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BIOSYNTHESIS OF TRITERPENOIDS FROM AMINO ACIDS IN *PISUM SATIVUM*. THE DISTRIBUTION OF THE RADIOACTIVITY IN SQUALENE BIOSYNTHESIZED FROM RADIOISOTOPICALLY LABELLED L-LEUCINE AND L-VALINE

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Key Word Index—*Pisum sativum*; Leguminosae; biosynthesis; squalene; β-amyrin; leucine; valine; labelling patterns.

Abstract—Radioisotopically labelled L-leucine and L-valine were fed to *Pisum sativum* and incorporated into squalene and β-amyrin. Chemical degradation of the radioactive squalene revealed an equal distribution of the radioactivity in the isopentenyl pyrophosphate(IPP)-derived and the 3,3-dimethylallyl pyrophosphate(DMAPP)-derived moieties of the squalene molecule, unlike the unbalanced distribution in favour of the DMAPP-derived moiety of a monoterpenoid molecule biosynthesized from these amino acids by higher plants.

INTRODUCTION

During the biosynthesis of monoterpenoids from mevalonate-[2-^{14}C] by plant leaves, radioactivity was predominantly incorporated into the IPP-derived moiety [1–4]. By contrast the radioactivity from mevalonate-[2-^{14}C] was equally distributed in the IPP- and DMAPP-derived moieties of squalene biosynthesized by rat liver [5–7] and peas [8, 9]. Since the biosynthetic pathway to steroids from leucine via MVA in animal tissues has been described [10–13], such a pathway may be probable for higher plants, but the incorporation of leucine into monoterpenoids in higher plants has not been established [3, 14, 15]. However, we recently reported the first evidence of the incorporation of radioisotopically labelled leucine, valine and alanine into monoterpenoids in higher plants [16–19], as well as the localization of the radioactivity from leucine and valine in favour of the DMAPP-derived moiety of the monoterpenoids [16–18], in contrast with the uptake of alanine [19] and mevalonate [1–4]. We have now tested the incorporation of radioisotopically labelled L-leucine and L-valine into squalene and β-amyrin in *Pisum sativum*, and determined the distribution of the radioactivity of tracer in the IPP- and DMAPP-derived moieties of squalene.

RESULTS AND DISCUSSION

Leucine-[U-^{14}C], leucine-[4,5-^{3}H(N)] and valine-[U-^{14}C] were fed to germinating pea seeds maintained in the dark. After appropriate time intervals from the start of feeding, several germinating seeds were minced and immersed in acetone for 2 days. The acetone solution was evaporated, followed by extraction with ether. The ether-soluble material was saponified and extracted with ether. Squalene and β-amyrin were isolated from the non-saponifiable lipid by methods described in Experimental. GLC analysis showed isolated squalene and β-amyrin to be in a high state of purity. Their radioactivities are listed in Tables 1 and 2. These data indicate that L-leucine and L-valine are incorporated into squalene and β-amyrin, though at a low level, in germinating seeds of *Pisum sativum*.

Time-course studies of the incorporation of MVA-[2-^{14}C] into squalene and β-amyrin showed that these compounds are formed actively *in vivo* from MVA in the germinating pea seeds, and the radioactivity which appears in squalene falls as the radioactivity accumulates in β-amyrin [9, 20–22]. However, time-course studies of the incorporation of leucine-[U-^{14}C] and leucine-[4,5-^{3}H(N)] in the germinating pea seeds (Fig. 1) indicated that the radioactivities of squalene and β-amyrin increased slowly up to 24 hr and then the radioactivity in squalene decreased rapidly in contrast to the constant radioactivity achieved in β-amyrin. The same tendency was also observed for squalene and β-amyrin in feeding of valine-[U-^{14}C].

The distributions of the radioactivity in the IPP-derived and the DMAPP-derived moieties of squalene obtained by feeding leucine-[U-^{14}C], leucine-[4,5-^{3}H(N)] and valine-[U-^{14}C], respectively, were determined by chemical degradation. All the samples of squalene obtained from the time-course studies of incorporation were combined. After addition of unlabelled squalene as carrier, the squalene was ozonized to yield acetone, levulinic acid and succinic acid (Scheme 1). Acetone and levulinic acid were converted to the thiosemicarbazone derivatives and succinic acid to its benzylamide. On the basis of the

Table 1. Incorporation of labelled amino acids into squalene after various incorporation periods

Precursors	(μCi)	Feeding time (hr)	Sp. act.* (dpm/mmol)	Incorp. (%)
Leucine-[U-^{14}C]	10	6	4.10×10^{5}	4.3×10^{-3}
Leucine-[U-^{14}C]	10	18	1.62×10^{6}	1.7×10^{-2}
Leucine-[U-^{14}C]	10	24	8.11×10^{5}	9.0×10^{-3}
Leucine-[U-^{14}C]	10	36	1.14×10^{5}	1.2×10^{-3}
Leucine-[4,5-^{3}H]	60	6	1.32×10^{6}	2.4×10^{-3}
Leucine-[4,5-^{3}H]	60	12	4.50×10^{6}	7.9×10^{-3}
Leucine-[4,5-^{3}H]	60	18	1.80×10^{6}	6.4×10^{-3}
Leucine-[4,5-^{3}H]	60	24	8.87×10^{6}	1.6×10^{-2}
Leucine-[4,5-^{3}H]	60	36	2.46×10^{4}	6.0×10^{-5}
Valine-[U-^{14}C]	15	12	1.13×10^{4}	3.9×10^{-4}
Valine-[U-^{14}C]	15	24	1.98×10^{5}	5.0×10^{-4}
Valine-[U-^{14}C]	15	36	4.06×10^{4}	1.7×10^{-4}

* Values cannot be compared between different batches of seeds as different quantities of carrier and/or tracer were used.

Table 2. Incorporation of labelled amino acids into β-amyrin after various incorporation periods

Precursors	(μCi)	Feeding time (hr)	Sp. act.* (dpm/mmol)	Incorp. (%)
Leucine-[U-^{14}C]	10	6	2.60×10^{6}	3.3×10^{-2}
Leucine-[U-^{14}C]	10	18		
Leucine-[U-^{14}C]	10	24		
Leucine-[U-^{14}C]	10	36		
Leucine-[4,5-^{3}H]	60	6	2.05×10^{5}	2.9×10^{-3}
Leucine-[4,5-^{3}H]	60	12	2.63×10^{6}	1.4×10^{-2}
Leucine-[4,5-^{3}H]	60	18	7.38×10^{6}	1.6×10^{-2}
Leucine-[4,5-^{3}H]	60	24	1.19×10^{5}	5.4×10^{-2}
Leucine-[4,5-^{3}H]	60	36	4.99×10^{5}	2.3×10^{-2}
Valine-[U-^{14}C]	15	12	5.83×10^{4}	3.1×10^{-4}
Valine-[U-^{14}C]	15	24	7.91×10^{4}	4.6×10^{-4}
Valine-[U-^{14}C]	15	36	1.63×10^{5}	6.6×10^{-4}

* Values cannot be compared among different batches of seeds as different quantities of carrier and/or tracer were used.

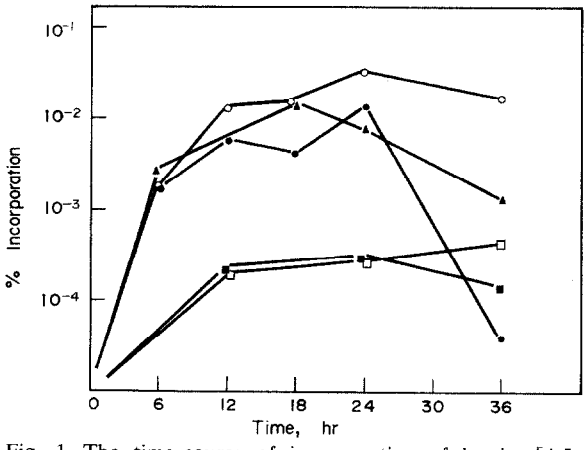
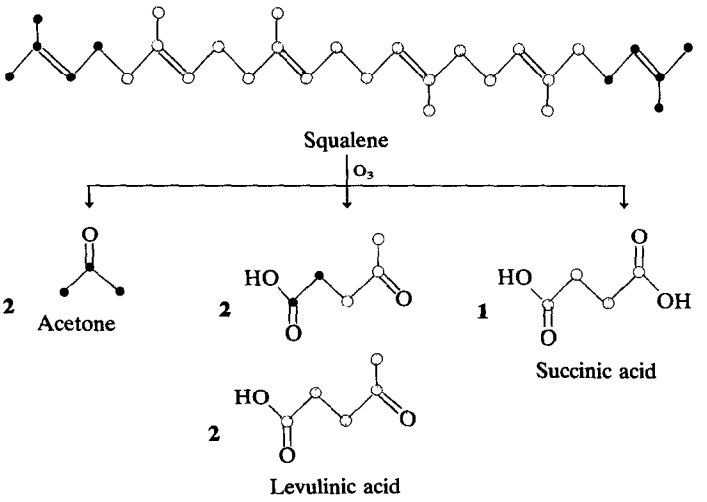


Fig. 1. The time-course of incorporation of leucine-[4,5-^{3}H(N)], leucine-[U-^{14}C] and valine-[U-^{14}C] into squalene and β-amyrin in *Pisum sativum*. Incorporations into squalene: (●—●) leucine-[4,5-^{3}H(N)], (▲—▲) leucine-[U-^{14}C], (■—■) valine-[U-^{14}C]. Incorporations into β-amyrin: (○—○) leucine-[4,5-^{3}H(N)], (□—□) valine-[U-^{14}C].

labelling pattern (Table 3), the distributions of the radioactivity in the IPP- and DMAPP-derived moieties of the squalene molecule were determined by following the procedures described in footnotes to Tables 3 and 4; the distributions are shown in Table 4. When ^{14}C-labelled and ^{3}H-labelled leucine and ^{14}C-valine were fed to the germinating pea seeds, the distributions of the radioactivity from the tracers in the IPP- and DMAPP-derived moieties of the squalene molecule were found to be almost equal. This agrees with the equal distribution of the radioactivity in the IPP- and DMAPP-derived moieties of squalene obtained by giving MVA-[2-^{14}C] to rat liver [5–7] and peas [8, 9], whereas it is not compatible with the preferential distribution of the radioactivity in the IPP-derived moiety of monoterpenoids biosynthesized from MVA-[2-^{14}C] by higher plants [1–4], or with the predominant location of the radioactivity in the DMAPP-derived moiety when radioisotopically labelled leucine and valine were fed to higher plants [16–18].

The above described and previously documented results of the equal or the unequal distributions of



Scheme 1. Chemical degradation of radioactive squalene. Open circles represent the carbon atoms theoretically derived from IPP and closed circles the carbon atoms theoretically derived from DMAPP.

Table 3. Distributions of radioactivity in squalene and its degradation products after uptake of leucine-[U-^{14}C], leucine-[4,5-^{3}H(N)] and valine-[U-^{14}C]

Precursors	Specific radioactivity (dpm/mmol) and the % of total radioactivity* Squalene	Acetone	Levulinic acid	Succinic acid
Leu-[U-^{14}C]	1.36×10^{4} (100)	1.16×10^{3} (17.0)	1.86×10^{3} (54.8)	1.36×10^{3} (10.0)
Leu-[4,5-^{3}H(N)]	2.30×10^{4} (100)	2.97×10^{3} (25.8)	3.91×10^{3} (68.0)	7.48×10^{2} (3.3)
Val-[U-^{14}C]	2.85×10^{3} (100)	2.71×10^{2} (19.0)	4.73×10^{2} (66.4)	3.80×10^{2} (13.3)

* The % of the total radioactivity (shown in brackets) was calculated as follows: (Sp. act. of product × mol of product per mol of squalene/sp. act. of squalene) × 100. Each mol of squalene yields 2 mol of acetone, 4 mol of levulinic acid and 1 mol of succinic acid.

radioactivity observed in terpenoids during the incorporations of radioisotopically labelled MVA and amino acids, such as alanine, leucine and valine, seem to support the previous conclusion that the site of triterpenoid biosynthesis differs from that of the mono- and sesquiterpenoid biosyntheses in higher plants [9], as well as our proposal that there is a possibility of the participation of the amino acids in the biosynthesis of monoterpenoids by directly giving rise to DMAPP [16–18].

Table 4. Distribution of radioactivity in the IPP- and DMAPP-derived moieties of squalene*

Precursors	Distributions (%)† IPP	DMAPP
L-Leucine-[U-^{14}C]‡	13	14
L-Leucine-[4,5-^{3}H(N)]§	17	13
L-Valine-[U-^{14}C]‡	16	17

* The carbon framework of squalene is biogenetically composed of 4 IPP-derived portions and 2 DMAPP-derived portions. This table shows the per cent distributions of the radioactivity in each unit of the IPP- or the DMAPP-derived portions, respectively.

† The errors are within ±2%.

‡ In the uptake of these amino acids, the DMAPP-derived moiety contains 1.66 times the radioactivity of the acetone molecule and the IPP-derived moiety has 1.25 times that of the succinic acid molecule.

§ In this case, all the radioactivity located in the DMAPP-derived moiety appears in the acetone molecule and all the radioactivity in the IPP-derived moiety in the levulinic acid molecule.

EXPERIMENTAL

General methods. GLC employed FID and a glass column (2.0 m × 3 mm) packed with OV-17 (2%) or OV-101 (2%) on Chromosorb W (80–100 mesh). Squalene was analysed at 260° on OV-17 and OV-101 and β-amyrin at 290° on OV-17. The RR_t's were compared with those of authentic samples of these compounds. TLC was on Si gel (0.75 mm). Materials on the plate were visualized by vaniline–sulfuric acid, followed by heating to 120°. Radioactivity was assayed by liquid scintillation counting using Bray's scintillation solvent [23]. The counting error was within ±2%.

Materials. L-Leucine-[U-^{14}C] (251 mCi/mmol), L-leucine-[4,5-^{3}H(N)] (32 Ci/mmol) and L-valine-[U-^{14}C] (225 mCi/mmol) were products of New England Nuclear Co., Boston.

Administration of radioactive precursors. The *Pisum sativum* seeds (5 g), the coats of which were partly broken to enhance a uniform uptake of H_2O, were germinated in petri dishes containing the radioactive precursor in the dark at 25° for 6, 10, 18, 24 and 36 hr, from the start of feeding. The seeds were minced and immersed in Me_2CO for 2 days at room temp. The Me_2CO soln was concd at red. pres. and extracted with Et_2O. The Et_2O-soluble material (80 mg) was saponified with 5% KOH–EtOH (5 ml) and extracted, after

removal of the EtOH, with 3×50 ml Et_2O. This Et_2O extract (40 mg) was subjected to PLC on Si gel (0.75 mm) with hexane to give radioactive squalene (1 mg) and β-amyrin (2 mg), which were further purified to homogeneity (by GLC) and to constant sp. act. as follows. The squalene, after addition of unlabelled squalene, was purified by way of the thiourea adduct. The β-amyrin was also diluted with a carrier sample and purified by PLC on Si gel (0.25 mm) with hexane–EtOAc (10:3), followed by 10% $AgNO_3$-impregnated Si gel (0.25 mm) with C_6H_6–EtOAc–MeOH (90:9:1).

Ozonolysis of squalene. The ozonolysis of squalene to give Me_2CO, levulinic acid and succinic acid was performed essentially according to the methods described [9, 24]. Squalene (100 mg) in hexane (3 ml) was ozonized for 3 hr at 0° and, after addition of HOAc (0.55 ml), ozonization was continued for another 3 hr. To the reaction mixture, 30% H_2O_2 (0.11, 0.11 and 0.55 ml, successively) was added and the mixture was warmed to 70° for 3 hr. After adjustment to pH 5, the mixture was distilled under N_2 into ice-cold aq. thiosemicarbazide and sodium acetate to isolate Me_2CO as the thiosemicarbazone, mp 177–178°. The aq. residue of the distillation was acidified with M H_2SO_4 and treated with $FeSO_4$ to decompose excess H_2O_2. Continuous extraction of the aq. residue with Et_2O for 16 hr gave a mixture of succinic acid and levulinic acid. The latter was separated by washing the mixture with $CHCl_3$, then converted first to the methyl ester, followed by transformation to its thiosemicarbazone, mp 140–141° (PLC on Si gel with hexane–EtOAc and recrystallized from MeOH–H_2O, then hexane–EtOAc–MeOH). Succinic acid was converted to the benzylamide, mp 215–216° (from Me_2CO–H_2O and then EtOH) for purification.

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