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PHYTOENE BIOSYNTHESIS: POSSIBLE MECHANISMS FOR THE COUPLING OF GERANYLGERANYL PYROPHOSPHATE*

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Abstract—A cell-free enzyme system, prepared from *Phycomyces blakesleeanus* mutant albino 10, converted (^{14}C)geranylgeranyl pyrophosphate into phytoene. Two mechanisms proposed for the coupling of two molecules of geranylgeranyl pyrophosphate to form phytoene were investigated, and it was also established that phytoene was the first C_{40} polyene formed and not lycopersene (C_{40} analogue of squalene). Free geranyllinaloyl pyrophosphate was not detected when the cell-free enzyme system was incubated with (^{14}C)geranylgeranyl pyrophosphate. A labelled enzyme-bound intermediate, isolated from the reaction mixture was converted to phytoene when incubated with the cell-free enzyme system. The two proposed coupling mechanisms are discussed in the light of these results.

INTRODUCTION

RADIOTRACER and enzymatic studies have established that carotenes may be synthesized from acetate via the general isoprenoid pathway.[1–4] It is generally agreed that the colourless polyene phytoene is the first C_{40} compound formed and that the other acyclic and cyclic carotenes are formed by dehydrogenation and cyclization of phytoene. The formation of phytoene and other carotenes from the C_{20} intermediate geranylgeranyl pyrophosphate has previously been demonstrated in a cell-free enzyme system prepared from *Phycomyces blakesleeanus* mutant R_1,[5] but little data is available on the mechanism by which two geranylgeranyl pyrophosphate molecules couple to form phytoene.

By analogy with squalene (C_{30})synthesis two types of mechanisms have been invoked for the coupling of two geranylgeranyl pyrophosphate molecules to form phytoene[6,7] but they have not been tested experimentally. The mechanism most favoured at present postulates that two molecules of geranylgeranyl pyrophosphate are coupled via a sulphonium ylide (Fig. 1). This type of mechanism requires a thio-ether grouping at the active site of the

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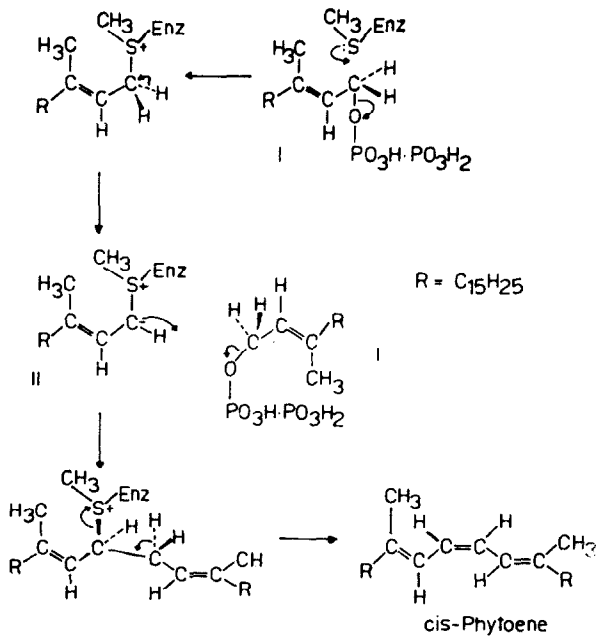

FIG. 1. MECHANISM OF PHYTOENE BIOSYNTHESIS FROM GERANYLGERANYL PYROPHOSPHATE VIA AN ENZYME-BOUND INTERMEDIATE (Adapted from Williams *et al.*[7]).

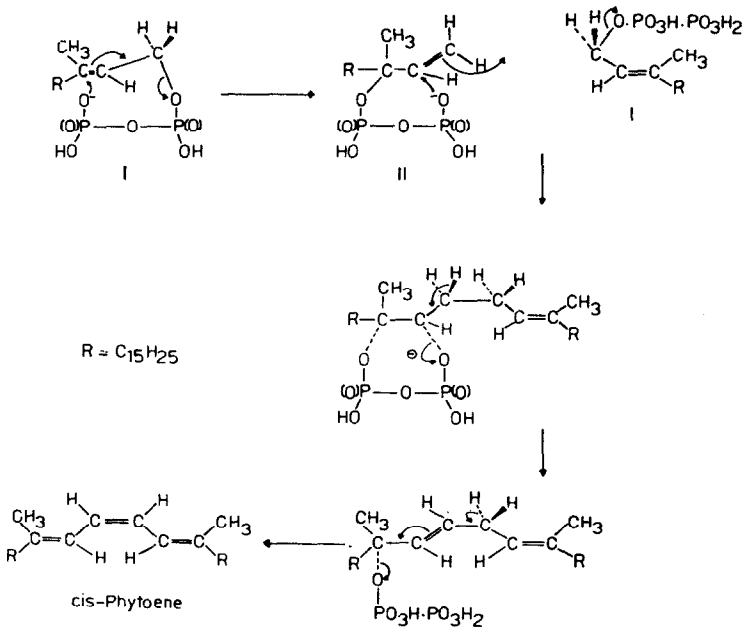

FIG. 2. MECHANISM OF PHYTOENE BIOSYNTHESIS FROM GERANYLGERANYL PYROPHOSPHATE VIA FREE GERANYLLINALOYL PYROPHOSPHATE (Adapted from Williams *et al.*[7]).

enzyme. The second type of mechanism for the formation of phytoene from geranylgeranyl pyrophosphate envisages free geranyllinaloyl pyrophosphate as an intermediate[6] (Fig. 2) but no evidence is available to eliminate this mechanism.

One of the available mutants of *P. blakesleeanus*, albino 10, synthesizes large amounts of phytoene[8] and thus is considered suitable for studying the proposed coupling mechanisms. A cell-free enzyme system prepared from this mutant can synthesize phytoene from (^{14}C)-geranylgeranyl pyrophosphate. Attempts to isolate the proposed intermediates of this coupling reaction are detailed in this report.

RESULTS AND DISCUSSION

Lycopersene as an Intermediate in Phytoene Synthesis

Many workers[9–12] have been unable to confirm the report of Grob and Boschetti[13] that lycopersene is the first C_{40} compound formed during carotene biosynthesis. A further attempt has been made to detect lycopersene formation in normal and diphenylamine-treated cultures of *P. blakesleeanus* mutant albino 10 which accumulate 1649 and 4770 μg phytoene/g dry wt. of mycelia respectively.[8] These amounts of phytoene are many times greater than those found in other carotenogenic systems previously examined for lycopersene formation.

(2-^{14}C)Mevalonic acid was supplied to normal and diphenylamine-treated albino 10 cultures. After 7 days growth, carrier lycopersene was added and the phytoene and lycopersene extracted and purified twice on silica gel thin layers developed with petroleum ether–benzene (96:4, v/v). The lycopersene spot contained no radioactivity after the second purification (Table 1). A similar result was obtained when a cell-free enzyme system prepared from albino 10 mycelia was incubated with (^{14}C)geranylgeranyl pyrophosphate (Table 2). In all three systems carotene synthesis was operating as phytoene was strongly labelled.

It is difficult to prove conclusively that lycopersene is not present in a carotenogenic system even though in these experiments the label from (2-^{14}C)geranylgeranyl pyrophosphate was not incorporated into lycopersene. Under the conditions of the experiments as little as 20 counts/min could easily be counted and as little as 0·05 μg of lycopersene could be detected on the TLC plates with iodine staining. Thus an extract of approximately 5 g dry

TABLE 1. INCORPORATION OF (2-^{14}C)MEVALONIC ACID* INTO PHYTOENE AND LYCOPERSENE BY UNTREATED AND DIPHENYLAMINE-TREATED ALBINO 10 MYCELIA†

	Radioactivity (dis/min)			
	Untreated		Diphenylamine-treated (10^{-4}M)	
Compound	1st TLC‡	2nd TLC‡	1st TLC‡	2nd TLC‡
Phytoene	4216	2208	8540	6970
Lycopersene	74	0	10	0

* 30×10^6 dis/min added to each flask.
† Each flask contained 4·92 g dry wt. of mycelia after 7 days of growth.
‡ See Experimental.

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[13] E. C. GROB and A. BOSCHETTI, *Chimia* **16**, 15 (1962).

TABLE 2. INCORPORATION OF (^{14}C)GERANYLGERANYL PYROPHOSPHATE INTO PHYTOENE AND LYCOPERSENE BY THE CELL-FREE ENZYME SYSTEM*

Compound	Radioactivity (dis/min) 1st TLC†	2nd TLC†
Phytoene	3820	3702
Lycopersene	20	0

* Standard system (see Experimental) incubated for 24 hr with (^{14}C)geranylgeranyl pyrophosphate (8×10^4 dis/min).
† See Experimental.

wt. of albino 10 mycelia contained less than 0·05 μg of lycopersene. Therefore, the concentration of lycopersene did not exceed 0·01 μg/g dry wt. of mycelia compared to the normal concentrations of phytoene in untreated mycelia (1649 μg/g dry wt.) or in diphenylamine-treated mycelia (4770 μg/g dry wt.). One can reasonably conclude that lycopersene is not an intermediate for carotene synthesis in this organism.

Geranyllinaloyl Pyrophosphate as an Intermediate in Phytoene Synthesis

Three experiments were carried out in which the cell-free enzyme system was incubated with (^{14}C)geranylgeranyl pyrophosphate for 2 and 8 hr and with (2-^{14}C)mevalonic acid for 8 hr. In each experiment no label was found in the geranyllinalool zone (terpene liberated from geranyllinaloyl pyrophosphate by enzymatic hydrolysis) on the TLC plates (Table 3) or in the geranyllinaloyl pyrophosphate fraction eluted from DEAE columns.

TABLE 3. FAILURE OF CELL-FREE ENZYME SYSTEM TO INCORPORATE (^{14}C)GERANYLGERANYL PYROPHOSPHATE AND (2-^{14}C)MEVALONIC ACID INTO GERANYLLINALOYL PYROPHOSPHATE.

Substrate	Experiment 1* (^{14}C)GGPP†	Experiment 2* (^{14}C)GGPP†	Experiment 3* (2-^{14}C)MVA†
Incubation time (hr)	2	8	8
Added dis/min $\times 10^4$	4	4	210
Geranylgeraniol‡ (dis/min)	9870	5210	280
Geranyllinalool‡ (dis/min)	0	0	0

* Experiments 1 and 2 used the standard system. In experiment 3, 2000 μmole tris HCl buffer (pH 7·8), 100 μmole ATP, 15 μmole NADH, 15 μmole NADPH, 15 μmole NAD, 750 μmole $MgCl_2$, 500 μmole dithiothreitol and 680 mg enzyme protein were contained in 40 ml.
† Abbreviations: GGPP, geranylgeranyl pyrophosphate; MVA, mevalonic acid.
‡ Geranylgeranyl pyrophosphate and geranyllinaloyl pyrophosphate were detected and estimated as geranylgeraniol and geranyllinalool, respectively.

These results indicate that free geranyllinaloyl pyrophosphate is not an intermediate in the conversion of geranylgeranyl pyrophosphate to phytoene by the cell-free enzyme system. It is interesting to note that nerolidyl pyrophosphate, the C_{15} analogue of geranyllinaloyl pyrophosphate, was not detected when farnesyl pyrophosphate was converted to squalene by yeast microsomes,[14] and in addition (1-$^3H_2$)nerolidyl pyrophosphate was not converted

[14] S. S. SOFER and H. C. RILLING, *J. Lipid Res.* **10**, 183 (1969).

to squalene by the same system. We have been unable to prepare unlabelled or (^{14}C)geranyllinaloyl pyrophosphate to determine whether a cell-free enzyme system can convert this compound to phytoene through an enzyme complex, or whether the addition of unlabelled geranyllinaloyl pyrophosphate will have a dilution effect on the conversion of (^{14}C)geranylgeranyl pyrophosphate to phytoene.

Conversion of Geranylgeranyl Pyrophosphate to Phytoene via an Enzyme-Bound Intermediate

It has been postulated that geranylgeranyl pyrophosphate may be converted to phytoene via an enzyme-bound intermediate (Fig. 1). Such a coupling would require a thio-ether or sulphydryl group for enzyme activity. The sulphydryl group stabilizing agent dithiothreitol has been found to stabilize the activity of the enzyme (Table 4) and furthermore, the

TABLE 4. EFFECT OF SULPHYDRYL GROUP INHIBITORS AND PROTECTORS ON THE INCORPORATION OF (^{14}C)GERANYLGERANYL PYROPHOSPHATE INTO PHYTOENE BY THE CELL-FREE ENZYME SYSTEM.

System	Radioactivity incorporated into phytoene (dis/min)	Inhibition (%)
1 Complete system*	3980	0
2 Without dithiothreitol	1988	50·0
3 *p*-Chloromercuribenzoate (5×10^{-3}M) added	80	98·0
4 Iodacetamide (10^{-3}M) added	0	100·0
5 N-Ethylmaleimide (10^{-3}M) added	38	99·0

* Standard system (see Experimental) incubated for 24 hr with (^{14}C)geranylgeranyl pyrophosphate (8×10^4 dis/min). In experiments 3-5, dithiothreitol was absent from the system. The enzyme was preincubated with the inhibitors for 30 min. Substrates were then added and the reaction continued.

sulphydryl group inhibitors *p*-chloromercuribenzoate, iodoacetamide and N-ethylmaleimide prevented the conversion of geranylgeranyl pyrophosphate to phytoene by the cell-free enzyme system.

Further support for the participation of an enzyme-bound intermediate was derived from the isolation of a labelled enzyme complex from the reaction mixture when (^{14}C)geranylgeranyl pyrophosphate was incubated with the cell-free enzyme system from albino 10 mycelia for 8 hr. The (^{14}C)enzyme-bound intermediate was initially purified on a Sephadex G-50 column and finally on a Sephadex G-100 column (Fig. 3). The (^{14}C)enzyme-bound intermediate was converted to phytoene on incubation with the cell-free enzyme preparation (Table 5). The above evidence appears to favour the type of mechanism shown in Fig. 1, although the identity of the compound bound with the enzyme and also the nature of the binding are unknown.

An enzyme-bound intermediate has been isolated during the conversion of farnesyl pyrophosphate to squalene,[15,16] however, there now exists considerable controversy over the structure of the squalene precursor and the nature of the association with the enzyme. Both

[15] G. KRISHNA, D. H. FELDBRUEGGE and J. W. PORTER, *Biochem. Biophys. Res. Commun.* **14**, 363 (1964).
[16] G. KRISHNA, H. W. WHITLOCK, D. H. FELDBRUEGGE and J. W. PORTER, *Archs Biochem. Biophys.* **114**, 200 (1966).

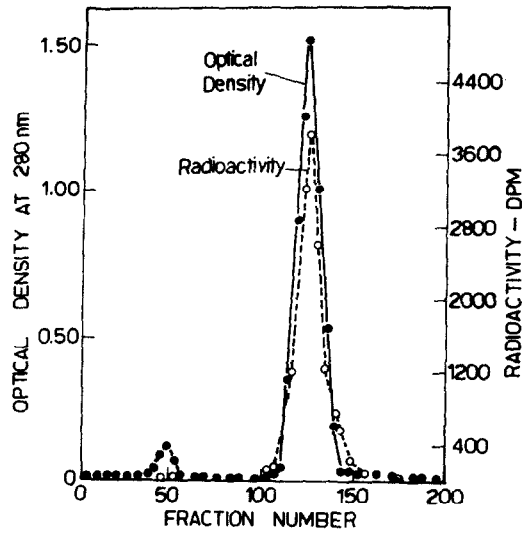

FIG. 3. GEL FILTRATION OF A (^{14}C)ENZYME-BOUND INTERMEDIATE FORMED DURING CONVERSION OF (^{14}C)GERANYLGERANYL PYROPHOSPHATE TO PHYTOENE.

Standard system (see Experimental) was incubated for 8 hr with (^{14}C)geranylgeranyl pyrophosphate (6×10^4 dis/min). The (^{14}C)enzyme-bound intermediate was purified as described in Experimental section.

Rilling[17,18] and Popjak[19] have isolated C_{30}-cyclic pyrophosphate esters as intermediates in squalene biosynthesis by liver and yeast microsomes but have not been able to agree on the exact structure of these compounds. Rilling[20] has also been able to show that the squalene precursor is tightly, but not covalently, bound to the microsomes. Thus, considerable effort is needed to identify the immediate precursors of both squalene and phytoene.

TABLE 5. CONVERSION OF (^{14}C)ENZYME-BOUND INTERMEDIATE INTO PHYTOENE BY THE CELL-FREE ENZYME SYSTEM*

	Radioactivity (dis/min) 1st TLC†	2nd TLC†
Phytoene	1607	1479

* Standard system (see Experimental) incubated for 24 hr with (^{14}C)enzyme-bound intermediate (3×10^4 dis/min).
† See Experimental.

EXPERIMENTAL

Microorganisms and cultural conditions. Phycomyces blakesleeanus mutant albino 10 was grown in shake culture.[8] Diphenylamine was added to the culture flasks to give a final concentration of 10^{-4}M.

Preparation of (^{14}C)*geranylgeranyl pyrophosphate and cell-free enzyme system.* (^{14}C)Geranylgeranyl pyrophosphate was synthesized enzymatically from (2-^{14}C)mevalonic acid by a soluble enzyme fraction of a

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[19] G. POPJAK, J. EDMOND, K. CLIFFORD and V. WILLIAMS, *J. Biol. Chem.* **244**, 1897 (1969).
[20] H. C. RILLING, *J. Lipid Res.* **11**, 480 (1970).

homogenate of endosperm tissue of immature *Echinocystis macrocarpa* Greene seed.[21] (^{14}C)Geranylgeranyl pyrophosphate was purified and assayed according to Oster and West.[21] The cell-free enzyme system was prepared from 4–5-day-old mycelial mats of *P. blakesleeanus* mutant albino 10.[5] The standard reaction mixture contained 2000 μmoles tris HCl buffer (pH 7·8), 500 μmoles dithiothreitol, 750 μmoles $MgCl_2$, 680 mg enzyme protein and (^{14}C)geranylgeranyl pyrophosphate. Where the contents of the reaction mixture varied from that of the standard mixture, details are given in the Results and Discussion section. The reaction mixtures were incubated at room temp. (25°).

Isolation and purification of (^{14}C)*phytoene and* (^{14}C)*lycopersene.* Incubation was stopped by the addition of 3 vols EtOH and carrier polyenes were added before extraction of the reaction mixture. Carrier phytoene was extracted from albino 10 mycelia and purified.[22,5] Phytoene and lycopersene extracted from the reaction mixtures were chromatographed twice on silica gel plates developed with petroleum ether–benzene (96:4, v/v).

Extraction and purification of terpenoid pyrophosphates. The reaction mixture was boiled for 3 min and the terpenoid pyrophosphates extracted with collidine and transferred to 0·01 M $NaHCO_3$.[21] Half of the sample was hydrolyzed with bacterial alkaline phosphatase after addition of carrier geranylgeraniol and geranyllinaloyl and the liberated terpenes chromatographed on silica gel TLC plates developed firstly with hexane to 15 cm and secondly in the same direction with benzene–ethyl acetate (9:1, v/v) to 10 cm.[21] Authentic terpenes were run on all plates. Terpenes were visualized on the plates with iodine vapour. Geranylgeraniol and geranyllinalool, tertiary and primary alcohols of equivalent chain length, were satisfactorily resolved. The remaining half of the sample was chromatographed on a DEAE Sephadex A-25 column.[23] The column was washed with 50 ml of 0·02 M NH_4HCO_3 after loading of the sample and developed with 500 ml of a gradient of NH_4HCO_3 ranging from 0·02 to 0·66 M. Peaks of pyrophosphate esters were identified by, (a) enzymic hydrolysis and cochromatography of liberated terpenes with authentic compounds, and (b) comparison of the retention volume of the individual peaks with those of Oster and West.[21]

Purification of enzyme-bound intermediate. The reaction mixture was concentrated to a small volume and passed through a column of Sephadex G-50 which was developed with 0·01 M tris HCl buffer (pH 8·0) containing 0·2 M NaCl after equilibration with this solution. Fractions (3 ml) were collected from the column effluent and assayed for radioactivity with a liquid scintillation spectrometer and for protein by absorption at 280 nm. The (^{14}C)enzyme-bound intermediate eluted from the Sephadex G-50 was further purified on a Sephadex G-100 column equilibrated and developed as above. The collected fractions (3 ml) were assayed for radioactivity and protein.

Acknowledgements—The authors gratefully acknowledge the cooperation of Drs M. O. Oster and C. A. West for their aid in obtaining *E. macrocarpa* seeds, invaluable suggestions for preparation of (^{14}C)geranylgeranyl pyrophosphate, and provision of an authentic sample of geranylgeranyl pyrophosphate. *Phycomyces blakesleeanus* mutant albino 10 was kindly provided by Dr. M. Delbruck of the California Institute of Technology, Pasadena, California. Authentic samples of lycopersene, geranylgeraniol, geranyllinalool, nerol, geraniol, farnesol and nerolidol were the gift of Dr. W. E. Scott, Hoffmann-La Roche, Inc., Nutley, New Jersey. The work was supported by U.S. Public Health Service Grant No. AM 11665 to C.O.C.

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Key Word Index—Phycomyces blakesleeanus; Fungi; biosynthesis; phytoene; geranylgeranyl pyrophosphate coupling.