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# CONVERSION OF 4-ANDROSTENE-3,17-DIONE TO TESTOSTERONE BY *PISUM SATIVUM*

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**Key Word Index**—*Pisum sativum*; Leguminosae; steroid metabolism; 4-androstene-3,17-dione; testosterone; 5α-androstane-3β,17β-diol.

**Abstract**—4-Androstene-3,17-dione-[4-$^{14}$C] was applied to the leaves of growing pea plants, *Pisum sativum*. Within a week, 28% of the administered steroid was specifically reduced to testosterone. Part of the testosterone was present in esterified form, and 5α-androstane-3β,17β-diol was also identified as a metabolite, but neither epitestosterone nor estrogens were detected.

## INTRODUCTION

Various $C_{19}$ steroids have been isolated from higher plants: 5α-androstane-3β,16α,17α-triol from *Haplopappus heterophyllus* [1], rubrosterone from *Achyranthes rubrofusca* [2], and testosterone, epitestosterone and androstenedione from *Pinus sylvestris* [3]. Although some work on the metabolism of $C_{19}$ steroids in plant tissue cultures [4, 5] and potato tuber slices [6, 7] has been reported, there is no information about their fate in intact plants. Evidence from other work on steroid hormones in plants [8] make it appear reasonable that they are metabolized by reactions analogous to those known from animal experiments [9].

## RESULTS

4-Androstene-3,17-dione-[4-$^{14}$C] (10 μCi) was applied to the leaves of *Pisum sativum* at different stages of growth. After 7 days, the whole plants were homogenized and boiled in acid. The neutral fraction contained 65% and the acidic fraction contained 3% of the total radioactivity. The TLC radiochromatogram of the neutral fraction on a Si Gel G plate in $CH_2Cl_2$–MeOH (49:1) showed 5 major radioactive peaks corresponding to 5α-androstane-3β,17β-diol ($R_f$ 0.24, 3% of the radioactivity of the neutral fraction), testosterone (17β-hydroxy-4-androsten-3-one) ($R_f$ 0.35, 36%), 4-androstene-3,17-dione ($R_f$ 0.66, 45%), testosterone ester ($R_f$ 0.89, 7%) and 5α-androstane-3β,17β-diol diester ($R_f$ 0.99, 4%). There was no significant difference between the radiochromatograms from plants treated during the vegetative, flowering, pod development, and seed development stages.

Positive identification of testosterone-[4-$^{14}$C] was made by TLC and by crystallization of testosterone and testosterone acetate to constant specific radioactivity. No radioactivity was detectable in epitestosterone after carrier dilution and triple development with $CH_2Cl_2$–EtOAc (9:1) to separate it from testosterone. Thus, the 17-keto group of 4-androstene-3,17-dione had been rapidly and specifically reduced to a 17β-hydroxyl group by the pea plants.

5α-Androstane-3β,17β-diol was also identified by carrier dilution, chromatography and crystallization of the compound and its acetate to constant specific radioactivity. Analogous methods failed to detect radioactivity in 17β-hydroxy-5α-androstan-3-one, 3β-hydroxy-5α-androstan-17-one, 5α-androstane-3,17-dione and 5β-androstane-3,17-dione. Other minor metabolites appearing in the radiochromatogram were more polar than 5α-androstane-3β,17β-diol and were not identified.

Alkaline hydrolysis of the radioactive zones $R_f$ 0.89 and $R_f$ 0.99 and TLC of the hydrolysates and their acetates showed that they were due to esters of testosterone and 5α-androstane-3β,17β-diol diesters, respectively. No radioactive epitestosterone was detected in the alkaline hydrolysate.

Carrier dilution and co-crystallization of estrone, estradiol, 16-epiestriol and estriol with appropriate zones from TLC of the acidic fraction failed to yield radioactive estrogens from pea plants during the vegetative, flowering, pod development and seed development stages.

In a control experiment with a boiled pea plant, radioactive testosterone, 5α-androstane-3β,17β-diol and their esters were not detected.

## DISCUSSION

In theory, 4-androstene-3,17-dione could be converted to at least 20 known $C_{19}$ steroids by permutations of α- and β-reductions at C-3, C-5 and C-17. The specific reduction to testosterone (17β-hydroxy-4-androsten-3-one) is all the more remarkable because testosterone has the highest androgenic and anabolic activity of all

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possible reduction products. No effort has been made so far to determine whether untreated pea plants contain testosterone, but they obviously have the ability to synthesize this hormone from a more common steroid metabolite, androstenedione. The known occurrence of $C_{19}$ steroids in plants belonging to different families makes it at least possible that Leguminosae may also contain androgens [8].

The degradation of sterols to androstenedione has been observed in several microorganisms, e.g. *Nocardia restrictus* [10], *Mycobacterium phlei* [11], *Arthrobacter simplex* [12], *Proactinomyces asteroides* [13] and *Fusarium solani* [14]. In higher plants, the degradation of sterols has so far been followed only as far as the $C_{21}$ steroids [15], but some information is available about the metabolism of $C_{21}$ and $C_{19}$ steroids [8].

Stohs and El-Olemy [4] have observed that 4-androstene-3,17-dione is converted to 3β-hydroxy-5α-androstan-17-one and 5α-androstane-3β,17β-diol by cell suspension cultures of *Dioscorea deltoidea* and have suggested that the $\Delta^4$-reductase initially acts on the substrate to form 3β-hydroxy-5α-androstan-17-one, which is subsequently metabolized by a 17β-hydroxysteroid dehydrogenase to produce 5α-androstane-3β,17β-diol. In our experiments with intact pea plants we were unable to detect incorporation into 3β-hydroxy-5α-androstan-17-one, 17β-hydroxy-5α-androstan-3-one, 5α-androstane-3,17-dione and 5β-androstane-3,17-dione at a level of more than 0.1%, but testosterone was identified as the major metabolite and 5α-androstane-3β,17β-diol as a minor metabolite. In experiments with tobacco cell suspension cultures, Hirotani and Furuya [5] have also observed that 4-androstene-3,17-dione was converted to testosterone and 5α-androstane-3β,17β-diol. The incubation of dehydroepiandrosterone with potato tuber slices resulted in the hydroxylation of the substrate [6, 7].

## EXPERIMENTAL

*Methods.* Commercially prepared TLC plates (250 μm) of Si Gel G and Si Gel H were used without activation. Radioactivity on 5 × 20 cm plates was detected with a radiochromatogram scanner and isolated zones were analysed with a liquid scintillation counter, having an efficiency of 90% in 10 ml of a soln of 6 g PPO and 150 mg POPOP per l. of toluene.

*Administration of 4-androstene-3,17-dione-[4-$^{14}$C].* The dwarf pea plants, *Pisum sativum* var. Progress No. 9, were grown in a greenhouse. 4-Androstene-3,17-dione-[4-$^{14}$C] (10 μCi, 57.5 mCi/mmol, New England Nuclear) was dissolved in 25 μl of 95% EtOH containing 0.1% DL-α-tocopherol and 0.1% of silicone oil DC-200 [16]. The soln was applied to the upper leaf surface of each plant at different growth stages: vegetative (*ca* 2 weeks old), flowering (*ca* 4 weeks old), pod development and seed development. In a control experiment, we used a pea plant which had been boiled in $H_2O$ for 10 min.

*Extraction of radioactive metabolites.* Seven days after the administration, the entire plant, in 100 ml $H_2O$, was homogenized in a blender. The homogenate was refluxed with 25 ml $C_6H_6$ and 25 ml conc HCl for 3 hr. One mg of each of the non-radioactive carriers, 4-androstene-3,17-dione, testosterone, estrone, estradiol, 16-epiestriol and estriol was added. The mixture was extracted with 3 × 100 ml $Et_2O$ (neutral fraction). The $Et_2O$ extract was then extracted with 4 × 50 ml of N aq. NaOH. After the aq. extract had been acidified with conc HCl, it was extracted with 3 × 100 ml $Et_2O$ (acidic fraction). About 65% of the administered radioactivity was in the neutral fraction and *ca* 3% of the radioactivity was in the acidic fraction.

*TLC of the neutral fraction.* An aliquot (0.5%) of the neutral fraction was chromatographed on a Si Gel G plate (5 × 20 cm) in $CH_2Cl_2$–MeOH (49:1). When the plate was scanned, the radiochromatogram showed 5 major radioactive zones at $R_f$ 0.24 (3% of the radioactivity of the neutral fraction), $R_f$ 0.35 (36%), $R_f$ 0.66 (45%), $R_f$ 0.89 (7%) and $R_f$ 0.99 (4%). Each zone was scraped off and assayed in a liquid scintillation counter. The radioactive peaks at $R_f$ 0.24, 0.35 and 0.66 corresponded to 5α-androstane-3β,17β-diol, testosterone and 4-androstene-3,17-dione, respectively, when the reference compounds were added to the neutral fraction before TLC. Depending on subsequent steps, exposure to $I_2$ vapors or spraying with 50% $H_2SO_4$ followed by charring were used for detection.

*Identification of testosterone-[4-$^{14}$C].* Testosterone (1 mg) and 1% of the neutral fraction were co-chromatographed on a Si Gel H plate (20 × 20 cm) in $CH_2Cl_2$–MeOH (97:3). The testosterone band was eluted with $Me_2CO$. Testosterone (10 mg) was added to the eluate and sequentially recrystallized from $CH_2Cl_2$–hexane, $Et_2O$–hexane and EtOAc–hexane until constant sp. act. (*ca* 4100 cpm/mg) was obtained. After acetylation of the product, the testosterone acetate was recrystallized from MeOH–$H_2O$, $Me_2CO$–$H_2O$, and MeCN–$H_2O$ until the acetate also gave constant sp. act.

*Absence of epitestosterone.* Testosterone and epitestosterone are not well separated by TLC in $CH_2Cl_2$–MeOH (49:1), but complete separation is achieved by triple development with $CH_2Cl_2$–EtOAc (9:1). When the testosterone zone from 1% of the neutral fraction was eluted with $Me_2CO$ and co-chromatographed with testosterone and epitestosterone, there was no detectable radioactivity in the epitestosterone.

*Identification of other radioactive steroids.* Co-crystallizations performed with 5α-androstane-3β,17β-diol, 17β-hydroxy-5α-androstan-3-one and 3β-hydroxy-5α-androstan-17-one were similar to that with testosterone, except that 4% of the neutral fraction was used in each case. The solvents used for 5α-androstane-3β,17β-diol were $Me_2CO$–hexane, EtOH–hexane and THF–hexane. The solvents used for 17β-hydroxy-5α-androstan-3-one and 3β-hydroxy-5α-androstan-17-one were $CH_2Cl_2$–hexane, EtOAc–hexane and $CHCl_3$–hexane. For the diacetate the same solvents were used as in recrystallization of testosterone acetate. The crystals of 5α-androstane-3β,17β-diol, being insoluble in the scintillation fluid, were first dissolved in 200 μl MeOH and then 10 ml of the scintillation fluid was added. Constant sp. act. of 5α-androstane-3β,17β-diol (*ca* 400 cpm/mg) was obtained. There was no radioactivity associated with either 17β-hydroxy-5α-androstan-3-one or 3β-hydroxy-5α-androstan-17-one. No radioactivity was detected in 5α-androstane-3,17-dione and 5β-androstane-3,17-dione by co-chromatography with $CH_2Cl_2$–MeOH (99:1). Since the $R_f$ values of other androstanediols and androstenediols were close to that of 5α-androstane-3β,17β-diol in $CH_2Cl_2$–MeOH (97:3) and since the radioactive peak for 5α-androstane-3β,17β-diol was not sharp, the presence of additional diols cannot be excluded.

*Identification of testosterone-[4-$^{14}$C] ester.* A 4% aliquot of the neutral fraction was chromatographed on a Si Gel H plate (20 × 20 cm) in $CH_2Cl_2$–MeOH (49:1) and the radioactive peak at $R_f$ 0.89 was eluted with $Me_2CO$. The residue was hydrolyzed in 1 ml 5% methanolic KOH in a Reacti-Vial (Pierce) at room temp. for 24 hr. After partition between $Et_2O$ and $H_2O$, the residue from the $Et_2O$ phase was co-chromatographed with testosterone by triple development with $CH_2Cl_2$–EtOAc (9:1). The radiochromatogram showed a radioactive peak corresponding to testosterone, whereas epitestosterone was not

detected. After acetylation, the radiochromatogram with $CH_2Cl_2$–MeOH (99:1) showed a radioactive peak corresponding to testosterone acetate.

*Identification of 5α-androstane-3β,17β-diol-[4-$^{14}$C] diester.* The radioactive zone at $R_f$ 0.99 was isolated by the same procedure as that for the testosterone ester. The residue was hydrolysed at 65° for 3 hr. The $Et_2O$-soluble residue was co-chromatographed with 5α-androstane-3β,17β-diol in $CH_2Cl_2$–MeOH (24:1). The peak in the radiochromatogram corresponded to 5α-androstane-3β,17β-diol. TLC of the acetate in $CH_2Cl_2$–MeOH (99:1) showed a radioactive zone corresponding to 5α-androstane-3β,17β-diol diacetate.

*Absence of radioactive estrogens.* The acidic fraction containing 1 mg of each reference compound (estrone, estradiol, 16-epiestriol and estriol) was chromatographed on a Si Gel H plate in $CH_2Cl_2$–MeOH–conc $NH_4OH$ (90:10:1). The estrone, estradiol, 16-epiestriol and estriol bands were eluted with $Me_2CO$. Each fraction was co-crystallized after the addition of 10 mg of reference material. The solvents used for sequential recrystallization of estrone were $CHCl_3$–hexane, EtOAc–hexane and $CH_2Cl_2$–hexane, for estradiol they were $Me_2CO$–hexane, $Et_2O$–hexane and EtOAc–hexane, for 16-epiestriol they were THF–hexane, EtOH–hexane and $Me_2CO$–hexane and for estriol they were THF–hexane, $Me_2CO$–hexane and EtOH–hexane. The acetates of these 4 estrogens were recrystallized from MeOH–$H_2O$, $Me_2CO$–$H_2O$, and MeCN–$H_2O$. The crystals of estriol or 16-epiestriol were first dissolved in 200 μl MeOH and then 10 ml Aquasol-2 (New England Nuclear) was added for counting. The crystals of estradiol were first dissolved in 200 μl MeOH and then 10 ml scintillation fluid was added for counting. Estrone may be dissolved in the scintillation fluid by warming it in a water bath. No radioactivity was associated with those estrogens.

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