METABOLISM OF SPIROLACTONES BY THE RAT TESTIS IN VITRO

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ABSTRACT

Spironolactone (SPIR) has been shown in numerous clinical studies to produce sexual disorders. We studied the metabolism of canrenone (CAN), the main metabolite of SPIR, and of the analogue 6,7-dihydrocanrenone (DHC) by the rat testis in vitro. The metabolites produced during a 4 h incubation period were isolated by HPLC and identified by nmr-, ms-, ir- and uv-spectrometry.

SPIR was not metabolised in a detectable amount. CAN was converted to canrenoic acid, several hydroxylated (15β-, 16α-, 19-, 20R- and 21S-OH-CAN) and one reduced metabolite (3α-OH-CAN). When DHC was incubated, an analogous pattern was detected.

It is concluded that CAN and DHC serve as substrates for steroid metabolism in the rat testis.

INTRODUCTION

Spironolactone (SPIR) and potassium canrenoate are widely used aldosterone antagonists. Their clinical use, however, is limited by adverse endocrine effects. These unwanted effects include decreased libido, impotence, and gynecomastia in men, breast enlargement and menstrual irregularities in women. Because of the structural similarity between the spirolactones and the naturally occurring steroid hormones, there are different possibilities by which the aldosterone antagonists of the spirolactone type could cause these side effects:

1.) direct interaction with the receptor site both on androgen (1,2,3) and estrogen receptors (4);

2.) alteration of the peripheral metabolism of testosterone resulting in changes in the ratio of testosterone to estradiol-17β (5); or

3.) reduction of the testicular synthesis of testosterone (6-9).

All these possible mechanisms are attributed to SPIR or its main metabolite canrenone (CAN). Numerous studies have shown that SPIR is extensively metabolised in experimental animals and man (for review: 10). Most of these metabolites were found in plasma, urine and feces. However, nothing is known about the metabolism of spirolactones by the steroid producing endocrine glands.

Therefore, we investigated the metabolism of CAN, DHC (6,7-dihydrocanrenone) and SPIR in rat testis in vitro to look for metabolites which might contribute to the endocrine side effects of spirolactones.

MATERIALS and METHODS

Substances

SPIR, DHC and (1,2,6,7-3H-)DHC were gifts from Boehringer Mannheim (FRG) and (20,21-3H-)SPIR from Hoechst (Frankfurt, FRG). CAN and (20,21-3H-)CAN were synthesized by alkaline hydrolysis of SPIR and (20,21-3H-)SPIR according to the Organikum (11). All these steroids were purified of HPLC before use. The tritiated substances were diluted to a specific activity of 5 µCi/µmol resulting in a final concentration of 100 µmol/l in the incubation medium. The other chemicals were obtained from Merck (Darmstadt, FRG).

Incubation

For each experiment five adult Sprague-Dawleys rats (Fa. Charles River Wiga, Sulzfeld, FRG) weighing 250-300 g were killed by dislocation of the neck. The testes were immediately removed and decapsulated. The tissue was minced

by a pair of scissors and transferred to a flask containing 100 ml of Krebs-Ringer bicarbonate buffer of pH 7.4 with 1.5 mg/ml glucose. Incubations were performed under O_2-CO_2 atmosphere (95:5, v:v) at 34°C for 4 h with shaking at 90 cycles/min. The spirolactones were added in ethanol to a final concentration of 100 µmol/l of spirolactone and 5.2 mmol/l of ethanol, and the tissue was incubated at 34°C for 4 h.

Isolation of Steroids

100 ml of the incubation medium were homogenised by means of an Ultra Turrax (Jahnke & Kunkel, Staufen, FRG) and extracted twice with 400 ml of dichloromethane. The organic phase was evaporated to dryness, the residue redissolved in 10 ml toluene and passed through a silica catridge (Sep-Pak, Waters, USA) to remove lipids. The steroids were eluted with 10 ml methanol and again evaporated to dryness. The residue was dissolved in approximately 500 µl of the mobile phase and samples of 100 µl were injected onto the HPLC column.

HPLC

The HPLC system consisted of a Hewlett-Packard model 1084B liquid chromatograph with a variable UV-VIS wavelength detector and an autosampler. The first separation was performed on a semi-preparative column (250x8 mm) filled with Lichrosorb RP-18, 10 µm (Fa. Knauer, Berlin, FRG) at a column temperature of 40°C. Elution was done with a linear gradient of 40 to 80% methanol in water within 40 min at a flow rate of 6 ml/min.

The eluates containing the metabolites were extracted twice with dichloromethane, evaporated to dryness and purified on an analytical column (250x4 mm) filled with a Lichrosorb RP-18, 5 µm material (Fa. Merck, Darmstadt, FRG) with a linear gradient of 25 to 55% acetonitrile in water within 48 min at a flow rate of 1.1 ml/min and a column temperature of 40°C. Thereafter the metabolites were pure enough for the identification by spectroscopic methods.

Identification of the Metabolites

Nuclear magnetic resonance spectra were obtained on a Bruker WH-270-spectrometer (Fa. Bruker, Karlsruhe, FRG) in deuterochloroform solutions. Chemical shifts were recorded in ppm (σ) values relative to chloroform at 7.27 ppm. Multiplicities of signals were designated as follows: s, singlet; d, doublet; dd doublet of doublets; ddd, doublet of doublet of doublets; dt, doublet of triplets; q, quartet; m, multiplet. Nuclear magnetic resonance data are listed in table 1.

Mass spectrometry was done with an AEI model MS-30 spectrometer (AEI, Manchester, England) at an electron energy of 70 eV. High resolution mass spectroscopy was

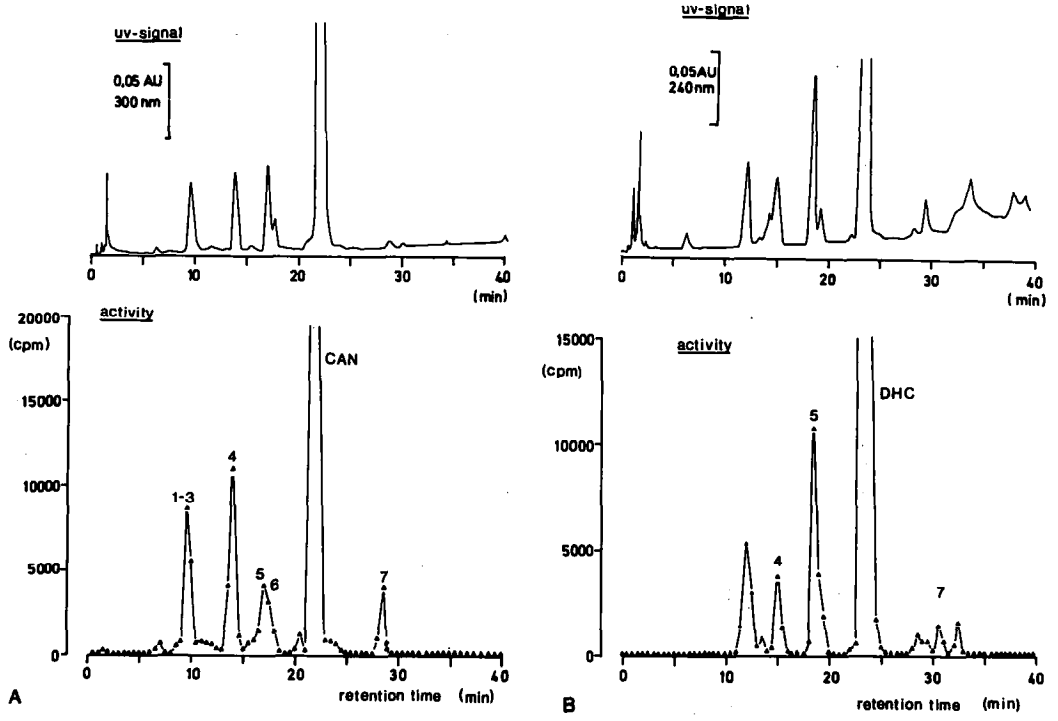

Fig. 1 Resolution of CAN and its metabolites (A) and DHC and its metabolites (B) on a Lichrosorb RP-18 column (250x4 mm) using a linear gradient of 40 to 80 % methanol in water within 40 min. Flow rate: 6 ml/min.
1: canrenoic acid; 2: 15β-OH-CAN; 3: 19-OH-CAN; 4: 16α-OH-CAN
5: 20R-OH-CAN; 6: 21S-OH-CAN; 7: 3α-OH-CAN or DHC respectively

performed with the same spectrometer by peak matching.

IR spectra were measured as potassium bromide microdisks with an IR-521 spectrometer (Perkin Elmer, Überlingen, FRG).

UV absorption spectra were obtained in methanol with a DMR-10 spectrometer (Zeiss, Oberkochen, FRG).

Measurement of Radioactivity

All radioactive determinations were done using a Mark II liquid scintillation spectrometer (Nuclear Chicago, Illinois, USA) in 15 ml of a Triton X-100, PPO, POPOP, toluene (210ml/4g/0.1g/790ml) scintillation solution.

RESULTS

Identification of Canrenone Metabolites

During incubation 10.5 % of (20,21-3H-)CAN was converted into six identified metabolites and 2.8% canrenoic acid (Fig.1A). A control incubation with inactivated tissue (30 min at 95°C) showed that CAN was stable under the conditions used, since only one peak corresponding to CAN was present. A total of 30 incubations with 10 testes each were performed and collected to get enough material for the identification of the metabolites.

Canrenone

CAN was prepared from SPIR by alkaline hydrolysis. Its identity was confirmed by nmr and ms spectrometry and the data agreed with those reported by Karim et al. (12,13). The structures of the mass fragmentation ions described throughout this paper were taken partly from Karim et al. (13), Zaretskii (14) and Spiteller-Friedmann & Spiteller (15).

ms: (m/e) 340 (68; M^+); 325 (20; $M^+ - CH_3$); 322 (4; M^+-

Tabl.1 Nuclear magnetic resonance data of the spirolactones and metabolites

Compound	C-4 H	C-6 H	C-7 H	C-18 H	C-19 H	Others
CAN	5.69-1H-s	6.15-1H-dd(10,3Hz)	6.09-1H-d(10Hz)	1.04-3H-s	1.13-3H-s	
DHC	5.74-1H-s			1.00-3H-s	1.21-3H-s	
3α-OH-CAN	5.63-1H			1.02-3H-s	0.95-3H-s	4.15 C-3β H
15β-OH-CAN	5.71-1H-s	6.18-1H-dd(10,3Hz)	6.30-1H-d(3Hz)	1.30-3H-s	1.18-3H-s	4.47-1H-m($w_{1/2}$=11Hz) C-15α H
16α-OH-CAN	5.70-1H-s	6.15-1H-dd(10,3Hz)	6.02-1H-d(10Hz)	1.07-3H-s	1.13-3H-s	1.92-1H-d(4Hz) C-16 OH 4.70-1H-dt(10,5,4Hz) C-16β H
16α-OH-DHC	5.74-1H-s			1.01-3H-s	1.20-3H-s	4.66-1H-dd(10,4Hz) C-16β H
19-OH-CAN	5.84-1H-s	6.19-1H-dd(10,3Hz)	6.12-1H-d(10Hz)	1.05-3H-s	3.83-1H-d(10Hz) 3.92-1H-d(10Hz)	
20R-OH-CAN	5.68-1H-s	6.12-2H-m($w_{1/2}$ = 4Hz)		1.06-3H-s	1.13-3H-s	2.58-1H-dd(18,4Hz) C-21 H_{eq} 2.86-1H-dd(18,7Hz) C-21 H_{ax} 4.45-1H-q(5Hz) C-20 H
20R-OH-DHC	5.74-1H-s			1.00-3H-s	1.20-3H-s	2.55-1H-dd(18,4Hz) C-21 H_{eq} 2.85-1H-dd(18,4Hz) C-21 H_{ax} 4.42-1H-dd(7,4Hz) C-20 H
21S-OH-CAN	5.71-1H-s	6.16-1H-dd(10,3Hz)	6.10-1H-d(10Hz)	1.15-3H-s	1.02-3H-s	2.05-1H-dd(13,10Hz) C-20 H_{ax} 2.83-1H-dd(13,8Hz) C-20 H_{eq} 4.49-1H-ddd(10,9,3Hz) C-21H

H_2O);307 (7; $M^+ - H_2O - CH_3$); 267 (100; Fig.2A); 227 (22; Fig.2B); 136 (30; Fig.2C)

ir: (cm^{-1}) 1728 (22-C=O); 1655, 1620, 1582 (3-oxo-$\Delta^{4,6}$)

uv: λ_{max}= 284 nm

Canrenoic acid

This substance was identified by the conversion to the lactone with hydrochloric acid (1N for 30 min at ambient temperature). CAN was confirmed by the HPLC retention time and by the mass spectrum.

15β-Hydroxycanrenone

This metabolite was produced by the testicular tissue in an amount of about 5 % of the total CAN metabolites. The molecular peak of the mass spectrum at m/e 356 (confirmed as $C_{22}H_{28}O_4$ by high resolution mass spectrometry), the peak at m/e 338 and the multiplet in nmr spectrum at 4.47 ppm indicated a hydroxylated metabolite of CAN. As the typical fragmentation ions indicating an intact A,B,C and D ring of the spirolactone were present at m/e 265, the hydroxylation must have taken place at one of these rings. The existence of the C-4, C-6 and C-7 proton signals in the nmr spectrum excluded the hydroxylation at these three positions.

Comparison of the additional chemical shift values of the C-10 and C-13 methyl groups and the signal at

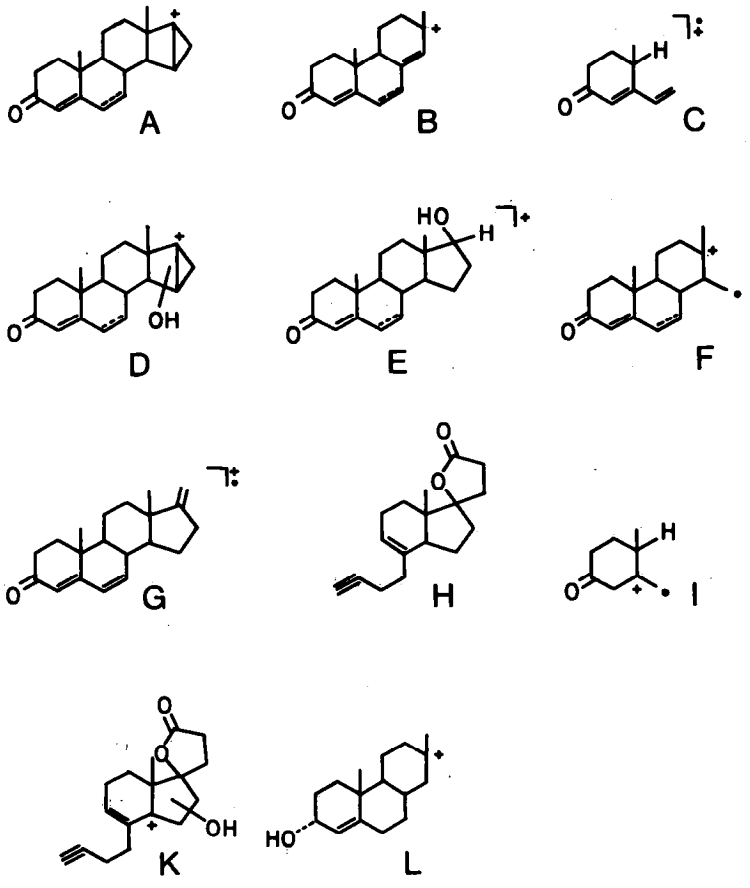

Fig. 2 Structure of fragmentation ions mentioned in the text

4.47 ppm with those published by Brigdeman et al. (16, table 2) limited the possibilities to 15β- and 16β-OH-CAN. The strong deshielding influence of the hydroxyl group on the C-7 proton signal (0.21 ppm to lower field) proved the 15β-position. This metabolite was found in the urine of male rats by Karim et al. (17), and our spectroscopic data agree with their data.

ms: (m/e) 356 (13; M^+;HR: $C_{22}H_{28}O_4$; precision: 5 ppm); 338 (80; $M^+ - H_2O$); 323 (19; $M^+ - H_2O - CH_3$); 265 (73; Fig.2D - H_2O; HR: $C_{19}H_{21}O$; precision: 0.5 ppm); 136 (34; Fig.2C)

ir: (cm^{-1}) 3420 (OH); 1770 (22-C=O); 1655, 1617, 1578 (3-oxo-$\Delta^{4,6}$)

uv: λ_{max} = 283 nm

19-Hydroxycanrenone

19-OH-CAN occured in an amount of 4 % of the total metabolites. Canrenoic acid and 15β-OH-CAN were separated on the analytical HPLC column using the acetonitrile in water gradient as described above. The molecular peak at m/e 356 and the signal at 3420 cm^{-1} in the ir spectrum indicated a hydroxylated product. The fragmentation peaks $M^+ - 30$ and $M^+ - 31$ together with the presence of only one methyl signal in the nmr spectrum indicated that the hydroxylation took place at one of the angular methyl groups. Comparison of the additional chemical shift values

Table 2 Additional shift values and range of σ-values of CH-OH signals

Substituent	C-19 H	C-18 H	CH-OH	C-4 H	Ref
1α-OH	+0.01	0.00	3.6		B
1β-OH	+0.05	0.00	3.4-3.7		B
2α-OH	+0.02	0.00	3.8-3.9		B
2β-OH	+0.26	+0.01	4.2		B
3-oxo-Δ^4	+0.42	+0.08			B
3α-OH-Δ^4	+0.21	+0.04	4.0-4.1		B
3β-OH-Δ^4	+0.29	+0.05	3.5-3.7		B
11α-OH	+0.12	+0.03	3.9-4.1	+0.01	B,T
11β-OH	+0.23	+0.24	4.3-4	-0.04	B,T
12α-OH	+0.01	+0.05	3.8	+0.01	B,T
12β-OH	+0.01	+0.07	3.3-3.6		B
15α-OH	+0.01	+0.05	4.0-4.2	0	B,T
15β-OH	+0.02	+0.28	4.3	0	B,T
16α-OH	-0.01	+0.01	4.4-4.5		B
16β-OH	+0.02	+0.25	4.4-4.5		B
18-OH	0.00			+0.02	T
19-OH		+0.02		+0.14	T
3α-OH-CAN	-0.18	+0.02	4.15	-0.06	
15β-OH-CAN	+0.05	+0.26	4.47	+0.02	
16α-OH-CAN	0.00	+0.03	4.70	+0.01	
16α-OH-DHC	+0.01	-0.01	4.66	0.01	
19-OH-CAN		+0.01		+0.15	

B = Bridgeman et al. (16); 5α,14β-steroids
T = Tori & Kondo (18); 3-oxo-Δ^4-steroid
A positive value indicates a shift to lower fields

of the C-4 proton and the remaining methyl group with the data published by Tori & Kondo (18, table 2) confirmed 19-OH-CAN.

ms: (m/e) 356 (5; M^+; HR: $C_{22}H_{28}O_4$; precision: 2 ppm); 338 (5; $M^+ - H_2O$); 326 (30; $M^+ - CH_2O$; HR: $C_{21}H_{26}O_3$; precision: 9 ppm); 325 (41; $M^+ - HOCH_2$; HR: $C_{21}H_{25}O_3$; precision: 1 ppm)

ir: (cm^{-1}) 3420 (OH); 1770 (22-C=O); 1657, 1615, 1577 (3-oxo-$\Delta^{4,6}$)

uv: λ_{max} = 284 nm

16α-Hydroxycanrenone

This metabolite represented about 32 % of the total CAN metabolites. The mass spectrum indicated a hydroxylated product (m/e 356 (M^+); 338 (M^+- H_2O)) substituted in the C-15 or C-16 position, since m/e 283 and 265 excluded the presence of a lactonic ring and m/e 227 and 136 the rings A,B and C. The additional chemical shift values of these metabolites were compared with those of the literature summarized in table 2. It was found that only 15α- or 16α-hydroxycanrenone were possible. After the exchange of the OH proton in D_2O the signal at 1.92 ppm in the nmr spectrum disappeared and a doublet of triplets at 4.70 ppm changed into a doublet of doublets with coupling constants of 5 and 10 Hz, confirming the hydroxylation at the 16α-position, since only the 16β- and not the 15β-proton has two vicinal protons resulting in a doublet of doublets. In addition the coupling constants of 10 and 5 Hz agree well with 9 and 3 Hz reported by Nambara *et al.* (19) for the 16β-proton of a 16α,17β-dihydroxyandrostane derivative.

ms: (m/e) 356 (85; M^+; HR: $C_{22}H_{28}O_4$; precision: 5 ppm); 338 (100; M^+- H_2O); 323 (61; M^+- H_2O - CH_3); 283 (57; Fig.2D; HR: $C_{19}H_{23}O_2$; precision: 2 ppm); 265 (56; Fig.2D - H_2O; HR: $C_{19}H_{21}O$; precision: 2 ppm); 227 (64; Fig.2B; HR: $C_{16}H_{19}O$; precision: 3 ppm); 136 (68; Fig.2C);

ir: (cm^{-1}) 3430 (OH); 1775 (22-C=O); 1655, 1620, 1582

(3-oxo-$\Delta^{4,6}$)

uv: λ_{max} = 283 nm

20R-Hydroxycanrenone

20R-OH-CAN was the main metabolite representing 40 % of the total metabolites. About 1.3 mg were isolated.

The same criteria, as discussed in connection with the other metabolites, show that hydroxylation took place. The signal in the mass spectrum at m/e 267 indicated that the hydroxylation had occurred at the lactonic ring. Provided the uv extinction coefficent of the 3-oxo-$\Delta^{4,6}$ structure is not changed by hydroxylation at the lactonic ring, 2/3 to 3/4 of the 3H-label was lost during incubation (Fig.1A). This large extent of label loss can only be explained by hydroxylation leading to 20-OH-CAN. Only a hydroxylation of the propionic acid sidechain in the β-position can lead to a proton exchange at the C-H activated C-21 position. Hydroxylation at the α-position of the propionic acid sidechain would decrease the specific activity by about 25 %, as will be discussed for the next metabolite. Furthermore, the coupling constants of 18 Hz of the two signals at 2.58 and 2.86 ppm in the nmr spectrum indicate two protons in the C-21 position.

The vicinal coupling constants of 7 and 4 Hz between the two C-21 protons and the remaining C-20 proton must be due to the 20R configuration. These coupling constants are only possible with a C-20 proton in the equatorial position. An

equatorial proton of 20S-OH-CAN would lead to a steric hinderance between the hydroxyl group and the C-12α proton so that the lactonic ring would change its conformation resulting in an axial C-20 proton with vicinal coupling constants of about 10 to 12 Hz. Therefore, only the lactonic ring of 20R-OH-CAN with an equatorial C-20 proton and an axial OH-group is stable and allows coupling constants of 4 and 7 Hz.

.High resolution mass spectrometry showed two different fragments of the m/e 312 peak in the mass spectrum with the formula $C_{21}H_{28}O_2$ and $C_{20}H_{24}O_3$, indicating that the lactonic ring of this metabolite is disintegrated in two steps: decarboxylation and cleavage of CH_2=CHOH to the fragment with m/e 268. Both sequences are possible.

ms: (m/e) 356 (36; M^+; HR: $C_{22}H_{28}O_4$; precision: 1 ppm); 341 (6; M^+- CH_3); 338 (M^+- H_2O); 323 (21; M^+- H_2O - CH_3); 312 (22; M^+- CO_2; HR: $C_{21}H_{28}O_2$ precision: 12 ppm and M^+- CH_2=CHOH; HR: $C_{20}H_{24}O_3$; precision: 7 ppm); 285 (19; Fig.2E; HR: $C_{19}H_{25}O_2$; precision: 3 ppm); 268 (37; M^+- CO_2 - CH_2=CHOH; HR: $C_{19}H_{24}O$; precision: 2 ppm); 267 (41; Fig.A; HR: $C_{19}H_{23}O$; precision: 2 ppm); 242 (24; Fig.2F); 227 (100; Fig.2B); 136 (77; Fig.2C);

ir: (cm^{-1}) 3360 (OH); 1785 (22-C=O); 1642, 1616, 1580 (3-oxo-$\Delta^{4,6}$)

uv: λ_{max} = 283 nm

21S-Hydroxycanrenone

About 12 % of the CAN metabolites is represented by this substance.

The molecular peak at m/e 356 and the signal at 4.49 ppm indicated a hydroxylated product. The mass fragments at m/e 136, 227 and 267 indicated unchanged rings A,B,C and D. The decrease of the specific activity of about 25 % indicated a hydroxylation at C-21 as discussed for 20R-OH-CAN. This position is confirmed by the methylen coupling constants of 13 Hz of the C-20 protons at 2.05 and 2.83 ppm in the nmr spectrum.

The configuration could not be definitively determined. Assuming that the lactonic ring is in the same arrangement as shown for 20R-OH-CAN, the coupling constants of 10 and 8 Hz between the C-20 protons and the remaining C-21 proton suggest the structure of 20S-OH-CAN.

ms: (m/e) 356 (75; M^+; HR: $C_{22}H_{28}O_4$; precision 2 ppm); 341 (22; $M^+ - CH_3$); 338 (12; $M^+ - H_2O$); 312 (12; $M^+ - CO_2$ or $M^+ - CH_2{=}CHOH$); 282 (24; Fig.2G; HR: $C_{20}H_{26}O$; precision: 5 ppm); 268 (47; $M^+ - CO_2 - CH_2{=}CHOH$);267 (100; Fig.2A; HR: $C_{19}H_{23}O$, precision: 1 ppm); 227 (28; Fig.2B); 136 (87; Fig.2C)

ir: (cm^{-1}) 3290 (OH); 1775 (22-C=O); 1640, 1612, 1582 (3-oxo-$\Delta^{4,6}$)

uv: λ_{max} = 283 nm

3α-Dihydrocanrenone

This metabolite was produced in about 6 % of the total metabolites. As it was not uv-active at the wavelength used, the recovery was low, resulting in a final amount of about 100 µg.

The nmr spectrum was not quite conclusive, but nevertheless there is no doubt that the C-6 and C-7 protons are in the range of 5.9 to 6.1 ppm, the C-4 proton at about 5.63 ppm and the 3β-proton at about 4.15 ppm. The change of the uv-absorbance, the presence of the C-4, C-6 and C-7 proton signals in the lower field of the nmr spectrum and the molecular peak at 342 in the mass spectrum indicated that this metabolite is 3-OH-CAN. By means of the additional chemical shift data summarised in table 2, it is concluded that the hydroxyl group is in the α-position.

This assignment of the stereochemistry was confirmed by an incubation of this metabolite with the enzyme cholesterol oxidase, the substrate specificity of which has been well established by Smith & Brooks (20). Boreham et al. (21) confirmed the configuration of 3β-dihydrocanrenone by showing that this substrate was completely oxidised to canrenone by cholesterol oxidase. However, 3α-OH-CAN was not oxidised by cholesterol oxidase (E.C. 1.1.3.6.) from Nocardia erythropolis (Boehringer, Mannheim, FRG) using the incubation conditions of Smith & Brooks (20) and assaying the steroids by means of HPLC under the above mentioned

analytical conditions. A positive control with pregnenolone led to a complete conversion to progesterone.

In addition the chemical shift of the signal at about 4.15 ppm in the nmr spectra resulting from the 3β-proton is in agreement with the data of Brigdeman et al. (16, table 2) reporting 4.0 to 4.1 ppm.

ms: m/e 342 (100; M^+; HR: $C_{22}H_{30}O_3$; precision: 7 ppm); 327 (8; $M^+ - CH_3$); 324 (5; $M^+ - H_2O$); 309 (10; $M^+ - H_2O - CH_3$)

Identification of the 6,7-Dihydrocanrenone Metabolites

The incubation of (1,2,6,7-3H)-DHC with rat testis led to a metabolism of 17.4 % (Fig.1B). The chromatographic pattern was similar to that of CAN (Fig.1A) and only 10 incubations with 10 testes each were performed for the isolation of the metabolites. The yield was high enough to obtain nmr and ms spectra of the two main metabolites. The results given here are restricted to those substances which could be definitively identified. A control incubation with inactivated testes tissue showed that DHC was stable under the conditions used.

6,7-Dihydrocanrenone

The data of this substance are presented here because they are important for the discussion of its metabolites.

ms: m/e 342 (100; M^+); 327 (11; $M^+ - CH_3$); 324 (13; $M^+ - H_2O$); 309 (9; $M^+ - CH_3 - H_2O$); 300 (47; $M^+ - CH_2CO$);

269 (42; Fig.2A); 257 (19; Fig.2H); 244 (29, Fig.2F); 229 (30; Fig.2B); 124 (87; Fig.2I)

ir: (cm^{-1}) 1779 (22-C=O); 1670, 1625 (3-oxo-Δ^4)

uv: λ_{max} = 241 nm

6,7-Dihydrocanrenoic acid

This substance was identified in the same way as described for canrenoic aid.

16α-Hydroxy-6,7-dihydrocanrenone

This metabolite represented 14.4 % of the total DHC metabolites and had the same chromatographic behaviour as 16α-OH-CAN.

The molecular peak at m/e 358 together with the signal at 4.66 ppm in the nmr spectrum indicated a hydroxylated product. The fragments m/e 267 and 229 show, that the hydroxyl group was introduced at ring D and the doublet of doublets - typical for two vicinal protons - confirmed the C-16 position. The comparison of the additional chemical shift values of the angular methyl groups with those of Bridgeman et al. (16, table 2) led to 16α-OH-DHC.

ms: m/e 358 (100; M^+); 343 (12; $M^+ - CH_3$); 340 (27; $M^+ - H_2O$); 330 (16; $M^+ - CO$); 325 (12; $M^+ - CH_3 - H_2O$); 316 (32; $M^+ - CH_2CO$); 273 (23; Fig.2K); 267 (11; Fig.2D - H_2O); 229 (25; Fig.2B); 124 (85; Fig.2I)

ir: (cm^{-1}) 3450 (OH); 1772 (22-C=O); 1671, 1618

(3-oxo-Δ^4)

uv: λ_{max} = 244 nm

20R-Hydroxy-6,7-dihydrocanrenone

This metabolite represented 38 % of the total DHC-metabolites.

The hydroxylation (ms: m/e 358; 340; nmr: 4.42 ppm) must have taken place at the lactonic ring as shown by the fragments m/e 269, 229 and 124 representing unchanged rings A,B,C and D. The deshielding effect of the hydroxyl group to the vicinal proton signals caused a paramagnetic shift of the C-21 protons which can be identified by their coupling constants at 19 Hz. The steric assignment to 20R-OH-DHC was done in the same way as for 20R-OH-CAN.

Comparing the specific activity of this metabolite and that of 20R-OH-CAN, there was no loss of radioactivity in this case, due to the position of 3H at C-1, C-2, C-6 and C-7 of DHC.

ms: (m/e) 358 (84; M^+; HR:$C_{22}H_{30}O_4$; precision: 3 ppm); 343 (8; M^+- CH_3); 340 (66; M^+- H_2O); 330 (26; M^+- C=O); 325 (29; M^+- CH_3- H_2O); 316 (60; M^+- CH_2CO); 287 (94; Fig.2E; HR: $C_{19}H_{27}O_2$, precision: 4 ppm); 273 (25; Fig.2K); 269 (38; Fig.2A; HR: $C_{19}H_{25}O$; precision: 6 ppm); 244 (45; Fig.2F); 229 (68; Fig.2B); 124 (91; Fig.2I)

ir: (cm^{-1}) 3430 (OH); 1778 (22-C=O); 1662,1615 (3-oxo-Δ^4)

uv: λ_{max} = 244 nm

3α-6,7-Tetrahydrocanrenone

Although the yield of this metabolite was too low for nmr and ir spectra, this metabolite was analysed by means of the mass spectrum and by incubation with the enzyme cholesterol oxidase.

The molecular peak at m/e 344 and the loss of uv-activity indicated a metabolite with reduced A-ring. The high tendency to eliminate H_2O (m/e 326 and 215) indicated a hydroxyl group. A possible conversion of the 3-oxo-Δ^4-structure to a 3β-hydroxy-Δ^5-structure - as shown by Rosner et al. (22) with rabbit testis - could be excluded by means of high resolution mass spectrometry. Typical fragments with m/e 233 and 259 consisting of $C_{15}H_{21}O_2$ and $C_{17}H_{23}O_2$ would have indicated such a 3β-hydroxy-Δ^5-structure (14,15). Although the spectrum shows a peak at m/e 233, high resolution mass spectrometry assigned $C_{16}H_{25}O$ to this fragment, which must derive from the A, B and C ring. The configuration at C-3 was tested by the incubation of this metabolite with the enzyme cholesterol oxidase as described above. There was no conversion to DHC, thus proving the identity of 3α-OH-DHC.

ms: m/e 344 (49; M^+; HR: $C_{22}H_{32}O_3$; precision: 2 ppm); 329 (21; $M^+ - CH_3$); 326 (30; $M^+ - H_2O$); 311 (32; $M^+ - H_2O - CH_3$); 234 (79; Fig.2L + H; HR: $C_{16}H_{26}O$; precision: 6 ppm); 233 (50; Fig.2L; HR: $C_{16}H_{25}O$; precision: 3 ppm); 215 (84; Fig.2L - H_2O)

Incubation of Spironolactone

The incubation of (20,21-3H-)SPIR led to a small amount of several apolar substances. As a control incubation with inactivated testes tissue showed that the substrate was not stable under the conditions used, separation and identification of these substances was not performed.

DISCUSSION

Our data show that SPIR is not metabolised by rat testicuar tissue *in vitro* in a detectable scale.

In vivo SPIR is metabolised mainly to CAN with a short half-life of about 5 min (22). CAN is therefore regarded as

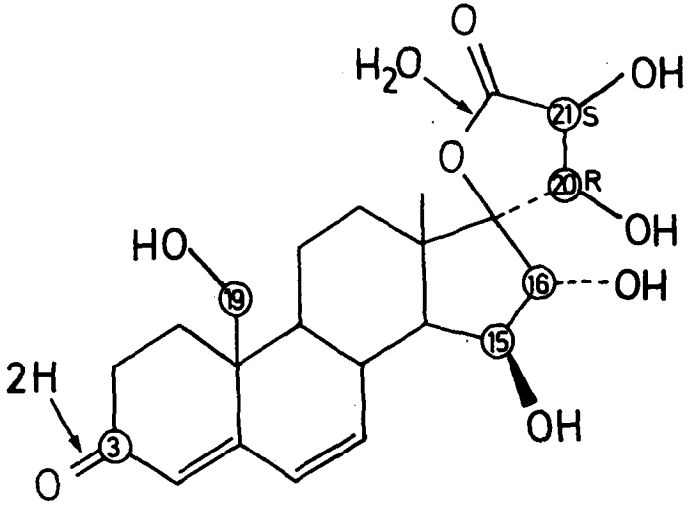

Fig. 3 Structure of canrenone (CAN) and the positions metabolised by the rat testis *in vitro*

the pharmacolgically active metabolite of SPIR (22,23). In contrast to SPIR, CAN contains no sulfur atom and was stable under our experimental conditions. CAN was metabolised by the rat testis to canrenoic acid and at least five hydroxylated and one reduced substance (Fig. 3).

15β-OH-CAN was detected in the urine of male rats by Karim et al.(13). 3α-OH-CAN and 16α-OH-CAN have not been described before. Metabolism at these positions was likely to occur, since the testis has 3α-hydroxysteroid dehydrogenase and 16α-hydroxylase activity (24). 20-OH-CAN was isolated by Finn et al. (25) and 21-OH-CAN by Karin et al. (13). However, the chemical configuration of these two metabolites was not described. In our experiments rat testicular tissue produced 20R- and 21S-OH-CAN. Up to now little is known about the biological activity of these metabolites of CAN.

19-OH-CAN was produced in our experiments in about 4 % of the total CAN-metabolites. Since hydroxylation in the C-19 position is the first step in the biosynthetic pathway of estrogens (24), it seems conceivable that spirolactones are converted to steroids with an aromatic A-ring. Braselton et al. (26) demonstrated that both 17β, 19-dihydroxy-17α-methyl-4-androsten-3-one and 4,6-androstadien-3,17-dione were converted to their corresponding 1,3,5(10)-estratrien derivatives by human placental microsomes. The configuration at the C-17 of CAN

is comparable to that of 17β,19-dihydroxy-17α-methyl-4-androsten-3-one, and the 3-oxo-$\Delta^{4,6}$ structure is part of both, CAN and 4,6-androstadien-3,17-dione. Thus a similar metabolism of CAN to a spirolactone with an aromatic A-ring could be expected. A CAN metabolite with an estrogen-like A-ring might be biologically active at low concentrations and contribute to the endocrine side effects of spirolactones.

The incubation of DHC with rat testis led to a pattern of metabolites similar to that of CAN (Fig. 1). The identification of 16α-OH-DHC, 20R-OH-DHC and 3α-OH-DHC showed the similarity of the metabolic pathways of CAN and DHC.

CAN was metabolised in about 14 % and DHC under the same conditions in about 20 %. The higher metabolic rate of DHC may be explained by the 3-oxo-Δ^4-structure of DHC which is identical with that of the endogenous steroids, while the 3-oxo-$\Delta^{4,6}$ structure of CAN could lead to some steric hindrance at the metabolising enzymes.

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Trivial names, abbriviations and systematic names of the spirolactones mentioned

Spironolactone, SPIR, 3-(7α-acetylthio-17β-hydroxy-3-oxo-4-androsten-17α-yl)propionic acid γ-lactone
Canrenone, CAN, 3-(17β-hydroxy-3-oxo-4,6-androstadien-17α-yl)propionic acid γ-lactone
6,7-Dihydrocanrenone, DHC, 3-(17β-hydroxy-3-oxo-4-androsten-17α-yl)propionic acid γ-lactone
Canrenoic acid, 3-(17β-hydroxy-3-oxo-4,6-androstadien-17α-yl)propionic acid
6,7-Dihydrocanrenoic acid, 3-(17β-hydroxy-3-oxo-4-androsten-17α-yl)propionic acid
3α-Dihydrocanrenone, 3α-OH-CAN, 3-(3α,17β-dihydroxy-4,6-androstadien-17α-yl)propionic acid γ-lactone
3α,6,7-Tetrahydrocanrenone, 3α-OH-DHC, 3-(3α,17β-dihydroxy-4-androsten-17α-yl)propionic acid γ-lactone
15β-Hydroxycanrenone, 15β-OH-CAN, 3-(15β,17β-dihydroxy-3-oxo-4,6-androstadien-17α-yl)propionic acid γ-lactone
16α-Hydroxycanrenone, 16α-OH-CAN, 3-(16α,17β-dihydroxy-3-oxo-4,6-androstadien-17α-yl)propionic acid γ-lactone
16α-Hydroxy-6,7-dihydrocanrenone, 16α-OH-DHC, 3-(16α,17β-dihydroxy-3-oxo-4-androsten-17α-yl)propionic acid γ-lactone
19-Hydroxycanrenone, 19-OH-CAN, 3-(17β,19-dihydroxy-3-oxo-4,6-androstadien-17α-yl)propionic acid γ-lactone
20R-Hydroxycanrenone, 20R-OH-CAN, (3R)-3-hydroxy-3-(17β-hydroxy-3-oxo-4,6-androstadien-17α-yl)propionic acid γ-lactone
20R-Hydroxy-6,7-dihydrocanrenone, (3R)-3-hydroxy-3-(17β-hydroxy-3-oxo-4-androsten-17α-yl)propionic acic γ-lactone
21S-Hydroxycanrenone, 21S-OH-CAN, 2(S)-2-hydroxy-3-(17β-hydroxy-3-oxo-4,6-androstadien-17α-yl)propionic acid γ-lactone

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