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BIOSYNTHESIS OF *HOLARRHENA* ALKALOIDS FROM PREGNENOLONE AND PROGESTERONE

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Abstract—Pregnenolone-4-^{14}C, but not progesterone-4-^{14}C, was converted to the steroidal alkaloids holaphylline and holaphyllamine by leaves of *Holarrhena floribunda*. Progesterone-4-^{14}C was converted to alkaloids of unknown structure.

INTRODUCTION

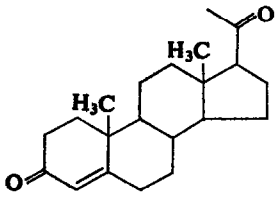
MANY African and Asian genera of the Apocynaceae contain alkaloids structurally related to the pregnane series of steroids. Several of these alkaloids have recently become important as starting materials for the synthesis of steroid hormones.[1] The leaves of *Holarrhena floribunda* contain the three related alkaloids holaphyllamine I, holaphylline II, and holamine III, which are 3β-amino-, 3β-methylamino-, and 3α-amino-Δ^5-pregnen-20-one, respectively.[2,3] If compounds of this type are formed, as has been suggested[4] by transamination of a ketone, the precursor should be Δ^4-pregnene-3, 20-dione (progesterone IV), which has recently been found in this plant.[4] We have found, however, that at least two of these alkaloids are biosynthesized not from progesterone but from pregnenolone V, which is known to be the direct precursor of progesterone in animals.[5]

RESULTS

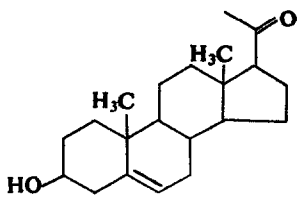
Progesterone-4-^{14}C was administered three times a week for three weeks to the leaves of a *Holarrhena floribunda* plant, by the technique previously described.[6] The alkaloid fraction obtained by extraction of the leaves contained 23 per cent of the radioactivity originally administered (Table 1). However, thin-layer chromatography (TLC) of this fraction revealed that no radioactivity was associated with the three known alkaloids, although holaphylline and holaphyllamine were present in considerable quantity (Figs. 1 and 2). Figure 1 shows that an unknown, much less polar alkaloid contained most of the radioactivity. The amount of this material was comparable to that of holaphylline and holaphyllamine. The radioactive peak corresponding to holaphylline in Fig. 1 was separated from the latter after acetylation (Fig. 2).

* 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.

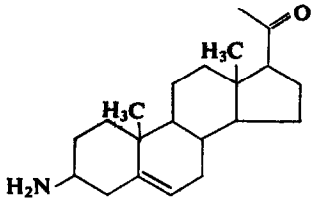
[1] R. GOUTAREL, *Bull. Soc. Chim. France* 1665 (1964).
[2] M.-M. JANOT, A. CAVÉ and R. GOUTAREL, *Bull. Soc. Chim. France* 896 (1959).
[3] M.-M. JANOT, A. CAVÉ and R. GOUTAREL, *Compt. Rend.* 251, 559 (1960).
[4] M. LEBOEUF, A. CAVÉ and R. GOUTAREL, *Compt. Rend.* 259, 3401 (1964).
[5] E. HEFTMANN and E. MOSETTIG, *Biochemistry of Steroids*, Reinhold, New York (1960).
[6] R. D. BENNETT and E. HEFTMANN, *Phytochem.* 4, 475 (1965).



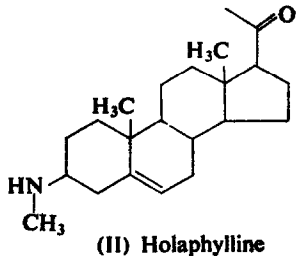
(IV) Progesterone



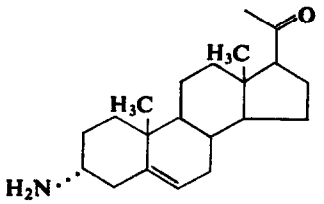
(V) Pregnenolone



(I) Holaphyllamine



(II) Holaphylline



(III) Holamine

TABLE 1. RADIOACTIVITY OF FRACTIONS FROM LEAVES TREATED WITH RADIOACTIVE STEROIDS

		Compound administered					
		Progesterone-4-^{14}C			Pregnenolone-4-^{14}C		
		Properties of fractions isolated					
Fraction	Character	Mg	Counts/min $\times 10^4$	Per cent of administered radioactivity	Mg	Counts/min $\times 10^4$	Per cent of administered radioactivity
A	Acidic and neutral glycosides	33	49·2	22	31	22·3	8
B	Basic glycosides	2	4·1	2	2	2·3	1
C	Neutral	77	31·0	14	37	149	53
D	Basic	4	50·7	23	4	24·9	9
Total		116	135	61	74	199	71

A second plant was then treated with pregnenolone-4-^{14}C in the same manner. Although the incorporation into the alkaloid fraction was lower in this case (9 per cent), the two known alkaloids present in quantity were radioactive, and the third, holamine, was probably also labelled (Fig. 3). The holaphylline and holaphyllamine were isolated by preparative TLC

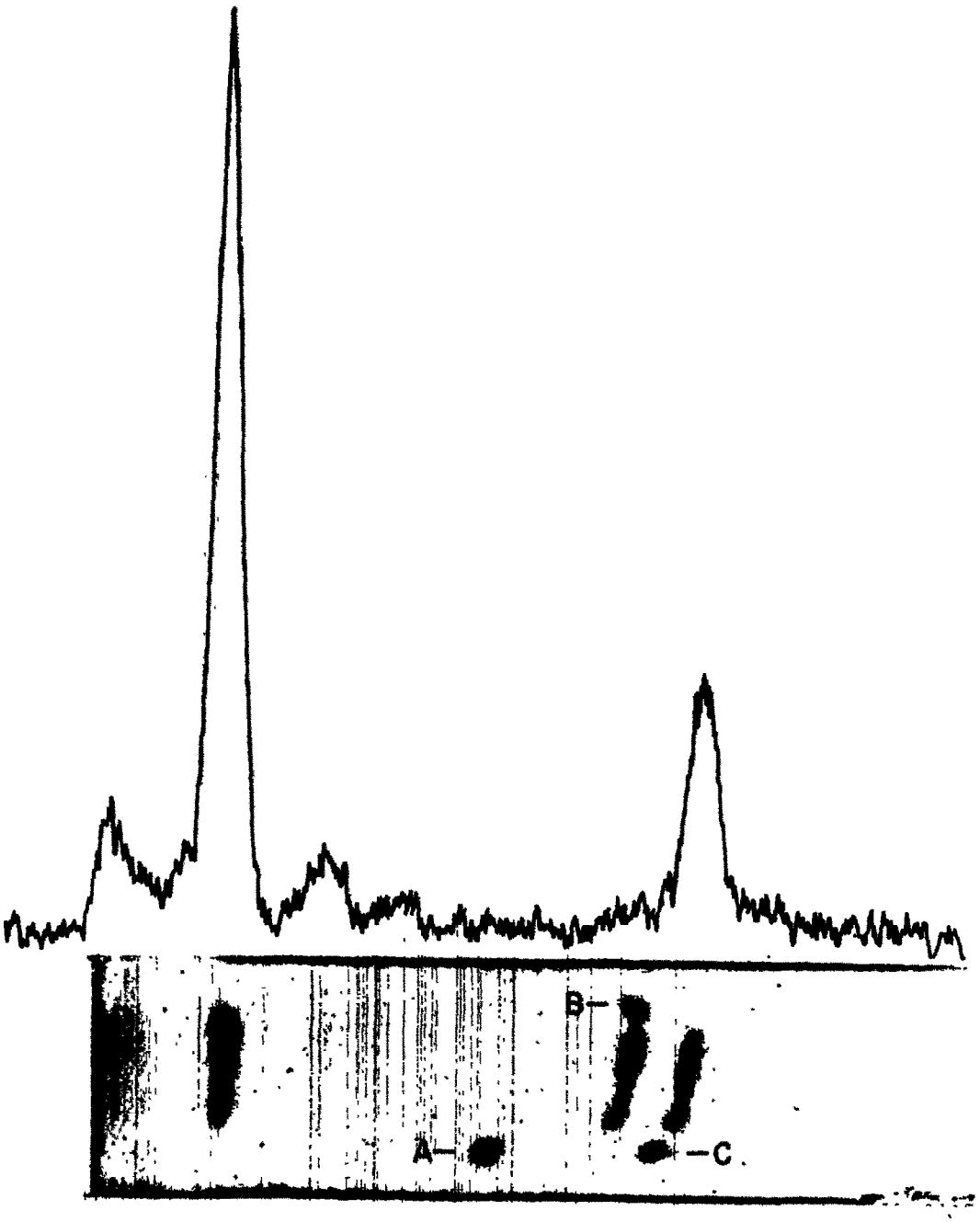


FIG. 1. RADIOCHROMATOGRAM OF BASIC EXTRACT OF HOLARRHENA LEAVES TREATED WITH PROGESTERONE-4-^{14}C.

Origin is at right, solvent front at left. Standards are: A, holamine; B, holaphyllamine; C, holaphylline. An alkaline Silica Gel G plate was developed twice with dichloromethane:2-propanol:methanol (15:3:2) and scanned at 0·75 in./hr, using a time constant of 100 sec and a slit width of 3 mm. The chromatogram was sprayed with 50% sulfuric acid and charred on a hot plate.

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FIG. 2. RADIOCHROMATOGRAM OF ZONE CORRESPONDING TO HOLAPHYLLINE, ISOLATED BY PREPARATIVE TLC (SEE FIG. 1), AFTER ACETYLATION.

Origin is at right, solvent front at left. Standards are both *N*-acetylholaphylline. Holaphylline remains at the origin in this system. A Silica Gel G plate was developed twice with dichloromethane: acetone (9:1) and scanned at 0·75 in./hr, using a time constant of 100 sec and a slit width of 3 mm.

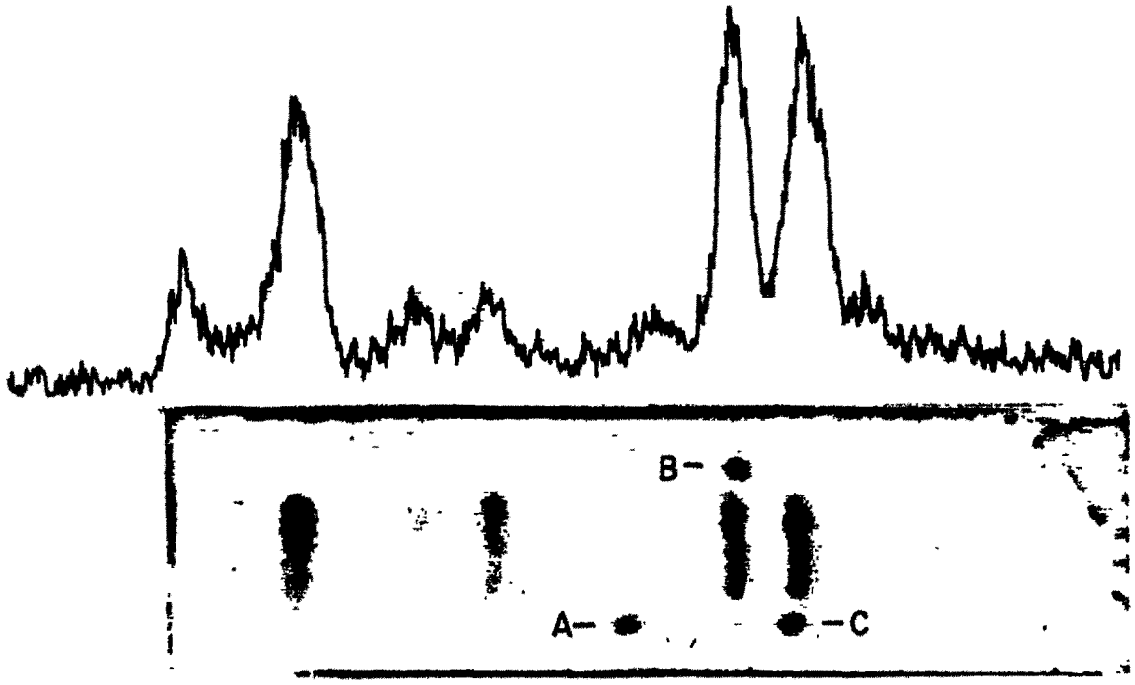

FIG. 3. RADIOCHROMATOGRAM OF BASIC EXTRACT OF HOLARRHENA LEAVES, TREATED WITH PREGNENOLONE-4-^{14}C. Conditions and standards as in FIG. 1.

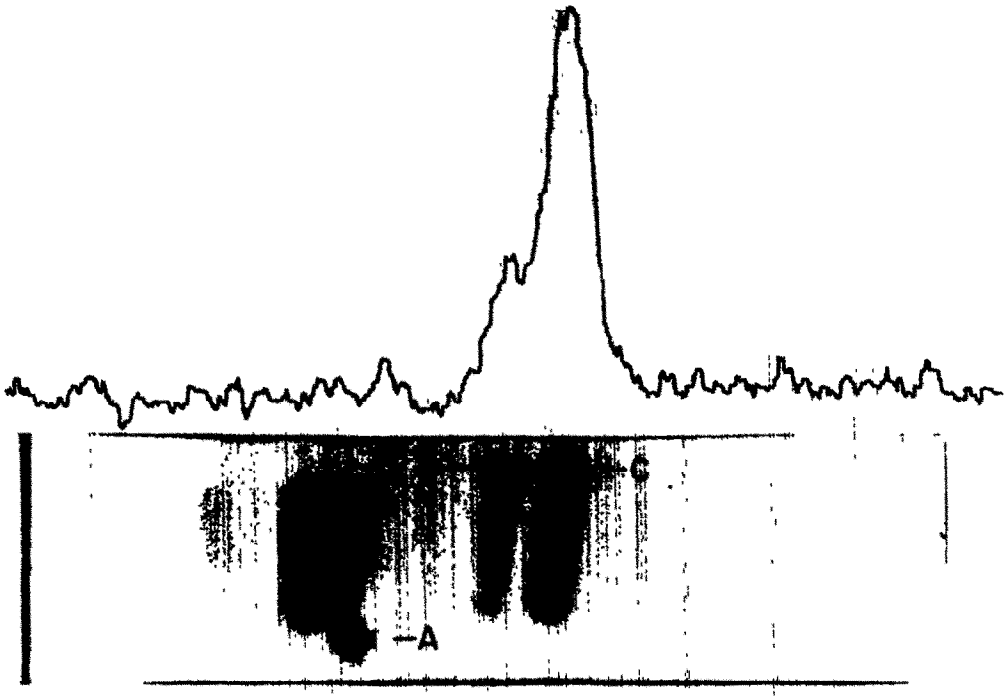

FIG. 4. RADIOCHROMATOGRAM OF ZONE CORRESPONDING TO *N*-ACETYLHOLAPHYLLINE, ISOLATED BY PREPARATIVE TLC, AFTER EPOXIDATION.

Origin is at right, solvent front at left. Standards are: A, *N*-acetylholaphylline; B and C, the two epoxides of *N*-acetylholaphylline. A Silica Gel G plate was developed twice with dichloromethane: acetone (7:3) and scanned at 0·75 in./hr, using a time constant of 300 sec and a slit width of 6 mm.

and acetylated. TLC showed that the former also contained a more polar radioactive material, apparently the same as that obtained in the progesterone experiment (Fig. 2). The radioactive *N*-acetylholaphylline and *N*-acetylholaphyllamine were each purified by preparative TLC. An aliquot of the *N*-acetylholaphylline fraction was epoxidized with *p*-nitroperbenzoic acid. A radiochromatogram of the product (Fig. 4) showed two radioactive peaks which corresponded both in mobility and approximate proportion to the two epoxides formed from authentic *N*-acetylholaphylline. Similar results were obtained upon epoxidation of the *N*-acetylholaphyllamine fraction.

The two acetylated alkaloid fractions were each diluted with authentic carrier material. The specific activities of these samples were not changed by recrystallization from three different solvents (Table 2).

TABLE 2. RECRYSTALLIZATION OF RADIOACTIVE *N*-ACETYL ALKALOIDS*

Compound	Solvent used for crystallization	Counts/min/μM
N-Acetylholaphylline		171 ± 8
	Hexane-acetone	174 ± 8
	Hexane-ethyl acetate	175 ± 8
	Hexane-benzene	178 ± 8
N-Acetylholaphyllamine		169 ± 8
	Hexane-acetone	162 ± 8
	Ethyl acetate	162 ± 8
	Benzene	168 ± 8

* 0·2-mg aliquots were plated from chloroform solutions on copper planchets over an area of 2·8 cm² and counted in duplicate, using a Nuclear Chicago Model D–47 counter with micromil window mounted in a Model C–110B sample changer, to the 0·9 level of confidence.

The radioactivity of the neutral fraction of the pregnenolone-treated leaves was almost entirely accounted for by unchanged pregnenolone, although TLC indicated some conversion to progesterone. Examination of the neutral fraction from the progesterone experiment, on the other hand, revealed that the latter had been extensively metabolized, largely to more polar material. Investigations into the nature of these products are now in progress.

DISCUSSION

From Fig. 1 it appears that five different alkaloids, all unknown, are formed from progesterone, while Fig. 3 indicates that pregnenolone is a precursor of these same five compounds and in addition the three known alkaloids. However, based on the relative incorporation of radioactivity in the two experiments, progesterone is probably a more direct precursor of the unknown alkaloids than is pregnenolone. This suggests that they are biosynthesized from pregnenolone via progesterone. The fact that the neutral fraction from the pregnenolone-treated leaves apparently contained radioactive progesterone is evidence in favor of this view.

The identity of the precursor which is aminated to form the known alkaloids is of considerable interest, since it is obviously not progesterone. Although amine biosynthesis often occurs by transamination of a ketone, in this instance pregnenolone appears to be directly converted to holaphyllamine. This would involve the replacement of a hydroxyl by an amino group, a reaction which is analogous to the amination of sugars and hydroxypurines by

ammonia or the amide group of glutamine.[7] An alternate possibility could be the transamination of Δ^5-pregnene-3, 20-dione, which might be the precursor both of the alkaloids and progesterone. However, Δ^5-3-ketones are easily isomerized to the more stable Δ^4-form and, to our knowledge, have never been found in nature.

The rapid turnover of progesterone in the leaves shows that it is functioning as a precursor of other compounds, whose identity and biological significance are as yet unknown. The possibility that progesterone itself has a physiological function in the plant also cannot be excluded.[8]

EXPERIMENTAL

Methods

Thin-layer chromatographic techniques were as described in previous papers,[6,9] except that alkaline Silica Gel G plates, made by substituting 0·1 N NaOH for water, were used for chromatography of free alkaloids. All solvent systems were saturated with water.[6] For repeated or continuous development and for preparative TLC an antioxidant, 2,6-di-*t*-butyl-*p*-cresol, was added in 0·002 per cent concentration to the solvent systems.[10]

Melting points were taken on a Kofler block and are corrected.

Aliquots of radioactive samples were counted on planchets at infinite thinness (except for material diluted with carrier) under a gas flow detector (see Table 2, legend, for details).

Materials

Progesterone-4-^{14}C and pregnenolone-4-^{14}C, both having a specific activity of 45·8 μc/μM, were purchased from New England Nuclear Corporation. *Holarrhena floribunda* plants were obtained from the Los Angeles County and State Arboretum, Arcadia, California, through the courtesy of Dr. W. S. Stewart. Samples of authentic holaphylline, holaphyllamine, and holamine were generously supplied by Dr. Robert Goutarel, C.N.R.S., Gif-sur-Yvette, France.

Administration of Radioactive Precursors

Progesterone-4-^{14}C ($2{\cdot}2 \times 10^5$ counts/min) or pregnenolone-4-^{14}C ($2{\cdot}8 \times 10^5$ counts/min) in acetone solution was applied to several leaves of a plant approximately 15 cm tall and growing in soil. The leaves were then sprayed with a petroleum ether solution of Silicone DC-200 (Chromatospray*). A total of ten such treatments were given, three times a week.

Extraction and Fractionation of Leaves

Three days 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 tissue grinder with Teflon pestle, using 40 ml of water and enough 2 N NaOH to keep the pH above 9. The solution was separated by centrifugation and the residue was re-extracted with four 10-ml portions of water. The aqueous solutions were combined, diluted with 50 ml of water, and extracted with three 80-ml portions of dichloromethane. Each extract was passed through two 20-ml portions of water; these were finally extracted with 10 ml of dichloromethane,

* Research Specialties Co., Richmond, California.

[7] A. MEISTER, in *The Enzymes* (Edited by P. D. BOYER, H. LARDY, and K. MYRBÄCK) (2nd Ed.) Vol. 6, p. 247, Academic Press, New York (1962).
[8] E. HEFTMANN, *Ann. Rev. Plant Physiol.* **14**, 225 (1963).
[9] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 577 (1965).
[10] J. J. WREN and A. D. SZCZEPANOWSKA, *J. Chromatog.* **14**, 405 (1964).

which was combined with the other dichloromethane extracts and evaporated. This extract was pooled with an extract obtained by boiling the leaf residue with 400-ml acetone for 3 hr (basic-neutral fraction).

The aqueous solutions from above were combined with the leaf residue, made 3 N by addition of conc. HCl, overlaid with 100 ml of benzene, and refluxed for 3 hr. The benzene layer was separated and the aqueous layer extracted with two 100-ml portions of dichloromethane. Each organic layer was passed through 25 ml of water, which was back-extracted with 10 ml of dichloromethane. The organic layers were combined and evaporated (Fraction A, Table 1).

The aqueous layer from above was combined with the water washes, made basic with KOH, and extracted with two 100-ml portions of dichloromethane. Each extract was passed through 50 ml of water, which was back-extracted with 20 ml of dichloromethane. The organic extracts were combined and evaporated (Fraction B, Table 1).

The basic-neutral fraction described earlier was taken up in 250 ml of benzene and extracted with two 50-ml portions of 0·5 N HCl and 25 ml of water. Each extract was passed through 25 ml of benzene, which was later combined with the benzene solution and evaporated (Fraction C, Table 1).

The aqueous extracts were made basic with KOH and extracted with two 50-ml portions of dichloromethane. Each extract was passed through 25 ml of water, which was back-extracted with 10 ml of dichloromethane. The dichloromethane extracts were combined and evaporated, and the residue was taken up in 50 ml of benzene. This solution was extracted with two 25-ml portions of 0·5 N HCl and one 10-ml portion of water. Each extract was passed through 10 ml of benzene; then they were combined, made basic with KOH, and extracted with two 25-ml portions of dichloromethane. Each extract was passed through 10 ml of water, which was back-extracted with 5 ml of dichloromethane. The extracts were combined and evaporated (Fraction D, Table 1).

Isolation of Alkaloids

TLC of an aliquot of Fraction D from the progesterone-treated leaves showed the presence of two major radioactive components, one of which corresponded to holaphylline (Fig. 1). An aliquot representing one-tenth of this fraction was subjected to preparative TLC in the same system, and the zone corresponding to holaphylline was removed and eluted with acetone-methanol, giving 5900 counts/min. TLC of an aliquot of this material on an alkaline Silica Gel G plate, developed continuously[11] with dichloromethane:methanol (93:7) for 6 hr, revealed that the radioactivity was actually associated with a more polar material, rather than with holaphylline. This was confirmed by acetylation. After treatment with acetic anhydride:pyridine (1:1) for 2 days at 25°, TLC showed that the *N*-acetylholaphylline migrated ahead of the radioactive peak (Fig. 2).

Since TLC indicated that both holaphylline and holaphyllamine were radioactive in Fraction D from the pregnenolone-treated plant (Fig. 3), nine-tenths of this fraction was subjected to preparative TLC in the system given in Fig. 1. The zones corresponding to these two alkaloids were removed and eluted: holaphylline, 0·6 mg, 38,000 counts/min; holaphyllamine, 0·9 mg, 38,000 counts/min.

TLC of an aliquot of the holaphylline fraction on an alkaline Silica Gel G plate by continuous development with dichloromethane:methanol (23:2) for 7 hr showed two radioactive peaks, one corresponding to holaphylline and the other to slightly more polar material.

[11] R. D. Bennett and E. Heftmann, *J. Chromatog.* **12**, 245 (1963).

The remainder of this fraction was acetylated as above. TLC of an aliquot of the acetylated material, by developing a Silica Gel G plate twice with dichloromethane: acetone (9:1), also revealed two radioactive peaks, one with the same mobility as *N*-acetylholaphylline and the other corresponding roughly to the radioactive material in Fig. 2. The *N*-acetylholaphylline was isolated by preparative TLC in the same system, giving 0·6 mg (10,000 counts/min). An aliquot of this material was dissolved in 2 ml of benzene: ether (1:1), 1 mg of *p*-nitroperbenzoic acid[12,13] was added, and the solution was allowed to stand overnight. After addition of 1 ml 10% Na_2CO_3 and thorough mixing, the organic layer was separated and the aqueous layer was extracted with two 1-ml portions of ether. The organic layers were combined and evaporated. A radiochromatogram (Fig. 4) of the product showed that the radioactivity was entirely associated with the epoxides, identified by comparison with material prepared by treatment of authentic *N*-acetylholaphylline as above.

An aliquot of the radioactive *N*-acetylholaphylline, representing 5000 counts/min, was diluted with 10·5 mg of authentic *N*-acetylholaphylline (for preparation see below) and recrystallized from three different solvents as shown in Table 2, without decrease in specific activity.

The holaphyllamine fraction described above appeared radiochemically pure by TLC of an aliquot on an alkaline Silica Gel G plate, developed continuously with dichloromethane: methanol (93:7) for 6 hr. After acetylation as above, an aliquot also gave only a single radioactive peak corresponding to *N*-acetylholaphyllamine by TLC on a Silica Gel G plate, developed twice with dichloromethane: acetone (17:3). By preparative TLC in the same system, the *N*-acetylholaphyllamine was freed of some non-radioactive contaminants, giving 0·5 mg (18,000 counts/min). An aliquot of this material was epoxidized as above; all of the radioactivity of the product was associated with the two epoxides of *N*-acetylholaphyllamine, as shown by TLC on a Silica Gel G plate developed twice with dichloromethane: acetone (17:3).

An aliquot of the radioactive *N*-acetylholaphyllamine, representing 5000 counts/min, was diluted with authentic *N*-acetylholaphyllamine (for preparation see below) and recrystallized from three different solvents as shown in Table 2, without decrease in specific activity.

Preparation of N-*Acetyl Alkaloids*

Holaphyllamine hydrochloride (19 mg) was dissolved in 3 ml of water, containing a few drops of methanol. The solution was made basic with 2 N NaOH and extracted with three 2-ml portions of benzene. The benzene extracts were combined, evaporated, and taken up in 1 ml of dry pyridine and 1 ml of acetic anhydride. The solution was kept in the dark at 25° for 2 days, after which it was added slowly to 25 ml of 1 N HCl. The mixture was extracted with three 10-ml portions of dichloromethane, each extract being passed through 10 ml of 10% Na_2CO_3 and filtered. The combined extracts on evaporation gave 18·9 mg of *N*-acetylholaphyllamine, homogeneous by TLC. After recrystallization from acetone its melting point was 227–228°.

Holaphylline (15 mg) was acetylated and the product isolated in the same way, giving 16·4 mg of *N*-acetylholaphylline, homogeneous by TLC. After recrystallization from acetone, its melting point was 202–203°.

Acknowledgement—The authors gratefully acknowledge the assistance of Mrs. Cornell Phillips in taking care of the plants.

[12] M. VILKAS, *Bull. Soc. Chim. France* 1401 (1959).
[13] C. MATHIS and G. OURISSON, *J. Chromatog.* 12, 94 (1963).

Note added in proof. We have now verified that pregnenolone is converted to progesterone by *H. floribunda* leaves,[14] as suggested in this paper (see Results).

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