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ISOLATION OF ESTRONE AND CHOLESTEROL FROM THE DATE PALM, *PHOENIX DACTYLIFERA* L.

RAYMOND D. BENNETT, SHUI-TZE KO and ERICH HEFTMANN

Western Regional Research Laboratory,* Albany, California, and Division of Biology, California Institute of Technology, Pasadena, California, U.S.A.

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Abstract—The presence of estrone in date palm seeds and pollen was demonstrated by thin-layer chromatography, isolation, i.r. spectrum, and reduction to estradiol. Cholesterol was obtained from date palm pollen and identified by thin-layer chromatography, isolation, i.r. spectrum, and conversion to cholesterol acetate.

INTRODUCTION

THE presence of steroidal estrogens in plants has been reported three times in the older literature. Butenandt and Jacobi[1] described the isolation of crystalline estrone from the press cake of palm kernels, the botanical source of which was not specified. Skarzynski[2] found a phenolic compound which he believed to be estriol in female willow flowers. Wafa *et al.*[3,4] obtained from date palm pollen a noncrystalline estrogenic substance which gave positive tests when subjected to three color reactions considered to be specific for estrone. Recently Jacobsohn *et al.*[5] were unable to detect any estrone in the seeds of the African oil palm (*Elaeis guineensis*). In view of the significance attributed to the occurrence of steroid hormones in plants,[6] we decided to re-investigate this problem, using more effective modern techniques for isolation and analysis. We chose the date palm, *Phoenix dactylifera* L. for this purpose and have found that estrone is present in both the pollen and seeds of this plant. A preliminary report of this work has appeared elsewhere.[7]

Hügel *et al.*[8] have inferred the presence of cholesterol, as well as other C_{27}, C_{28}, and C_{29} sterols, in pollen from various sources from mass spectra. Since cholesterol, the precursor of all the steroid hormones in animals, has been obtained in pure form from only two plants,[9–11] its occurrence in pollen required confirmation by more substantial evidence. Accordingly, we have investigated the sterol fraction of date palm pollen and isolated pure cholesterol from it.

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RESULTS

A ketonic fraction was isolated from date palm seeds by extraction, column chromatography of acidic material, Girard's separation, and preparative thin-layer chromatography (TLC). It contained a substance which had the same mobility as estrone by TLC in two solvent systems, as well as the same color and fluorescence when sprayed with 50% sulfuric acid. Upon reduction with lithium aluminum hydride it gave a compound with chromatographic mobility, color, and fluorescence corresponding to estradiol, the reduction product of estrone.

For actual isolation of pure estrone date palm pollen was used. Essentially the same methods were employed to concentrate the estrone, and it was then further purified by preparative TLC in three different solvent systems, followed by recrystallization. The material thus obtained had an i.r. spectrum very similar to that of estrone. When a portion of this was reduced with sodium borohydride, the i.r. spectrum of the product was identical with that of authentic estradiol.

The sterols of the date pollen extract were isolated by column chromatography of the neutral fraction, followed by preparative TLC. After acetylation an aliquot was subjected to TLC, using continuous development to separate the sterol acetates differing only in the side chain.[12] The two major components had the same mobilities as cochromatographed samples of β-sitosterol acetate and cholesterol acetate. The material corresponding to the latter was isolated by preparative TLC. It was shown to be a mixture by epoxidation with *m*-chloroperbenzoic acid,[13] which gave the expected two epoxides of cholesterol acetate but also two more polar products, apparently diepoxides. This finding was confirmed by TLC on silver nitrate-impregnated silica gel plates, which showed one spot corresponding to cholesterol acetate and a second, more polar component, in approximately equal amounts. The mixture was separated by preparative TLC in the same system and the material corresponding to cholesterol acetate was recrystallized. Its i.r. spectrum was identical with that of authentic cholesterol acetate. After hydrolysis, the free sterol had an i.r. spectrum not significantly different from that of cholesterol.

DISCUSSION

Our results, together with those previously reported,[1-4] leave little doubt that steroidal estrogens do occur in plants. Such questions as whether they are found in other species than those studied so far and whether they are physiologically active in plants require further investigation.

Since our original discovery of cholesterol in higher plants,[9-11] this sterol has been presumptively identified, without isolation, by mass spectrometry and or gas chromatography in several other species.[8, 14-17] Thus, it now appears that cholesterol may be present in many if not all plants, although in low concentrations. The occurrence of both cholesterol and estrone in date palm pollen suggests that they may be biosynthetically related.

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EXPERIMENTAL

Methods

Thin-layer chromatographic techniques were as described in previous papers.[18–20] Unless otherwise specified, Silica Gel G* plates were used. Vacuum zone collectors† were used to remove zones from chromatoplates. The thimble containing the adsorbent was placed in a filter funnel‡ equipped with an Alpha-6 Metricel membrane filter,‡ which had been previously covered with a layer of silica gel (particle size 0·2–0·05 mm), about 5 mm high, and washed with 100 ml of acetone. The funnel was filled to the top of the thimble with acetone and allowed to drain dry, and the process was repeated two or three times. The acetone eluate was evaporated, and the residue was taken up in a few ml of acetone and centrifuged to remove any adsorbent. The filter may be reused several times after washing with acetone.

Infrared spectra were obtained in KBr disks with a Beckman Model IR 7 spectrophotometer. Melting points were taken on a Kofler block and are corrected.

Materials

Seeds and pollen of the date palm, *Phoenix dactylifera* L. (var. Khadrawy), were generously supplied by Mr. Roy W. Nixon, Agricultural Research Service, U.S. Department of Agriculture, Indio, California.

Isolation of Estrone from Seeds

One kg of date seeds, ground in a pulverizing mill, was refluxed with 6 l. of 3 N hydrochloric acid for 3 hr. After filtration and washing with water, the dried filter cake was extracted in a Soxhlet apparatus with 3 l. of methanol for 24 hr and then 3 l. of acetone for 24 hr. The combined extracts were evaporated to dryness, and the residue was partitioned between 1 l. of dichloromethane and 1 l. of 2 N NaOH. The aqueous layer was separated, extracted with two 500-ml portions of dichloromethane, and acidified with conc. HCl. It was then extracted with three 500-ml portions of dichloromethane, which were combined, washed with three 300-ml portions of water and dried (Na_2SO_4). Upon evaporation, 1·15 g of acidic material was obtained. This was chromatographed on a 35 g column of neutral alumina, Grade III.§ The first fraction, eluted with 200 ml of benzene–methanol (1:1) and weighing 142 mg, was rechromatographed on a 4 g column of alumina, eluting first with 15 ml of benzene and then with 15 ml each of benzene–methanol (199:1) and (99:1). The latter two fractions, which contained substances similar in polarity to estrone (by TLC), were combined (16 mg). This material was refluxed with 1 g of Girard's reagent T, 1·5 ml of acetic acid, and 20 ml of methanol for 1 hr. After cooling in an ice-bath and diluting with 200 ml of ice-water, the solution was partially neutralized with 10% NaOH and extracted with three 150-ml portions of ice-cold ether. The combined extracts were washed with two 100-ml portions of ice-water, which were added to the aqueous layer, along with 25 ml of conc. HCl. After 2 hr at 25° the

* Brinkmann Instruments, Westbury, New York. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

† Brinkmann Instruments, Westbury, New York.

‡ Gelman Instrument Co., Ann Arbor, Michigan.

§ Woelm, Eschwege, Germany.

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solution was extracted with three 150-ml portions of ether. The combined extracts were washed with two 100-ml portions of 2·5% Na_2CO_3 and three 100-ml portions of water, dried (Na_2SO_4), and evaporated, yielding 3·5 mg of ketonic material. This was purified by preparative TLC with cyclohexane–ethyl acetate (1:1). The zone corresponding to co-chromatographed standards of estrone was removed and eluted (1·9 mg). TLC in the same system, and also with dichloromethane–acetone (7:3), showed that a component of this material had the same mobility as estrone, and, after spraying with 50% H_2SO_4 and heating, it gave the same characteristic orange color and greenish fluorescence.

About 0·8 mg of this fraction was dissolved in 2 ml of anhydrous ether and excess $LiAlH_4$ added. After 30 min the mixture was treated with one drop of water, followed by enough 3 N HCl to dissolve the precipitated alumina. The ether layer was removed, washed with 1 ml of water, and evaporated. TLC with dichloromethane–acetone (7:3) of the residue showed, after treatment with 50% H_2SO_4, a spot corresponding in mobility, color, and fluorescence to estradiol, the reduction product of estrone expected under these conditions.

Isolation of Estrone from Pollen

Date palm pollen (170 g) was worked up as in the preceding section. The ketonic fraction (29 mg) from the Girard's separation was purified by preparative TLC four times, each time developing with cyclohexane–ethyl acetate (1:1) and isolating the zone corresponding to an estrone standard. TLC of the product (4·5 mg) in the same system and also with dichloromethane–acetone (7:3) and subsequent treatment with 50% H_2SO_4 revealed that it contained a compound with the same mobility, fluorescence, and color as estrone. It also gave the same bluish fluorescence and brown color as estrone after spraying with Fast Black Salt K solution. After reduction of a portion of this fraction with $LiAlH_4$ as in the preceding section TLC with cyclohexane–ethyl acetate (1:1) showed the presence of a compound corresponding in mobility, color, and fluorescence to estradiol.

For further purification the estrone was again subjected to preparative TLC, first with dichloromethane–methanol (49:1), giving 1·0 mg, and then with dichloromethane–acetone (47:3), yielding 600 μg, which appeared homogeneous by TLC in the latter system. After recrystallization from hexane–benzene 230 μg was obtained. An i.r. spectrum of this material showed characteristic maxima at 3380 (3-OH), 1722 (17-keto), 1610, 1581, and 1500 (aromatic ring), 878, and 820 cm^{-1}. The spectrum of authentic estrone was very similar, with some differences in relative intensities probably due to polymorphism.

Half of the estrone from pollen was dissolved in 0·2 ml of tetrahydrofuran, to which was added a solution of 0·5 mg of $NaBH_4$ in 0·2 ml of tetrahydrofuran and 0·1 ml of water. After 20 min, 0·5 ml of 3 N HCl was added, and the solution was extracted with two 0·3-ml portions of ether. The extracts were combined and concentrated to 0·1 ml, 0·3 ml of water was added, and the product was again extracted with two 0·2-ml portions of ether. The extracts were combined and evaporated. The i.r. spectrum of the residue was identical in all features with the spectrum of authentic estradiol, showing characteristic maxima at 3200 and 3440 (3- and 17-hydroxyls), 1610, 1586, and 1498 (aromatic ring), 874, and 819 cm^{-1}. Some additional peaks were observed due to boric acid present as an impurity from the reduction with $NaBH_4$.

Isolation of Cholesterol From Pollen

The dichloromethane layer obtained on partitioning the extract in the preceding section with NaOH was evaporated (17·9 g) and chromatographed on a 500 g column of neutral

alumina, Grade III. Fractions of 1 l. each were collected, using the following eluents: Fraction **1**, hexane; **2**, 5%; **3**, 10%; **4**, 25%; **5**, 50% benzene in hexane; **6**, benzene; **7**, 5%; **8**, 10%; **9**, 25%, **10**, 50% ether in benzene; and **11**, ether. TLC with dichloromethane–acetone (93:7) indicated that fractions 5–9 contained sterols. These fractions were combined (total weight, 3·6 g) and a 28 mg portion was subjected to preparative TLC, with dichloromethane–acetone (93:7). The zone corresponding to cholesterol was removed, eluted, and acetylated with 3 ml of pyridine–acetic anhydride (1:1) for 16 hr at 25°. TLC of the sterol acetates on an Anasil B* plate, developed continuously with hexane–ether (99:1) for 5 hr, showed the presence of two major components, one corresponding to β-sitosterol acetate and the other one to cholesterol acetate. A third compound, in much smaller amount, had the same mobility as stigmasterol acetate. The sample was chromatographed on three Anasil B preparative plates in the same system and the material corresponding to cholesterol acetate was isolated. For further purification it was rechromatographed on one Anasil B plate, after which it appeared homogeneous by TLC in the same system and weighed 12·8 mg.

A 50 μg aliquot was treated with 500 μg of *m*-chloroperbenzoic acid in 1 ml of ether for 24 hr at 25°. After adding 1 ml of 10% Na_2CO_3 and mixing well, the ether layer was separated and evaporated. A portion of the residue was examined by TLC with dichloromethane–acetone (99:1). It contained two components corresponding to the two epoxides of cholesterol acetate prepared by the same reaction, and also two more polar compounds, with mobilities similar to the stigmasterol acetate epoxides, indicating the presence of a sterol with two double bonds.

TLC of the pollen sterol acetate on a $AgNO_3$-impregnated Silica Gel G plate,[21] developed with hexane–benzene (1:1), gave two spots, one corresponding in mobility to cholesterol acetate and the second to desmosterol acetate. However, the color of the second component on spraying with 50% H_2SO_4 was different from that of desmosterol acetate. The two sterol acetates were separated by preparative TLC in the same system and the cholesterol acetate (5·3 mg) was finally purified by recrystallization from methanol, yielding 3·0 mg, m.p. 117–119·5°. The melting point of a mixture of this material and authentic cholesterol acetate (m.p. 116·5–117·5°) was 115·5–118°. The i.r. spectrum matched that of a cholesterol acetate reference sample.

Half of the cholesterol acetate from pollen was converted to the free sterol by treatment with $LiAlH_4$ in ether.[10] The product melted at 144·5–146°. The mixed melting point with authentic cholesterol (m.p. 146–148°) was 145–147·5°. The i.r. spectrum was identical with that of reference cholesterol, except for some carbonyl impurity in the latter.

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* Analabs Inc., Hamden, Connecticut.

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