (M-(2 × 90 + 15)), 253, 243 and 231. The fragmentation pattern was similar to that of the TMS ether of reference 7β-hydroxyepiandrosterone, but differences are seen in the relative intensities of the peaks (see Fig. 5). The 3α-orientation of the hydroxyl group at carbon 3 was confirmed by oxidation with 3α-hydroxysteroid dehydrogenase. Reference 7β-hydroxyandrosterone was not available to us, but the RRT values of its TMS derivative were calculated from those of 7β-hydroxyepiandrosterone TMS ether by using the ratio between the RRT values of the TMS ethers of androsterone and epiandrosterone. The values obtained (QF-1: 1·70, SE-30:0·65) were very close to those of compound 3 (Table 1). These results show that compound 3 is 7β-hydroxyandrosterone.

Compound 4. The mass spectrum of the TMS ether derivative of compound 4 is presented in Fig. 4. The molecular ion is seen at m/e 450 and the base peak at m/e 271 (M-(90 + 89)). Other intense ions are seen at m/e 360 (M-90), 345 (M-(90 + 15)), 305, 270 (M-2 × 90), 255, 253, 231, 129 and 107. After boro-

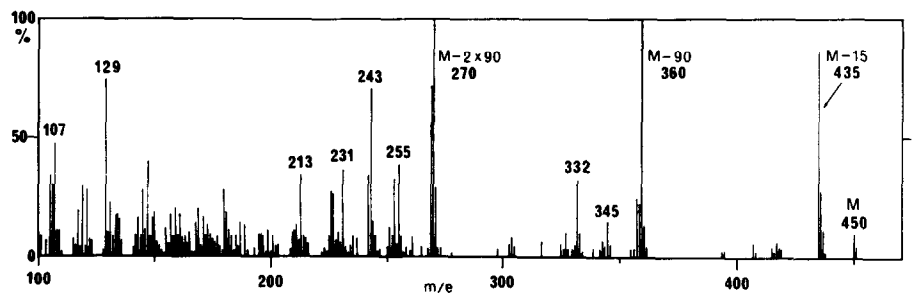

Fig. 3. Mass spectrum of the TMS ether derivative of compound 3 (7β-hydroxyandrosterone).

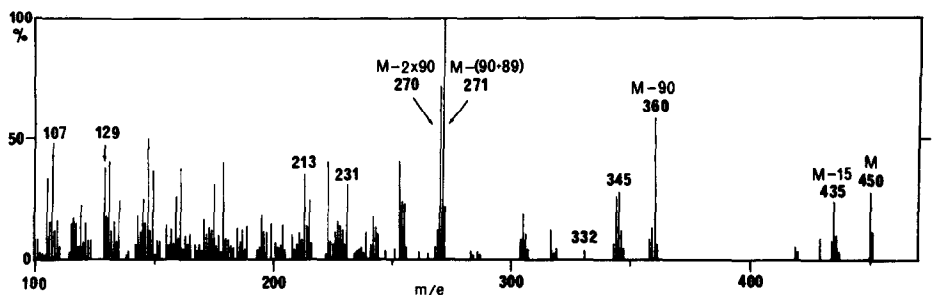

Fig. 4. Mass spectrum of the TMS ether derivative of compound 4 (7α-hydroxyepiandrosterone).

hydride reduction, the TMS ether derivative of the reduction product of compound 4 had the same RRT values as those reported by Gustafsson *et al.* for 5α-androstane-3β, 7α, 17β-triol[17]. The mass spectrum of the TMS ether of this reduction product had a molecular ion at m/e 524 and an intense base-peak at m/e 393 (M-131), indicating a structure of androstane-3,7,17-triol[16, 17]. After oxidation with 3α-hydroxysteroid dehydrogenase, compound 4 was unchanged, thus showing 3β-orientation of the hydroxyl group at carbon 3. The ratios between the RRT values of the TMS ethers of 7α- and 7β-hydroxydehydroepiandrosterone on QF-1 and SE-30 columns were 0·84 and 0·78, respectively. The same ratios were observed between the RRT values of the TMS ethers of compound 4 and of reference 7β-hydroxyepiandrosterone. Reference 7α-hydroxyepiandrosterone was not available to us, but the results obtained show that compound 4 is 7α-hydroxyepiandrosterone.

Compound 5. As TMS ethers this steroid and its reduction product had RRT values identical with the same derivatives of reference 7β-hydroxydehydroepiandrosterone and 5-androstene-3β,7β,17β-triol, respectively (Table 1). The mass spectra of the TMS ethers of compound 5 and of its reduction product were also identical with these reference steroids, and very similar to those of the TMS ethers of 7α-hydroxydehydroepiandrosterone (Fig. 2) and 5-androstene-3β, 7α, 17β-triol, respectively. In the light of these results, compound 5 was identified as 7β-hydroxydehydroepiandrosterone.

Compound 6. The RRT values (Table 1) and mass spectrum (Fig. 5) of the TMS ether derivative of this steroid were identical with those of the corresponding derivative of reference 7β-hydroxyepiandrosterone (Table 1). The 3β-hydroxy structure of compound 6 was confirmed by the fact that 3α-hydroxysteroid dehydrogenase did not oxidise this steroid. These results show that compound 6 is 7β-hydroxyepiandrosterone.

In conclusion, the following 7-hydroxylated C_{19} steroids were identified in the monosulphate fraction of the steroids in human urine: 7α- and 7β-hydroxyandrosterone, 7α- and 7β-hydroxyepiandrosterone and 7α- and 7β-hydroxydehydroepiandrosterone. None of these steroids could be found in the disulphate fraction of urinary steroids.

Although accurate quantitative analyses have not so far been carried out, it can be seen in Fig. 1 that Δ^5-unsaturated steroids are the most abundant compounds in the fraction of 7-hydroxylated C_{19} steroids. In the urine samples analysed, the ratio between 7α- and 7β-hydroxydehydroepiandrosterone was about 2–3 : 1.

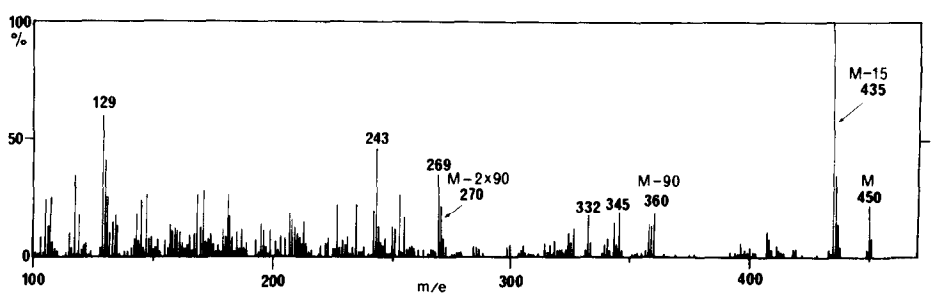

Fig. 5. Mass spectrum of the TMS ether derivative of compound 6 (7β-hydroxyepiandrosterone).

DISCUSSION

In the present study, the mono- and disulphate fractions of neutral steroids in urine were separated by chromatography on Sephadex LH-20. After solvolysis, a fraction containing 7-hydroxylated C_{19} steroids was isolated from other steroids in urine using silicic acid chromatography and TLC. The steroids were identified by GLC, GC-MS and some chemical as well as enzymatic reactions.

The presence of an enzyme system which hydroxylates dehydroepiandrosterone in position 7 has been found in adult human liver, adrenals and skin [18, 19], several foetal tissues [1, 2] and in the microsomal fraction of the liver of some animals [3, 20–22]. It has been shown that at least human foetal liver, testicular and adrenal tissues, and microsomes of rat liver are able to perform both 7α- and 7β-hydroxylation of dehydroepiandrosterone [1–3]. Dehydroepiandrosterone is not the only Δ^5-unsaturated hormonal steroid which can be hydroxylated in position 7, because 7-hydroxylation of pregnenolone, too, was found in rat liver microsomes [23]. Rat liver microsomes were also capable of carrying out 7α-hydroxylation of 5α-dihydrotestosterone [24]. As far as we know, no evidence for *in vitro* formation of saturated 7-hydroxylated C_{19} steroids by human tissues has hitherto been reported. After administration of etiocholanolone or testosterone to a human subject, 7β-hydroxyetiocholanolone has been identified in the urine [25].

Of the six 7-hydroxylated C_{19} steroids identified in the present study, only 7α-hydroxydehydroepiandrosterone has previously been found in human urine [4], and this steroid is present in blood plasma, too [26]. From the urine of a patient with adrenal carcinoma, another Δ^5-unsaturated 7α-hydroxylated C_{19} steroid has been isolated, namely 3β,7α,16α-trihydroxy-5-androsten-17-one [5]. In the present work this steroid could not be found in normal human urine. Two saturated 7-hydroxylated C_{19} steroids, 7α-hydroxyandrosterone and 5α-androstane-3α,7α,17β-triol, have been identified from faeces and urine of rats [16].

In this study, five 7-hydroxylated C_{19} steroids, which have not previously been found in human urine, were identified in the monosulphate fraction of urinary steroids. It remains to be established whether the precursors of the saturated 7-hydroxylated steroids are androsterone and epiandrosterone, respectively, or whether these steroids are metabolites of 7α- and 7β-hydroxydehydroepiandrosterone. It is neither clear whether there are separate enzymes for the 7α- and 7β-hydroxylation of dehydroepiandrosterone or whether a secondary epimerisation, direct or via 7-ketodehydroepiandrosterone, takes place after the formation of one of these metabolites [2, 3].

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REFERENCES

1. L. Stárka, J. Sulcová and J. E. Jirásek: *Excerpta med. int. Congr. Ser.* **170** (1968) 33.
2. J. Sulcová, A. Čapková, J. Ev. Jirásek and L. Stárka: *Acta endocr. Kbh.* **59** (1968) 1.

3. J. Sulcová and L. Stárka: *Steroids* **12** (1968) 113.
4. L. Stárka, J. Sulcová and K. Šilink: *Clin. chim. Acta* **7** (1962) 309.
5. M. Okada, D. K. Fukushima and T. F. Gallagher: *J. biol. Chem.* **234** (1959) 1688.
6. O. Jänne, T. Laatikainen, J. Vainio and R. Vihko: *Steroids* **13** (1969) 121.
7. O. Jänne and R. Vihko: *Ann. Med. exp. Fenn.* **46** (1968) 301.
8. O. Jänne and R. Vihko: *Steroids* **14** (1969) 235.
9. O. Jänne and R. Vihko: *Acta endocr. Kbh.* (1970). In press.
10. O. Jänne and R. Vihko: *Scand. J. clin. Lab. Invest.* **23**, Suppl. 108 (1969) 40.
11. S. Burstein and S. Lieberman: *J. biol. Chem.* **233** (1958) 331.
12. R. Vihko: *Acta endocr. Kbh* **52**, Suppl. 109 (1966).
13. T. Luukkainen, W. J. A. VandenHeuvel, E. O. A. Haahti and E. C. Horning: *Biochim. biophys. Acta* **52** (1961) 599.
14. J. Sjövall and K. Sjövall: *Steroids* **12** (1968) 359.
15. O. Berséus: *Eur. J. Biochem.* **2** (1967) 493.
16. J. -Å. Gustafsson and J. Sjövall: *Eur. J. Biochem.* **6** (1968) 227.
17. J. -Å. Gustafsson, B. P. Lisboa and J. Sjövall: *Eur. J. Biochem.* **6** (1968) 317.
18. L. Stárka: *Naturwissenschaften* **52** (1965) 1.
19. I. Faredin, A. G. Fazekas, I. Tóth, K. Kókai and M. Julesz: *J. invest. Derm.* **52** (1969) 357.
20. L. Stárka and H. Breuer: *Z. physiol. Chem.* **344** (1966) 124.
21. L. Stárka, E. Döllefeld and H. Breuer: *Z. physiol. Chem.* **348** (1967) 293.
22. W. L. Heinrichs, R. L. Mushen and A. Colás: *Steroids* **9** (1967) 23.
23. L. Stárka, J. Sulcová, K. Dahm, E. Döllefeld and H. Breuer: *Biochim. biophys. Acta* **115** (1966) 228.
24. J. -Å. Gustafsson, B. P. Lisboa and J. Sjövall: *Eur. J. Biochem.* **5** (1968) 437.
25. D. K. Fukushima: *J. biol. Chem.* **239** (1964) 1748.
26. L. Stárka and R. Hampl: *Naturwissenschaften* **51** (1964) 164.