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REDUCTION OF 4-ANDROSTENE-3,17-DIONE BY GROWING CUCUMBER PLANTS

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Key Word Index—*Cucumis sativus*; cucumber, steroid metabolism; androgens; 4-androstene-3,17-dione, testosterone, 3β-hydroxy-5α-andostan-17-one; 17β-hydroxy-5α-androstan-3-one, 5α-andostane-3β,17β-diol.

Abstract—4-[4-^{14}C]Androstene-3,17-dione was applied to the leaves of growing cucumber plants, *Cucumis sativus*, twice a week. Four weeks after the first application, 16% of the administered steroid was specifically reduced to testosterone. The following radioactive metabolites were also identified: 5α-androstane-3,17-dione; 5β-androstane-3,17-dione; 3α-hydroxy-5α-androstan-17-one; 3β-hydroxy-5α-androstan-17-one; 17β-hydroxy-5α-androstan-3-one; 17β-hydroxy-5β-androstan-3-one; 5α-androstane-3α,17β-diol; 5α-androstane-3β,17β-diol; and 5β-androstane-3β,17β-diol. No radioactive 17α-hydroxyandrostane derivatives were detected. The radioactivities of the 5α-androstane derivatives were higher than those of the 5β-androstane analogs and the radioactivities of the 3β-hydroxyandrostane derivatives were higher than those of the 3α-hydroxyandrostane analogs. Neither radioactive $C_{19}O$ and $C_{19}O_3$ androgens nor estrogens were detected. There was no significant difference in the metabolism of 4-[4-^{14}C]androstene-3,17-dione between growing monoecious and gynoecious cucumber plants.

INTRODUCTION

We have previously reported the metabolism of 4-[4-^{14}C]androstene-3,17-dione in pea plants (*Pisum sativum*) [1], the first androgen metabolism study in intact plants. Within a week, 28% of the administered 4-[4-^{14}C]androstene-3,17-dione was specifically reduced to radioactive testosterone. Another radioactive metabolite identified was 5α-androstane-3β,17β-diol. TLC and co-crystallization were used to identify the radioactive metabolites. Later, we used both normal-phase and reversed-phase HPLC [2] to study the metabolism of [4-^{14}C]progesterone in pea plants [3] and thus identified 11 radioactive reduction products. Realizing the power of HPLC we decided to apply it to a study of the metabolism of 4-[4-^{14}C]androstene-3,17-dione in higher plants. For this purpose we have developed both normal-phase and reversed-phase HPLC methods of separating 69 androgens [4]. The application of HPLC to the identification of possible oxidation and reduction products of androstenedione is now reported.

Some androgens have been identified in higher plants [5, 6]. A boar pheromone, 5α-androst-16-en-3-one, was recently identified in parsnip (*Pastinaca sativa*), celery (*Apium graveolens*) [7] and truffles (*Tuber melanosporum*) [8] by GC/MS. Testosterone, epitestosterone and androstenedione were identified in pollen of pine (*Pinus nigra*) by radioimmunoassay and fluorimetric methods [9]. Rubrosterone in *Achyranthes rubrofusca* [10] and 5α-androstane-3β,16α,17α-triol in Rayless goldenrod (*Haplopappus heterophyllus*) [11] were also identified. The biosynthetic pathway of androgens in higher plants has never been elucidated, but it may be assumed to be the same as that observed in animals and micro-organisms. Stohs and El-Olemy [12] have shown that androstenedione was converted into 3β-hydroxy-5α-androstan-17-one and 5α-androstane-3β,17β-diol by cell suspension cultures of *Dioscorea deltoidea*. Hirotani and Furuya [13] have reported that tissue cultures of *Nicotiana tabacum* are capable of transforming testosterone to 4-androstene-3,17-dione, 17β-hydroxy-5α-androstan-3-one, 5α-androstane-3β,17β-diol and 3β-hydroxy-5α-androstan-17-one. The tobacco cells are also capable of transforming 4-androstene-3,17-dione to testosterone and 17β-hydroxy-5α-androstan-3-one.

The presence of estrogens in higher plants has also been reported [5, 6]. Young *et al.* have shown that estradiol was biosynthesized by *Phaseolus vulgaris* from applied labeled mevalonic acid and estrone [14] and that estrone and estradiol are interconvertible in plant tissues [15]. The biosynthetic pathway of estrogens in higher plants may also be similar to that in animals and micro-organisms, but conversion of progesterone and 4-androstene-3,17-dione to estrogens has never been demonstrated in higher plants. Using previously developed methods of normal-phase and reversed-phase HPLC [16], a search was made for radioactive estrogens resulting from the metabolism of 4-[4-^{14}C]androstene-3,17-dione in both monoecious and gynoecious cucumber plants. Kopcewicz [17] has reported earlier that the topical application of estrogens to *Ecballium elaterium* (Cucurbitaceae) increased the number of female flowers, while androgens increased the number of male flowers. Alterations in the sex expression of *Cucumis sativus* have also been obtained by Gawienowski *et al.* [18]. We were, therefore, interested to determine whether there is a difference in the metabolism of 4-androstene-3,17-dione between monoecious and gynoecious cucumber plants.

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Table 1 HPLC retention times (min) of $C_{19}O_2$ androgens

	Substituents				Systems						
No	3	4	5	17	1	2	3	4	5	6	7
1	O	—	β	O	7 5	15.5	—	—	—	—	—
2	O	—	Δ	O	8	17.5	—	—	—	—	—
3	O	—	α	O	8	17.75	—	—	—	—	—
4	O	—	β	α	8 5	23	—	17	7 5	—	11 5
5	β	—	β	O	10	14 25	—	12 5	—	—	12 5
6	O	—	α	α	10 25	18	—	31	9 5	—	12 5
7	α	—	α	O	12 5	16 25	—	14 5	—	—	14 25
8	β	—	Δ	O	12 5	11.25	—	11	—	—	12 75
9	O	—	α	β	12.5	16	—	15	—	—	14 75
10	O	—	β	β	12 5	15 75	—	16 25	—	—	13 25
11	β	—	α	O	13	14 75	—	11 5	—	5	15
12	α	—	β	O	13	16 25	—	10 5	—	—	12
13	β	—	β	β	16 5	11 75	15	—	—	9	—
14	α	—	α	β	18	16 75	12 5	—	—	9	—
15	β	—	β	α	19 25	16	15	—	—	7 75	—
16	O	Δ	—	O	20	11 5	—	—	—	—	—
17	β	—	Δ	β	20	10 25	19 5	5	—	9 25	—
18	β	—	α	β	20	13 5	22	—	—	10 5	—
19	β	Δ	—	β	20 25	9 75	20 75	—	—	8	—
20	α	—	α	α	20 25	33	14 75	—	—	8 25	—
21	β	—	Δ	α	20 5	11 25	23 5	5 5	—	7	—
22	β	—	α	α	20 5	14.5	20 75	—	—	7	—
23	O	Δ	—	α	21 5	12	—	—	18 5	—	9 75
24	α	—	β	α	24	32	21 25	—	—	7	—
25	α	—	β	β	24	15 5	13	—	—	9	—
26	O	Δ	—	β	25 75	10 75	—	—	16 75	—	9 75

Hydroxyl groups are indicated by α and β, depending on orientation, at C-3 and C-17 However, at C-5, α and β are used to designate the orientation of hydrogen Keto groups are indicated by O, and double bonds by Δ. Free androgens were chromatographed in systems 1 and 2, acetates in all other systems. System 1 (see Fig. 1) normal-phase column, hexane–ethanol (97 3), system 2 (see Fig. 2) reversed-phase column, methanol–water (7 3), system 3, normal-phase column, hexane–ethanol (998 2), system, 4 normal-phase column, hexane–ethanol (995 5); system 5, normal-phase column, hexane–ethanol (99 1), system 6, reversed-phase column, methanol–water (9 1), system 7, reversed-phase column, methanol–water (8 2)

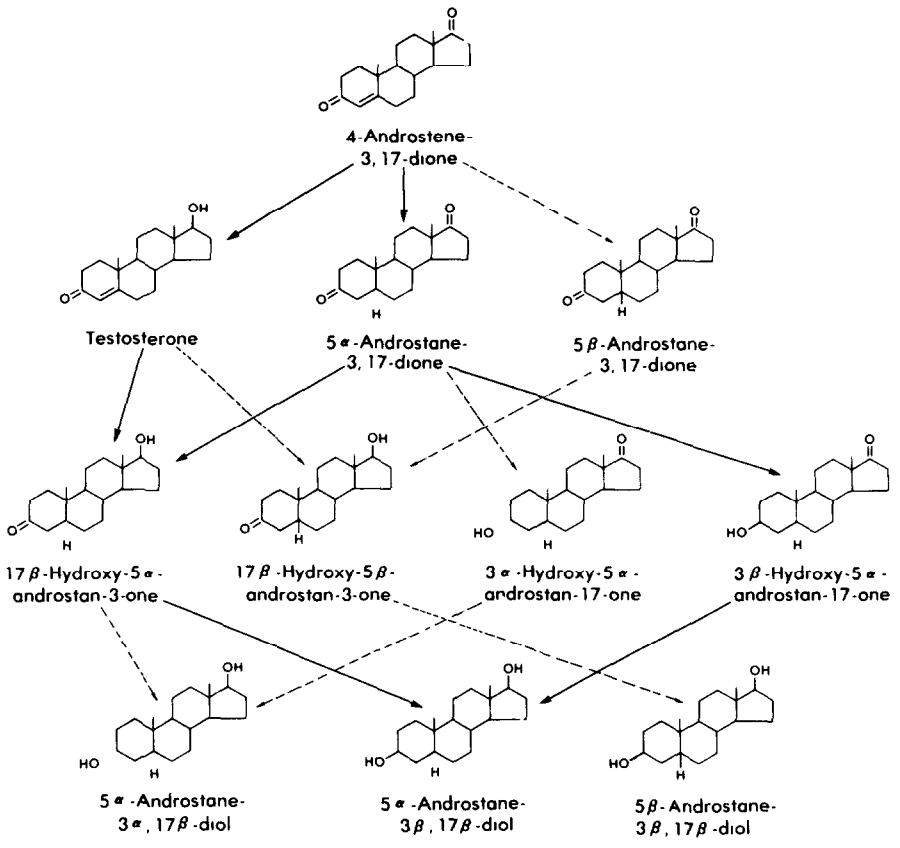
Table 2 Metabolites of 4-[4-^{14}C]androstene-3,17-dione in growing *C. sativus* and percent of administered radioactivity

Androgen	%
4-Androstene-3,17-dione	7 7
5α-Androstane-3,17-dione	0 06
5β-Androstane-3,17-dione	0 04
Testosterone	16 0
3α-Hydroxy-5α-androstan-17-one	0 06
3β-Hydroxy-5α-androstan-17-one	0 56
17β-Hydroxy-5α-androstan-3-one	0 28
17β-Hydroxy-5β-androstan-3-one	0 10
5α-Androstane-3α,17β-diol	0 16
5α-Androstane-3β,17β-diol	1 0
5β-Androstane-3β,17β-diol	0 06

RESULTS

4-[4-^{14}C]Androstene-3,17-dione was applied to the leaves of both monoecious and gynoecious cucumber plants. The plants were homogenized and boiled in acid The total lipid fractions were analysed by both normal-phase and reversed-phase HPLC before and after acetylation, as shown in Table 1 The radioactive metabolites of 4-[4-^{14}C]androstene-3,17-dione are shown in Table 2 The other androgens listed in Table 1 were not detected in radioactive form. If they were radioactive, they constituted less than 0 01 % of the administered radioactivity. No significant difference in the metabolism of 4-androstene-3,17-dione was observed between monoecious and gynoecious cucumber plants

The hypothetical pathway of 4-androstene-3,17-dione metabolism in growing cucumber plants is shown as Scheme 1 The heavy arrows show the major metabolic pathways The major metabolites were testosterone, 5α-androstane-3β,17β-diol, 3β-hydroxy-5α-androstan-17-one and 17β-hydroxy-5α-androstan-3-one. No 17α-hydroxy androgens were detected. Evidently the 17-keto group was specifically reduced to a 17β-hydroxyl group The reduction of the 3-keto group appears to be less specific than that of the 17-keto group. Reduction to a 3β-hydroxyl group was preferred over the 3α-hydroxyl group The reduction of the double bond at C-4 also showed some stereospecificity. Reduction to 5α-androstane derivatives was preferred over the 5β-androstane derivatives



Scheme 1 Hypothetical pathways of 4-androstene-3,17-dione metabolism in growing cucumber plants Heavy arrows show the major pathways.

All metabolites of 4-androstene-3,17-dione detected in growing cucumber plants belong to the $C_{19}O_2$ series. No metabolites were found in the $C_{19}O$ and $C_{19}O_3$ series, i.e. they were not produced above the 0.01% level of incorporation No conversion to known estrogens above the 0.002% level of incorporation was observed.

DISCUSSION

HPLC is a highly effective method for the purification and identification of radioactive metabolites. We have previously used normal-phase and reversed-phase HPLC as well as co-crystallization to constant specific radioactivity to identify the reduction products of progesterone [3]. That work required 10 mg of each reference compound for co-crystallization. Because of limited quantities of some androgens, normal-phase and reversed-phase HPLC techniques of androgen acetates were substituted for co-crystallization. HPLC of androgen acetates has not been reported previously. Table 1 is an almost complete list of all possible reduction products of 4-androstene-3,17-dione. The elution order of androgens in any one of the four HPLC systems in Table 1 is not exactly the same or the reverse of the other HPLC systems [4] Because these four HPLC systems complement each other, the identification of almost all compounds in Table 1 has now become possible. However, 5α-androstane-3,17-dione (No. 3 in Table 1) and 5-androstene-3,17-dione (No. 2) could not be separated by the two HPLC systems we used Since no metabolic double bond shift from C-4 to C-5 has been reported and no other Δ^5-metabolites were detected in either this study or our previous progesterone metabolism study [3], we assume that the radioactivity detected is associated with 5α-androstane-3,17-dione

As shown in Table 2, we have identified 10 radioactive metabolites of 4-[4-^{14}C]androstene-3,17-dione in growing cucumber plants. Only four of them, testosterone, 3β-hydroxy-5α-androstan-17-one, 17β-hydroxy-5α-androstan-3-one and 5α-androstane-3β,17β-diol, have previously been identified in tobacco [13] and *Dioscorea* [12] tissue cultures, and in pea plants [1]. In the present study, six additional metabolites, 5α-androstane-3-17-dione, 5β-androstane-3,17-dione, 3α-hydroxy-5α-androstan-17-one, 17β-hydroxy-5β-androstan-3-one, 5α-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol were identified in cucumber plants The 10 known metabolites of androstenedione fit into the metabolic pathways proposed in Scheme 1.

As previously observed in *Pisum sativum* [1], 4-[4-^{14}C]androstene-3,17-dione is specfically reduced to the most radioactive metabolite, testosterone. Radioactive epitestosterone (17α-hydroxy-4-androsten-3-one) was not detected in growing cucumber plants. In fact, none of the eight 17α-hydroxyandrostane derivatives in Table 1 were detected as metabolites of 4-[4-^{14}C]androstene-3,17-dione.

In the reduction of the double bond at C-4 (cf. Table 2) by *Cucumis sativus*, the formation of 5α-androstane

derivatives predominates over that of 5β-androstane derivatives This bias was also noted in the reduction of progesterone by *Pisum sativum* [3]. In the reduction of the 3-keto group of androstenedione by *Cucumis sativus* (cf. Table 2) the formation of 3β-hydroxyandrostane derivatives predominates over that of 3α-hydroxyandrostane derivatives, whereas the reduction of progesterone by *P sativum* [3] resulted in the predominant formation of 3α-hydroxypregnane derivatives The most common androstanediol in cucumber (cf Table 2) as well as pea plants [1] is 5α-androstane-3β,17β-diol and the most common pregnanediol in pea plants [3] is 5α-pregnane-3α,20β-diol, while in man, they are 5α-androstane-3α,17β-diol and 5β-pregnane-3α,20α-diol, respectively The most common reduction product of 4-androstene-3,17-dione in cucumber (cf Table 2), as well as pea plants [1], is testosterone, and the most common reduction product of progesterone in pea plants [3] is the highly reduced 5α-pregnane-3α.20β-diol

Part of the hydroxy steroids in plants are probably in the form of fatty acid esters and glycosides We have reported [1] that part of the metabolites of 4-androstene-3,17-dione, testosterone and 5α-androstane-3β,17β-diol, in pea plants were in the form of their fatty acid ester and diester, respectively. Hirotani and Furuya [13] have found a part of the metabolites of testosterone in *Nicotiana tabacum* tissue cultures in the form of palmitates and glucosides *Ca* 5 6 % of the administered radioactivity was in fractions 2–7 (Fig 1) This could be due to fatty acid esters not hydrolysed by hydrochloric acid

We have not detected any conversion of 4-[4-^{14}C]androstene-3,17-dione to estrogens by growing cucumber or pea plants However, Young *et al* [14] reported the conversion of mevalonic acid and of estrone to estradiol by *Phaseolus vulgaris* The conversion of estrone to estradiol involves the same reaction as the conversion of 3β-hydroxy-5α-androstan-17-one to 5α-androstane-3β,17β-diol Perhaps the reductases for both conversions are the same

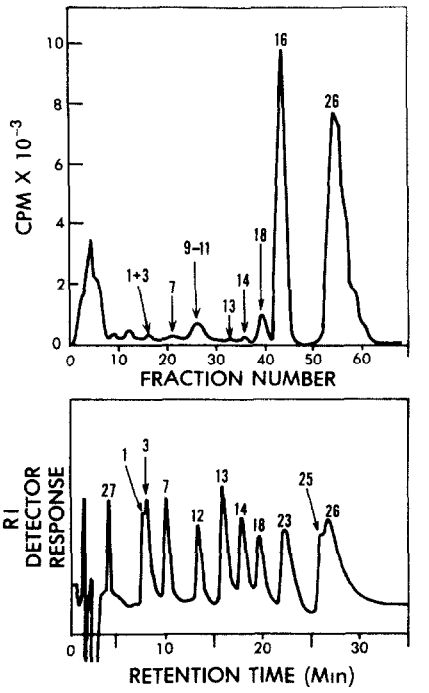


Fig 1 Normal-phase radiochromatogram of the neutral lipid fraction from two cucumber plants after the administration of 4-[4-^{14}C]androstene-3,17-dione (25 μCi) Sample, 5 % of the neutral lipid fraction combined with between 50 μg (5α-androst-16-en-3β-ol) and 300 μg (testosterone) of each carrier in 250 μl of the eluent Column, Zorbax BP-SIL, 25 × 0 46 cm, eluent, hexane–ethanol (97 3), flow-rate 2 ml/min, fraction volume, 1 ml, pressure, 200 psi; RI detector, sensitivity 16 ×, recorder, speed 12 cm/hr, span 10 mV For the identity of peaks, see Table 1, except 27 = 5α-androst-16-en-3β-ol

EXPERIMENTAL

Administration of 4-[4-^{14}C]*androstene*-3,17-*dione* 4-[4-^{14}C]Androstene-3,17-dione (50 μCi, 57 4 mCi/mmol, New England Nuclear, Boston, MA) was dissolved in 2 8 ml 95 % EtOH, containing 0 1 % DL-α-tocopherol and 0 1 % silicone oil, DC-200 [19] Monoecious (Samson) and gynoecious (Delila) cultivars of *Cucumis sativus* were raised in a greenhouse Two plants of each cultivar were used 38 days after planting, 100 μl of the soln was applied to the upper leaf surface of each plant The administration was repeated seven times, twice a week Thus, each plant received 12 5 μCi 4-[4-^{14}C]androstene-3,17-dione, corresponding to 2 4 × 10^7 cpm The plants were harvested when they were 65 days old One monoecious plant had 15 male flowers, 2 female flowers and 9 leaves when it was harvested, and the other monoecious plant had 15 male flowers, 2 female flowers, and 8 leaves The two monoecious plants together weighed 24 75 g One gynoecious plant had 6 female flowers, no male flowers, 11 leaves and 1 fruit, and the other gynoecious plant had 7 female flowers, no male flowers, 8 leaves and 1 fruit The two gynoecious plants together weighed 91 50 g The two fruits together weighed 51 g In a control expt we applied 4-[4-^{14}C]androstene-3,17-dione to a cucumber plant which had been boiled in H_2O for 10 min

Extraction of radioactive metabolites The two whole plants belonging to the same cv were homogenized in a blender with H_2O (500 ml) No carrier androgens or estrogens were added to the homogenate The homogenate was refluxed with 100 ml toluene and 100 ml conc HCl for 3 hr The mixture was extracted with 3 × 300 ml Et_2O, the vol of the total lipid extract was reduced to *ca* 300 ml, and then the acidic lipids were extracted with 3 × 100 ml 1 N aq NaOH The organic phase (neutral lipid fraction) was further fractionated (see below) After the aq extract had been acidified with conc HCl, it was extracted with 3 × 150 ml Et_2O (acidic lipid fraction) In both monoecious and gynoecious cucumber plants, *ca* 55 % of the administered radioactivity was recovered in the neutral lipid fraction and *ca* 1 6 % of the administered radioactivity was in the acidic lipid fraction

HPLC of androgens The HPLC apparatus was assembled from commercially available components Samples were dissolved in hexane when the normal-phase column was used, and in MeOH when the reversed-phase column was used *Ca* 100 μl of sample was injected An event marker recorded each change of the fraction collector on a recorder together with the UV detector response or RI detector response

The HPLC columns were packed in our laboratory with a slurry packer For normal-phase HPLC, Zorbax BP-SIL (7–8 μm, DuPont, Wilmington, DE) was packed into a column (25 × 0 46 cm) in a balanced-density slurry (tetrabromoethane–tetrachloroethane, 35 65) For reversed-phase HPLC, Zorbax BP-ODS (7–8 μm, DuPont) was packed into a column (25 × 0 46 cm) in a balanced density-slurry ($CHBr_3$ $CHCl_3$, 15 85)

The RI detector was used for free androgens, but the UV detector at 200 nm was used for androgen acetates The λ_{max} of 5α-androstane-3β,17β-diol diacetate being 200 nm, *ca* 2 μg of the Δ^4- or Δ^5-androgen acetates and *ca* 200 μg of the other androgen acetates were analysed by HPLC The R_ts (min, flow-rate 2 ml/min) of free androgens and androgen acetates, eluted with various solvent systems, are shown in Table 1

An aliquot of each fraction was counted in a liquid scintillation counter Conventional liquid scintillation fluid (5 ml, soln of 6 g PPO and 150 mg POPOP per l of toluene) with a counting efficiency of 86% was used for normal-phase HPLC Aquasol-2 (5 ml, New England Nuclear) with a counting efficiency of 88% was used for reversed-phase HPLC Filmware tubes (Nalge Co, Rochester, NY) were inserted in the liquid scintillation vials

Analysis of $C_{19}O$ *and* $C_{19}O_2$ *androgens* A 5% aliquot of the neutral lipid fraction was combined with nonradioactive androgens ($C_{19}O$ and $C_{19}O_2$) and the mixture was chromatographed as shown in Fig 1 Fractions were pooled according to individual radioactive peaks and R_ts of androgens (cf Table 1), as follows fractions 5–9, 13–18, 19–24, 25–29, 30–34, 35–37, 38–42, 43–49 and 50–65 The peaks in the RI chromatogram correspond to the added androgens and are numbered as in Table 1

Fractions 5–9 (cf Fig 1) were combined with four nonradioactive $C_{19}O$ androgens (5α-androst-16-en-3-one, 5α-androst-16-en-3α-ol, 5-androsten-3β-ol and 5α-androst-16-en-3β-ol) and the mixture was rechromatographed on a reversed-phase column with MeOH–H_2O (17 3) as the eluent [4] No radioactivity was associated with any of these $C_{19}O$ androgens Fractions 13–18 were combined with the nonradioactive androgens Nos 1–4, shown in Table 1, and the mixture was rechromatographed in system 2 (Fig 2) Both 5α-androstane-3,17-dione (No 3) and 5β-androstane-3,17-dione (No 1) were associated with radioactivity 17α-Hydroxy-5β-androstan-3-one (No 4) was not radioactive. More carrier was added to the two fractions and they were chromatographed separately on the normal-phase column with hexane–EtOH (99 1) as the eluent In this HPLC system, not shown in Table 1, the R_t of 5α-androstane-3,17-dione is 17 min, while that of 5β-androstane-3,17-dione is 16 min 5α-Androstane-3,17-dione (No 3) and 5-androstene-3,17-dione (No 2) cannot be separated by these HPLC systems

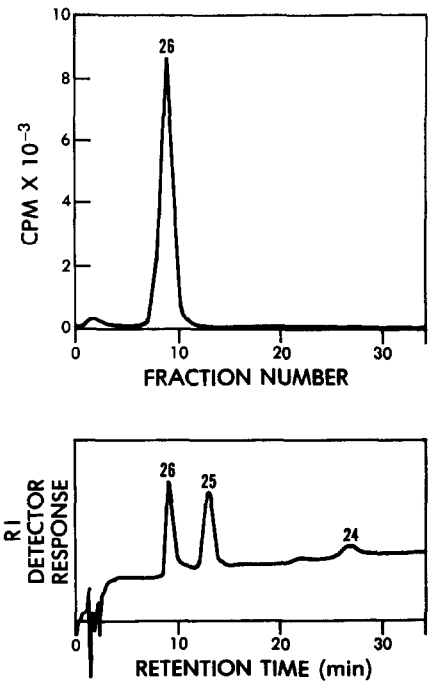


Fig 2. Reversed-phase radiochromatogram of the fractions 50–65 (Fig. 1) Sample, combined with 50 μg of each carrier, in 250 μl methanol Column, Zorbax BP-ODS, 25 × 0.46 cm; eluent, methanol–water (7:3); flow-rate, 2 ml/min, fraction volume, 2 ml, pressure, 1000 psi, RI detector, sensitivity 16 ×; recorder, speed 12 cm/hr, span 10 mV For the identity of peaks, see Table 1.

Fractions 50–65 (cf Fig 1) were combined with the nonradioactive androgens Nos 24–26 of Table 1 and were rechromatographed as shown in Fig 2 Only testosterone (No 26) was associated with radioactivity. The radioactive fractions were pooled and acetylated with 0 5 ml pyridine and 0 25 ml Ac_2O The acetate was then combined with carrier testosterone acetate and was chromatographed successively in systems 5 and 7 (Table 1) The other radioactive peaks in Fig 1 (fractions 19–24, 25–29, 30–34, 35–37, 38–42 and 43–49) were individually combined with appropriate nonradioactive androgens and chromatographed in system 2 (Table 1) Each radioactive peak obtained was acetylated, combined with the appropriate androgen acetates and then chromatographed in system 3, 4, or 5 (Table 1) (normal-phase column) The radioactive fractions thus obtained were combined with additional carrier material and rechromatographed in system 6 or 7 (Table 1) (reversed-phase column) The radioactive androgens, identified by the four HPLC systems and conversion to the acetyl derivatives, are shown in Table 2 None of the androgens in Table 2 was found in the control expt

Analysis of $C_{19}O_3$ *androgens* A 5% aliquot of the neutral lipid fraction was combined with 11 nonradioactive $C_{19}O_3$ androgens and the mixture was chromatographed on the normal-phase column with hexane–EtOH (93·7) as the eluent. The HPLC data of 39 $C_{19}O_3$ androgens have been published [4] and the UV chromatogram of these 11 carrier $C_{19}O_3$ androgens appears in that publication The radiochromatograms from both monoecious and gynoecious plants were similar to that from the control expt The fractions were pooled to form seven approximately equal groups Five of them represented five broad radioactive peaks Each pooled group was combined with appropriate nonradioactive $C_{19}O_3$ androgens and rechromatographed on a reversed-phase column with MeOH–H_2O (11 9) as the eluent [4]. The radiochromatograms showed low levels of radioactivity and were similar to those of the control expt Most of the radioactivity appeared to be associated with unspecified autoxidation products of 4-[4-^{14}C]androstene-3,17-dione

Analysis of estrogens A 20% aliquot of the acidic fraction was combined with nine estrogens (mono- and diols) and the mixture was chromatographed on a normal-phase column with hexane–EtOH (97 3) The UV (280 nm) chromatogram of these nine estrogens has been published [16]. Low radioactivities were associated with the fractions (1 ml/fraction·0.5 min) The fractions were pooled to form *ca* five equal groups according to the R_ts of known estrogens Another 20% aliquot of the acidic lipid fraction was combined with seven estrogens (tri- and tetraols) and the mixture was chromatographed on a normal-phase column with hexane–EtOH (9 1) The UV (280 nm) chromatogram of these seven estrogens has also been published [16] The fractions were pooled to form three groups according to the R_ts of known estrogens Each group was combined with appropriate estrogens and the mixture was chromatographed on a reversed-phase column with 35% aq acetonitrile (for mono- and diols) or 25% aq. acetonitrile (for tri- and tetraols). No radioactivity was associated with the 24 known estrogens [16]

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