Phytochemistry, 1970, Vol. 9, pp. 565 to 568. Pergamon Press. Printed in England.

BIOSYNTHESIS OF PLANT STEROLS AND TRITERPENOIDS. THE INCORPORATION OF (3RS)-[2-^{14}C, (4R)-4-$^{3}H_1$] MEVALONATE INTO α-SPINASTEROL AND β-AMYRIN IN *CAMELLIA SINENSIS*

RAMESHWAR K. SHARMA

Veterans Administration Hospital and Department of Biochemistry, College of Basic Medical Sciences, University of Tennessee, Memphis, Tennessee 38104, U.S.A.

(*Received* 22 *July* 1969)

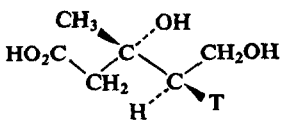
Abstract—The biosynthesis of α-spinasterol and β-amyrin has been investigated in *Camellia sinensis*. Administration of doubly labeled 4R,4-^{3}H-2-^{14}C-mevalonic acid led to the formation of radioactive α-spinasterol and β-amyrin. In the case of α-spinasterol, the $^{3}H/^{14}C$ ratio was found to be 2·44:5·00 rather than 3:5. The $^{3}H/^{14}C$ ratio of the biosynthesized β-amyrin was found to be 1·0:1·0, which is consistent with an earlier study in *Pisum sativum*.

INTRODUCTION

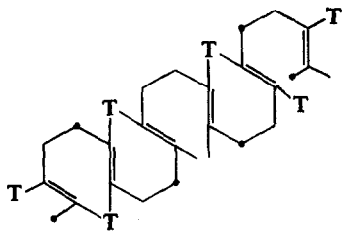
A CONSIDERABLE amount of research has been devoted to understanding the processes by which plant sterols are biosynthesized in plants. Recent investigations have indicated that the formation of steroids in plants follow the same pathways as in animals.[1,2] Although it has been tacitly assumed that the pattern of distribution of radioactivity in the triterpenoids and plant sterols is the same as in animals, it is important that the study be conducted in a range of plants in order to confirm this.

Relatively little information is available on the biosynthesis of C-29 phytosterols which have the ethyl side-chain linked to C-24. Incorporation of radioactive acetate and mevalonate into phytosterols has been demonstrated in some plant systems[3-6] and it appears that C-29 phytosterols are formed by the route mevalonic acid (I) → squalene (II) → either cycloartenol (III) or lanosterol (IV) → C-29 phytosterols. However, the steps after lanosterol are still not clear. Desmosterol and 24-methylenecholesterol are probably the intermediates through which these sterols are biosynthesized after the cycloartenol stage.[7] Recently it has been shown that 1,2-H-migration takes place during the alkylation of the Δ^{24}-bond.[8,9] It was of interest to determine whether there is the possibility of the operation of several routes after the cycloartenol (or lanosterol) stage. To decide between these possibilities, it is necessary to determine the distribution of the radioactivity of the incorporated precursors (especially that of mevalonic acid) in the biosynthesized phytosterols and triterpenoids, in our case specifically α-spinasterol and β-amyrin.

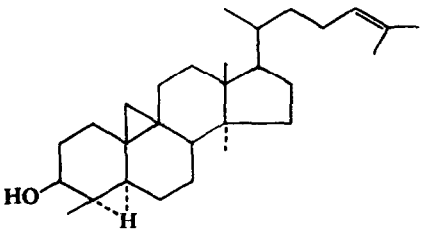
[1] E. HEFTMANN, *Lloydia* **30**, 209 (1967).
[2] I. D. FRANTZ and G. J. SCHROEPFER, JR., *Ann. Rev. Biochem.* **36**, 691 (1967).
[3] S. BADER, L. GUGLIELMETTI and D. ARIGONI, *Proc. Chem. Soc.* 16 (1964).
[4] D. BAISTED, E. CAPSTACK and W. R. NES, *Biochemistry* **1**, 537 (1962).
[5] H. J. NICHOLAS, *Nature* **189**, 143 (1961).
[6] A. R. H. SMITH, L. J. GOAD and T. W. GOODWIN, *Chem. Comm.* 926 (1968).
[7] E. LEDERER, *Biochem. J.* **93**, 449 (1964).
[8] K. H. RAAB, J. DESOUZA and W. R. NES, *Biochim. biophys. Acta* **152**, 742 (1968).
[9] M. AKHTAR, P. F. HUNT and M. A. PARVEZ, *Biochem. J.* **103**, 1616 (1967).



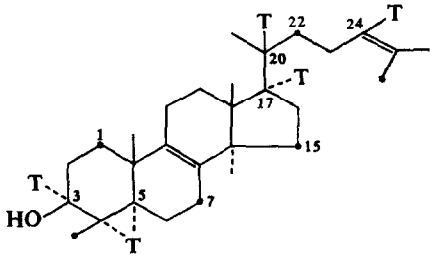
Mevalonic acid (I)



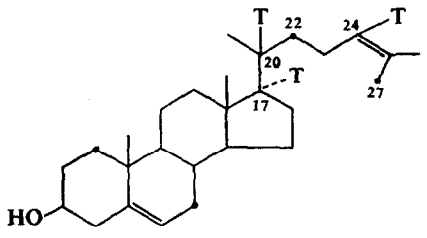
Squalene (II)



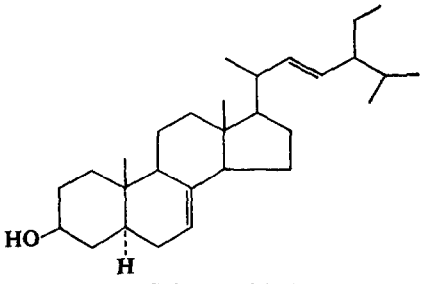
Cycloartenol (III)



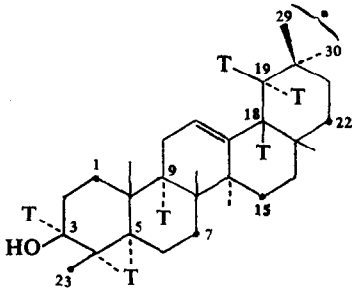
Lanosterol (IV)



Desmosterol (V)



α-Spinasterol (VI)



β-Amyrin (VII)

• = ^{14}C
T = ^{3}H

With a view to obtaining more information regarding the biogenetic processes by which plants elaborate phytosterols and triterpenoids, a mixture of (3RS)-(2-C^{14})-mevalonic acid and (3RS)-(4R)-4-$^{3}H_1$-mevalonic acid dibenzylethylenediamine salt, was administered to *Camellia sinensis*, a plant not previously used for biosynthetic studies.

RESULTS AND DISCUSSION

A mixture of (3RS)-[(4R)-4-$^{3}H_1$]-mevalonic acid dibenzylethylenediamine salt (250 μc) and (3RS)-(2-^{14}C)-mevalonic acid (50 μc) ($^{3}H/^{14}C$ ratio 5·37) was administered by the "brush"

method. The tea plant was harvested after 5 weeks and processed in the manner described earlier.[10, 11] α-Spinasterol (VI) and β-amyrin (VII) were isolated from the ligroin extract. The $^3H/^{14}C$ ratios of the isolated α-spinasterol and β-amyrin were determined (Table 1).

TABLE 1. THE $^3H/^{14}C$ RATIOS OF THE PRODUCTS RECOVERED AFTER THE ADMINISTRATION OF (3RS)-[(4R)-4-$^3H_1$]-MEVALONIC ACID (250 μc) AND (3RS)-(2-^{14}C)-MEVALONIC ACID (50 μc) ($^3H/^{14}C$ RATIO 5·37) TO *Camellia sinensis*

	$^3H/^{14}C$ Ratio
β-Amyrin	
First crystallization	5·68
Second crystallization	5·46
Third crystallization	5·38
Fourth crystallization	5·46
α-Spinasterol	
First crystallization	1·73
Second crystallization	2·09
Third crystallization	2·24
Fourth crystallization	2·62
Fifth crystallization	2·67

Desmosterol (V) or the analogous C-27 intermediate formed in the biogenetic sequence from (3RS)-[2-^{14}C(4R)-4-$^3H_1$]-mevalonic acid (I) ($^3H/^{14}C$ ratio 5·37), should have three positions labeled with ^3H (at C-17, C-20 and C-24) and five with ^{14}C (at positions 1, 7, 15, 22 and 27), with a theoretical $^3H/^{14}C$ ratio of $5{\cdot}37 \times 3/5 = 3{\cdot}22$. The same ratio should be observed even if 24-methylene derivative is formed as an intermediate, since such an intermediate will involve a carbonium ion at C-25 and ^3H from C-24 position will be transferred to the C-25 position.[8, 9] On the other hand, if the ^3H does not shift to C-25 during the carbonium ion intermediate, the complete loss of ^3H should occur during the formation of 24-methylene intermediate. Then in the α-spinasterol only two ^3H atoms (at C-17 and C-20) and five ^{14}C labeled atoms (at positions 1, 7, 15, 22 and 27) should persist. The calculated $^3H/^{14}C$ ratio should therefore be $5{\cdot}37 \times 2/5 = 2{\cdot}15$. The experimentally determined ratio of 2·67 appears to indicate that either none of the above routes operates, at least in the biosynthesis of α-spinasterol, or alternatively that the formation of α-spinasterol proceeds simultaneously by the two routes; one route involving the intermediate in which ^3H at C-24 is lost, possibly during the formation of 24-methylene intermediate and the other route in which ^3H is transferred to C-25 through the carbonium ion mechanism. Both pathways operating simultaneously at equal rates should give a ^3H and ^{14}C distribution of $5{\cdot}37 \times 2{\cdot}5/5 = 2{\cdot}68$. The experimental value found is 2·67. The observed value of $^3H/^{14}C$ ratio of 2·67 corresponding to the 2·44 atoms of ^3H and five atoms of ^{14}C is quite significant, since Rees *et al.*[12] found a different $^3H/^{14}C$ ratio (of 3:5) for β-sitosterol synthesized in pea seedlings. The results of Rees *et al.*[12] are consistent with those observed in the metabolism of liver cholesterol.[13] In view of the present findings with α-spinasterol in *Camellia sinensis*, we question

[10] J. A. F. WICKRAMSINGHE, E. P. BURROWS, R. K. SHARMA, J. B. GREIG and E. CASPI, *Phytochem.* **8**, 1433 (1968).
[11] J. VON EUW and T. REICHSTEIN, *Helv. Chim. Acta* **47**, 711 (1964).
[12] H. H. REES, E. I. MERCER and T. W. GOODWIN, *Biochem. J.* **99**, 726 (1966).
[13] J. W. CORNFORTH, R. H. CORNFORTH, C. DONNINGER, G. POPJÁK, Y. SHIMIZU, S. ICHII, E. FORSCHIELLI and E. CASPI, *J. Am. Chem. Soc.* **87**, 3224 (1965).

whether the mechanism of alkylation is different for different phytosterols or if it is the function of the plant being studied. Experiments to clarify this point are continuing. However, involvement of more than one pathway, at least in the biosynthesis of cardenolides, has been observed before.[10]

In the biosynthesis of β-amyrin in *C. sinensis*, if the biosynthetic steps take place as proposed by Eschenmoser *et al.*[14] six ^3H labeled atoms (at C-3, 5, 9, 18, 19, 19) and six ^{14}C-labeled atoms (at positions 1, 7, 15, 22, 29 and 23) should persist; the expected $^3H/^{14}C$ ratio of 5·37 is in good agreement to the observed value of 5·46. Recently, Rees *et al.*[15] in investigating the biosynthesis of β-amyrin in pea seedlings, have also confirmed the hypothesis of Eschenmoser *et al.*[14]

EXPERIMENTAL

Characterization of α-spinasterol and β-amyrin. The characterization of cold α-spinasterol and β-amyrin isolated from *Camellia sinensis* leaves was done by high resolution NMR and mass spectroscopy.[16] Both of these compounds were further checked for their purity by GLC.

Chromatography. Silica gel (Merck $HF_{254+366}$) was used for TLC in the indicated solvent systems. Thin-layer plates were used for the final purification of α-spinasterol and β-amyrin. Chromatographically homogeneous products were further checked for purity and identity by cocrystallization to constant specific activity and constant $^3H/^{14}C$ ratio.

Counting. Counting was carried out in a Nuclear Chicago, automatic liquid scintillation counter, Unilux II. The samples were dissolved in 15 ml of a scintillation solution of toluene containing 4 g 2,5-diphenyloxazole and 100 mg of *p*-bis-2-(5-phenyloxazolyl)-benzene, per 1000 ml.

Administration of (3*RS*)-[2-^{14}C, (4*R*)-4-$^3H_1$]-*mevalonic acid to* Camellia sinensis. (3RS)-[(4R)-4-$^3H_1$]-mevalonic acid dibenzylethylenediamine salt (250 μc), prepared according to the procedure of Cornforth *et al.*,[17] was mixed with (3RS)-(2-^{14}C)-mevalonic acid (50 μc) ($^3H/^{14}C$ ratio 5·37) and administered to a *C. sinensis* plant by the "brush" method. The plant was illuminated and watered as necessary. After 5 weeks the plant was harvested and processed as previously described.[10,11]

Isolation of α-spinasterol. The isolation of α-spinasterol was carried out on the hydrolysate of the ligroin extract by TLC using benzene–EtOAc–MeOH (90:9:1). The α-spinasterol zone was extracted, then purified by TLC on silica gel impregnated with 10% (v/v) $AgNO_3$ using the above solvent. The radioactivity corresponding to the α-spinasterol was extracted. This extract was diluted with non-radioactive α-spinasterol (9·7 mg) and rechromatographed sequentially on silica gel-impregnated $AgNO_3$ plates in $CHCl_3$ and in the benzene–EtOAc–MeOH mixture. The purified radioactive zone of α-spinasterol was extracted and crystallized several times from absolute ethanol until the specific activity and $^3H/^{14}C$ ratio was constant (Table 1). On the basis of the ^3H and ^{14}C present in the α-spinasterol crystallized to constant specific activity and constant ratio, the α-spinasterol in the total ligroin extract contained $1·833 \times 10^4$ dpm of ^{14}C (equivalent to the incorporation of 0·0167% of ^{14}C) and $4·894 \times 10^4$ (equivalent to the incorporation of 0·008898% of ^3H).

Isolation of β-amyrin. β-amyrin was isolated from the hydrolysate of the ligroin extract by TLC using the same benzene mixture. The β-amyrin zone was located under u.v. light, and the area of radioactivity corresponding to the zone was extracted from the chromatogram. The extract was further purified by TLC as for α-spinasterol. The purified β-amyrin was diluted with non-radioactive β-amyrin (8·12 mg) and crystallized several times from acetone until the specific activity and $^3H/^{14}C$ ratio was constant (Table 1). On the basis of ^3H and ^{14}C present in the β-amyrin cocrystallized to constant specific activity and constant ratio, the β-amyrin in the total ligroin extract contained $2·1112 \times 10^4$ dpm of ^{14}C (equivalent to the incorporation of 0·0028% of ^{14}C) and $11·633 \times 10^5$ dpm of ^3H (equivalent to the incorporation of 0·02115% of ^3H.

Acknowledgement—The author thanks Professor William R. Nes, Drexel Institute of Technology, Philadelphia, Pennsylvania, for stimulating discussion during this study.

[14] A. ESCHENMOSER, L. RUZICKA, O. JEGER and D. ARIGONI, *Helv. Chim. Acta* **38**, 1850 (1955).
[15] H. H. REES, G. BRITTON and T. W. GOODWIN, *Biochem. J.* **106**, 659 (1968).
[16] R. K. SHARMA, to be published.
[17] J. W. CORNFORTH, R. H. CORNFORTH, C. DONNINGER and G. POPJÁK, *Proc. Roy. Soc.* Ser. B **163**, 492 (1966).