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Stereochemistry of Phytoene Biosynthesis by Isolated Chloroplasts

By M. J. BUGGY, G. BRITTON and T. W. GOODWIN
Department of Biochemistry, University of Liverpool, L69 3BX

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The incorporation of [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA* and [2-^{14}C,5-$^{3}H_2$]MVA into geranylgeraniol and phytoene by a preparation of 'non-aqueous' bean leaf chloroplasts has been studied. In the formation of phytoene from two molecules of geranylgeranyl pyrophosphate, the loss of hydrogen is stereospecific, the hydrogen atom lost from C-1 of each molecule of geranylgeranyl pyrophosphate being that which was originally the *pro-S* hydrogen atom from C-5 of mevalonate. All the *pro-R* hydrogen atoms from C-5 of mevalonate are retained. These results with a cell-free system confirm and extend the observations made in previous work with tomato slices.

As part of their work on the stereochemistry of squalene biosynthesis, Cornforth, Popják and their co-workers (Popják, Goodman, Cornforth, Cornforth & Ryhage, 1961; Donninger & Popják, 1966; Cornforth, Cornforth, Donninger & Popják, 1966) used [5-$^{2}H_2$]MVA and (5*R*)-[5-$^{3}H_1$]MVA to show that the condensations between isopentenyl pyrophosphate and dimethylallyl pyrophosphate or geranyl pyrophosphate occur with inversion of configuration at the carbon atoms from which the pyrophosphate groups leave, i.e. at the carbon atoms that arise from C-5 of MVA. In this way, the orientation of the hydrogen atoms from C-5 of MVA was found, for farnesyl pyrophosphate, to be as shown in structure (I). On the basis of these investigations, the C_{20} homologue of farnesyl pyrophosphate, geranylgeranyl pyrophosphate, produced by condensation between farnesyl pyrophosphate and isopentenyl pyrophosphate, should have the hydrogen atoms arising from C-5 of MVA orientated as shown in structure (II).

Cornforth *et al.* (1966) also studied the stereochemical course of the reaction in which two molecules of farnesyl pyrophosphate condense to form squalene, and found that the *pro-R* hydrogen atom from C-5 of MVA at C-1 of each molecule of farnesyl pyrophosphate was retained, whereas one *pro-S* hydrogen atom was eliminated. In carotenoid biosynthesis, two molecules of geranylgeranyl pyrophosphate condense to give phytoene, probably the first C_{40} compound formed (Goodwin, 1965), and two hydrogen atoms are lost from C-1 (originally C-5 of MVA) of each geranylgeranyl pyrophosphate molecule. To study the stereochemistry of this and other reactions in carotenoid biosynthesis in tomato fruits and bean leaves, we prepared and used [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA (Williams, Britton & Goodwin, 1966; Williams, Britton, Charlton & Goodwin, 1967). In this work, however, the standard on which calculation of the $^{14}C/^{3}H$ atomic ratios of phytoene and other carotenes was based had to be the squalene synthesized in the same system, and the reasonable assumption was made that the situation in plant squalene was the same as in animal squalene.

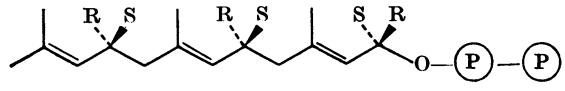
To eliminate this uncertainty, however, we investigated a cell-free preparation of chloroplasts isolated in organic media ('non-aqueous chloroplasts') that, in the presence of appropriate cofactors, will synthesize, from MVA, geranylgeraniol and phytoene as the main terpenoid products (Charlton, Treharne & Goodwin, 1967; M. J. Buggy, G. Britton & T. W. Goodwin, unpublished work). Experiments with this system have allowed us to make a direct comparison of the $^{14}C/^{3}H$ atomic ratio of phytoene with that of geranylgeraniol, from which, as geranylgeranyl pyrophosphate, phytoene is formed. Our cell-free system accumulates large amounts of geranylgeraniol, presumably because of the presence of an active pyrophosphatase that acts on geranylgeranyl pyrophosphate.

EXPERIMENTAL

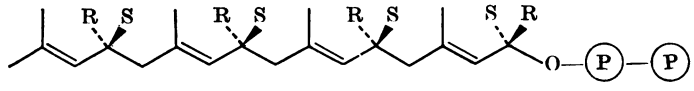
Radioactive substrates. [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA were from the batches prepared by Williams *et al.* (1967).

Preparation of chloroplasts. Seedlings of dwarf bean (*Phaseolus vulgaris* var. Lightning) were grown for 12–14 days at 20–24° in the dark, followed by 24hr. illumination. The leaf tissues were then freeze-dried and a preparation of developing chloroplasts was obtained by the non-aqueous technique described by Charlton *et al.* (1967).

* Abbreviation: MVA, mevalonic acid.



(I)



(II)

R and S represent *pro-R* and *pro-S* hydrogen atoms respectively from C-5 of MVA. Ⓟ, phosphate group.

Incubations. The chloroplast pellet was suspended in 0·1M-potassium phosphate buffer, pH 7·4, and subjected to ultrasonic disintegration for 1 min. ATP (10 μmoles), $MgCl_2$ (20 μmoles), GSH (20 μmoles) and either [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA or [2-^{14}C,5-$^{3}H_2$]MVA (as potassium salt; 2·0 μc of ^{14}C, 20 μc of ^{3}H) were then added. The mixture (final volume 1·5 ml.) was incubated at 25° for 6 hr.

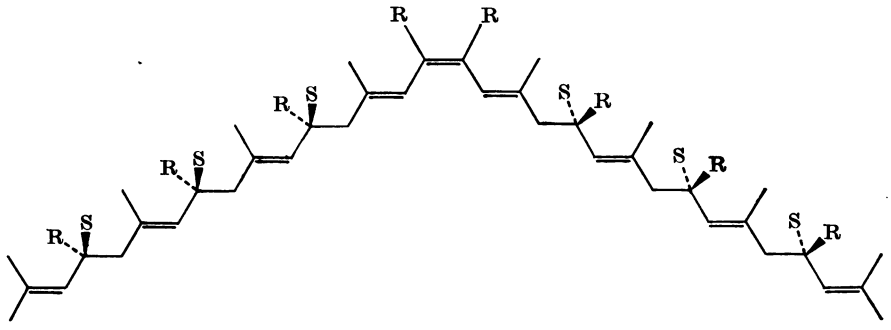
Extraction and purification of products. After incubation, the reaction mixtures were saponified and the unsaponifiable lipid material was extracted by standard procedures (Britton & Goodwin, 1969). In each case carrier phytoene (from tomatoes) and geranylgeraniol (a kind gift from Roche Products Ltd., Welwyn Garden City, Herts.) were added, and the unsaponifiable material was chromatographed on a 5 g. column of alumina (Brockmann grade III). Two fractions were obtained: fraction I, eluted with 0·5% (v/v) diethyl ether in light petroleum (b.p. 40–60°), contained phytoene; fraction II, eluted with 30% (v/v) diethyl ether in light petroleum, contained geranylgeraniol. Phytoene was purified by successive t.l.c. in three systems: (*a*) silica gel G, with 0·5% (v/v) diethyl ether in light petroleum as developing solvent; (*b*) MgO–Kieselguhr G (1:1, w/w), with light petroleum as developing solvent; (*c*) silica gel G, with 5% (v/v) benzene in light petroleum as developing solvent. Geranylgeraniol was purified: (*a*) by t.l.c. on silica gel G, with 50% (v/v) diethyl ether in light petroleum as developing solvent; (*b*) twice by reversed-phase t.l.c. on Kieselguhr G impregnated with liquid paraffin, with aq. 75% (v/v) acetone as developing solvent; (*c*) by chromatography on a small column (2 g.) of Brockmann grade III alumina, geranylgeraniol being eluted with 30% (v/v) diethyl ether in light petroleum, after elution of paraffin with 2% (v/v) diethyl ether in light petroleum. Further purification procedures applied to similarly prepared phytoene and geranylgeraniol failed to detect any radioactive impurities (M. J. Buggy, G. Britton & T. W. Goodwin, unpublished work).

Radioassay. The geranylgeraniol and phytoene samples were transferred to counting vials and, after removal of the solvent, were redissolved in toluene (5 ml.) and irradiated with u.v. light for 12 hr. After 2–3 days in the dark, a scintillator solution [5 ml. of a solution of 10 g. of 2,5-diphenyloxazole and 0·60 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene] was added, and the samples were assayed for ^{14}C and ^{3}H radioactivity in a Beckman LS200B liquid-scintillation system. Samples were counted for 20 min. with a 2σ counting error of 2·0%. Samples of squalene synthesized by maize [*Zea mays* var. Giant Hybrid (Rhodesia)] leaves in the presence of [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA in an experiment carried out at the same time by Mr T. J. Walton were also assayed. (The capacity of our isolated chloroplasts to synthesize squalene is slight.)

RESULTS AND DISCUSSION

Table 1 shows that phytoene biosynthesized from [2-^{14}C,5-$^{3}H_2$]MVA has ^{14}C/^{3}H atomic ratio 8:14 when compared with geranylgeraniol (^{14}C/^{3}H atomic ratio normalized to 4:8). Thus two hydrogen atoms originating from C-5 of MVA are lost in the formation of one molecule of phytoene from two molecules of geranylgeranyl pyrophosphate; this must be in the formation of the C-15–C-15′-double bond of phytoene. Table 2 shows that phytoene biosynthesized from [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA has ^{14}C/^{3}H atomic ratio 8:8 when compared with geranylgeraniol (^{14}C/^{3}H atomic ratio normalized to 4:4). Thus no hydrogen atoms labelled from [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA are lost in the formation of phytoene from geranylgeraniol. In particular, the two hydrogen atoms retained at C-15 and C-15′ of phytoene are those that are labelled from [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA, showing that the loss of hydrogen from these positions in the formation of phytoene is stereospecific. By taking the two experiments into consideration, it becomes clear that the hydrogen atoms lost must be those that were originally the *pro-S* hydrogen atoms at C-5 of MVA. The orientation in phytoene of the hydrogen atoms from C-5 of MVA is thus as shown in structure (III).

These results are in full agreement with the conclusions formed from previous work with tomato slices etc., in which the ^{14}C/^{3}H atomic ratios for phytoene were obtained by reference to the observed ^{3}H/^{14}C radioactivity ratios for squalene. The present work thus shows that in some systems, with some doubly-labelled species of MVA, the use of squalene as a reference compound



(III)

R and S represent *pro-R* and *pro-S* hydrogen atoms respectively from C-5 of MVA.

Table 1. *Comparison of the incorporations of* [2-^{14}C,5-$^{3}H_2$]*MVA into geranylgeraniol and into phytoene by isolated chloroplasts*

For incubation conditions see the text. The $^{14}C/^{3}H$ atomic ratios are based on the ratio 4:8 for geranylgeraniol.

Substance	Radioactivity (d.p.m.) ^{3}H	Radioactivity (d.p.m.) ^{14}C	$^{3}H/^{14}C$ radioactivity ratio	$^{14}C/^{3}H$ atomic ratio
Geranylgeraniol	2176	175	12·44	4:8
Phytoene	1265	121	10·47	8:13·6
Squalene (from maize leaves)	369309	33069	11·17	6:10·8

Table 2. *Comparison of the incorporations of* [2-^{14}C,(5*R*)-5-$^{3}H_1$]*MVA into geranylgeraniol and into phytoene by isolated chloroplasts*

For incubation conditions see the text. The $^{14}C/^{3}H$ atomic ratios are based on the ratio 4:4 for geranylgeraniol.

Substance	Radioactivity (d.p.m.) ^{3}H	Radioactivity (d.p.m.) ^{14}C	$^{3}H/^{14}C$ radioactivity ratio	$^{14}C/^{3}H$ atomic ratio
Geranylgeraniol	1877	149	12·59	4:4
Phytoene	17380	1382	12·58	8:8
Squalene (from maize leaves)	752448	58996	12·57	6:6

for studies of the stereochemistry of carotenoid biosynthesis is valid. This is further substantiated by comparison of the $^{3}H/^{14}C$ radioactivity ratios obtained for geranylgeraniol and phytoene with those obtained for squalene biosynthesized from [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA in another experiment carried out at the same time. The $^{14}C/^{3}H$ atomic ratios calculated for squalene, based on the results obtained for geranylgeraniol and phytoene, are very close to the expected values (Tables 1 and 2).

The present work also confirms the validity of the conclusions previously reached about the stereochemistry at the centre of the phytoene molecule as it is formed from two molecules of geranylgeranyl pyrophosphate, i.e. that one hydrogen atom is eliminated from C-1 of each molecule of geranylgeranyl pyrophosphate and that both hydrogen atoms eliminated are those that originated as the *pro-S* hydrogen atoms from C-5 of MVA.

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