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INCORPORATION OF LABEL FROM GERANIOL-^{14}C INTO SQUALENE, β-AMYRIN AND β-SITOSTEROL IN GERMINATING PEA SEEDS*

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Abstract—Label from geraniol-^{14}C was incorporated into squalene, β-amyrin and sterol, principally β-sitosterol, to the extent of 0·41, 1·41 and 0·09 per cent respectively in germinating seeds of *Pisum sativum*. Modes of utilization of this substrate are discussed.

INTRODUCTION

THE participation of the pyrophosphates of the isoprenoid alcohols, dimethylallyl alcohol, isopentenyl alcohol, geraniol and farnesol in the biosynthesis of higher isoprenoids has been established.[1] With one exception, very low incorporations of the free alcohols into higher isoprenoids have been reported. Thus, light petroleum-soluble compounds, one of which was farnesol, obtained from mevalonic acid-2-^{14}C in the rat liver enzyme system have been incorporated into digitonin-precipitable sterols in the same system to the extent of 60 per cent, of which 4 per cent was found in cholesterol.[2] In contrast, Goodman and Popjak[3] reported that the incorporation of farnesol was insignificantly small into either the squalene or allyl pyrophosphate fractions. In yeast extract however, a small but significant incorporation (< 0·2%) of farnesol into squalene has been observed.[4]

The incorporation of both dimethylallyl alcohol-4,4'-^{14}C and dimethylacrylaldehyde-4, 4'-^{14}C into β-carotene by *Euglena gracilis* has been demonstrated by Steele and Gurin.[5] More recently, geraniol-^{14}C and citral-^{14}C have been shown[6] to be incorporated into β-carotene in carrot slices to the extent of 0·6 and 0·5 per cent respectively.

The bacterial degradation of the alcohols, citronellol, geraniol and farnesol has been shown[7, 8] to proceed in the bacterium *Pseudomonas citronellolis* by an initial oxidation of the primary alcohol function. Subsequent oxidation to acetate then occurs via coenzyme A intermediates.

The pea seed, known to be particularly efficient in the utilization of mevalonic acid-2-^{14}C

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[5] J. W. STEELE and S. GURIN, *J. Biol. Chem.* **235**, 2778 (1960).
[6] T. N. R. VARMA and C. O. CHICHESTER, *Arch. Biochem. Biophys.* **96**, 419 (1962).
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for the synthesis of β-amyrin[9] and squalene,[10] provides a useful system for examining the transformation of geraniol-^{14}C into these triterpenes. The present paper describes the incorporation of geraniol-^{14}C into the non-saponifiable fraction of pea seeds to the extent of 9·4% based on substrate utilized. Of this conversion 0·41% is found in squalene, 1·41% in β-amyrin and 0·09% in sterol, principally β-sitosterol.

RESULTS

Geraniol-^{14}C was applied to germinating seeds of *Pisum sativum* (Burpee's Blue Bantam). Absorption of substrate was enhanced by lightly spraying the seeds with a light petroleum solution of silicone oil. After 24 hr in continuous light, the seeds were washed with water and hexane, then extracted with hot acetone. The extract was evaporated and saponified. Radioactivity measurements were made on all extracts and gas–liquid radiochromatographic analyses for geraniol-^{14}C made on the acetone and non-saponifiable extracts. The results are shown in Table 1.

TABLE 1. DISTRIBUTION OF ^{14}C IN THE EXTRACTS MADE DURING THE ISOLATION OF THE NON-SAPONIFIABLE MATERIAL FROM PEA SEEDS GERMINATED IN THE PRESENCE OF GERANIOL-^{14}C

		Activity (dpm × 10^{-6})
I.	Activity administered	2·077
II.	Washings	0·042
III.	Acetone extract	1·706
IV.	Geraniol-^{14}C in III+	0·210
V.	Aqueous saponifiable	1·242
VI.	Non-saponifiable	0·211
VII.	Geraniol-^{14}C in VI+	0·040

Radioactivity in the extracts was measured on planchets at infinite thinness on a Tracerlab thin-window counter.

+ Geraniol separated gas chromatographically was trapped on silicone-coated anthracene then counted in a scintillation counter.

All counting was to the 0·9 level of confidence.

The non-saponifiable material was isolated and submitted to preparative thin-layer chromatography (TLC). The three major components, β-sitosterol, β-amyrin and squalene were isolated and diluted with carrier. The first two were crystallized to constant specific activity, whereas squalene was first converted to the hexahydrochloride before purification. The results are presented in Table 2. The incorporations of geraniol-^{14}C into the three components, estimated from the specific activity and total material present (carrier+ endogenous material*) are: squalene (7440 dpm); β-amyrin (25,700 dpm) and β-sitosterol (1700 dpm).

* Endogenous β-amyrin (1·1 mg) and β-sitosterol (5·5 mg) in the thirty seeds is calculated from the data presented in Ref. 9.

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[10] E. CAPSTACK, Jr., D. J. BAISTED, W. W. NEWSCHWANDER, G. BLONDIN, N. L. ROSIN and W. R. NES, *Biochemistry* **1**, 1178 (1962).

TABLE 2. CRYSTALLIZATION OF SQUALENE HEXAHYDROCHLORIDE, β-AMYRIN AND β-SITOSTEROL TO CONSTANT SPECIFIC ACTIVITY

Compound	Crystallization solvent	m.p. (°C)	Spec. act. dpm/mg
Squalene hexahydrochloride	Acetone	112·0–118·0	172 ± 6
	Acetone	107·0–109·0	135 ± 8
	Acetone	107·5–109·0	178 ± 10
	Acetone	107·5–109·0	176 ± 11
β-Amyrin	Chloroform–methanol	188·0–191·0	561 ± 23
	Methanol	193·5–195·0	655 ± 15
	Acetone–methanol	197·0–198·5	636 ± 11
	Methanol–water	197·5–198·0	652 ± 14
β-Sitosterol	Chloroform–methanol	138·5–139·5	96·2 ± 12·4
	Methanol	137·5–138·5	78·9 ± 3·8
	Acetone	137·5–138·5	75·6 ± 3·2
	Methanol–water	138·0–139·0	76·6 ± 3·5

Aliquots (0·30–0·60 mg) were plated from chloroform solution on aluminium planchets with area 8·0 cm^2 and counted on a Tracerlab thin-window counter to the 0·9 level of confidence.

DISCUSSION

The incorporation of label from geraniol-^{14}C into the three major higher isoprenoids of germinating pea seeds occurs to a significant extent. The preference for β-amyrin synthesis is in agreement with previous results.[9] It seems reasonable to assume that this transformation occurs by a pathway involving direct assimilation of geraniol via pyrophosphate derivatives. Such a pathway is supported by the recently indicated existence of a geraniol kinase in a higher plant.[12] That this is not the only metabolic route is revealed by inspection of Table 1: the aqueous saponifiable fraction ($1{\cdot}242 \times 10^6$ dpm) represents the largest proportion of the administered radioactivity ($2{\cdot}077 \times 10^6$ dpm). Furthermore, only $0{\cdot}17 \times 10^6$ dpm (IV–VII, Table 1) of this aqueous fraction can be attributed to free geraniol. It is unlikely that this fraction contains the pyrophosphate intermediates of the isoprenoid pathway in view of the considerable capacity for synthesis of higher isoprenoids known to exist at this stage of development of the pea seed.[11] Consequently, an additional and presumably dominant pathway(s) for the metabolism of geraniol appears to be operating. A route involving degradation to acetate would be in accord with the facts and would have a precedent in the work of Seubert and Remberger.[7] Such a degradation would involve an oxidation of geraniol to geranic acid which, via the CoA derivative and involvement of CO_2, would be degraded by the repeated removal of C_2 units. It is important to note that dimethylacrylyl CoA would be an intermediate in this pathway.

Interestingly, the unsaturation of geraniol and, in particular, the allylic nature of the alcohol function endows the substrate with a susceptibility towards photolytic oxidation. The geraniol to geranic acid transformation might very well occur by this process. It is

[11] W. R. NES, D. J. BAISTED, E. CAPSTACK, Jr., W. W. NEWSCHWANDER and P. T. RUSSELL, *Stereochemical and Evolutionary Aspects of Isopentenoid Biosynthesis*, Proceedings of the NATO Advanced Study Inst. on the Biochemistry of the Chloroplast, Aberystwyth, Wales, August 1965. Academic Press, New York, in press (1966).

[12] J. BATTAILLE and W. D. LOOMIS, *Phytochem.* 5, 423 (1966).

possible therefore that some transformation of the substrate may have occurred by these non-enzymatic routes prior to metabolism by the seed.

Though it is not possible to determine the contribution of the degradative pathway(s) to the incorporation of label into non-saponifiable material, it is probable that it is not substantial. Thus, the incorporation of label from geraniol-^{14}C into the non-saponifiable fraction is 9·4 per cent (see Experimental). It was earlier shown that incorporation of mevalonic acid-2-^{14}C under essentially the same conditions was about 35 per cent.[11] A comparison of the incorporations of mevalonic acid-2-^{14}C and dimethylacrylic acid-1-^{14}C over a 5-day period revealed incorporations of 29 and 0·05 per cent respectively.[9] We might conclude therefore that the degradative pathway(s) does not contribute substantially to the non-saponifiable fraction but is most likely responsible for the large proportion of radioactivity found in the aqueous saponifiable fraction.

The poor incorporations of isoprenoid alcohols into higher isoprenoids previously reported[3–6] may be accounted for by the dominance of such a degradative pathway rather than one involving direct assimilation via pyrophosphate derivatives.

It is noteworthy that an investigation of geraniol-^{14}C metabolism by *Pelargonium graveolens* (rose geranium) also resulted in a high proportion of water-soluble radioactivity.[13]

EXPERIMENTAL

Materials

Geraniol-^{14}C was prepared biosynthetically from *Pelargonium graveolens* grown in an atmosphere of $^{14}CO_2$.[13] The purified product showed a single radioactive spot, corresponding with authentic geraniol, by TLC on 0·25 mm thick layers of Silica Gel G in ethyl acetate–hexane (12–88, v/v). When subjected to gas chromatography (see Methods) 99 per cent of the recoverable radioactivity was associated with the geraniol peak. The specific activity was 0·73 μ c/mg.

Methods

Preparative TLC was carried out on a 1 mm layer of Silica Gel G spread on a 200 × 300 mm plate. The sample was applied as a band 15 cm wide and 15 mm from the bottom of the plate. Reference compounds were co-chromatographed each side of this band. The solvent mixture dichloromethane–acetone (99–1) was allowed to ascend to a line drawn 5 cm from the top of the plate. The chromatogram was scanned for radioactivity by placing a plastic mask with a rectangular slit (30 × 0·5 mm) on the plate and counting each successive area from origin to solvent front with a Tracerlab thin-window counter. Zones, identified with Rhodamine 6G, were found for β-sitosterol, R_f 0·21; β-amyrin, R_f 0·31; and squalene, R_f 0·95. The corresponding radioactive areas were scraped from the plate and each one extracted with a solution containing a known weight of the appropriate carrier in ether–ethanol (9–1) in centrifuge tubes. The adsorbents were extracted several more times with the solvent alone.

Aliquots of radioactive extracts were counted on planchets at infinite thinness. For specific activity determinations samples 0·30–0·60 mg were plated.

Gas chromatographic analyses for geraniol-^{14}C were carried out with a Beckman GC-2A equipped with a thermal conductivity detector. The column used was 6 ft × ¼ in. aluminium tubing, containing firebrick (100–120 mesh) coated with Quadrol and sucrose acetate isobutyrate, 4·2 and 1·6% (w/w) respectively. It was maintained at 144° with a helium flow of 60 ml/min. The geraniol fraction was trapped in a cartridge, 45 × 7 mm dia., containing

[13] D. J. BAISTED and W. D. LOOMIS, unpublished results.

silicone-coated anthracene* and plugged at one end with a cigarette filter. The cartridges, held in a Packard fraction collector, Tri-Carb Model 830, fitted tightly under the exit of the chromatograph. Radioactivity was counted on a Packard, Tri-Carb Model 314-DC scintillation counter.

Procedure

Thirty pea seeds were germinated for 15 hr in water containing 10 ppm of the fungicide "Phygon", then removed and partially dried with filter paper. A solution of geraniol-^{14}C ($2{\cdot}077 \times 10^6$ dpm) in hexane (0·1 ml) was uniformly distributed over the surface of the seeds. Immediately after the hexane had evaporated the seeds were lightly sprayed with a light petroleum solution of Silicone DC-200[14] (Chromatospray) and then kept moist for 24 hr under continuous illumination. The seeds appeared to suffer no injury and the rate of germination was unaffected.

Isolation of Non-saponifiable Material

The seeds were washed thoroughly with water and once with hexane to remove radioactivity (42×10^3 dpm) remaining on their surfaces. They were ground in a mortar with sand and acetone then transferred to a Soxhlet and extracted with the same solvent for 8 hr. A sample of the acetone extract† ($1{\cdot}706 \times 10^6$ dpm) was submitted to gas chromatography and the geraniol fraction trapped and counted ($0{\cdot}21 \times 10^6$ dpm). Thus, of the original $2{\cdot}077 \times 10^6$ dpm distributed over the seeds, a total radioactivity of $0{\cdot}252 \times 10^6$ dpm was recovered, presumably as unchanged geraniol. The non-saponifiable material was isolated from the evaporated acetone extract as previously described.[9] Of the radioactivity in the acetone extract, the aqueous saponifiable contained $1{\cdot}242 \times 10^6$ dpm and the ether extracted non-saponifiable $0{\cdot}211 \times 10^6$ dpm. Gas chromatographic analysis of a sample of the non-saponifiable extract revealed $0{\cdot}040 \times 10^6$ dpm associated with the geraniol peak. The transformation of geraniol-^{14}C into non-saponifiable material (NS) therefore occurs to the extent of 9·4 per cent.‡

The NS was subjected to preparative TLC along with squalene, β-amyrin and β-sitosterol as standards. Zones corresponding to the three were identified and extracted. The extraction of β-sitosterol was made with 16·5 mg of carrier and contained 4000 dpm; β-amyrin with 38·5 mg carrier and gave 30,100 dpm. Squalene (8900 dpm) was extracted along with 11·0 mg carrier to which an additional 11·0 mg was added. Squalene hexahydrochloride was isolated as previously described[10] and, diluted with additional squalene hexahydrochloride (equivalent to 5·5 mg squalene), was also crystallized to constant specific activity (see Table 1).

The geraniol-^{14}C present in the NS has R_f identical with β-sitosterol by TLC in the system used. It was necessary to establish that geraniol is removed during purification of β-sitosterol and β-amyrin. Crystallization of non-radioactive β-sitosterol (25 mg) in the presence of geraniol-^{14}C (57,900 dpm) removed all radioactivity after three crystallizations from methanol. That geraniol-^{14}C is likewise removed during the crystallization of β-amyrin was similarly established.

* Packard, Anthracene DC 550.

† Ten per cent of the acetone extract was held back for further investigations. Subsequent radioactivity data are corrected to account for this removal.

‡ Calculated from:

$$\frac{\text{activity in NS} - \text{activity of geraniol-}^{14}\text{C in NS}}{\text{activity administered} - (\text{activity in washings} + \text{activity of acetone extracted geraniol-}^{14}\text{C})} \times 100$$

[14] R. D. Bennett and E. Heftmann, *Phytochem.* 4, 577 (1965).