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The Stereochemistry of *trans*-Phytoene Synthesis. Some Observations on Lycopersene as a Carotene Precursor and a Mechanism for the Synthesis of *cis*- and *trans*-Phytoene†

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ABSTRACT: *trans*-Phytoene biosynthesized by a *Mycobacterium sp.* has been shown to retain one *pro-S* and one *pro-R* hydrogen from C-1 of the two molecules of geranylgeranyl pyrophosphate that constitute this carotene. This complements the finding of Williams *et al.* [Williams, R. J. H., Britton, G., Charlton, J. M., and Goodwin, T. W. (1967), *Biochem. J. 104*, 767–777] who demonstrated that two *pro-R* hydrogens were retained in *cis*-phytoene. Although Barnes *et al.* [Barnes, F. J., Qureshi, A. A., Semmler, E. J., and Porter, J. W. (1973), *J. Biol. Chem. 248*, 2768–2773] have presented evidence that lycopersene is a precursor to phytoene, a stereochemical analysis of phytoene synthesis shows that lycopersene can be a precursor to *cis*-phytoene only if two special and unlikely requirements are met. These considerations make it unlikely that lycopersene is a carotene precursor. We propose a mechanism for the synthesis of *cis*- and *trans*-phytoene directly from prephytoene pyrophosphate.

The stereochemical aspects of bond formation in polyterpenoid biosynthesis have been studied extensively (Popjak and Cornforth, 1966; Goodwin, 1971). In an investigation concerning carotene biosynthesis, Williams *et al.* (1967) and Buggy *et al.* (1969) established that both *pro-S* hydrogens are lost from C-1 of geranylgeranyl pyrophosphate during its conversion to *cis*-phytoene. One would anticipate a retention of different hydrogens during the synthesis of the trans isomer if *cis*- and *trans*-phytoenes are synthesized from common intermediates by a similar mechanism. We have examined the stereochemistry of hydrogen retention during the synthesis of *trans*-phytoene from geranylgeranyl pyrophosphate in a bacterial system and have found that 1 *pro-R* and 1 *pro-S* hydrogen are retained during this transformation as predicted. These results in conjunction with those of Williams *et al.* (1967) and Buggy *et al.* (1969) lead us to postulate a consistent mechanism for *cis*- or *trans*-phytoene synthesis from prephytoene pyrophosphate.

A stereochemical analysis of phytoene synthesis reveals that lycopersene can be a precursor of *cis*-phytoene only if either of two requirements, which are considered unlikely, can be met. These and other considerations have led us to conclude that lycopersene is probably not a normal precursor to carotenes.

Materials and Methods

all-trans-Geranylgeraniol, a generous gift from Dr. L. J. Altman, was oxidized by MnO_2. The resulting aldehyde was then reduced with NaB^3H_4 to form [1-$^3H_2$]geranylgeraniol (75 Ci/mol). [1-^{3}H]Geranylgeranial, prepared from [1-$^3H_2$]-geranylgeraniol by MnO_2 oxidation, was stereoselectively reduced by NADH and liver alcohol dehydrogenase to yield (1*S*)-[1-^{3}H]geranylgeraniol (38 Ci/mol) (Donninger and Ryback, 1964). The pyrophosphate esters of the alcohols were prepared and isolated by methods previously described (Gregonis and Rilling, 1973). [4-^{14}C]Isopentenyl pyrophosphate (4.2 Ci/mol) was prepared by the method of Tchen (1963). [4-^{14}C]Geranylgeranyl pyrophosphate was enzymatically prepared from farnesyl pyrophosphate and [4-^{14}C]isopentenyl pyrophosphate. The enzyme used was derived from a photoinduced *Mycobacterium sp.* by ammonium sulfate precipitation (35%) of a 100,000*g* supernatant fraction. The ammonium sulfate was removed by dialysis against 0.05 M potassium phosphate–1 mM $MgCl_2$ (pH 7.4). The incubation mixture contained 11 mg of enzyme protein and 0.4 μM isopentenyl pyrophosphate, 1.6 μM *trans*-farnesyl pyrophosphate, 0.05 M potassium phosphate (pH 7.4), and 1 mM $MgCl_2$ in a volume of 4 ml. The [4-^{14}C]geranylgeranyl pyrophosphate was extracted into 1-butanol and purified by ion exchange chromatography (Gregonis and Rilling, 1973). For the experiments described, it was combined with (1*S*)-[1-^{3}H]-geranylgeranyl pyrophosphate. [1-$^3H_2$,4-^{14}C]Geranylgeranyl pyrophosphate was prepared from *trans*-farnesyl pyrophosphate and [1-$^3H_2$,4-^{14}C]isopentenyl pyrophosphate in the same manner. [^{3}H]NADPH was prepared by chemical reduction of NADP by [^{3}H]$NaBH_4$ as described by Chaykin (1965). The specific activity was 35 Ci/mol.

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TABLE I: Incorporation of Radioisotopes from [1-$^3H_2$,4-^{14}C]Geranylgeranyl Pyrophosphate into Phytoene.

Expt	Geranylgeranyl Pyrophosphate $^3H/^{14}C$ (A)	*cis*-Phytoene DPM ^{14}C	*cis*-Phytoene $^3H/^{14}C$ (B)	B/A	*trans*-Phytoene DPM ^{14}C	*trans*-Phytoene $^3H/^{14}C$ (C)	C/A
1	0.89	2080	0.44	0.49	620	0.46	0.52
2	11.6	2073	4.6	0.40			
3	10.4				76	5.0	0.48

Extracts from either a *Mycobacterium sp.* or C_5 mutant of *Phycomyces blakesleeanus* were used respectively for the biosynthesis of *trans*- and *cis*-phytoene from geranylgeranyl pyrophosphate. Whole homogenates of *Mycobacteria sp.* were prepared as before (Gregonis and Rilling, 1973). For the *Phycomyces* preparation, 1 g of freshly harvested mycelial mat was suspended in 8 ml of 0.05 M potassium phosphate buffer (pH 7.4) containing 1 mM $MgCl_2$, and dispersed with a Polytron homogenizer. The suspension was then passed through a French pressure cell at 15,000 psi. The pellet obtained by centrifugation at 100,000*g* for 1 hr was suspended in 4 ml of the $MgCl_2$ containing buffer. Typically, the incubation mixtures contained 1 nmol of geranylgeranyl pyrophosphate and for *trans*-phytoene synthesis 2 mg of Mycobacterial protein in 0.2 ml, while incubation mixtures for *cis*-phytoene synthesis contained 0.5 mg of *Phycomyces* protein in 0.1 ml. Incubations were for 2 hr at 30°.

The incubation mixtures were extracted with 2 ml of 1-butanol, and the appropriate carrier phytoene was added. The butanol was removed under a stream of nitrogen. The phytoenes were separated by chromatography on a 1 × 13 cm column of 1% deactivated alumina (Gregonis and Rilling, 1973). Columns were developed with a gradient of Skellysolve B to 5% diethyl ether in Skellysolve B (for *cis*-phytoene) or to 9% diethyl ether in Skellysolve B (for *trans*-phytoene). Column eluates were monitored for both phytoene absorbance at 286 nm and radioactivity. In all experiments there was a coincidence of the absorbancy of phytoene and radioactivity.

Results

As anticipated, *cis*- or *trans*-phytoene synthesized from [1-$^3H_2$,4-^{14}C]geranylgeranyl pyrophosphate by enzymes from either *Phycomyces* or *Mycobacteria* retained two of four labeled hydrogens (Table I). This experiment establishes that both hydrogens on the central two carbons of *trans*-phytoene come from geranylgeranyl pyrophosphate rather than from another source such as NADPH. This was confirmed by synthesizing phytoene from [4-^{14}C]geranylgeranyl pyrophosphate in the presence of [^3H]NADPH. The phytoene recovered from alumina chromatography contained 3390 dpm ^{14}C and 44 dpm ^3H. If one hydrogen from NADPH had been incorporated into the phytoene about 9000 dpm ^3H would have been found in these fractions. When (1*S*)-[1-^3H]geranylgeranyl pyrophosphate was used as substrate, the *cis*-phytoene synthesized by the *Phycomyces* extracts retained none of the labeled hydrogens (Table II). This observation confirms the finding of Williams *et al.* (1967) and Buggy *et al.* (1969) that the *pro-S* hydrogens of geranylgeranyl pyrophosphate are lost during *cis*-phytoene synthesis and also extends the generality of this observation to *Phycomyces*. When (1*S*)-[1-^3H]geranylgeranyl pyrophosphate was used as substrate with the mycobacterial preparation, one of two hydrogens was retained in *trans*-phytoene (Table II). This established that a *pro-R* and a *pro-S* hydrogen are retained in this sequence of reactions.

Discussion

In an earlier study on the mechanism of phytoene synthesis, we demonstrated that a 40-carbon cyclopropylcarbinyl pyrophosphate (prephytoene pyrophosphate) was an intermediate between geranylgeranyl pyrophosphate and phytoene (Altman *et al.*, 1972). These results extended the generality of the mechanism of head-to-head terpene condensations first demonstrated for squalene synthesis (Epstein and Rilling, 1970; Rilling *et al.*, 1971; Altman *et al.*, 1971; Edmond *et al.*, 1971; Popjak *et al.*, 1973). At that time we proposed a mechanism for the synthesis of phytoene that was similar to that proposed for squalene synthesis from farnesyl pyrophosphate except for a proton loss rather than a hydride reduction for the final step (Altman *et al.*, 1971). Since then, work from Porter's laboratory has demonstrated the same pyrophosphorylated intermetiate is involved in carotene synthesis in tomato preparations (Qureshi *et al.*, 1972; Barnes *et al.*, 1973). Since these workers found that lycopersene, 15,15′-dihydrophytoene, was synthesized by their enzyme preparations, they termed the intermediate prelycopersene pyrophosphate. They also demonstrated that lycopersene was converted to phytoene by an enzyme preparation from tomatoes, providing the most definitive evidence that this compound is an intermediate in carotene synthesis. In contrast, since the initial report of the existence of lycopersene by Grob *et al.* (1961), many laboratories have failed in their attempts to detect this compound as an intermediate in carotene synthesis. (This is summarized by

TABLE II: Incorporation of Radioisotopes from (1*S*)-[1-^3H,4-^{14}C]Geranylgeranyl Pyrophosphate into Phytoene.

Expt	Geranylgeranyl Pyrophosphate $^3H/^{14}C$ (A)	*cis*-Phytoene DPM ^{14}C	*cis*-Phytoene $^3H/^{14}C$ (B)	B/A	*trans*-Phytoene DPM ^{14}C	*trans*-Phytoene $^3H/^{14}C$ (C)	C/A
1	12.8	700	0.21	0.016	4820	7.3	0.57
2	12				439	5.1	0.43
3	11.6				312	6.0	0.52
4	13.3	1150	0.22	0.017			

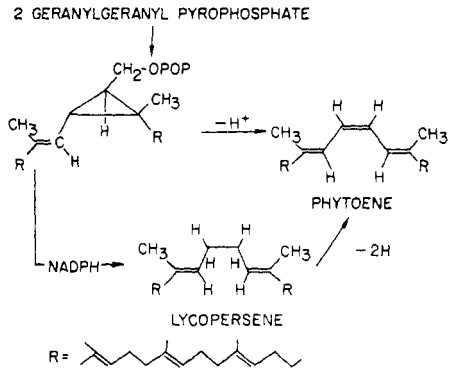

FIGURE 1: Two possible routes for phytoene synthesis from geranylgeranyl pyrophosphate.

identity is maintained only if the molecule cannot rotate. If lycopersene would be free to leave the enzyme or to rotate on the enzyme surface during its transit from its site of synthesis to the site of dehydrogenation, the stereochemical identity of these atoms will be lost as a result of symmetry and these carbons will become equivalent.[1]

During its conversion to phytoene, two of the four hydrogens on the central two carbons of lycopersene would be lost. Three distinct factors will govern which hydrogens are retained: the mode of dehydrogenation (cis or trans), the configuration of the product (cis or trans), and the randomization or nonrandomization of lycopersene. The consequences of these variables on the hydrogens retained in *cis*- and *trans*-phytoene are shown in Figures 2 and 3. The observed retention

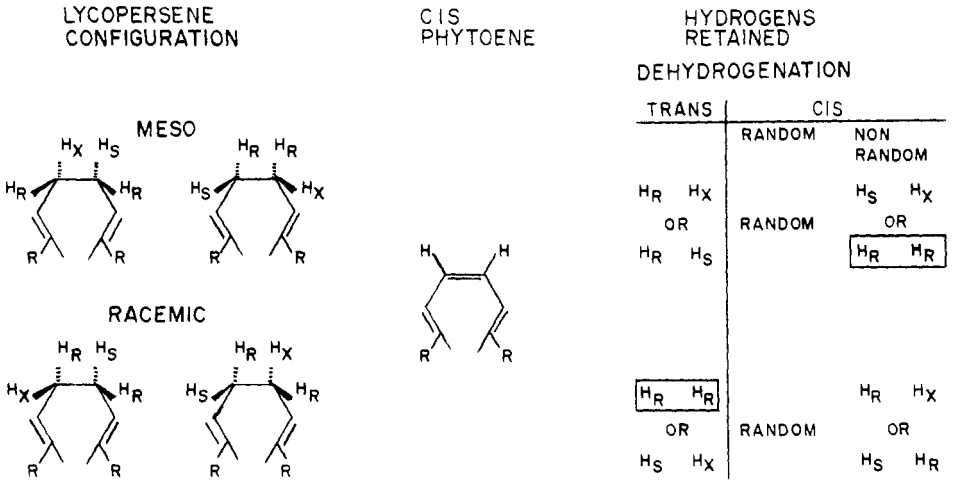

FIGURE 2: The sterochemical consequences of hydrogen retention in *cis*-phytoene if lycopersene is an intermediate. The observed retention of hydrogen is boxed.

Barnes *et al.* (1973)). Thus, it seems that two routes from geranylgeranyl pyrophosphate are possible (Figure 1). However, we reasoned that if lycopersene is an intermediate one would anticipate that a portion of the hydrogens on the central carbons of phytoene would come from NADPH. Our experiment showing a lack of incorporation of ^3H from NADPH into *trans*-phytoene and the experiments from Goodwin's laboratory (Williams *et al.*, 1967; Buggy *et al.*, 1969), which show that these hydrogens of *cis*-phytoene are *exclusively* derived from a *pro-R* hydrogen of (5*R*)-[5-^3H]-mevalonate, rule out this possibility. This consideration, along with the observed retention of two *pro-R* hydrogens from C-1 of geranylgeranyl pyrophosphate during its conversion to *cis*-phytoene and our finding that 1 *pro-R* and 1 *pro-S* hydrogen are retained in *trans*-phytoene, provide stereochemical requirements that must be met in this sequence of reactions. The following analysis of these requirements shows it unlikely that lycopersene is an intermediate in phytoene synthesis.

Lycopersene, like its analog squalene, is a symmetrical compound. However, biosynthetic squalene is not biologically symmetrical since two of the four hydrogens on the central two carbons were *pro-R* hydrogens and one a *pro-S* hydrogen of the condensing farnesyl pyrophosphates that constitute this symmetrical terpene (Popjak and Cornforth, 1966). The remaining hydrogen comes from NADPH by a reduction. The central carbons of lycopersene are expected to be similarly substituted. Even though the central carbons of lycopersene and squalene are asymmetrically substituted, their separate

of hydrogen in *cis*- and *trans*-phytoene are boxed in the figures.

For *trans*-phytoene to arise from lycopersene, the observed hydrogen retention would result from cis dehydrogenation of a meso form that was not restricted from randomizing (Figure 3). These are not unusual or special requirements since the analog of lycopersene, squalene, is of meso configuration. However, as Figure 2 shows, the synthesis of *cis*-phytoene from lycopersene requires either that lycopersene be racemic rather than meso or that it not be permitted to randomize. The possibility of these alternatives is now considered.

Presqualene pyrophosphate and the carotene precursor have been shown by physical methods to have the same absolute orientation (Barnes *et al.*, 1973), and the stereochemistry of the hydrogens on the carbinyl carbon can be assumed to be the same as in geranylgeranyl pyrophosphate. If these molecules are reduced to their hydrocarbon products by a similar mechanism, lycopersene and squalene would have the same configuration. However, it is not necessary for analogous reactions to proceed by stereochemically identical pathways unless a mechanistic advantage is gained by so doing (Rose,

[1] For the purposes of this discussion, we will assume the lycopersene and/or squalene molecules being considered have been so labeled and therefore can be assigned a meso or racemic configuration, depending upon the arrangement of these hydrogens. Also, H_R and H_S will represent the *pro-R* and *pro-S* hydrogens at C-1 from either farnesyl or geranylgeranyl pyrophosphate and H_X the hydrogen from NADPH.

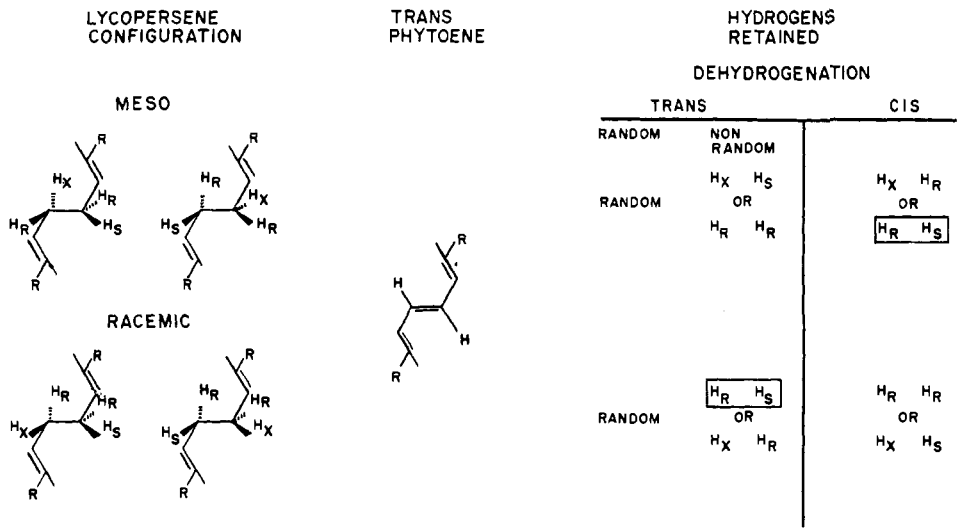

FIGURE 3: The stereochemical consequences of hydrogen retention in *trans*-phytoene if lycopersene is an intermediate. The observed retention of hydrogen is boxed.

1972). Squalene derived from either (1*R*)-[$^3H_1$]- or (1*R*)-[1-$^2H_1$]-farnesyl pyrophosphate is meso as established by the elegant studies of Popjak and Cornforth (1966). If lycopersene were to be synthesized from its precursor by a mechanism that differs from the mechanism of squalene synthesis by a change in number of inversions, then it could have a racemic rather than a meso configuration. An examination of the sequence of events leading from presqualene pyrophosphate to squalene reveals three steps at which an inversion could be gained or lost. These are during the ionization of the carbon–oxygen bond of the pyrophosphate ester, during the initial cationic rearrangement of the C_1–C_3 bond, and during the reduction of the final carbonium ion intermediate by NADPH. For stereochemical and stereoelectronic reasons which are fully developed elsewhere by Poulter *et al.* (1974), it is improbable that any of these steps would proceed differently than in squalene synthesis. Thus, it seems most unlikely that a lycopersene other than the meso compound could be obtained from (1*R*)-[1-$^3H_1$]geranylgeranyl pyrophosphate.

The second consideration is of the possible randomization of lycopersene. Several laboratories have investigated a similar problem—the randomization of squalene during sterol biosynthesis. By examining the distribution of ^3H in lanosterol synthesized from farnesyl pyrophosphate in the presence of NADP^3H, Etemadi *et al.* (1969) concluded that the biosynthesized squalene had randomized. Ebersole *et al.* (1973) incubated (3*R*)-[2-^{14}C]-(5*S*)-[5-^3H]mevalonate with cultures of *Fusidium coccineum*. The distribution of the radioactivity in the fusidic acid subsequently isolated left no doubt that squalene had completely randomized; that is, it was cyclized from either end. Also, if lycopersene were not randomized during conversion to phytoene, one would expect its existence to be fleeting and its concentration to be less than the concentrations of the enzymes that synthesize and metabolize it. Although this expectation is consistent with the failure of many investigators to detect lycopersene, Barnes *et al.* (1973) found the biosynthesis of significant quantities of lycopersene produced by cell-free preparations of tomatoes. Also, in the experiments of Barnes *et al.* (1973), the concentration of lycopersene rose continually throughout 4 hr of incubation. These observations are not consistent with a fleeting existence for lycopersene.

In summary, lycopersene could be converted to *cis*-phytoene with the observed hydrogen retention by either of two pathways. One would involve racemic lycopersene, the "wrong" isomer, with no restraints on randomization before dehydrogenation. The other path would involve *meso*-lycopersene but would not permit randomization of the molecule. Neither alternative seems likely.

If lycopersene is not an intermediate, how can the observation that lycopersene is synthesized and converted to phytoene by a carotene-synthesizing system be rationalized? The synthesis is easily accounted for since squalene synthetase, which will synthesize lycopersene from geranylgeranyl pyrosphate (Qureshi *et al.* (1973)), is present in this carotene-synthesizing system (C. Subbarayan and H.

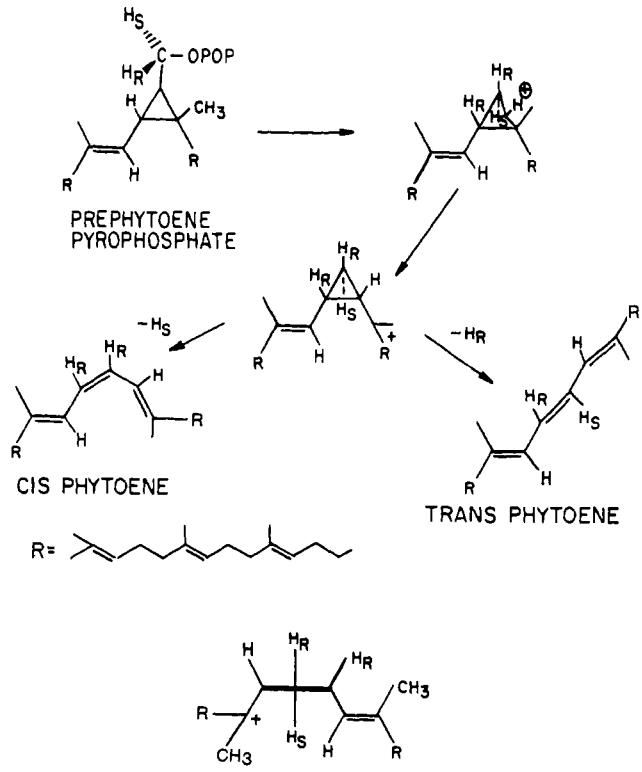

FIGURE 4: A mechanism for *cis*- or *trans*-phytoene synthesis from prephytoene pyrophosphate. The bottom structure is an edge-on view of the tertiary cyclopropylcarbinyl cation showing the equivalent positions of H_r and H_s.

C. Rilling, unpublished observation). One would need to invoke nonspecific dehydrogenation of lycopersene to account for its fortuitious incorporation into phytoene. To resolve the question of lycopersene as an intermediate, it will be necessary to purify phytoene synthetase free of squalene synthetase and then examine it for lycopersene synthetase activity.

Since it is doubtful that lycopersene is an obligatory intermediate in phytoene synthesis, we present an alternate mechanism, Figure 4. In this mechanism, *cis*- and *trans*-phytoene arise by a trans or cis hydrogen removal from a common tertiary cyclopropylcarbinyl cation. This mechanism is consistent with and extends the mechanism proposed by Poulter *et al.* (1973) for squalene synthesis.

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