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BIOSYNTHESIS OF PREGNENOLONE FROM CHOLESTEROL IN *PUNICA GRANATUM*

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Abstract—Following administration of cholesterol-4-^{14}C to leaves of *Punica granatum* plants, radioactive pregnenolone was isolated and identified by thin-layer and gas chromatography and the formation of the acetate derivative.

INTRODUCTION

THE PRESENCE of cholesterol in higher plants was first reported by Johnson *et al.*[1] Since then, this sterol, which had earlier been thought to be a characteristic animal product, has since been detected in a large variety of plants.[2,3] The biosynthesis of pregnenolone from cholesterol has been demonstrated by Bennett and Heftmann[7] in *Haplopappus heterophyllus* and by Caspi *et al.*[5] in *Digitalis purpurea.* Pregnenolone has been found in four plants.[6–9] The conversion of pregnenolone to progesterone has been demonstrated in *Holarrhena floribunda*[10] and in *D. lanata.*[11] This study is an attempt to show the *in vivo* conversion of cholesterol-4-^{14}C to pregnenolone during the growth and development of the plant *Punica granatum.*

RESULTS

Following extraction the various fractions were counted for total radioactivity. Of the $8{\cdot}4 \times 10^{-6}$ cpm applied to the leaves the fractions contained: (A) $4{\cdot}6 \times 10^{3}$ cpm, (B) $4{\cdot}4 \times 10^{3}$ cpm, and (NF) $3{\cdot}4 \times 10^{6}$ cpm, representing a 50 per cent recovery of the administered cholesterol-4-^{14}C. The ketonic fraction contained $4{\cdot}6 \times 10^{5}$ cpm, and the non-ketonic fraction $2{\cdot}0 \times 10^{6}$ cpm, representing 73 per cent recovery from the neutral fraction. The ketonic fraction was divided into four aliquots for analysis:

1. The sample was run on TLC in system I, and the pregnenolone (R_f 0·34) was eluted and counted. The acetate derivative of this pregnenolone was formed and chromatographed in system V, giving a pregnenolone acetate spot (R_f 0·57) with radioactivity and having a peak on the gas chromatograph at 12·6 min, the same as pregnenolone acetate.

[1] D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 231 (1966).
[2] E. HEFTMANN, *Lloydia* **30**, 209 (1967).
[3] E. HEFTMANN, *Lloydia* **31**, 293 (1968).
[4] A. M. GAWIENOWSKI and C. C. GIBBS, *Steroids* **12**, 545 (1968).
[5] E. CASPI, D. O. LEWIS, D. M. PIATAK, K. THIMANN and A. WINTER, *Experientia* **22**, 506 (1966).
[6] R. D. BENNETT, SHUI-TZE KO and E. HEFTMANN, *Phytochem.* **5**, 231 (1966).
[7] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).
[8] R. TSCHESCHE and G. SNATZKE, *Ann. Chem.* **636**, 105 (1960).
[9] R. ELBER, cited in J. VONEUV and T. REICHSTEIN, *Helv. Chim. Acta* **47**, 711 (1964).
[10] R. D. BENNETT and E. HEFTMANN, *Science* **149**, 652 (1965).
[11] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **6**, 1521 (1967).

2. After chromatography in system I, pregnenolone ($1{\cdot}2 \times 10^4$ cpm) was isolated. The acetate derivative of this pregnenolone contained radioactivity after TLC in system V. It also gave a peak on the gas chromatography at 11·4 min, the same as the standard, and on another column (2·5 per cent SE-30) it gave a peak at 19·9 min, the same as the standard.

3. Following chromatography in system I in the first dimension, and systems III and II in the second dimension, radioactivity was associated with pregnenolone. On a 2·5 per cent SE-30 column this pregnenolone had a retention time of 14·3 min, the same as the standard. Following acetylation and development in system V the retention time of the sample and standard on a 2·5 per cent SE-30 column was 19·0 min and it was radioactive.

4. 300 μg of pregnenolone was added to this sample before chromatography in system I. The radioactive pregnenolone was transferred to another plate and run in system II giving a pregnenolone spot (R_f 0·47). This pregnenolone was acetylated and one-half run in system V was radioactive and had a retention time of 11·4 min, the same as the standard. The other half run in system IV (R_f 0·48) had a retention time of 11·4 min and was also radioactive.

The total radioactivity associated with pregnenolone in the four aliquots was $3{\cdot}2 \times 10^4$ cpm, and with pregnenolone acetate it was $4{\cdot}6 \times 10^3$ cpm. The range of radioactivity in the four aliquots was from $1{\cdot}2 \times 10^4$ cpm to $3{\cdot}5 \times 10^3$ cpm for pregnenolone and from $1{\cdot}25 \times 10^3$ cpm to $3{\cdot}2 \times 10^2$ cpm for pregnenolone acetate.

DISCUSSION

Heftmann[3] believes that the pathways of steroid biosynthesis in plants are similar to those of animals. When we found cholesterol and progesteron[12] and, especially, estrone in apple seeds,[13] we became interested in research on the pomegranate plant. Its seeds are the richest plant source of estrogens yet found (17 mg/kg).[14] Since the biosynthesis of pregnenolone from cholesterol has been shown in two different plant[4, 5] it seemed logical that we study this biosynthetic pathway in the pomegranate plant. Our per cent conversion of cholesterol-4-^{14}C to radioactive pregnenolone acetate was 0·053 while that of Bennett and Heftmann[7] was 0·041.

EXPERIMENTAL

Materials

Pomegranate (*Punica granatum*) plants were germinated from seeds of a single fruit and planted in soil for 4 months until they reached a height of 50–55 cm. Cholesterol-4-^{14}C with a specific activity of 146 μc/mg (New England Nuclear Co.) was applied to the leaves of twelve plants at five different times over a period of 2 weeks according to the procedure of Bennett and Heftmann.[14] The total amount of cholesterol-4-^{14}C applied to the twelve plants was $8{\cdot}4 \times 10^6$ cpm.

Procedure

Three days after the last treatment the leaves (21 g) were homogenized with methanol. The homogenate was filtered and the filter cake was extracted for 8 hr with benzene:methanol (3:1), followed by 5 hr with acetone. These extracts were added to the original filtrate, evaporated to dryness, the residue refluxed with benzene:water:conc. HCl (25:55:20) for 3 hr and the benzene and aqueous phases were separated. The latter was extracted with CH_2Cl_2 which was washed twice with water and evaporated to dryness. The residue was dissolved in benzene, added to the original benzene extract, and extracted twice with 2 per cent $NaHCO_3$ (A), washed with water and twice with 0·5 N NaOH, and then with 0·5 N HCl (B). The neutral fraction (NF) was separated into ketonic and non-ketonic fractions with Girard's Reagent T(2).

Pregnenolone, isolated from the ketonic fraction by TLC was acetylated with 0·2 ml pyridine and 0·2 ml acetic anhydride at room temperature overnight.

[12] A. M. GAWIENOWSKI and C. C. GIBBS, *Phytochem.* **8**, 685 (1969).
[13] R. A. MASARACCHIA and A. M. GAWIENOWSKI, *Steroids* **11**, 718 (1968).
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Silica gel (Camag, DF-5) with fluorescent indicator was used for the application of a 0·3 μ layer to 200-mm square plates for TLC. Several systems were used for the isolation and purification of the neutral ketonic fraction: I-CH_2Cl_2–MeOH (97:3); II-CH_2Cl_2–ether (5:2); III-hexane–EtOAc (5:2); IV-CH_2Cl_2–acetone (99:1); V-benzene: EtOAc (4:1). Samples and co-chromatographed standards were applied as spots and following development were eluted from the silica gel according to the method of Masaracchia and Gawienowski.[13]

A Barber Colman 5000 series gas chromatograph with a flame ionization detector and a U-shaped 6 ft × $\frac{1}{8}$ in. glass column was used. Samples purified by TLC were analyzed on a 3 per cent SE–30, Chromosorb W-DMCS, 80/100 mesh column at 230° with nitrogen maintained at 20 psi, unless mentioned otherwise in the Results section. Retention times were calculated by comparing extract peaks with a standard.

Samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3310. 9 g of PBD and 0·05 g of POPOP/l. of toluene was used as the scintillation fluid.

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