Biochem. J. (1972) **129**, 657–663
Printed in Great Britain

Studies on the Biosynthesis *in vivo* and Excretion of 16-Unsaturated C_{19} Steroids in the Boar

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(*Received* 30 *March* 1972)

1. In one experiment [7α-^{3}H]pregnenolone was infused continuously for 12min into the left spermatic artery of a sexually mature boar and blood was collected during this period by continuous drainage from the spermatic vein. After infusion, the testis was removed and immediately cooled to −196°C. 2. From both the testicular tissue and the spermatic venous plasma, ^{3}H-labelled 16-unsaturated C_{19} steroids were isolated and characterized and their radiochemical purity was established. 5α-Androst-16-en-3α- and 3β-ol occurred mainly as sulphate conjugates and to a lesser extent as free steroids. Only traces of these alcohols occurred as glucosiduronate conjugates. 5α-Androst-16-en-3-one was found in the free (ether-extractable) fraction. 3. The isotope concentration of each of the ^{3}H-labelled 16-unsaturated C_{19} steroids in testicular tissue was different from that in spermatic venous plasma. 4. The ratios of tritiated 5α-androst-16-en-3α- and 3β-ol (free steroids) to their respective sulphate conjugates in the testicular tissue were less than the ratios of the same compounds in the spermatic venous plasma. The possibility that the sulphates are partially hydrolysed by testicular sulphatases before secretion is discussed. 5. In a second experiment, a continuous close-arterial infusion of [7α-^{3}H]pregnenolone into the left testis was performed over a 200min period and all the urine that accumulated during the infusion was collected for analysis. 6. No ^{3}H-labelled 16-unsaturated C_{19} steroids were detected in the urine as free steroids. Only a trace of 5α-androst-16-en-3α-ol was detected conjugated as glucosiduronate, whereas the corresponding 3β-alcohol occurred mainly as glucosiduronate and to a lesser extent as sulphate. 7. The absence of 5α-androst-16-en-3β-ol glucosiduronate in the spermatic venous blood and its presence in considerable amount in the urine may be attributed to hepatic glucuronyl transferase activity.

A group of 16-unsaturated C_{19} steroids were isolated from boar testes by Prelog & Ruzicka (1944), and the results of quantitative studies of these compounds have been published (Booth, 1970; Claus, 1970). The analysis of boar spermatic venous plasma (Gower *et al.*, 1970) revealed the presence of 5α-androst-16-en-3α-ol (an-α) and 5α-androst-16-en-3β-ol (an-β) predominantly as sulphate conjugates and, to a lesser extent, 5α-androst-16-en-3-one and an-α in the free steroid fraction. Further, boar urine contains an-β conjugated as glucosiduronate (Gower *et al.*, 1970).

Studies of the biosynthesis of 16-unsaturated C_{19} steroids in boar testicular tissue have been undertaken recently (for review, see Gower, 1972). It is now clear that the C_{21} steroids, pregnenolone and progesterone, are the parent compounds and that these are converted first into 5,16-androstadien-3β-ol and 4,16-androstadien-3-one respectively by a microsomal enzyme system (Gower & Loke, 1971). These steroids are then further metabolized via 5α-androst-16-en-3-one to an-α and an-β (Brophy & Gower, 1972).

In view of the possible physiological significance of the 16-unsaturated C_{19} steroids in the boar (Melrose *et al.*, 1971), the present studies *in vivo* were undertaken to investigate the biosynthesis, secretion and excretion of these compounds. A preliminary account of this work has been published (Gower *et al.*, 1972).

Experimental

Materials

Reagents, steroids and enzyme. Authentic 16-unsaturated C_{19} steroids, solvents and materials for alumina-column chromatography and g.l.c. were as described by Brooksbank & Gower (1970) and Gower *et al.* (1970). All reagents, unless otherwise stated, were of analytical grade. [7α-^{3}H]Pregnenolone (specific radioactivity 6.9Ci/mmol in boar E and 14.7Ci/mmol in boar C) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Ox liver β-glucuronidase (Ketodase) was obtained from

William R. Warner and Co. Ltd., Eastleigh, Hants., U.K.

Animals. Boar E (Lydney Courier 6th.) was a 2½-year-old Landrace male weighing 208kg. Boar C (Meltonhouse King David 22nd.) was an 18-months-old Large White male weighing approx. 200kg. Both animals were sexually mature stud boars from the Babraham pig herd.

Methods

Anaesthesia and operative procedures. Both animals were prepared for experiment in the following way. Preliminary sedation was produced by intramuscular injection of 400mg (boar E) or 500mg (boar C) of phencyclidine hydrochloride (Sernylan; Parke, Davis and Co. Ltd., Hounslow, Middx., U.K.). Anaesthesia was induced by open-mask administration of halothane (Fluothane; I.C.I. Ltd., Macclesfield, Cheshire, U.K.) and oxygen. After endotracheal intubation with a cuffed Magill tube, anaesthesia was maintained with fluothane–oxygen mixtures in a closed-circuit re-breathing system.

Sterile operative procedures were used for catheterization of the arterial and venous supply of the testis. The left scrotum was incised to expose the left testicle. The left spermatic artery was catheterized beneath the surface of the tunica albuginea by a technique similar to that of Herd & Barger (1964). A mersuture needle (30mm, curved, round-bodied) was attached to a length of fine polytetrafluorethylene catheter (no. 6423, ultra-thin wall) and passed into and along the artery to emerge about 1cm beyond the point of entry. After removal of the needle, the catheter was pulled back until the free tip was in the lumen of the artery and could be advanced along the vessel for a short distance before the artery divided to supply the testicle. Gentle pressure on the artery rapidly closed the hole in the wall and the testicle was repositioned in the scrotum, after the catheter had been anchored to the tunica with silk mersuture.

The left spermatic vein was exposed through a mid-ventral incision of the abdomen. Procaine anaesthetic solution (Procaine hydrochloride, British Pharmacopoeia grade; 2%, w/v) was dripped round the vessel to reduce spasm and the vessel was catheterized with a re-entrant loop of vinyl tube (NT 6 SH 90; Portex Plastics Ltd., Hythe, Kent, U.K.) so that the complete venous drainage from the testis could be diverted or sampled during the infusion. Heparin, 10000i.u., was injected intravenously to decrease the risk of clot formation in the loop. Peripheral blood samples were collected during the long infusion into boar C from a catheter in an arterial branch in the sublingual region of the head.

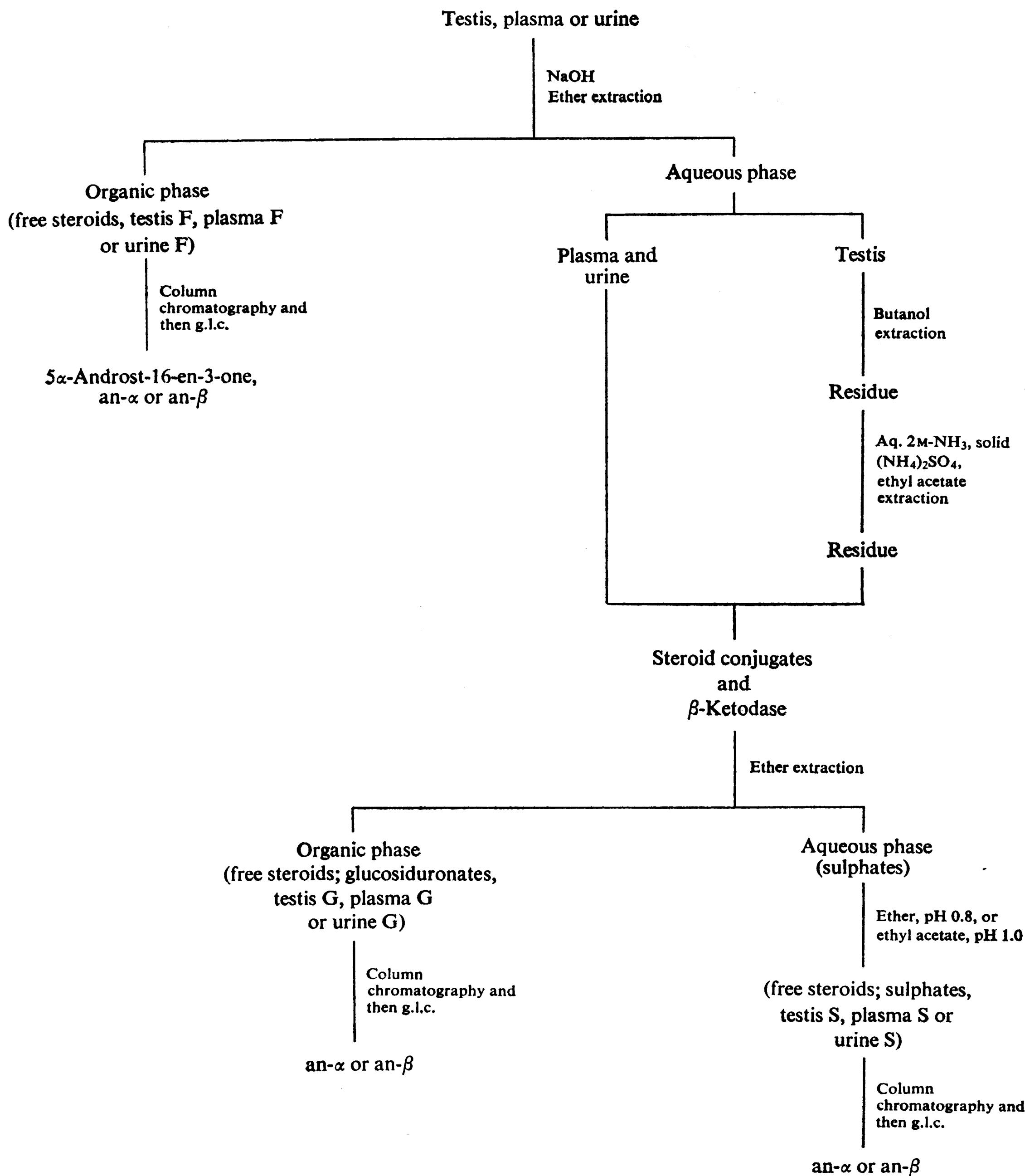
Infusions. [7α-^{3}H]Pregnenolone was infused close-arterially into the left testis. In boar E the radioactively-labelled steroid (250μCi) was dissolved in 0.5ml of ethanol and diluted to 6ml with Sterivac saline (Allen and Hanbury Ltd., Bethnal Green, London E.2, U.K.) before infusion. A small portion of this was used for measurement of radioactivity (see below) and the remainder (5.8ml) was infused into the spermatic artery at approx. 0.5ml/min for 12min. The blood draining from the infused testicle was collected continuously, serially, into heparinized bottles. At the end of the infusion the testis was quickly removed and immediately cooled to −196°C in liquid N_2. The blood samples were centrifuged at 3000g for 15min in a refrigerated centrifuge and the plasma was separated and stored at −20°C. In boar C the radioactively labelled steroid (250μCi) was dissolved in ethanol (0.4ml) and diluted with 40ml of sterile saline. This solution was infused continuously for 200min at 0.190ml/min by using a Palmer syringe-pump. The urinary bladder was catheterized (Foley latex catheter) through a stab incision and a purse-string suture in the fundus and drained. The urine that accumulated during the infusion of the radioactive compound was subsequently drained and stored at −20°C until analysed.

Measurement of radioactivity. A Beckman automatic scintillation spectrometer (model 1650) was used for radioactivity measurements. Samples were dissolved in 1,4-dioxan scintillant (5ml) (Bray, 1960) and under these conditions the counting efficiency for ^{3}H was 36% with a background of 133d.p.m.

Column chromatography. The crude extracts, together with authentic 16-unsaturated steroid carriers (100–200μg), were submitted to column chromatography on alumina (5g or 10g). The steroids were eluted with mixtures of benzene–light petroleum (b.p. 80–100°C) (1:1, v/v or 1:9, v/v) either in 5ml or in 30ml fractions. Recoveries of 5α-androst-16-en-3-one, an-α and an-β from the columns were 79–83%, 74–84% and 72–81% respectively.

Gas–liquid chromatography. 16-Unsaturated C_{19} steroids were identified by g.l.c. with a series 104, model 24, dual flame ionization chromatograph (Pye–Unicam Ltd., Cambridge, U.K.). The column used (1.5m long ×3.5mm internal diam.) was packed with Gaschrom Q (100–200 mesh) coated with cyclohexanedimethanol succinate (CHDMS; 0.6%–methyl polysiloxane gum (JXR; 0.75%). Analyses were performed at 196°C with a carrier gas (argon) flow of 50ml/min (Gower *et al.*, 1970).

Extraction of 16-unsaturated C_{19} steroids from testicular tissue, plasma and urine. The testicular tissue was thawed at room temperature, homogenized with 0.6M-NaOH in an MSE overhead homogenizer and the suspension centrifuged at 3000g for 20min. The precipitate was re-homogenized with 0.6M-NaOH, centrifuged again and the pellet discarded. The supernatants were combined and extracted with peroxide-free ether (3 vol.). The combined ether extracts were washed with water (50ml), and



Scheme 1. *Flow diagram for the fractionation and purification of* 16-*unsaturated* C_{19} *steroids from testis, spermatic venous plasma and urine of boars, after testicular infusion of* [7α-^{3}H]*pregnenolone*

For details see the Experimental section.

then with 5% (v/v) acetic acid (50ml) and finally with water (2×50ml). The washed extract was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under reduced pressure (water pump). This extract was designated (testis F) 'free fraction'. Recoveries were in the range 80–90%.

The aqueous alkaline portion was extracted with *n*-butanol (2vol.) and the extract was evaporated to dryness under reduced pressure. The residue was dissolved in 20ml of aq. 2M-NH_3 soln., and solid $(NH_4)_2SO_4$ was added to give a concentration of 2M; the solution was extracted with ethyl acetate (3×40ml). The extract was washed with 6ml of aq. 2M-NH_3 soln. and then evaporated to dryness. This portion was designated 'testis conjugates'. The residue was dissolved in 30ml of 4M-sodium acetate buffer, pH4.5, and incubated with 1ml of Ketodase (approx. 5000i.u.) for 48h at 38°C. The steroids released after this incubation were extracted with ether (3×50ml) and the combined extracts washed first with 10% (w/v) $NaHCO_3$ soln. (2×10ml) and then with water. The extracts were dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation to give the steroids hydrolysed from glucosiduronates (testis G). The aqueous phase containing the sulphate conjugates was made pH1 with conc. HCl and adjusted to a salt concentration of about 20% (w/v) with solid NaCl; the mixture was extracted with ethyl acetate (2vol.) and the extract incubated for 48h at 38°C (Burstein & Lieberman, 1958). The extract was evaporated to dryness, and the residue dissolved in 0.25M-NaOH (30ml) and extracted with peroxide-free ether (3×50ml) as described by Gower *et al.* (1970). The ether extract was evaporated to dryness, giving the residue designated (testis S) 'sulphate fraction'. These procedures are summarized in Scheme 1. No correction for analytical losses was made because of the lack of authentic 16-unsaturated C_{19} steroid conjugates.

For the extraction of 16-unsaturated C_{19} steroids from spermatic venous plasma and urine the methods described by Gower *et al.* (1970) were followed (see Scheme 1).

Results

Identification of 16-unsaturated C_{19} steroids in spermatic venous plasma and testicular tissue

The plasma F fraction (see the Experimental section and Scheme 1) was chromatographed on alumina with benzene–light petroleum (b.p. 80–100°C) (1:1, v/v) as eluent. Measurements of radioactivity and weight of carrier steroids in the 5ml fractions obtained showed that two peaks were eluted (Fig. 1): the first containing 5α-androst-16-en-3-one and an-α carriers, and the second mainly an-β. Fractions 4–8 were therefore combined and rechromatographed on alumina with benzene–light petroleum (b.p. 80–100°C) (1:9, v/v) as eluent (Katkov & Gower, 1968). Fig. 2 shows that 5α-androst-16-en-3-one was clearly separated from an-α and the constancy of specific radioactivity over each peak indicated the radiochemical purity of these metabolites. Fractions 9–16 (Fig. 1) were submitted to further column chromatography on alumina with benzene–light petroleum (b.p. 80–100°C) (1:1, v/v) as eluent. Two peaks of radioactivity were then obtained (Fig. 3), the minor peak containing tritiated an-α and the major peak containing tritiated an-β. Radiochemical purity was again established.

The same procedures were used for the purification and identification of the ^3H-labelled 16-unsaturated C_{19} steroid metabolites in the testis F fraction. A similar pattern of peaks was obtained.

For the characterization of steroid-conjugate fractions, an-α and an-β were the only carriers used and one column chromatography step on alumina, with benzene–light petroleum (b.p. 80–100°C) (1:1, v/v) as eluent, was sufficient to separate them. The presence of tritiated an-α and an-β was then demonstrated in the testis S and plasma S fractions. Only traces of

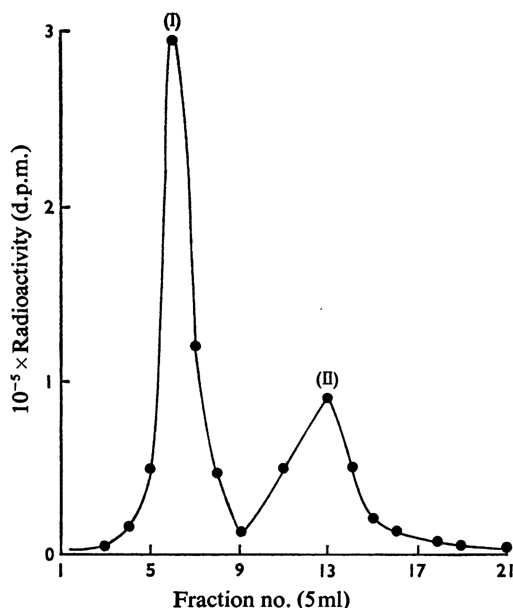


Fig. 1. *Column chromatography on alumina (10g), with benzene–light petroleum (1:1, v/v) as eluent, of the free steroid (plasma F) fraction*

The free steroid fraction was obtained from spermatic venous plasma in an experiment in which boar E received a short-term (12min) testicular infusion of [7α-^{3}H]pregnenolone (242μCi). Radioactivity was measured by liquid-scintillation counting.

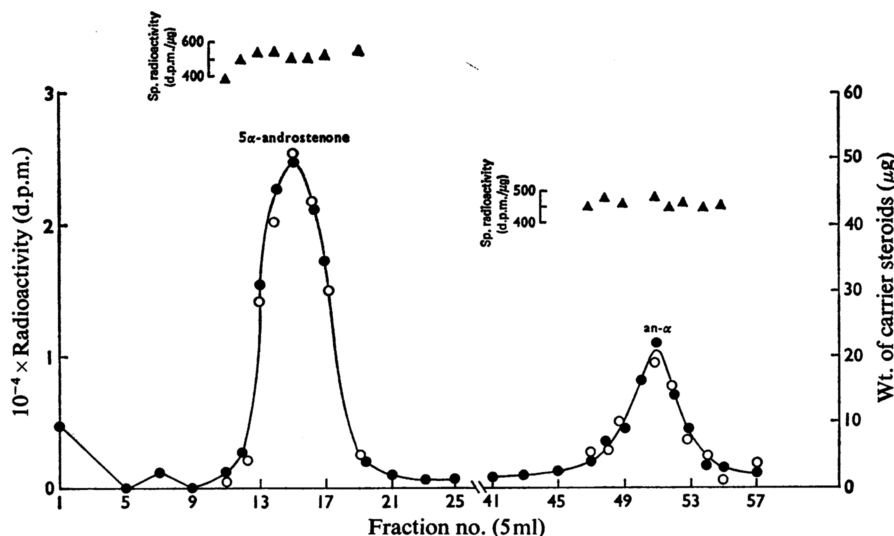


Fig. 2. *Column chromatography on alumina* (10*g*), *with benzene – light petroleum* (1:9, *v*/*v*) *as eluent*, *of the pooled fractions* 4–8 (*peak* I *of Fig.* 1)

The weight of carrier steroid (o) was estimated by g.l.c. and radioactivity (●) by liquid-scintillation counting. ▲, specific radioactivity (d.p.m./µg).

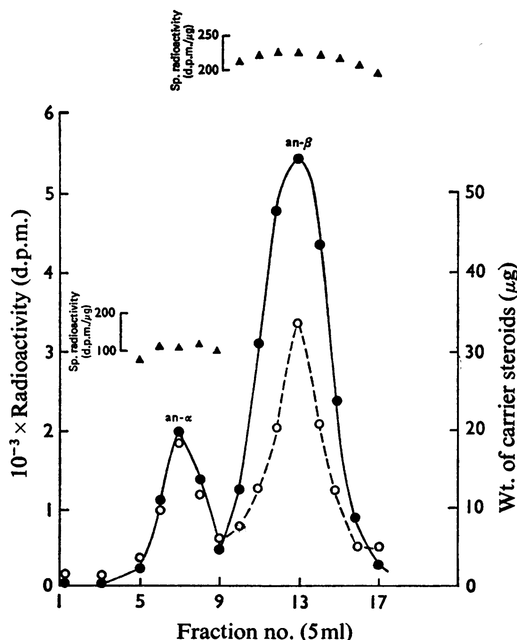


Fig. 3. *Column chromatography on alumina* (10*g*), *with benzene–light petroleum* (1:1, *v*/*v*) *as eluent*, *of the pooled fractions* 9–16 (*peak* II *of Fig.* 1)

The weight of carrier steroid (o) was estimated by g.l.c. and radioactivity (●) by liquid-scintillation counting. ▲, Specific radioactivity (d.p.m./µg).

these androstenols were detected in the corresponding glucosiduronate fractions and these could not be fully characterized.

Table 1 summarizes the concentrations of ^{3}H-labelled 16-unsaturated steroid metabolites found in spermatic venous plasma, testis and urine. The relative amounts of radioactivity show an-β>an-α>5α-androst-16-en-3-one in the free steroid fractions of testicular tissue. In contrast the relative yields of the metabolites in the free steroid fractions of plasma were 5α-androst-16-en-3-one>an-α>an-β. Nearly four times as much tritiated an-β sulphate as an-β was found in both testicular tissue and spermatic venous plasma. The an-α sulphate:an-α ratios were 2.8:1 and 1.8:1 for testicular tissue and spermatic venous plasma respectively.

No ^{3}H-labelled 16-unsaturated steroid metabolites were found in the free steroid fraction of urine obtained from boar C in the long infusion experiment (Table 1). Only traces of an-α glucosiduronate were detected, whereas a considerable amount of an-β glucosiduronate was isolated and characterized (Fig. 4) and, to a lesser extent, an-β sulphate. No an-α sulphate was detected (Table 1).

Discussion

The aim of the present work was to study the testicular biosynthesis *in vivo* of 16-unsaturated steroids, their secretion into the spermatic venous blood and their excretion in the urine.

Table 1. *Conversion in vivo of [7α-^{3}H]pregnenolone into 16-unsaturated C_{19} steroid metabolites in boars*

Boar E received a testicular infusion (12min) of [7α-^{3}H]pregnenolone and the spermatic venous blood was collected continuously during this period. The infused testis was then removed and immediately cooled to −196°C. Boar C received a testicular infusion (200min) of [7α-^{3}H]pregnenolone, and urine accumulating in the bladder during this period was collected. Spermatic venous plasma (401ml), the infused testis (461g) and urine (1100ml) were then processed (see Scheme 1). Results are uncorrected for analytical losses.

	Boar E								Boar C			
	Radioactivity in testicular tissue (d.p.m./g)				Radioactivity in spermatic venous plasma (d.p.m./ml)				Radioactivity in urine (d.p.m./ml)			
Fraction	Total extracted	5α-Androst-16-en-3-one	An-α	An-β	Total extracted	5α-Androst-16-en-3-one	An-α	An-β	Total	5α-Androst-16-en-3-one	An-α	An-β
Free steroids	43660	236	534	876	30190	340	200	176	0	0	0	0
Glucosiduronates	2210	0	20	4	38	0	7	3	3740	0	0	230
Sulphates	57050	0	1490	3330	38920	0	365	633	3850	0	0	95

The preliminary results, summarized in Table 1, demonstrate the conversion of pregnenolone into 5α-androst-16-en-3-one, an-α and an-β as free steroids, in both testicular tissue and spermatic venous plasma. These findings are in agreement with the detection of these compounds in boar spermatic venous plasma (Gower *et al.*, 1970). In another experiment in which a boar testis was infused with radioactive pregnenolone, little or no ^{3}H-labelled 16-unsaturated C_{19} steroids were isolated from the peripheral arterial blood (D. B. Gower, unpublished work). In the present experiment (boar E), where labelled pregnenolone was infused close-arterially for 12min and spermatic venous blood was collected continuously, no labelled precursor could have entered the general circulation. These findings provide direct evidence for the testicular formation of 16-unsaturated steroids from pregnenolone in the boar.

The secretion of 5α-androst-16-en-3-one into the spermatic venous blood may be related to its presence in boar fat, to which it gives a strong taint (Patterson, 1968), since this steroid is highly lipophilic. The lower concentration of labelled 5α-androst-16-en-3-one in

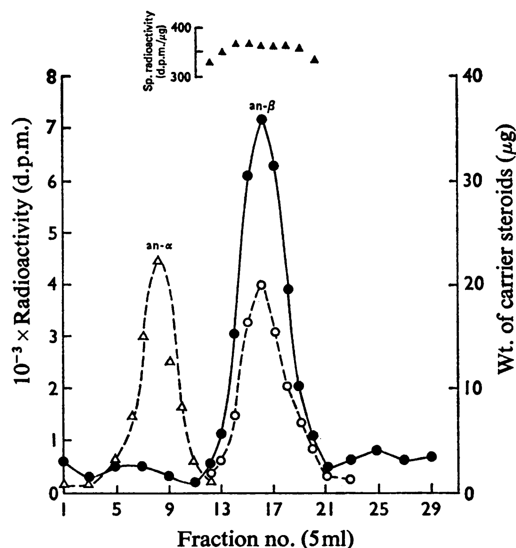


Fig. 4. *Column chromatography on alumina* (10*g*), *with benzene–light petroleum* (1:1, *v/v*) *as eluent, of the hydrolysed glucosiduronate fraction of boar urine*

This fraction was obtained from urine of boar C during a long-term (200min) testicular infusion of [7α-^{3}H]pregnenolone (250μCi). The weight (○) of carrier steroids added before chromatography (see the text) was determined by g.l.c., and radioactivity (●) by liquid-scintillation counting. ▲, Specific radioactivity (d.p.m./μg).

the testicular tissue compared with spermatic venous plasma (Table 1) may be explained as follows: the fat-soluble 5α-androst-16-en-3-one may pass readily into the spermatic venous blood, or be rapidly metabolized by testicular reductases to an-α and an-β (Brophy & Gower, 1972). Both these androstenols have been isolated from boar testes as free steroids (Prelog & Ruzicka, 1944; Booth, 1970) and, together with 5α-androst-16-en-3-one, are well-known to be biosynthesized in the same tissue *in vitro* (Gower, 1972). However, a more probable explanation may be related to the conditions of the experiment, which was not performed in the steady state.

In the present work, the specific radioactivities of an-α and an-β, both as free steroids and as sulphates, were higher in the testicular tissue than in the spermatic venous plasma. This may be explicable on the assumption that there is more than one site of production for these compounds in boar testis. As far as androgen metabolism is concerned, the Leydig cells of testis interstitial tissue are well known to be the principal site of metabolism. However, some androgen biosynthesis occurs in the seminiferous tubules of rat (Christensen & Mason, 1965) and rabbit (Collins, 1968) testes. Recent experiments, in which canine testes were infused with [4-^{14}C]pregnenolone (van der Molen & Eik-Nes, 1971), also indicate more than one compartment for androgen synthesis, since the specific radioactivities of metabolites in the testis were not equal to those of corresponding metabolites in the spermatic venous plasma.

The results in Table 1 show that the ratio an-α sulphate:an-α and the ratio an-β sulphate:an-β in the testicular tissue were greater than those of the corresponding compounds isolated from spermatic venous plasma. Similar results have been reported for other C_{19} steroids in the boar by Baulieu *et al.* (1967); a value of 5 was found for the ratio dehydroepiandrosterone sulphate:dehydroepiandrosterone in the testicular tissue, whereas in the spermatic venous plasma the ratio was only 2. This may be attributed to sulphatase activity, for it is well known that testes from several species contain a steroid sulphatase (Burstein & Dorfman, 1963) capable of hydrolysing dehydroepiandrosterone sulphate. It is possible therefore that partial hydrolysis may occur before secretion and that the sulphates of the 16-unsaturated steroids are a potential source of unconjugated steroids in the spermatic venous plasma.

The results of the second experiment (boar C) demonstrate the further metabolism of 16-unsaturated C_{19} steroids. A considerable amount of labelled an-β glucosiduronate and, to a lesser extent an-β sulphate, have been isolated from the urine. However, the former conjugate was detected in only trace amounts in both the infused testis and the spermatic venous plasma; moreover, neither an-α glucosiduronate nor an-α sulphate was detected in the urine. These results would be explicable if an-α were converted into an-β by an hepatic epimerase, an-β then being conjugated to an-β glucosiduronate and finally excreted in the urine. The smaller amount of an-β sulphate excreted may be due to a lowered clearance of this conjugate from the blood and kidneys. The more rapid renal clearance of steroid glucosiduronates, compared with that of sulphates, is well known (Jayle *et al.*, 1962).

We are grateful to Miss G. Needham for her invaluable assistance; Y. A. S. expresses sincere thanks to the Agricultural Research Council for generous financial support (Grant no. AG 35/18).

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