Phytochemistry, 1965, Vol. 6, pp. 747 to 754. Pergamon Press Ltd. Printed in England

BIOSYNTHESIS OF PREGNENOLONE FROM CHOLESTEROL IN *HAPLOPAPPUS HETEROPHYLLUS*

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(*Received* 1 *January* 1966)

Abstract—After administration of cholesterol-4-^{14}C (I) to the leaves of a *Haplopappus heterophyllus* plant, radioactive pregnenolone (II) was isolated and purified to constant specific activity by chromatography and recrystallization of both pregnenolone and its acetate. Cholesterol was identified as a natural constituent of the plant by thin-layer and gas–liquid chromatography.

INTRODUCTION

RECENTLY, Zalkow *et al.*[1] provided the first evidence for the presence of C_{19} steroids in plants by isolating 5α-androstane-3β,16α,17α-triol (III) from *Haplopappus heterophyllus* Blake. In animals, compounds of this type are formed from C_{21} steroids, which are now known to be widely distributed in plants.[2] We have undertaken a study of the biosynthesis and metabolism of this androstanetriol in *H. heterophyllus* on the assumption that a C_{21} steroid would be a likely precursor. To ascertain whether compounds of this type are present in the plant, we therefore administered cholesterol-4-^{14}C and have now isolated radioactive pregnenolone.

RESULTS

Cholesterol-4-^{14}C (I) was applied twice a week for 4 weeks to the leaves of a *H. heterophyllus* plant. Most of the radioactivity recovered by extraction was found in the neutral fraction, a radiochromatogram of which is shown in Fig. 1. The ketonic fraction obtained by Girard's separation of this material appeared to contain radioactive pregnenolone (II) (Fig. 2), which was isolated by preparative TLC and purified by rechromatographing in a different system (Fig. 3). The pregnenolone was then acetylated, and the product was subjected to TLC to obtain chromatographically homogeneous pregnenolone acetate (Fig. 4). This material was shown to be radiochemically pure by dilution with authentic pregnenolone acetate and recrystallization from two solvents, hydrolysis to pregnenolone, and two more recrystallizations. The specific activity remained constant throughout these operations (Table 1).

To demonstrate the presence of cholesterol in *H. heterophyllus*, the sterols obtained from a second plant were acetylated and purified by chromatography. TLC of the sterol acetate fraction revealed a component corresponding in mobility to authentic cholesterol acetate,

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Work conducted under a cooperative agreement with the California Institute of Technology.

[1] L. H. ZALKOW, N. I. BURKE and G. KEEN, *Tetrahedron Letters* 217 (1964).
[2] R. TSCHESCHE, *Bull. Soc. Chim. France* 1219 (1965).

TABLE 1. RECRYSTALLIZATION OF PREGNENOLONE ACETATE AND PREGNENOLONE*

Compound	Solvent used for crystallizations	Counts/min/μM†
Pregnenolone acetate		31·4 ± 1·6
	Hexane	30·2 ± 1·6
	Methanol	30·7 ± 1·6
Pregnenolone	Hexane–acetone	29·7 ± 1·6
	Methanol	29·8 ± 1·6

* 0·2-mg portions were plated from chloroform solutions on ringed planchets over an area of 12·7 cm² and counted in duplicate on a Beckman Widebeta II instrument. Counter efficiency was 34% and background was 1·0–1·5 counts/min.
† 90% confidence level.

and a gas–liquid chromatogram showed a peak with the same retention time as cholesterol acetate.

DISCUSSION

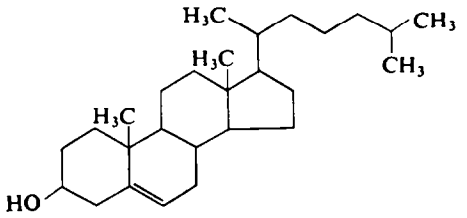
Axelrod *et al.*[3] have demonstrated that a combination of chromatography, derivative formation, and recrystallization is the most effective method for purification of radioactive steroids to constant specific activity. It is apparent from Figs. 3 and 4 and Table 1 that most of the original radioactivity remained associated with pregnenolone during these operations.

Previous studies have shown that acetate, mevalonic acid,[4,5] and squalene[6] are precursors of plant steroids. We have also demonstrated the conversion by plants of cholesterol to steroidal sapogenins[7] and alkaloids,[8] and conversion of pregnenolone to progesterone,[9] and the reversible interconversion of pregnenolone and *Holarrhena* alkaloids.[10,11] It is also known from the work of others that pregnenolone is a precursor of cardenolides[12] and bufadienolides.[13] Thus, the available evidence suggests that steroids are in a dynamic state in plants, just as they are in animals.[14]

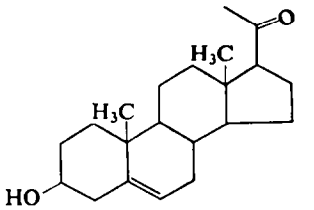
Pregnenolone (II) has been known to be a constituent of two plants, *Xysmalobium undulatum*[15] and *Trachycalymna fimbriatum*,[16] and we have recently obtained evidence of its occurrence in *Holarrhena floribunda*.[11] *Haplopappus heterophyllus* is thus the fourth plant source of this steroid. Our identification of cholesterol by two chromatographic methods adds another plant to the increasing list[17] in which this important sterol may be found.

[3] L. R. AXELROD, C. MATTHIJSSEN, J. W. GOLDZIEHER and J. E. PULLIAM, *Acta Endocrinol.*, Suppl. **99**, 1 (1965).
[4] E. HEFTMANN, *Ann. Rev. Plant Physiol.* **14**, 225 (1963).
[5] G. WILLUHN, *Pharm. Ztg.* **110**, 96 (1965).
[6] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).
[7] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 577 (1965).
[8] R. D. BENNETT and E. HEFTMANN, *Arch. Biochem. Biophys.* **112**, 616 (1965).
[9] R. D. BENNETT and E. HEFTMANN, *Science* **149**, 652 (1965).
[10] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 873 (1965).
[11] R. D. BENNETT, E. HEFTMANN and S.-T. KO, *Phytochem.* **5**, 517 (1966).
[12] R. TSCHESCHE and G. LILIENWEISS, *Z. Naturforsch.* **19b**, 265 (1964).
[13] R. TSCHESCHE and B. BRASSAT, *Z. Naturforsch.* **20b**, 707 (1965).
[14] E. HEFTMANN and E. MOSETTIG, *Biochemistry of Steroids*. Reinhold, New York (1960).
[15] R. TSCHESCHE and G. SNATZKE, *Ann. Chem.* **636**, 105 (1960).
[16] R. ELBER, Dissertation, Basel, 1964; cited in J. VON EUW and T. REICHSTEIN, *Helv. Chim. Acta* **47**, 711 (1964).
[17] R. D. BENNETT, S.-T. KO and E. HEFTMANN, *Phytochem.* **5**, 231 (1966).

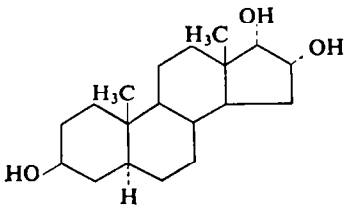
The conversion of cholesterol (I) to pregnenolone (II) is a key step in the biosynthesis of all steroid hormones in animals.[14] The occurrence of cholesterol, pregnenolone, and possibly androstanetriol (III), together with our demonstration of the cholesterol–pregnenolone



(I) Cholesterol



(II) Pregnenolone



(III) Androstanetriol

conversion in the same plant, supports the hypothesis[4] that steroid metabolism in plants in many ways resembles that in animals. However, earlier attempts to demonstrate this reaction in *Holarrhena floribunda* were unsuccessful.[8] Undoubtedly, alternate pathways of pregnenolone biosynthesis may also exist in plants, as some of the other sterols present could likewise yield pregnenolone on degradation of their side chains.

So far, we have been unable to detect 5α-androstane-3β,16α,17α-triol (III) in our specimens of *Haplopappus heterophyllus* at any stage of development. Nor was any radioactivity, corresponding to this compound, observed in chromatograms of plants which had received cholesterol-4-^{14}C by foliar application. Since the plant used by Zalkow *et al.*[1] was referred to as "rayless goldenrod", there is some possibility that it may have been *H. tenuisectus* (Greene) Blake. Both of these plants have been called "rayless goldenrod", and they are often confused.[18]

EXPERIMENTAL

Methods

Thin-layer chromatographic techniques were as described in previous papers,[7,8] except that silver membrane filters* (0·45 μ pore diameter) were used during elution of zones. Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details).

We are indebted to Dr. James A. Waters, National Institutes of Health, Bethesda, Maryland, for the gas–liquid chromatograms, which were obtained with a Barber-Coleman Series 5000 instrument, equipped with a column of 1% SE-30 on Gaschrom P, at 230°.

* Selas Flotronics, Spring House, Penn. 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.

[18] F. H. TSCHIRLEY and S. C. MARTIN, *Ariz. Univ., Agr. Expt Sta. Tech. Bull.* No. 146 (1961).

Materials

Cholesterol-4-^{14}C, having a specific activity of 58 μc/μM, was purchased from Atomic Accessories, Valley Stream, N.Y.

Haplopappus heterophyllus (A. Gray) Blake (jimmyweed) plants were raised from seeds generously supplied by Dr. Charles Mason, Jr., Curator of the University of Arizona Herbarium, Tucson, Arizona.

Administration of Radioactive Cholesterol

Cholesterol-4-^{14}C ($2{\cdot}62 \times 10^5$ counts/min) was administered to several upper leaves of a plant, about 3 months old and 50 cm long, by the technique previously described.[6] Two such treatments were given per week until nine doses had been administered. During this period the plant grew to a length of 96 cm and flowered.

Isolation and Purification of Pregnenolone

Two days after the final treatment, the plant was cut off above the soil line, frozen in liquid nitrogen, and lyophilized. The dry material (4·4 g) was homogenized with 150 ml of water. The homogenate was filtered and the filter cake was washed with three 50-ml portions of water. The combined filtrates were extracted with two 100-ml portions of dichloromethane, and the extracts were passed through 50 ml of water, combined, and evaporated. This material was pooled with the residue from an extract obtained by boiling the filter cake with 400 ml of acetone for 3 hr. The combined residues were taken up in 200 ml of benzene, and extracted with 100 ml of 1 N NaOH and two 100-ml portions of water. Each extract was

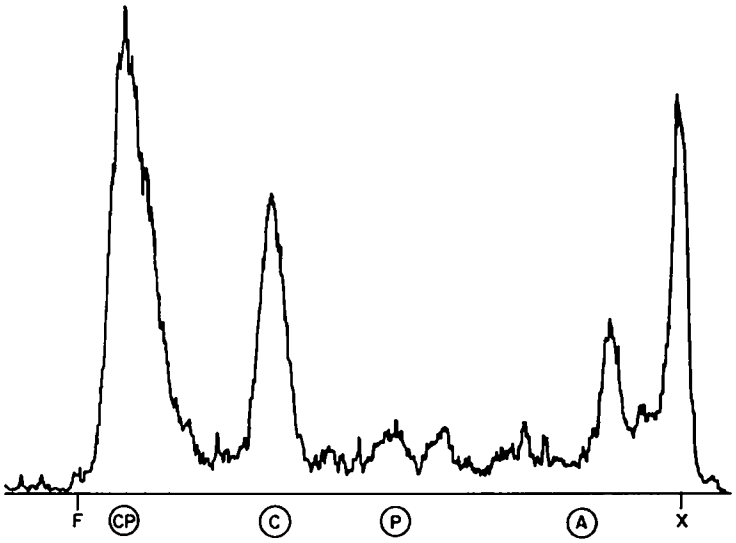


FIG. 1. RADIOCHROMATOGRAM OF NEUTRAL EXTRACT OF *H. heterophyllus*, TREATED WITH CHOLESTEROL-4-^{14}C.

Letters indicate positions of zones corresponding to standards: F, solvent front; CP, cholesterol palmitate; C, cholesterol; P, pregnenolone; A, androstanetriol; X, origin. A 5 × 20-cm Silica Gel G plate was developed, first, with benzene for 50 mm and, then, with cyclohexane–ethyl acetate (1 : 1) to a height of 170 mm. The chromatogram was scanned at 0·75 in./hr, with a time constant of 100 sec and a slit width of 3 mm, and later sprayed with 50% sulfuric acid and charred on a hot plate.

passed through 50 ml of benzene, and the two benzene layers were combined, filtered, and evaporated to yield a neutral fraction of 105 mg ($1{\cdot}29 \times 10^6$ counts/min) (see Fig. 1).

This material was refluxed with 500 mg of Girard's Reagent T and 1 ml of acetic acid in 20 ml of methanol for 1 hr. The reaction mixture was cooled to 5° and, after addition of 250 ml of ice-water and 10 ml of 10% NaOH, extracted with three 150-ml portions of cold ether. The extracts were washed with 75 ml of ice-water, combined, and evaporated to give a nonketonic fraction of 84 mg ($9{\cdot}80 \times 10^5$ counts/min).

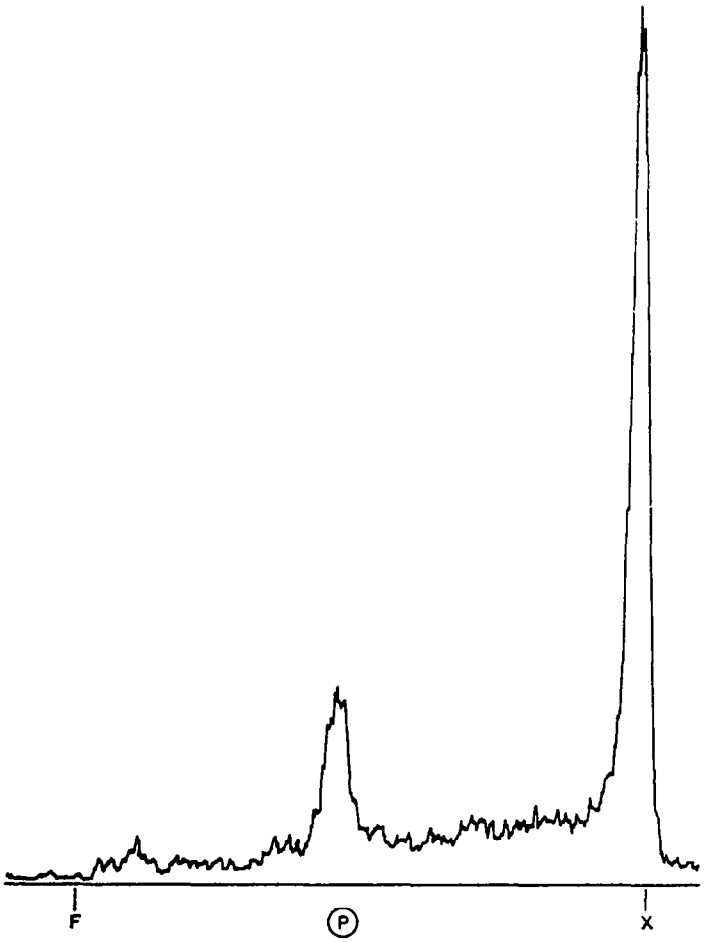


FIG. 2. RADIOCHROMATOGRAM OF KETONIC FRACTION FROM NEUTRAL EXTRACT (see Fig. 1.)
Letters and conditions as in Fig. 1.

The aqueous layers were combined and treated with 50 ml of conc. HCl. After 2 hr at 25°, the solution was extracted with two 100-ml portions of dichloromethane. The extracts were passed through 50 ml of water, combined, and evaporated to give a ketonic fraction of 9·7 mg ($1{\cdot}22 \times 10^5$ counts/min). One-fourth of this was subjected to preparative TLC as in Fig. 2 and the zone corresponding to pregnenolone was removed and eluted (0·8 mg; 4000 counts/min). Authentic pregnenolone (20 μg) was added as carrier and this material was chromatographed as shown in Fig. 3. The pregnenolone zone was isolated (2250 counts/min)

and acetylated with pyridine–acetic anhydride (1:1). The product was subjected to TLC as shown in Fig. 4 and the pregnenolone acetate zone removed and eluted (960 counts/min). After dilution with 10·0 mg of authentic pregnenolone acetate, it was recrystallized twice as shown in Table 1. The material from the second crystallization was refluxed with 1·5 ml of 0·1 N NaOH in 80% methanol for 15 min. The methanol was removed by azeotropic distillation with benzene, and the aqueous residue was extracted with three 1-ml portions of benzene. The extracts were combined and evaporated to give chromatographically homogeneous pregnenolone, which was recrystallized as shown in Table 1.

Isolation and Identification of Sterols

A second untreated plant was harvested, frozen in liquid nitrogen, and lyophilized. The dried material (5·1 g) was homogenized in a blender with 200 ml of methanol. The homogenate was filtered and the filter cake was refluxed with 300 ml of benzene–methanol (3:1) for

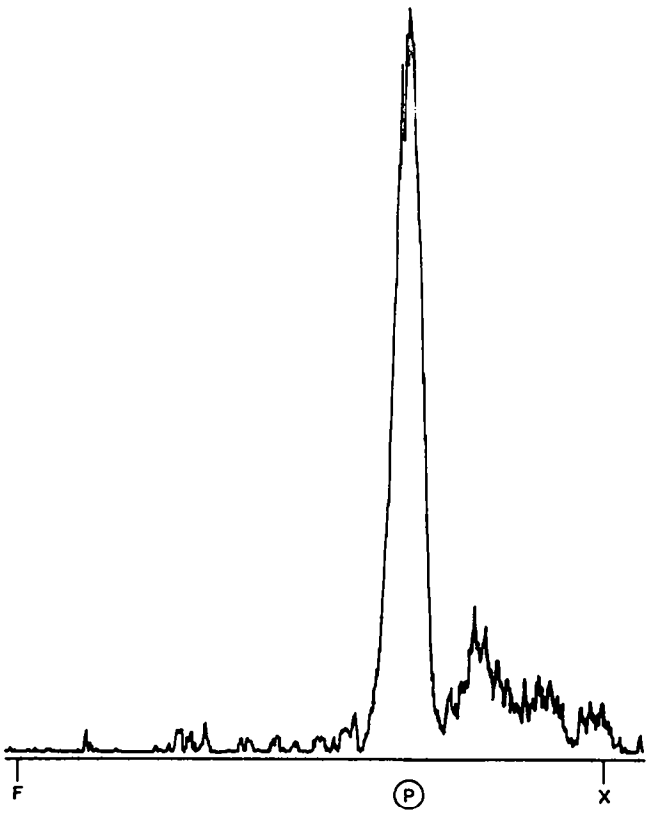


FIG. 3. RADIOCHROMATOGRAM OF ZONE CORRESPONDING TO PREGNENOLONE (see Fig. 2), ISOLATED BY PREPARATIVE TLC.

Letters as in Fig. 1. A Silica Gel G plate was developed with dichloromethane–acetone (24:1) and then scanned at 6 in./hr, with a time constant of 30 sec and a slit width of 4 mm. The chromatogram was sprayed with 0·1% Rhodamine 6G and examined under short-wave u.v. light.

4 hr. The mixture was filtered and the filter cake was washed with two 100-ml portions of benzene. The combined filtrates were pooled with the methanolic filtrate from above and

evaporated. The residue was then refluxed with 300 ml of 3 N HCl and 100 ml of benzene for 3 hr. The benzene layer was separated and the aqueous layer was extracted with two 100-ml portions of dichloromethane. The organic layers were washed with 50 ml of water, combined, and evaporated. The residue was taken up in 200 ml of benzene and extracted with 100 ml of 1 N NaOH and two 100-ml portions of water. The aqueous layers were passed through 50 ml of benzene, and the two benzene layers were combined, filtered, and evaporated, yielding a neutral fraction of 666 mg.

This neutral material was subjected to Girard's separation as above to obtain a non-ketonic fraction of 612 mg, which was chromatographed on a 100-g column of silica gel (particle size 0·05–0·2 mm). Elution with 500 ml of dichloromethane–acetone (99:1) gave 258 mg of material which contained sterols (by TLC). This was then chromatographed on an 8-g column of non-alkaline alumina,* Grade III. Fractions of 30 ml each were collected

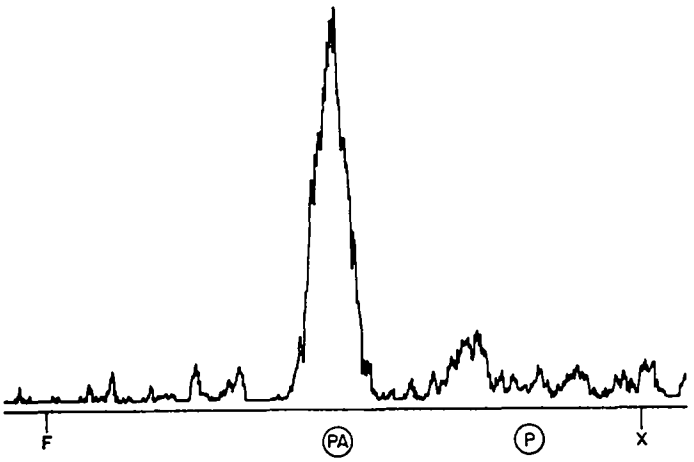


FIG. 4. RADIOCHROMATOGRAM OF ZONE CORRESPONDING TO PREGNENOLONE, ISOLATED BY TLC (see Fig. 3), AFTER ACETYLATION.

PA, Pregnenolone acetate; other letters as in Fig. 1. A Silica Gel G plate was developed with dichloromethane–acetone (99:1) and then scanned and visualized as in Fig. 3.

with the following eluents: Fraction *1*, hexane; *2*, 5%; *3*, 10%; *4*, 25%; *5*, 50% benzene in hexane; *6*, benzene; and *7*, 5% ether in benzene. The fractions were monitored by TLC and the last two, which contained the sterols, were combined (53 mg). This material was subjected to preparative TLC on Silica Gel G with dichloromethane-acetone (24:1) and the zone corresponding to cholesterol was removed, eluted, and acetylated. The sterol acetates (15 mg) were freed of more polar material by preparative TLC on Silica Gel G, developed twice with hexane–dichloromethane (7:3), giving 4·5 mg.

The methods used up to this point do not separate sterols differing only in the side chain. For this purpose the sterol acetates were chromatographed on an Anasil B† plate, developed continuously[19] with hexane–ethyl acetate (499:1) for 2 hr. One of the two major constituents had the same mobility as stigmasterol acetate, and the other moved slower than any of the

* Woelm, Eschwege, Germany.

† Analabs, Hamden, Conn.

[19] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* In press.

sterol acetates available to us as standards. Much smaller spots corresponding to β-sitosterol acetate and cholesterol acetate were also observed. A fraction richer in the latter two components was obtained by preparative TLC on an Anasil B plate, developed continuously[20] with hexane–ethyl acetate (499:1) for 5 hr (1·2 mg). TLC of this material on Anasil B, developed continuously with hexane–ethyl acetate (999:1) for 5 hr, revealed three spots, corresponding to β-sitosterol acetate, cholesterol acetate, and stigmasterol acetate. A gas–liquid chromatogram showed four peaks, with retention times of 22·1, 31·7, 37·1, and 42·0 min. The first three corresponded in retention times to standards of cholesterol acetate, stigmasterol acetate, and β-sitosterol acetate, respectively.

Acknowledgements—The authors gratefully acknowledge the assistance of Mrs. Shui-Tze Ko, Miss Ellen R. Lieber, Mrs. Cornell C. Phillips, and Mr. James Cunningham.

[20] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **12**, 245 (1963).