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The Stereospecific Biosynthesis of Phytoene and Polyunsaturated Carotenes

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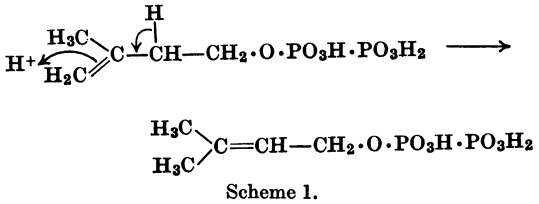
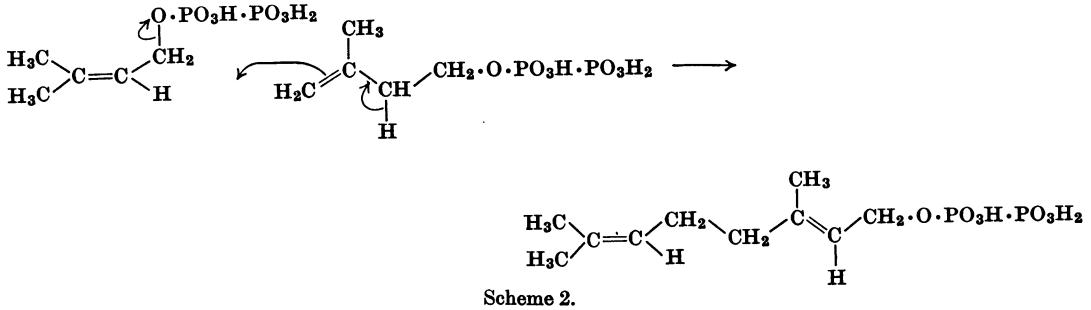
1. The incorporation of [2-^{14}C,(5*R*)-5-^{3}H$_1$]mevalonic acid and [2-^{14}C,5-^{3}H$_2$]-mevalonic acid into phytoene, phytofluene, ζ-carotene, neurosporene, α-, β-, γ- and δ-carotene and lycopene by slices of fruit from two tomato mutants (*delta* and *tangerine*) and into α- and β-carotene by bean leaves has been studied. 2. In the formation of phytoene, all the *pro-R*-hydrogen atoms from C-5 of mevalonic acid are retained whereas two *pro-S*-hydrogen atoms are lost. 3. Possible mechanisms for the condensation of two molecules of all-*trans*-geranylgeranyl pyrophosphate are outlined. 4. In each dehydrogenation step from phytoene to the fully unsaturated carotenes, one *pro-R*-hydrogen atom from C-5 of mevalonic acid is lost, indicating that the sequential dehydrogenation is stereospecific and in the same sense at each step.

In the biosynthesis of terpenoids, the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate involves the expulsion of a proton from what was originally C-4 of MVA‡ (Scheme 1). The condensation of a second molecule of isopentenyl pyrophosphate with dimethylallyl pyrophosphate to form geranylgeranyl pyrophosphate (C_{10}) also involves loss of a proton from C-4 of MVA (Scheme 2). Subsequent chain elongation to farnesyl pyrophosphate (the C_{15} precursor of squalene) and geranylgeranyl pyrophosphate (the C_{20} precursor of phytoene) (Wells, Schelbe & Porter, 1964) involves the further loss of protons from the same source. Phytoene is the first C_{40} compound in the biosynthetic sequence leading to the fully unsaturated carotenes, such as α-, β-, γ- and δ-carotene and lycopene (see Goodwin, 1965).

As there are two hydrogen atoms attached to C-4 of MVA, the question arose as to whether there



Scheme 1.



Scheme 2.

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‡ Abbreviation: MVA, mevalonic acid.

was stereospecific enzymic removal of one hydrogen atom, and, if so, which.

Cornforth and Popják and their colleagues (Cornforth, Cornforth, Donninger & Popják, 1966; Popják & Cornforth, 1966) elegantly demonstrated with the use of (3*RS*)-[2-^{14}C,(4*R*)-4-$^{3}H_1$]MVA and (3*RS*)-[2-^{14}C,(4*S*)-4-$^{3}H_1$]MVA, that, in the formation of squalene, all the eliminations of hydrogen from C-4 (of MVA) have the same stereospecificity; the *pro-R*-hydrogen atom is retained and the *pro-S*-hydrogen atom is lost [in the Cahn & Ingold (1951) nomenclature, as extended by Hanson (1966) to naming the paired ligands *g* and *g* at a tetrahedral atom X*ggij*]. With the same substrates, kindly provided by Dr Cornforth and Dr Popják, we showed that, in the formation of phytoene by a number of organisms, the stereospecificity of the removal of hydrogen from C-4 of MVA is the same as in squalene biosynthesis (Goodwin & Williams, 1965*a*,*b*, 1966; Williams, Britton & Goodwin, 1966*a*).

Further consideration of carotenoid biosynthesis (Scheme 3) indicates that in the formation of phytoene two hydrogen atoms are lost from the two central carbon atoms, which originate from C-5 of MVA.

Further, one hydrogen atom from C-5 of MVA is lost at each step during the conversion of phytoene into the fully unsaturated carotenes, exemplified by lycopene in Scheme 3; the carbon atoms involved are marked with an asterisk. C-5 of MVA carries two hydrogen atoms, as does C-4 of MVA, so the problem of whether or not the removal of hydrogen in phytoene formation from geranylgeranyl pyrophosphate and the removal of hydrogen from phytoene are stereospecific processes, and, if so, in what sense, also arises. (The exact point in the sequence at which cyclization takes place to form α-, β-, γ- and δ-carotene is not relevant to the present problem, since the cyclization reaction does not involve hydrogen atoms from C-5 of MVA, and therefore should not affect the $^{3}H/^{14}C$ ratios.)

To study these problems we have synthesized (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and (3*RS*)-[2-^{14}C, 5-$^{3}H_2$]MVA and followed their incorporation into phytoene, a number of intermediates between phytoene and lycopene and a number of cyclic carotenes in the fruit of two tomato mutants, *delta* and *tangerine*, and in bean leaves.

A preliminary account of some of this work has already appeared (Williams, Britton & Goodwin, 1966*b*).

EXPERIMENTAL

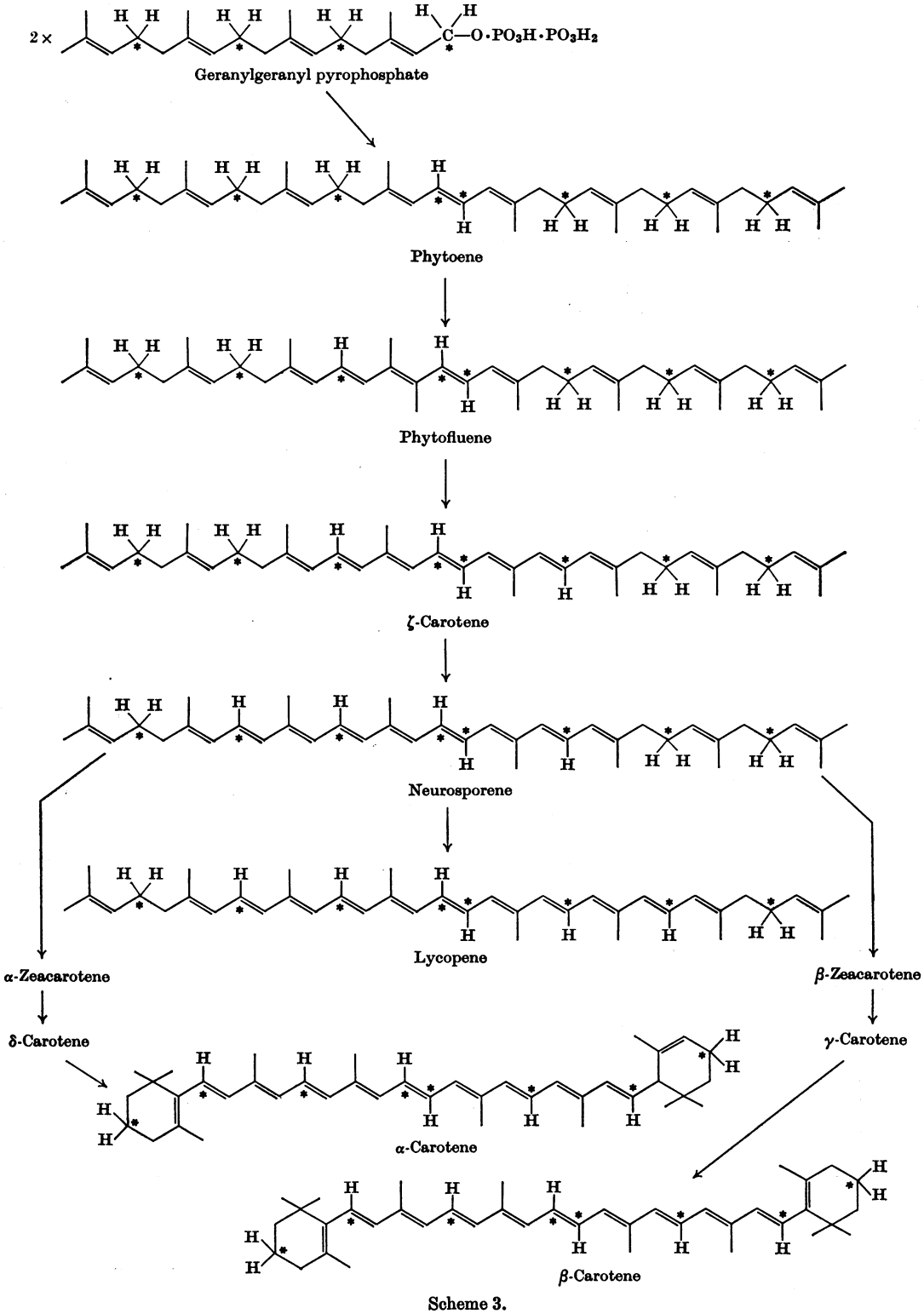
Preparation of (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]*mevalonic acid.* The method of Donninger & Popják (1966) in which mevaldate is reduced enzymically with mevaldate reductase in the presence of ^{3}H-labelled NADH was used, with the modification that the ^{3}H-labelled NADH was prepared chemically and not enzymically. The NAD^+ was reduced with NaB^3H_4 (Chaykin & Meissner, 1964; Chaykin, Chakraverty, King & Watson, 1966). These workers have shown that the reduction gives a mixture of [1,2-$^{3}H_2$]-, [1,4-$^{3}H_2$]- and [1,6-$^{3}H_2$]-NADH, but that alcohol reductase utilizes only the 1,4-isomer in stereospecifically transferring ^{3}H from the 'A' side of the reduced nicotinamide residue to acetaldehyde to produce stereospecifically labelled ethanol. Mevaldate reductase exhibits the same stereospecificity (Donninger & Popják, 1966). Potassium mevaldate was prepared from *NN'*-dibenzylethylenediammonium bis-(3*RS*)-3-hydroxy-5,5-dimethoxy-3-methylpentanoate by the method of Knauss, Brodie & Porter (1962); the starting material was prepared by the procedure of Shunk *et al.* (1957).

Mevaldate reductase (mevaldate–NAD oxidoreductase) was prepared from pig liver (Schlesinger & Coon, 1961).

(3*RS*)-[(5*R*)-5-$^{3}H_1$]MVA was obtained from an incubation mixture consisting of ^{3}H-labelled NADH (500μmoles containing approx. 150μmoles of [1,4-$^{3}H_2$]NADH) in water (8ml.), potassium mevaldate (400μmoles) in water (5ml.) and enzyme (72mg. of protein/ml.) in 10mM-tris–HCl buffer, pH 8·0 (8ml.), the whole being made up to 35ml. with 40mM-phosphate buffer, pH 6·0. The mixture was incubated for 1hr. at 27 ± 1° when the oxidation of NADH, determined by ΔE_{340}, had become negligible. The mixture was then adjusted to pH 1 with 5N-HCl and the solution saturated with $(NH_4)_2SO_4$ to diminish the solubility of the MVA lactone, before extraction with ether (ten times) and chloroform (five times). The aqueous residue was then extracted with boiling chloroform for 24hr. The combined extracts were filtered and evaporated to dryness under reduced pressure, and the residue, dissolved in chloroform (10ml.), was chromatographed on a Celite–0·5N-H_2SO_4 column (15cm. × 1·2cm.; 10g. of support and 8ml. of 0·5N-H_2SO_4) with chloroform saturated with 0·5N-H_2SO_4 as developer (Cornforth, Hunter & Popják, 1953). Fractions (25ml.) were collected and a small sample was chromatographed on paper in ethanol–aq. NH_3–water (16:2:3, by vol.) as developing solvent. The radioactive MVA was located by means of a Desaga radioactive plate scanner by comparison of its position with that of authentic [2-^{14}C]MVA used as a marker. The fractions found to contain MVA were combined and reduced in volume to 10ml. The product (30μmoles; specific activity approx. 90μc/μmole) was characterized by taking a small known sample, diluting it with unlabelled carrier MVA (50mg.) and converting it into the benzhydrylamide (Cornforth, Cornforth, Popják & Youhotsky-Gore, 1958) and recrystallizing to constant m.p. (97–98°) and specific activity (0·004μc/μmole).

(3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA was prepared by mixing the (3*RS*)-[(5*R*)-5-$^{3}H_1$]MVA lactone (1mc) with (3*RS*)-[2-^{14}C]MVA lactone, of specific activity 5·03μc/μmole (The Radiochemical Centre, Amersham, Bucks.) (100μc), in benzene solution to give a $^{3}H/^{14}C$ ratio approx. 10:1. This product was also characterized by its benzhydrylamide. The specific activity of the final product was 32·15μc of ^{3}H/μmole and 3·22μc of ^{14}C/μmole.

Preparation of (3*RS*)-[2-^{14}C,5-$^{3}H_2$]*mevalonic acid.* [5-$^{3}H_2$]MVA was synthesized by reduction of mevaldate with NaB^3H_4. To a solution of potassium mevaldate (see above) (200μmoles) in water (3ml.) was added $NaBH_4$ (2mg.) followed by a cold aqueous solution (2ml.) of


2×
Geranylgeranyl pyrophosphate
Phytoene
Phytofluene
ζ-Carotene
Neurosporene
Lycopene
α-Zeacarotene
β-Zeacarotene
δ-Carotene
γ-Carotene
α-Carotene
β-Carotene

Scheme 3.

Table 1. *Chromatographic adsorption and identification of polyenes of mutant tomato strains*

Fraction no.	Column chromatography: On MgO–Celite (1:1)	Column chromatography: On MgO–Celite (5:2)	Thin-layer chromatography on Kieselgel G	$\lambda_{max.}$ in light petroleum (mμ)	Compound	Criteria of identification in addition to $\lambda_{max.}$
1	1% acetone in light petroleum	1% acetone in light petroleum	5% benzene or 2% acetone in light petroleum	276, 285, 300	Phytoene	Adsorptive power
2				415, 438, 468	'ϵ-Carotene' (tentative)	Adsorptive power
3		2% acetone in light petroleum		332, 348, 368	Phytofluene	Green fluorescence in u.v. light
4		2·5–3% acetone in light petroleum	6% benzene or 3% acetone in light petroleum	420, 440, 474	α-Carotene	Co-chromatography with authentic α-carotene from carrots on thin-layer chromatography
5		Extrusion and elution of pigments with ether	10% benzene in light petroleum	330·5, 348, 367	'*cis*-Phytofluene'	Thin-layer chromatography with authentic phytofluene before and after isomerization
6				397, 419, 447	'α-Zeacarotene' (tentative)	Adsorptive power
7			6% benzene or 3% acetone in light petroleum	424, 452, 479	β-Carotene	Co-chromatography with authentic β-carotene on thin-layer chromatography
8	Extrusion and elution of pigments with ether or ether–ethanol		10% benzene or 5% acetone in light petroleum	362, 379, 400, 425	ζ-Carotene	Co-chromatography with authentic all-*trans*-ζ-carotene
9			10% acetone in light petroleum	378, 398, 423	*cis*-ζ-Carotene	Co-chromatography with isomer of I_2-isomerized ζ-carotene
10				428, 459, 488	δ-Carotene	Adsorptive property; δ-carotene is main pigment in *delta* tomato
11				436, 460, 490	γ-Carotene	Adsorptive affinity
12				414, 436, 463	Prolycopene	Characteristic shift to 442, 469 and 500 mμ on I_2 treatment
13				414, 439, 467	'*cis*-Neurosporene' (tentative)	
14			40% benzene or 15% acetone in light petroleum	415, 440, 470	Neurosporene	Co-chromatography with authentic pigment on thin-layer chromatography
15				446, 470, 502	Lycopene	Characteristic pigment of normal tomatoes

Table 2. *Quantitative analysis of carotenes in fruit of three tomato strains*

Tomato strain	Wet wt. (g.)	Dry wt. (g.)	Wt. of unsaponifiable material (mg.)	Carotene (μg./g. dry wt.)														
				Phytoene	Phytofluene	'cis-Phytofluene'	ζ-Carotene	'cis-ζ-Carotene'	Neurosporene	'cis-Neurosporene'	'α-Zeacarotene'	δ-Carotene	γ-Carotene	ε-Carotene	α-Carotene	β-Carotene	Prolycopene	Lycopene	Total
Ordinary	176·0	3·574	412·7	91·35	44·76		8·39		6·58				23·22		1·88	162·9		803·0	1142·0
delta	79·0	3·265	216·7	249·6	72·5		3·3				3·4	468·5	200·9	4·8	25·7	72·8		345·0	1447·0
tangerine	136·2	5·061	84·8	488·7	97·6	16·3	414·4	6·1		38·4					4·1	46·0	255·8	173·5	1541·0

NaB^3H_4 (100mc; specific activity 721mc/m-mole). After 30min. a further 5mg. of unlabelled $NaBH_4$ was added to complete the reduction. After standing for 1hr. the solution was adjusted to pH1 with 5N-HCl and extracted with ether (16 × 10ml.) and chloroform (5 × 10ml.). The extract was evaporated to dryness under reduced pressure and the product characterized by its i.r. spectrum (identical with that of MVA lactone), chromatographic properties and by preparation, after addition of carrier, of its benzhydrylamide, which was recrystallized to constant m.p. (97–98°) and specific activity (0·0009μc/μmole). The undiluted [5-$^3H_2$]MVA (150μmoles) had a specific activity of 4μc/μmole. The possibility that the borohydride reduction might show some stereoselectivity and lead to a product with a preponderance of either the (5*R*)-[5-$^3H_1$]- or (5*S*)-[5-$^3H_1$]-isomer was considered but the results of the present and other experiments give no indication of any significant stereoselectivity.

Tomato strains. The seeds of the two mutant strains, *delta* and *tangerine*, were gifts from Dr G. Mackinney (University of California, Berkeley, Calif., U.S.A.) and Dr M. L. Tomes (Purdue University, Lafayette, Ind., U.S.A.). They were germinated and grown in the greenhouse. Normal tomatoes were bought from a local shop.

Incubation. Ripe fruit were used and thin slices taken from the central portions were immediately floated on a medium containing 0·1M-tris–HCl (Sigma 7–9) buffer, pH7·2–7·4, and the appropriate MVA species, in a Petri dish, enclosed in cellophan to minimize external contamination. Incubations were continued for 30–60hr.

Extraction and separation of pigments. The unsaponifiable material was prepared by our usual method (Goodwin, 1955) and dissolved in a small volume of light petroleum (b.p. 40–60°) (free from aromatic hydrocarbons) and stored at −20°; the precipitated sterol was removed before chromatography on an MgO–Celite (1:1, w/w) column with 1% (v/v) acetone in light petroleum (b.p. 60–80°). The first fraction was collected in the eluate, and the remainder formed discrete zones, which were separated mechanically from the extruded column, eluted and purified by rechromatography and identified as indicated in Table 1. Not all the pigments listed were observed in every strain of tomato. The quantitative distribution of carotenes in the three strains grown under our conditions is given in Table 2. These results are generally in agreement with those of Tomes (1963), but we noted two additional pigments in each of the mutant strains. In the *delta* tomato, α-zeacarotene and ε-carotene were tentatively identified, and in the *tangerine* tomato a *cis*-ζ-carotene and an isomer of phytofluene (probably a di-*cis*-phytofluene) were detected.

Table 2 indicates the importance of the availability of these mutants to our studies: they synthesize far more of the intermediates between phytoene and the fully unsaturated carotenes than do normal tomatoes. They have also been useful for our studies on the cyclization reactions involved in the production of α-, β-, γ- and δ-carotene and on the formation of the poly-*cis*-carotene prolycopene (R. J. H. Williams, G. Britton & T. W. Goodwin, unpublished work).

It is essential that the polyenes are radiochemically as well as chromatographically pure. An experiment was carried out in which the polyenes extracted from *delta*-tomato slices incubated with [2-^{14}C]MVA (potassium salt) were separated as indicated in Table 1 and then subjected

to further purification on Kieselgel G plates in one of two solvent systems: (*a*) light petroleum containing varying amounts of benzene; (*b*) light petroleum containing varying amounts of acetone. The purified fractions from each system were assayed spectrophotometrically and then for radioactivity; specific activities could thus be calculated. The solvent systems used are summarized in Table 3. Fractions more polar than ζ-carotene were rechromatographed on an alumina column (Brockmann grade III; Woelm) and eluted with 0·2–0·8% (v/v) ether in light petroleum, before the final purification by thin-layer chromatography. This procedure rendered the fractions free from sterols. The specific activities of the polyenes so obtained (Table 4) indicate that the specific activities of the pigments purified in two different ways were very similar, and within the limits of accuracy of the experiment.

Table 3. *Solvent systems used for purifying polyenes by thin-layer chromatography on Kieselgel G*

Polyene	Systems: Concn. of benzene (% in light petroleum)	Systems: Concn. of acetone (% in light petroleum)
Phytoene	5	2
Phytofluene	6	3
ζ-Carotene	10	—
α-Zeacarotene	6	3
δ-Carotene	25	8
γ-Carotene	32	10
ε-Carotene	5	2
α-Carotene	6	3
β-Carotene	6	3
Lycopene	50	10

Bean-leaf preparations. Dwarf-French-bean seedlings were germinated for 7 days at 27±1° in the light. The leaves (about 20/experiment) were harvested, the main veins were removed and the laminar tissue was thinly sliced into conical flasks containing the labelled MVA in 1·5ml. of tris–HCl buffer, pH7·4. The flask was incubated with shaking at 26±1° in the light for 6hr. and in the dark for a further 12hr. The polyenes, squalene, α-carotene and β-carotene were extracted, separated and purified as described above for tomato fruit.

Measurement of radioactivity. Samples were assayed for ^{14}C and ^{3}H in a Packard Tri-Carb series 314E liquid-scintillation spectrometer, fitted with a Packard model 500D automatic control and automatic sample changer (Packard Instrument Co., La Grange, Ill., U.S.A.). Samples were transferred to counting vials and, after removal of the solvent, were redissolved in toluene (5ml.), decolorized, if necessary, in sunlight, and the scintillator [5ml. of a toluene solution of 10g. of 2,5-diphenyloxazole and 0·60g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l.] was added. The ^{3}H and ^{14}C radioactivities were calculated by use of the equation of Okita, Kahara, Richardson & Le Roy (1957). The results from the bean-leaf experiments were processed in an IBM 1620 computer with a programme prepared by Miss S. G. Lutkins and Mr H. H. Rees. The efficiency of counting was checked with standard solutions and the virtual absence of quenching demonstrated. Each sample was assayed three to five times on four separate occasions, from which the final average count and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Three experiments were carried out on tomatoes with (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA, two on the

Table 4. *Radioactivity and specific activity of polyenes from* delta *tomatoes incubated with* [2-^{14}C]*mevalonic acid*

Tomato slices (79·0g. wet wt.) were incubated with 2μc of (3*RS*)-[2-^{14}C]MVA for 36hr.; pigments were extracted and separated as described in the Experimental section and purified in two thin-layer systems. For details of solvent systems see Table 3.

Polyene	Thin-layer chromatography on Kieselgel G: Benzene–light petroleum: Total activity (disintegrations/min.)	Benzene–light petroleum: Specific activity (disintegrations/min./μg.)	Acetone–light petroleum: Total activity (disintegrations/min.)	Acetone–light petroleum: Specific activity (disintegrations/min./μg.)
Phytoene	2880	22·2	2110	24·1
Phytofluene	122	4·1	144	3·9
ζ-Carotene†	105	18·4	—	—
α-Zeacarotene†	45	15·0	—	—
δ-Carotene	1660	7·6	1260	7·8
γ-Carotene	435	9·3	323	9·6
ε-Carotene†	—	—	60	25·1
α-Carotene	339	33·9	1133	36·3
β-Carotene	1139	16·2	1026	15·9
Lycopene	614	10·7	547	11·4

† Insufficient material for two purifications.

Table 5. *General conditions of experiments for incorporation of* (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]-*mevalonic acid into tomato polyenes*

Tomato strain	Incubation period (hr.)	Wet wt. (g.)	Wt. of unsaponifiable material isolated (mg.)	Radioactivity added (μc)	
				^{14}C	^{3}H
delta (I)	32	86	77·1	4·0	40·0
delta (II)	64	80	94·1	4·0	40·0
tangerine	49·5	100	142·5	6·0	60·0

Table 6. *Comparison of incorporation of* (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]*mevalonic acid into squalene and carotenoid polyenes by slices of* tangerine *tomatoes*

For general details of the experiment see Table 5. The ^{3}H/^{14}C atomic ratios are based on the ratio 6:6 for squalene. Standard errors of the means of counting are given in parentheses.

Substance	Radioactivity (disintegrations/min.)		Mean ^{3}H/^{14}C radioactivity ratio	Mean ^{3}H/^{14}C atomic ratio
	^{3}H	^{14}C		
MVA	41476	3924	10·57	—
Squalene	312260	26485	11·79	6:6
Phytoene	4001	330	12·12	8·23 (±0·08):8
ζ-Carotene	5622	594	9·47	6·43 (±0·045):8
β-Carotene	8346	1440	5·78	3·92 (±0·02):8
Lycopene	8079	1578	5·12	3·50 (±0·01):8

Table 7. *Comparison of incorporation of* (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]*mevalonic acid into squalene and carotenoid polyenes by slices of* delta *tomatoes*

For general details of the experiment see Table 5. The ^{3}H/^{14}C atomic ratios are based on the ratio 8:8 for phytoene. Standard errors of the means of counting are given in parentheses.

Substance	Radioactivity (disintegrations/min.)		Mean ^{3}H/^{14}C radioactivity ratio	Mean ^{3}H/^{14}C atomic ratio
	^{3}H	^{14}C		
Expt. I				
MVA	41830	4061	10·30	
Squalene	281220	24600	11·43	6:6
Phytoene	24380	2056	11·86	8:8
δ-Carotene	28665	4330	6·62	4·46 (±0·09):8
γ-Carotene	2486	370	6·72	4·54 (±0·05):8
α-Carotene	4334	673	6·44	4·36 (±0·11):8
β-Carotene	14985	2345	6·39	4·30 (±0·06):8
Lycopene	5643	890	6·34	4·23 (±0·13):8
Expt. II				
MVA	41570	4040	10·29	
Squalene	60720	5280	11·50	6:6
Phytoene	126940	10500	12·09	8:8
δ-Carotene	102680	16750	6·13	4·05 (±0·02):8
γ-Carotene	6573	1050	6·26	4·16 (±0·02):8
α-Carotene	8818	1420	6·21	4·11 (±0·03):8
β-Carotene	15475	2460	6·29	4·16 (±0·01):8
Lycopene	25320	4546	5·57	3·70 (±0·03):8

Table 8. *Comparison of incorporation of* [2-^{14}C,5-$^{3}H_2$]*mevalonic acid into squalene and carotenoid polyenes by slices of* tangerine *tomatoes*

Tomato slices (70g. wet wt.) were incubated for 50hr. with [2-^{14}C,5-$^{3}H_2$]MVA (4·80μc of ^{14}C, 52·80μc of ^{3}H). The ^{3}H/^{14}C atomic ratios are based on the ratio 11:6 for squalene. Standard errors of the means of counting are given in parentheses.

Substance	Radioactivity (disintegrations/min.) ^{3}H	^{14}C	Mean ^{3}H/^{14}C radioactivity ratio	Mean ^{3}H/^{14}C atomic ratio
MVA	43000	3760	11·43	
Squalene	352950	33550	10·52	11:6
Phytoene	132090	12870	10·26	14·31 (±0·09):8
β-Carotene	14710	1960	7·50	10·46 (±0·24):8
Lycopene	32030	4490	7·13	9·94 (±0·24):8
Prolycopene	5010	684	7·32	10·20 (±0·26):8

Table 9. *Incorporation of* (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]*mevalonic acid into squalene and α- and β-carotene by bean-leaf slices*

Bean-leaf slices (0·38g. dry wt.) were incubated for 18hr. at 26° with [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA (6·0μc of ^{14}C, 60μc of ^{3}H). The ^{3}H/^{14}C atomic ratios are based on the ratio 6:6 for squalene.

Substance	Radioactivity (disintegrations/min.) ^{3}H	^{14}C	Mean ^{3}H/^{14}C radioactivity ratio	Mean ^{3}H/^{14}C atomic ratio
Squalene	225350	18800	11·89	6:6
α-Carotene	47770	7910	6·04	4·06:8
β-Carotene	17550	2810	6·25	4·21:8

Table 10. *Incorporation of* (3*RS*)-[2-^{14}C,5-$^{3}H_2$]*mevalonic acid into squalene and α- and β-carotene by bean-leaf slices*

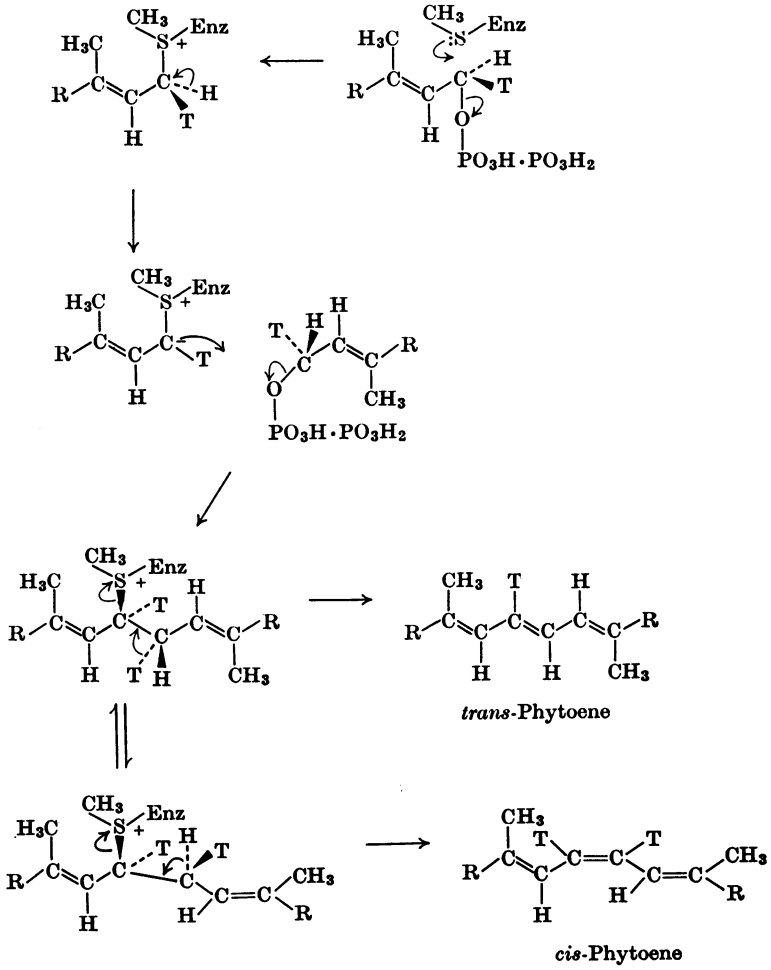
Bean-leaf slices (0·38g. dry wt.) were incubated for 18hr. at 26° with [2-^{14}C,5-$^{3}H_2$]MVA (4·8μc of ^{14}C, 52·8μc of ^{3}H). The ^{3}H/^{14}C atomic ratio are based on the ratio 11:6 for squalene.

Substance	Radioactivity (disintegrations/min.) ^{3}H	^{14}C	Mean ^{3}H/^{14}C radioactivity ratio	Mean ^{3}H/^{14}C atomic ratio
Squalene	49940	4740	10·48	11:6
α-Carotene	68050	9610	7·18	10·06:8
β-Carotene	34350	4680	7·30	10·20:8

delta strain and one on the *tangerine* strain. The general data for the experiments are given in Table 5, and the radioactivity results in Tables 6 and 7. The results of an experiment with [2-^{14}C,5-$^{3}H_2$]MVA and slices of the *tangerine* tomato are given in Table 8. It will be seen from the results with the *tangerine* tomato that the ^{3}H/^{14}C ratio of the phytoene formed from (3*RS*)-[2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA is the same as that of squalene, i.e. no *pro-R*-hydrogen atom is lost from the centre of the molecule during the formation of phytoene (in this section '*pro-R*'- and '*pro-S*'-hydrogen atoms refer to the hydrogen atoms at C-5 of MVA). The same result was obtained with the *delta* tomato (Table 7). Experiments with MVA labelled non-stereospecifically with ^{3}H at C-5 confirm this conclusion and indicate that two *pro-S*-hydrogen atoms are lost. In the formation of squalene from six mevalonic acid residues with a total of 12 hydrogen atoms from C-5, one *pro-S*-hydrogen atom is lost (Donninger & Popják, 1966), i.e. the ^{3}H/^{14}C atomic ratio with [2-^{14}C,5-$^{3}H_2$]MVA as substrate is 11:6 in squalene (Popják, Goodman, Cornforth, Cornforth & Ryhage, 1961). Since the observed ratio of radioactivity in squalene represents an atomic ratio 11:6 then the observed ratio in phytoene should be 14:8, if two

pro-S-hydrogen atoms are lost from the centre. The observed value is 14·3:8 (Table 8). The ratios observed with ζ-carotene, β-carotene and lycopene in the *tangerine* tomatoes (Table 6) indicate that the removal of hydrogen from what was C-5 of MVA during the stepwise conversion of phytoene (Scheme 3) into the fully unsaturated polyenes is stereospecific and in the same sense in each case. The *pro-R*-hydrogen atom is lost and the *pro-S*-hydrogen atom retained (see Scheme 3). No values were available for phytofluene or neurosporene; the former gave aberrant results, which may be due to the fact that it is highly fluorescent, and there was insufficient of the latter for reliable measurements of radioactivity. The loss of four *pro-R*-hydrogen atoms originating from C-5 of MVA is also demonstrated in the conversion of phytoene into α-, β-, γ- and δ-carotene and lycopene in the *delta* tomatoes (Table 7). This conclusion was again confirmed by the experiment with [2-^{14}C,5-$^{3}H_2$]MVA; the loss of four *pro-R*-hydrogen atoms when phytoene is converted into the fully unsaturated carotenes should result in a change of $^{3}H/^{14}C$ ratio from 14:8 to 10:8. The results (Table 8) show that all the ratios are within the range 10·06–10·46:8. The results with α- and β-carotene were confirmed in experiments with bean leaves (Tables 9 and 10). In these experiments the compound taken as a reference was squalene; it was known from the tomato experiments to have the same $^{3}H/^{14}C$ ratio as phytoene, and green tissues accumulate insignificant amounts of phytoene.

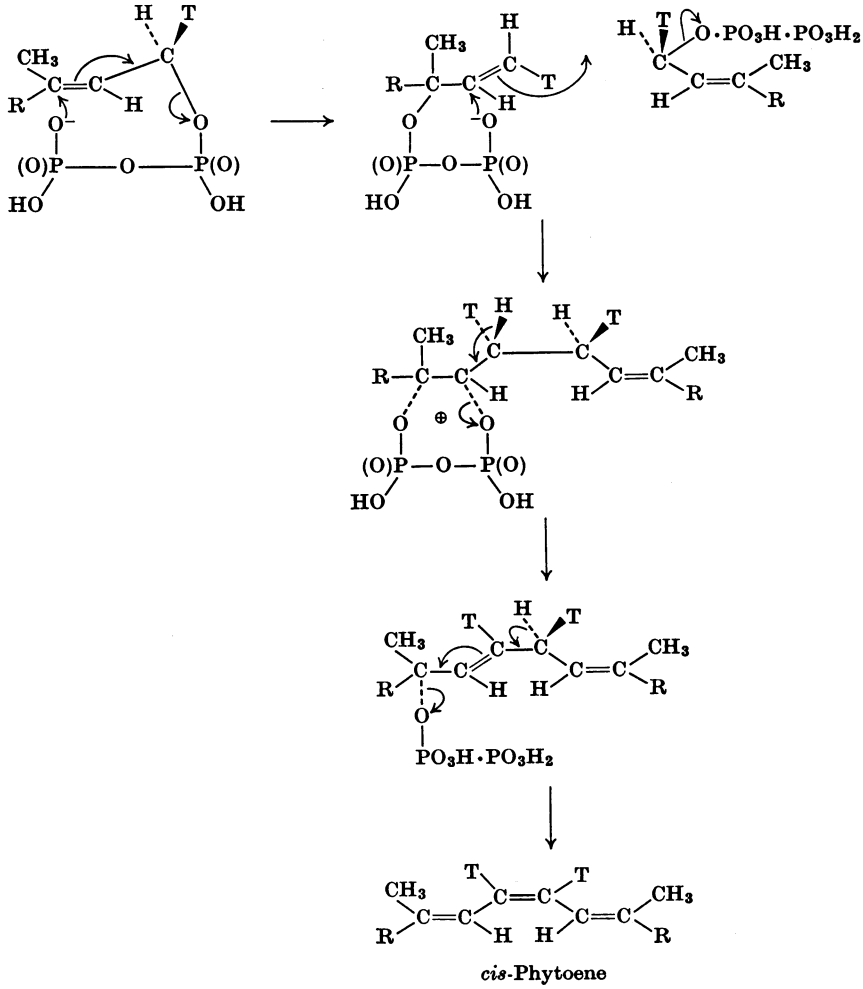
The ratios observed for prolycopene biosynthesized in *tangerine* tomatoes from [2-^{14}C,(5*R*)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA (and also [2-^{14}C,-



Scheme 4.

(4*R*)-4-$^3H_1$]MVA; R. J. H. Williams, G. Britton & T. W. Goodwin, unpublished work) are the same as those for lycopene. These results show that the stereochemistry of the biosynthesis of the poly-*cis*-prolycopene is the same as that of all-*trans*-lycopene.

Mechanism of formation of phytoene. In their elegant work on the stereospecific biosynthesis of squalene, Popják and Cornforth have discussed a number of possible mechanisms for the condensation of two molecules of farnesyl pyrophosphate to form squalene. Two of these mechanisms can, with minor modifications, explain the stereospecific biosynthesis of phytoene, the central double bond of which appears to have the *cis*-configuration (Rabourn & Quackenbush, 1956; Davis, Jackman, Siddons & Weedon, 1966; Jungalwala & Porter, 1965). In Scheme 4 (based on Cornforth *et al.* 1966) the geranylgeranyl residues are connected via a sulphonium ylide, a mechanism that requires the presence of a thio ether grouping (e.g. a methionine residue) at the active centre of phytoene synthetase. This thio ether group displaces the pyrophosphate from a molecule of geranylgeranyl pyrophosphate, by an S_N2 substitution reaction involving inversion of configuration at C-1 of the geranylgeranyl group, to give a sulphonium ion. The hydrogen atoms at C-1, situated between a double bond and S^+, now have a tendency to ionize with the formation of an ylide. This alkylates a second molecule of geranylgeranyl pyrophosphate (again with inversion at C-1) to give a lycopersyl-sulphonium ion (lycopersene is the C_{40} homologue of squalene). Now, instead of



Scheme 5.

a nucleophilic displacement of the –S–enzyme by H^- from the 'B' side of NADPH, which occurs in squalene formation, the incipient positive charge on the carbon atom from which the –S–enzyme is lost is neutralized by loss of a proton from the adjacent methylene group, thus introducing the central double bond of phytoene.

Scheme 5 (based on Popják *et al.* 1961) is essentially the alkylation of a double bond and resembles the chain-elongation process in the earlier stages of terpenoid biosynthesis. The first step is an anionotropic rearrangement of a geranylgeranyl group to yield a geranyl-linaloyl derivative. Alkylation, with inversion, of a second molecule of geranylgeranyl pyrophosphate, occurs, with the pyrophosphate group of the first molecule stabilizing the resulting electron deficiency at C-2. Normal *trans*-elimination then regenerates the double bond. In the final stage, the pyrophosphate group is removed, but, instead of the intermediate undergoing reduction with NADPH as in the formation of squalene, the positive charge that builds up as the pyrophosphate departs is neutralized by the loss of a proton by a *cis*-1,4-elimination with migration of the π electrons of the double bond, to yield phytoene. Theoretical considerations indicate that both *pro-R*-hydrogen atoms at the centre of the molecule would be retained only if the configuration of the central double bond formed is *cis*. As indicated above, the central double bond of natural phytoene appears to be *cis*. No experiments have yet been reported on phytoene biosynthesis to test either of these hypotheses, but it has been noted that, in the formation of phytoene in broken chloroplasts prepared by non-aqueous technique, NAD^+ or $NADP^+$ is not required (Charlton, Treharne & Goodwin, 1966). This would fit in with the view that a proton rather than H^- is the final unit eliminated.

In the squalene field, Scheme 5 is not consistent with the observation by Krishna, Whitlock, Feldbruegge & Porter (1966) that nerolidyl pyrophosphate (replaces geranyl-linaloyl pyrophosphate in Scheme 5 when squalene is under consideration) inhibits squalene synthetase, whereas their observation that the enzyme is inhibited by thiol inhibitors supports Scheme 4. On the other hand the report of Rilling (1966), in which he considers that an intermediate in squalene biosynthesis is a pyrophosphate ester of a C_{30} alcohol with a cyclopropane ring, may require a major rethinking on the type of mechanism involved.

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REFERENCES

Cahn, R. S. & Ingold, C. K. (1951). *J. chem. Soc.* p. 612.
Charlton, J. M., Treharne, K. J. & Goodwin, T. W. (1966). *Biochem. J.* **101**, 7P.
Chaykin, S., Chakraverty, K., King, L. & Watson, J. G. (1966). *Biochim. biophys. Acta*, **124**, 1.
Chaykin, S. & Meissner, L. (1964). *Biochem. biophys. Res. Commun.* **14**, 233.
Cornforth, J. W., Cornforth, R. H., Donninger, C. & Popják, G. (1966). *Proc. Roy. Soc.* B, **163**, 492.
Cornforth, J. W., Cornforth, R. H., Popják, G. & Youhotsky-Gore, I. (1958). *Biochem. J.* **69**, 146.
Cornforth, J. W., Hunter, G. D. & Popják, G. (1953). *Biochem. J.* **54**, 597.
Davis, J. B., Jackman, L. M., Siddons, P. T. & Weedon, B. C. L. (1966). *J. chem. Soc.* C, p. 2154.
Donninger, C. & Popják, G. (1966). *Proc. Roy. Soc.* B, **163**, 465.
Goodwin, T. W. (1955). In *Modern Methods of Plant Analysis*, vol. 3, p. 372. Ed. by Paech, K. & Tracey, M. V. Heidelberg: Springer-Verlag.
Goodwin, T. W. (1965). In *Biosynthetic Pathways in Higher Plants*, p. 37. Ed. by Pridham, J. B. & Swain, T. London: Academic Press (Inc.) Ltd.
Goodwin, T. W. & Williams, R. J. H. (1965*a*). *Biochem. J.* **94**, 5C.
Goodwin, T. W. & Williams, R. J. H. (1965*b*). *Biochem. J.* **97**, 28C.
Goodwin, T. W. & Williams, R. J. H. (1966). *Proc. Roy. Soc.* B, **163**, 515.
Hanson, K. R. (1966). *J. Amer. chem. Soc.* **88**, 2731.
Jungalwala, F. B. & Porter, J. W. (1965). *Arch. Biochem. Biophys.* **110**, 291.
Knauss, H., Brodie, J. D. & Porter, J. W. (1962). *J. Lipid Res.* **3**, 197.
Krishna, G., Whitlock, H. W., Feldbruegge, D. H. & Porter, J. W. (1966). *Arch. Biochem. Biophys.* **114**, 200.
Okita, G. I., Kahara, J. J., Richardson, F. & Le Roy, G. V. (1957). *Nucleonics*, **15**, 111.
Popják, G. & Cornforth, J. W. (1966). *Biochem. J.* **101**, 553.
Popják, G., Goodman, De W. S., Cornforth, J. W., Cornforth, R. H. & Ryhage, R. (1961). *J. biol. Chem.* **236**, 1934.
Rabourn, W. J. & Quackenbush, F. W. (1956). *Arch. Biochem. Biophys.* **61**, 111.
Rilling, H. C. (1966). *J. biol. Chem.* **241**, 3233.
Schlesinger, M. J. & Coon, M. J. (1961). *J. biol. Chem.* **236**, 2421.
Shunk, C. H., Linn, B. O., Huff, J. W., Gilfillan, J. L., Skeggs, H. R. & Folkers, K. (1957). *J. Amer. chem. Soc.* **79**, 3294.
Tomes, M. L. (1963). *Bot. Gaz.* **124**, 180.
Wells, L. W., Schelbe, W. J. & Porter, J. W. (1964). *Fed. Proc.* **23**, 426.
Williams, R. J. H., Britton, G. & Goodwin, T. W. (1966*a*). *Biochim. biophys. Acta*, **124**, 200.
Williams, R. J. H., Britton, G. & Goodwin, T. W. (1966*b*). *Biochem. J.* **101**, 7P.