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IN VIVO CONVERSION OF A STEROIDAL ALKALOID, HOLAPHYLLAMINE, TO PREGNENOLONE

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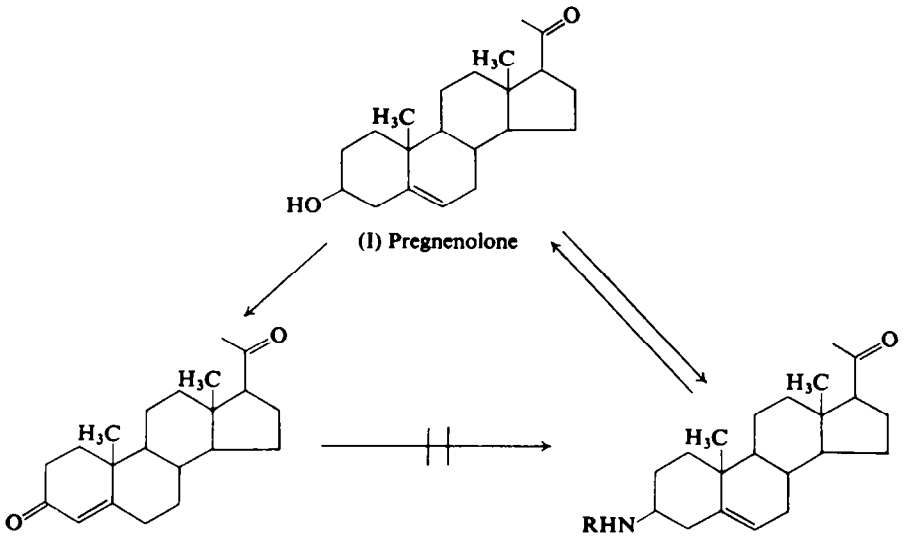
Abstract—After administration of holaphyllamine-4-^{14}C to the leaves of a *Holarrhena floribunda* plant, radioactive pregnenolone was isolated and shown to be radiochemically pure by chromatography, by crystallization of its acetate to constant specific activity, and by subsequent hydrolysis to free pregnenolone of the same specific activity. This experiment indicates that pregnenolone may be a natural constituent of this plant and that the previously observed conversion of pregnenolone to holaphyllamine is reversible.

INTRODUCTION

IN PREVIOUS work on the biosynthesis of the steroids of *Holarrhena floribunda*, we found that pregnenolone-4-^{14}C (I) administered to the leaves was converted to holaphyllamine (III, 3β-amino-Δ^5-pregnen-20-one), holaphylline (IV, 3β-methylamino-Δ^5-pregnen-20-one),[1] and progesterone[2] (II). It then became of interest to ascertain whether pregnenolone may be a



(I) Pregnenolone

(II) Progesterone

(III) R = H; Holaphyllamine

(IV) R = CH_3; Holaphylline

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[1] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 873 (1965).

[2] R. D. BENNETT and E. HEFTMANN, *Science* **149**, 652 (1965).

natural constituent of this plant. In the case of progesterone (II) this was accomplished by actual isolation, starting from 16 kg of dried leaves.[3] We were unable to use this method because of insufficient plant material, and therefore we hoped to approach the problem by observing the conversion of some radioactive precursor to pregnenolone in *H. floribunda*. When cholesterol-4-^{14}C was administered, however, no radioactive pregnenolone (I) could be detected, although the alkaloids (III and IV) were both labelled.[4] We have now found that holaphyllamine is metabolized to pregnenolone, thus reversing the reaction previously observed.

RESULTS

Radioactive holaphyllamine (III), prepared biosynthetically from pregnenolone-4-^{14}C and purified by chromatography, was applied to the leaves of a *Holarrhena floribunda* plant. The alkaloid fraction of the leaves contained only 11 per cent of the radioactivity administered, while 55 per cent was found in the neutral fraction. Thin-layer chromatography (TLC) of the latter revealed a wide distribution of radioactivity from the origin to the solvent front.

TABLE 1. RECRYSTALLIZATION OF PREGNENOLONE ACETATE AND PREGNENOLONE*

Compound	Solvent used for crystallization	Counts/min/μmole†
Pregnenolone acetate		11·4±0·5
	Hexane	11·6±0·5
	Methanol	11·2±0·5
Pregnenolone	Hexane–acetone	11·1±0·5
	Methanol	11·3±0·5

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

† 90 per cent confidence level.

The less polar material, which was isolated by preparative TLC, appeared to contain radioactive pregnenolone (I) and was subjected to Girard's separation. The pregnenolone in the ketonic fraction was purified by TLC in two solvent systems and acetylated. The pregnenolone acetate was purified by TLC, diluted with carrier material, and shown to be radiochemically pure by crystallization from two solvents (Table 1). After hydrolysis to pregnenolone, the specific activity was unchanged by further recrystallizations.

DISCUSSION

The conversion of holaphyllamine to pregnenolone indicates that the latter may be a natural constituent of *H. floribunda* and provides evidence for the biological significance of the previously observed transformations of pregnenolone in this plant.[1,2] However, no radioactive pregnenolone could be detected when cholesterol-4-^{14}C was administered,[4] although the specific activity of the cholesterol was about 200 times that of the holaphyllamine

[3] M. LEBOEUF, A. CAVÉ and R. GOUTAREL, *Compt. Rend.* **259**, 3401 (1964).
[4] R. D. BENNETT and E. HEFTMANN, *Arch. Biochem. Biophys.* **112**, 616 (1965).

used in this experiment and the total radioactivity administered was about 100 times as great. An attempt to trap pregnenolone by administering a mixture of radioactive cholesterol and nonradioactive pregnenolone to the leaves also failed to yield any radioactivity in the isolated pregnenolone. Furthermore, no conversion of holaphyllamine (III) to holaphylline (IV) could be demonstrated, although methylation could have been expected.

Obviously, failure to observe reactions expected on the basis of biochemical principles does not prove the principles wrong, but indicates that many factors must be accounted for in experiments *in vivo*. These are particularly: turnover rates, pool sizes, compartmentalization and activation. Reactions which are observed, on the other hand, give good indication that their products are natural constituents, even if they are present in amounts too small to permit direct isolation.

This is, to our knowledge, the first time that the formation of an alkaloid by amination of a neutral precursor has been found to be reversible. LeBoeuf *et al.*[3] suggested that *H. floribunda* might contain a transaminase which could mediate both the synthesis and deamination of the alkaloids, but they assumed that the neutral compound involved was progesterone, rather than pregnenolone. We previously found that progesterone is not a precursor of holaphyllamine and holaphylline,[1] and the present experiment indicates that it is not a major metabolite of holaphyllamine. The possibility that some radioactive progesterone was formed from holaphyllamine was not investigated, since under the conditions of our experiment a direct formation from the alkaloid could not be differentiated from an indirect biosynthesis via pregnenolone.

Pregnenolone has previously been found in two plants, *Xysmalobium undulatum*[5] and *Trachycalymna fimbriatum*,[6] and it has been shown to be a precursor of cardenolides in *Digitalis lanata*[7] and of a bufadienolide in *Helleborus atrorubens*.[8]

EXPERIMENTAL

Methods

Thin-layer chromatographic techniques were as described in previous papers.[1, 4] Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas flow detector (see Table 1 for details).

Materials

Pregnenolone-4-^{14}C, having a specific activity of 45·8 μc/μmole, was purchased from New England Nuclear Corporation.* *Holarrhena floribunda* plants were raised from seeds generously supplied by the curator of the Botanical Garden, University of Ibadan, Ibadan, Nigeria.

Preparation and Administration of Radioactive Holaphyllamine

A plant was grown from seed in the greenhouse, and about one month after germination, pregnenolone-4-^{14}C ($3{\cdot}76 \times 10^5$ counts/min) was applied to several upper leaves by the

* 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.

[5] R. TSCHESCHE and G. SNATZKE, *Ann. Chem.* **636**, 105 (1960).
[6] R. ELBER, Dissertation, Basel (1964); from J. VON EUW and T. REICHSTEIN, *Helv. Chim. Acta* **47**, 711 (1964).
[7] R. TSCHESCHE and G. LILIENWEISS, *Z. Naturforsch.* **19b**, 265 (1964).
[8] R. TSCHESCHE and B. BRASSAT, *Z. Naturforsch.* **20b**, 707 (1965).

technique previously described.[9] Three such treatments were given per week until ten doses had been administered. On the day after the last treatment the leaves were removed, frozen in liquid nitrogen, and lyophilized. The dried leaves, weighing 0·5 g, were homogenized in a blender with 50 ml of methanol containing 1 per cent of ammonia. The homogenate was transferred to a flask with 150 ml of methanol and refluxed for 4 hr. The mixture was filtered and the filtrate was evaporated to dryness. The evaporation residue was taken up in 100 ml of benzene and extracted with two 20-ml portions of 0·5 N HCl and two 20-ml portions of water. The extracts were passed through 10 ml of benzene, combined, made basic to pH 10 with KOH, and extracted with two 20-ml portions of dichloromethane. The extracts were passed through 10 ml of water, combined, and evaporated, giving an alkaloid fraction of 5·9 mg ($6{\cdot}96 \times 10^5$ counts/min). Holaphyllamine was isolated by preparative TLC on alkaline Silica Gel G[1] and then rechromatographed to yield 0·2 mg ($8{\cdot}5 \times 10^4$ counts/min). An aliquot of this showed only one peak, corresponding to authentic holaphyllamine, when chromatographed as above and scanned for radioactivity, and an acetylated aliquot was also found to be chromatographically homogeneous *N*-acetylholaphyllamine by the same method.

One-half of the holaphyllamine-4-^{14}C was taken up in 300 μl of 3 per cent acetic acid and centrifuged. The solution was separated from a small amount of insoluble material and 300 μl of ethanol was added. An aliquot of this solution was subjected to TLC with dichloromethane:methanol (97:3) and scanned; no radioactivity was observed corresponding to pregnenolone. The remainder of the solution ($3{\cdot}6 \times 10^4$ counts/min) was divided into three equal doses and applied every third day to several upper leaves of a plant grown as above. Prior to the first treatment the leaves were rinsed with a 0·1 % solution of Tween 20.

Extraction and Fractionation of Leaves

Four days after the final treatment the leaves were removed, frozen in liquid nitrogen, and lyophilized. The dried leaves, weighing 0·5 g, were homogenized in a blendor with 100 ml of methanol, and the homogenate was filtered. The filter cake was refluxed with 100 ml of benzene–methanol (3:1) for 4 hr, the mixture was filtered, and the filter cake was washed with two 50-ml portions of benzene. This filtrate was combined with the previous one and evaporated to dryness. The residue was taken up in 200 ml of benzene and extracted with two 50-ml portions of 0·5 N HCl and 50 ml of water, each extract being passed through 25 ml of benzene. The benzene solutions were combined, filtered, and evaporated, giving 54 mg of neutral and acidic material ($1{\cdot}9 \times 10^4$ counts/min).

The aqueous extracts were combined, made basic to pH 10 with KOH, and extracted with two 50-ml portions of dichloromethane. The extracts were passed through 25 ml of water, combined, and evaporated, giving an alkaloid fraction of 6·8 mg ($3{\cdot}9 \times 10^3$ counts/min). TLC of an aliquot showed that the radioactivity was largely associated with holaphyllamine.

Isolation and Purification of Pregnenolone

The neutral and acidic fraction was subjected to preparative TLC by developing first with benzene halfway up the plate and then, after a drying period, the full length of the plate with dichloromethane–methanol (97:3). The adsorbent from the upper half of the plate was removed and eluted, yielding 14·5 mg ($7{\cdot}2 \times 10^3$ counts/min). 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 to 5°, dilution with 200 ml of ice-water, and partial neutralization with 10 % NaOH,

[9] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).

the solution was extracted with three 150-ml portions of ice-cold ether. The combined extracts were washed with 100 ml of ice-water, which was combined with the aqueous solution, and then with two 100-ml portions of 2·5% sodium carbonate solution and three 100-ml portions of water. The ether solution was dried over sodium sulfate, filtered, and evaporated to dryness, giving 8.0 mg of nonketonic material ($1{\cdot}2 \times 10^3$ counts/min).

The aqueous solution from above was treated with 25 ml of conc. HCl. After 2 hr at 25° the solution was extracted with three 150-ml portions of ether. The extracts were combined and washed with two 100-ml portions of 2·5% sodium carbonate solution and three 100-ml portions of water. The ether solution was dried over sodium sulfate, filtered, and evaporated to dryness, giving a ketonic fraction of 1·5 mg ($4{\cdot}9 \times 10^3$ counts/min).

Authentic pregnenolone (100 μg) was added to the ketonic fraction, half of which was chromatographed on a $50 \times 200 \times 0{\cdot}3$ mm Silica Gel G plate with dichloromethane–methanol (97:3). Scanning of the plate revealed a distribution of radioactivity similar to that found in the partially purified material above. The zone corresponding to pregnenolone was removed and eluted. The other half of the ketonic fraction was chromatographed in the same manner, and the two pregnenolone aliquots were combined ($1{\cdot}05 \times 10^3$ counts/min). This material was chromatographed on a plate as above with cyclohexane–ethyl acetate (1:1). The pregnenolone zone, which coincided with the major radioactive peak, was removed and eluted (900 counts/min). After acetylation with pyridine–acetic anhydride (1:1), the product was chromatographed with dichloromethane–acetone (49:1) and scanned. No radioactivity was associated with the area of the chromatogram corresponding to pregnenolone (R_f 0·33). The only peak observed coincided with pregnenolone acetate (R_f 0·72), which was removed and eluted (725 counts/min). An aliquot of this material was diluted with authentic pregnenolone acetate and crystallized as shown in Table 1. The material from the second recrystallization (6·7 mg) 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 dryness, yielding 5·8 mg of pregnenolone, homogeneous by TLC with dichloromethane–acetone (19:1), which was recrystallized as shown in Table 1.

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