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Studies on the Metabolism of 5α-Androst-16-en-3-one in Boar Testis *in vivo*

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1. [5α-^{3}H]5α-Androst-16-en-3-one (5α-androstenone) was infused at a constant rate for 180min into the spermatic artery of a sexually mature boar. Samples of spermatic-venous blood were collected at 1min intervals for the first 10min of the infusion and thereafter at 15min intervals for the first hour, then at 64, 125, 155 and 172min. After infusion, the testis was removed and immediately cooled to −196°C. 2. From both the testicular tissue and the spermatic-venous plasma, endogenous and ^{3}H-labelled androst-16-enes were isolated, characterized and quantitatively determined and their specific radioactivity was calculated. 3. The specific radioactivities of 5α-androstenone, 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol (an-α and an-β) in testicular tissue were different from those in the spermatic-venous plasma, suggesting that these compounds may be present in more than one compartment of the testis and differentially secreted into the spermatic-venous blood. 4. The ratios of the specific radioactivities of an-α and an-β to their respective sulphate conjugates in the testicular tissue were less than the ratios of the same compounds in the spermatic-venous plasma. 5. The patterns of secretion of these labelled compounds in the spermatic-venous blood during the period of infusion were demonstrated. 6. The urine that accumulated during the infusion was analysed and found to contain ^{3}H-labelled an-β, conjugated as both glucuronide and sulphate, the specific radioactivities of which were determined. Little or no androst-16-enes occurred as free steroids. 7. The presence of an-β glucuronide in the urine is discussed.

Earlier studies have shown that in boar testicular tissue, androst-16-enes are produced in high yield from pregnenolone (3β-hydroxypregn-5-en-20-one) or progesterone (pregn-4-ene-3,20-dione) (for references see Gower, 1972). Further, studies *in vivo* (Saat *et al.*, 1972) have confirmed these results, since when [7α-^{3}H]pregnenolone was infused into a boar testis, ^{3}H-labelled androst-16-enes were isolated from the spermatic-venous-blood plasma. The metabolites characterized were an-α (5α-androst-16-en-3α-ol) and an-β (5α-androst-16-en-3β-ol), which occurred largely as sulphates, and 5α-androstenone (5α-androst-16-en-3-one), which was found in the ether-extractable fraction.

The ketone 5α-androst-16-en-3-one is known to be readily reduced in boar testicular preparations to a mixture of an-α and an-β (Brophy & Gower, 1972), and in boar salivary glands mainly to an-α (Katkov *et al.*, 1972). Both 5α-androst-16-en-3-one and an-α have been found in adipose tissue of mature boars (Patterson, 1968; Beery & Sink, 1971) and, by virtue of their characteristic smell, give rise to the well-known boar taint. The present work is concerned with the way in which 5α-androst-16-en-3-one is produced and metabolized in boar testis *in vivo*. A preliminary account of the present work has been published (Saat *et al.*, 1973).

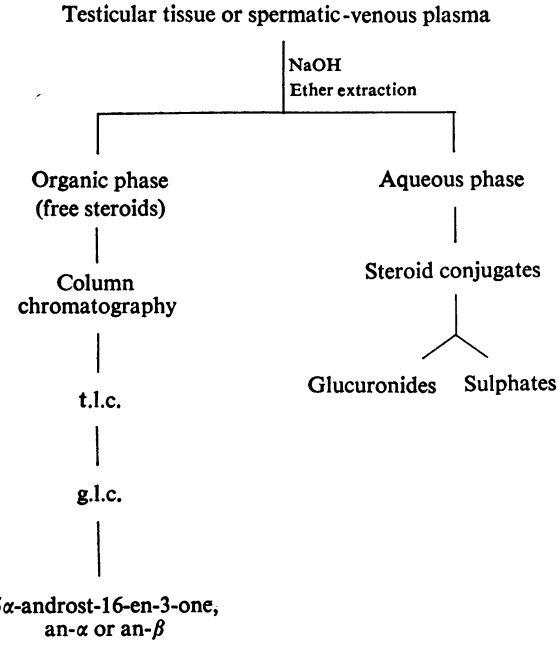
Experimental

Materials

Reagents, steroids and enzyme. Authentic androst-16-enes, 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. MN-Kieselgel G-HR was purchased from Camlab, Cambridge, U.K. Amberlite XAD-2, a neutral cross-linked polystyrene polymer, was obtained from Rohm and Haas Co., Philadelphia, Pa., U.S.A. Ox liver β-glucuronidase (Ketodase) was obtained from William R. Warner and Co. Ltd., Eastleigh, Hants., U.K.

Purification of [5α-^{3}*H*]*androst-16-en-3-one.* [5α-^{3}H]-5α-Androst-16-en-3-one (radiochemical purity 90%; specific radioactivity 15.01Ci/mmol) was kindly supplied by Dr. W. Hafferl, Institute of Organic Chemistry, Syntex, Palo Alto, Calif., U.S.A. This material was purified first by column chromatography on alumina in benzene–light petroleum (b.p. 80–100°C) (1:3, v/v), then by t.l.c. in benzene–methanol (9:1, v/v).

Animal. A sexually mature stud boar (Meltonhouse King David 34th) of the Large White breed, aged 2 years and weighing 222kg, was used.



Scheme 1. *Flow diagram for the fractionation and characterization of androst-16-enes from testicular tissue and spermatic-venous plasma after continuous infusion of boar testis with* [5α-^3H]5α-*androst-16-en-3-one*

See the Experimental section for details.

Methods

Anaesthesia and operative procedures. These were as described by Saat *et al.* (1972). Early in the operative preparation, an intravenous drip of glucose–saline (4.3% dextrose, 0.18% NaCl) was given during the experiment (500ml/h).

Infusion and experimental protocol. [5α-^{3}H]5α-Androst-16-en-3-one (1mCi) was infused close-arterially into the right spermatic artery at a constant rate of 1.607 μCi/min for 180min from a glass syringe mounted on a Harvard syringe injector (Harvard Apparatus Co. Inc., Dover, Mass., U.S.A.).

Complete drainage of the right spermatic-venous blood was commenced at the start of the radioisotope infusion, and samples were collected at 1min intervals for the first 10min. Thereafter, blood was returned to the animal via the intravenous drip and samples were collected at 15, 30, 45, 64, 125, 155 and 172min. Blood flow was measured by weighing timed collections. Arterial blood samples were taken from a catheter in the left anterior tibial artery at 130, 157 and 180min. Immediately after the infusion was stopped, the infused testis was rapidly removed and immediately cooled to −196°C and stored at −20°C. Urine that had accumulated during the time of infusion was also collected and stored at −20°C. The blood samples were centrifuged at 3000*g* for 15min in a refrigerated centrifuge and the plasma was separated and stored at −20°C.

Measurement of radioactivity. A Beckman scintillation spectrometer (model 1650) was used for the determination of radioactivity. Free steroids were dissolved in scintillant A (5ml) which consisted of xylene containing 2,5-diphenyloxazole (0.3%, w/v); counting efficiencies for ^{3}H and ^{14}C were 52 and 92% respectively. Steroid conjugates were dissolved in scintillant B (5ml), which was prepared as described by Bray (1960); efficiencies for ^{3}H and ^{14}C under these conditions were 27 and 80% respectively.

Extraction of androst-16-ene derivatives from testicular tissue and plasma. By the procedures described by Saat *et al.* (1972), the free androst-16-ene derivatives were purified and characterized by column chromatography on alumina and by t.l.c. on $AgNO_3$-impregnated Kieselgel G (Katkov & Gower, 1970) or on MN-Kieselgel G-HR, and finally by g.l.c. (Scheme 1). It has been shown previously that when these procedures are followed, radiochemical purity of these metabolites is established (Brophy & Gower, 1972; Saat *et al.*, 1973).

The aqueous phase containing the steroid conjugates was adjusted to pH4.5 with 4M-sodium acetate buffer and incubated with β-glucuronidase (Ketodase)

for 48h at 38°C. The steroid glucuronides released as free steroids after this incubation were treated as described by Saat *et al.* (1972). The aqueous phase, still containing the sulphate conjugates, was adjusted to pH 1 with conc. HCl and to a NaCl concentration of about 20% (w/v); the mixture was extracted with ethyl acetate (2vol.) and the extract incubated for 48h at 38°C (Burstein & Lieberman, 1958). The free steroids released were treated as described by Gower *et al.* (1970).

Extraction of androst-16-ene derivatives from boar urine. The urine (780ml), which had accumulated during the time of infusion (180min), was thawed to room temperature and its pH measured (pH 5.8–6.1). A column (length 30cm, inner diam. 2cm) was packed with Amberlite XAD-2 resin (80ml). This was then washed successively with 3vol. of water, 1vol. of methanol and finally 3vol. of water. The urine was adjusted to pH 9 with NaOH and passed through the column at a rate of 5ml/min. The column was then washed with 50ml of water at a rate of 1.5ml/min (Osawa & Slaunwhite, 1970). Steroids were eluted with methanol at a rate of 1.5ml/min and the combined eluate was evaporated to dryness, the residue dissolved in 0.5M-NaOH (15ml) and the androst-16-ene derivatives were extracted and characterized by the procedures used for testicular tissue and spermatic-venous plasma.

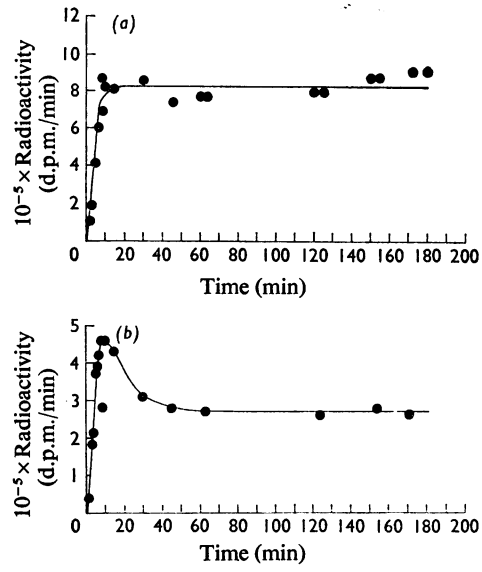


Fig. 1. *Radioactivity (d.p.m./min) obtained from the spermatic-venous plasma during continuous infusion (180min) of a boar testis with [5α-^{3}H]5α-androst-16-en-3-one*

(*a*) Total radioactivity; (*b*) radioactivity after extraction of free steroids. See the Experimental section for details.

Results

The total radioactivity in each sample of spermatic-venous plasma collected during the continuous infusion of tritiated 5α-androst-16-en-3-one was measured, and the pattern of radioactivity showed that the amount of ^{3}H-labelled compounds increased markedly during the first 10min of infusion and reached a plateau from 10 to 180min after the start of the infusion (Fig. 1*a*). During this latter period a constant proportion of about one-third of the total radioactivity was not extracted by diethyl ether, indicating the synthesis of conjugated compounds from 5α-androst-16-en-3-one.

The pattern of the individual labelled free androst-16-enes in the spermatic vein during the time of infusion is given in Fig. 2(*a*). This shows that tritiated 5α-androst-16-en-3-one increased rapidly during the first 10min, and then declined, the amount remaining constant after about 45min. In contrast, the amounts of an-α and an-β did not show any marked increase during the first 5min of infusion, but thereafter their concentration rose sharply, reaching a steady state after about 30min, forming a plateau until about 120min, with a final increase during the last 60min of the infusion. The specific radioactivities (d.p.m./μg) of tritiated 5α-androst-16-en-3-one, an-α and an-β in the spermatic-venous plasma were greater than those of the corresponding compounds in the testicular tissue (Table 1). During the last 2h of the infusion more than 70% of labelled 5α-androstenone was extracted by the testis.

After the extraction of the free steroids, the remaining radioactivity (steroid conjugates) in each sample of spermatic-venous blood collected during the time of infusion was measured. This was assumed to be due to the presence of sulphates, since little or no androst-16-ene glucuronides occur (Saat *et al.*, 1972). Fig. 1(*b*) shows that there was a sharp increase of radioactivity associated with the conjugate fraction during the first 20min, followed by a plateau from 30 to 180min. The pattern of labelled androst-16-ene sulphates secreted into the spermatic vein (Fig. 2*b*) revealed a sharp increase of labelled an-α and an-β during the first 10 and 15min of the infusion respectively, followed by a rapid decline from 15 to 60min and, in the case of labelled an-β sulphate, a slight decrease until the end of the infusion at 180min. During the last 2h of the infusion, an-α and an-β sulphates accounted for about 30% of the conjugated compounds. The specific radioactivities of an-α and an-β sulphates in the spermatic-venous plasma were again greater than the corresponding specific radioactivities in the testicular tissue (Table 1).

The percentage conversion ratio (Longcope *et al.*, 1968) of 5α-androst-16-en-3-one into free an-α

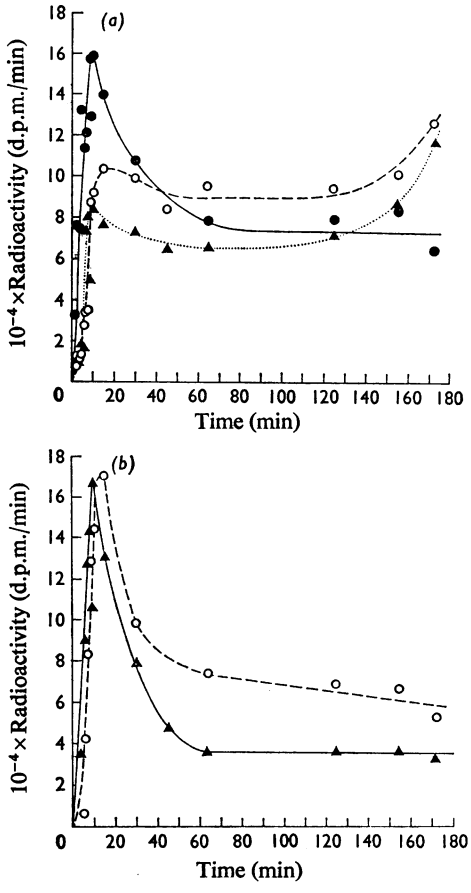


Fig. 2. *Radioactivity (d.p.m./min) of (a) free androst-16-enes and (b) androst-16-en-3-ol conjugates secreted into the spermatic-venous blood during the continuous infusion of a boar testis with [5α-^3H]5α-androst-16-en-3-one*

●, 5α-Androstenone; ▲, an-α; ○, an-β. See the Experimental section for details.

and an-β was 89.6 and 116.5% respectively during the infusion period 45–155 min after the start of the experiment. The percentage conversion ratio for 5α-androstenone into an-α and an-β sulphates was 48.4 and 86.5% respectively.

It is significant that only traces of radioactivity were found in samples of blood taken from the anterior tibial artery during the infusion at 130, 157 and 180 min, indicating that there was little recirculation of tritiated 5α-androst-16-en-3-one and its metabolites during the infusion.

The results obtained from the boar urine that had accumulated during the period of infusion showed that an insignificant amount of free radioactive compounds had been excreted. Further, only traces of an-α glucuronide and sulphate were detected, the radioactivity excreted being mainly an-β glucuronide and to a lesser extent an-β sulphate (Table 1).

Discussion

The close-arterial infusion of [5α-^{3}H]5α-androstenone into a testis of a mature boar has provided new information on the synthesis and metabolism of this odoriferous compound. Whereas the amount of total radioactivity in the spermatic vein was constant during the major part of the infusion, a steady-state situation applied over a relatively short period in respect of the amounts of 5α-androstenone, an-α and an-β. The two predominant metabolites of 5α-androstenone recovered in a free form were an-α and an-β, a finding which confirms the results of previous studies carried out *in vitro* (Brophy & Gower, 1972). These labelled metabolites appeared in the spermatic vein in small amounts during the first 6 min of the infusion, but thereafter their concentration and rate of secretion increased dramatically 1 min and 3 min later for an-α and an-β respectively (Fig. 2*a*). The formation of these

Table 1. *Quantitative determination of the endogenous androst-16-ene derivatives and their specific radioactivity in spermatic-*

The same determinations were performed for the infused tissue

		5α-Androst-16-en-3-one			An-α		
Source	Time (min)	(μg/100 ml)	(μg/min)	10^{-5}×Sp. radioactivity (d.p.m./μg)	(μg/100 ml)	(μg/min)	10^{-5}×Sp. radioactivity (d.p.m./μg)
Spermatic-venous plasma	125	1.45	0.16	5.0	0.83	0.09	7.9
	155	1.21	0.14	5.8	0.97	0.11	7.3
	172	0.92	0.11	5.1	1.04	0.13	8.6
Mean±S.E.M.		1.19±0.15	1.14±0.01	5.3±0.3	0.95±0.06	0.11±0.01	7.9±0.4
Testicular tissue		26*		0.66	48*		0.58
Urine							
Glucuronides	—	—	—	—	Traces		—
Sulphates	—		—	—	—		—

* μg/100g wet wt. of testicular tissue.

hydroxylated epimeric metabolites of 5α-androst-16-en-3-one apparently reached a steady state between about 30 and 120 min, the conversion into an-β being slightly greater than that into an-α, but after this time both metabolites were secreted in greater amounts. The time-course of the formation of labelled an-α and an-β sulphates was superficially similar to that of the free metabolites, but although the amount of an-α sulphate was constant during the last 3 h, this was not true of an-β sulphate (Fig. 2*b*). During the period when a steady state was established between 40 and 140 min of infusion, approx. 42% of the total radioactivity in plasma could be recovered in 5α-androst-16-en-3-one, an-α, an-β and the sulphates of an-α and an-β. This indicates that an appreciable proportion of infused 5α-androstenone was metabolized into other compounds not identified in the present study.

These findings can be best interpreted by the postulates that the synthesis and metabolism of 5α-androst-16-en-3-one, an-α and an-β involve more than one intra-testicular compartment, and that an-α and an-β are not formed exclusively from one single precursor. This interpretation is supported by the specific radioactivities of 5α-androst-16-en-3-one, an-α and an-β, which were considerably greater in spermatic-venous blood than in testicular tissue. If free an-α and an-β were formed only from 5α-androst-16-en-3-one, and if only one pool of these androstenols existed in the testis, the specific radioactivities of all three compounds should be the same. Similar findings have been reported from experiments in which canine testes were infused with labelled pregnenolone (van der Molen & Eik-Nes, 1971). Further, Bell *et al.* (1971) showed that, although testosterone was synthesized mainly in the Leydig cells of rat testicular preparations, there was also some synthesis in the seminiferous tubules. This has been confirmed by Galena & Terner (1974), who showed that non-flagellate germinal cells (spermatocytes and young spermatids), isolated from rat seminiferous tubules, can actively form androgens from progesterone.

Alternative precursors of free an-α and an-β in spermatic-venous blood are the corresponding androstenol sulphates. In keeping with this suggestion the ratios in the testis of an-α sulphate to an-α, and of an-β sulphate to an-β, were 1.6 and 1.7 respectively (Table 1), whereas the ratios of the corresponding compounds in spermatic-venous plasma were somewhat lower, 1.1 and 1.3 respectively. 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 of dehydroepiandrosterone sulphate to dehydroepiandrosterone in testicular tissue, whereas in the spermatic-venous plasma the ratio was only 2. This may be attributed to sulphatase activity, and, as shown in Fig. 2(*b*), there is a sharp fall in the secretion of an-α and an-β sulphates after 15 min of the infusion, with a continued slight decline until the end of the experiment. It is possible, therefore, that partial hydrolysis of the sulphates may occur before secretion. It is well known that testes from various species contain steroid sulphatase (Burstein & Dorfman, 1963; Aakvaag *et al.*, 1964) capable of hydrolysing steroid sulphates to free steroids.

Since the specific radioactivities of 5α-androst-16-en-3-one, an-α and an-β in the spermatic-venous blood are higher than those of the corresponding compounds in the testicular tissue, it seems most likely that no real equilibrium is attained between the secretory testicular pool(s) and spermatic-venous blood, and it is probably only part of the total tissue content of the metabolites that is in equilibrium with the infused [5α-^{3}H]androstenone. If this is so, only part would contribute to steroid secretion into the venous blood. Thus the results from this present experiment support the idea that androst-16-ene steroids in the boar testis may be present in different

venous plasma obtained during the last 60 *min of continuous infusion* (180 *min*) *of boar testis with* [5α-^{3}*H*]5α-*androst*-16-*en*-3-*one* and urine collected during the whole period of the infusion.

An-α sulphate			An-β			An-β sulphate		
(μg/100 ml)	(μg/min)	10^{-5}×Sp. radioactivity (d.p.m./μg)	(μg/100 ml)	(μg/min)	10^{-5}×Sp. radioactivity (d.p.m./μg)	(μg/100 ml)	(μg/min)	10^{-5}×Sp. radioactivity (d.p.m./μg)
1.02	0.11	2.5	1.13	0.12	7.5	—	—	—
0.93	0.11	2.8	1.49	0.14	6.3	2.0	0.24	2.8
1.10	0.14	2.1	1.69	0.21	6.0	1.8	0.23	2.3
1.02±0.05	0.12±0.01	2.5±0.2	1.44±0.16	0.16±0.03	6.6±0.5	1.9	0.23	2.5
81*		0.34	181*		0.26	313*		0.41
—		—	19.14		0.14	—		—
Traces		—	—		—	6.21		0.08

anatomical compartments. If these compartments are not uniformly supplied with the infused radioactive 5α-androst-16-en-3-one, this may not contribute equally to the secretion of the substrate and the end products into the spermatic-venous blood.

The formation of urinary an-β glucuronide (Table 1) may be attributed to the rapid glucuronidation of free an-β by glucuronyltransferase, presumably in the liver. The relatively small quantities of an-β sulphate detected in the urine may be due to partial hydrolysis of this conjugate by the sulphatase usually found in mammalian livers (Roy, 1960). As a result of this hydrolysis, more an-β would be rapidly reconjugated to an-β glucuronide. It is relevant here to compare the rapid excretion of oestrogen glucuronides in humans after administration of both free and conjugated oestrogens (Sandberg & Slaunwhite, 1965; Inoue *et al.*, 1969*a*,*b*). Further, rapid clearance of steroid glucuronides compared with steroid sulphates is well known (Jayle *et al.*, 1962) and there is good evidence that the metabolic clearance rate of unconjugated dehydroepiandrosterone in man is approximately 100 times greater than that for dehydroepiandrosterone sulphate (Wang *et al.*, 1967). Although no information about the metabolic clearance rate of androst-16-ene sulphates is yet available, it is possible that these compounds, being C_{19} steroid sulphates, may have a low clearance rate, similar to that of dehydroepiandrosterone sulphate. If this were so it would help to explain the small quantities of an-α sulphate in boar urine (see Table 1 and Gower *et al.*, 1970) and human urine (Brooksbank & Haslewood, 1961).

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