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REVIEW

ENZYMIC ASPECTS OF THE BIOSYNTHESIS OF MONOTERPENES IN PLANTS*

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Key Word Index—Monoterpenes; prenyltransferase; carbocyclase; stereochemistry; allylic pyrophosphates; isoprenoids.

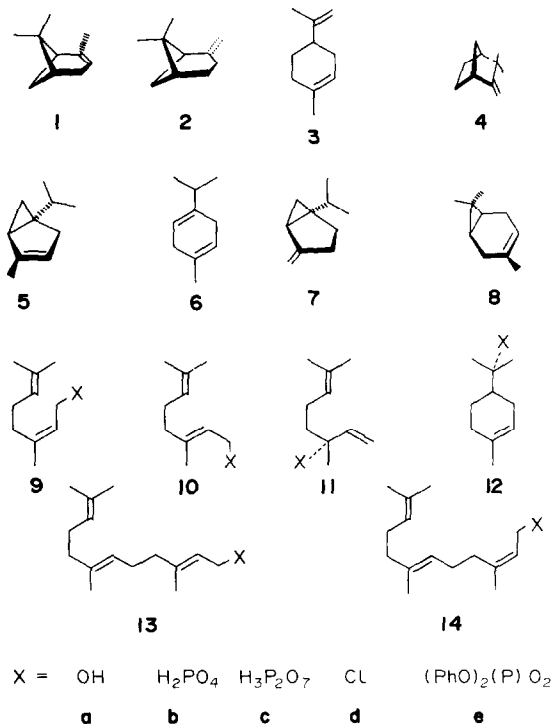
Abstract—The biosynthesis of monoterpenes in higher plants is reviewed, with particular emphasis on recent studies of the enzymology of biosynthesis.

INTRODUCTION

Members of the numerous family of compounds derived from isoprene, C_5H_8, are present in most, if not all, cell structures, but the variety of molecules present in plants is much larger than that in animal tissues. Almost any number of isoprene units may be incorporated, and there is scarcely a limit to the structural complications of the carbon skeleton. These compounds are collectively called isoprenoids or terpenoids.

With the exception of isoprene itself [1], the simplest end products of this biosynthetic pathway are the monoterpene hydrocarbons ($C_{10}H_{16}$) found almost exclusively in higher plants [2] as components of the essential oils. Stanley established in 1958 [3] that α-pinene was formed from [2-^{14}C]mevalonic acid by needles of *Pinus nigra*, and this work started the biochemical exploration of monoterpene biosynthesis, whose general pattern had been proposed by Ruzicka [4]. Some of the more frequent carbon skeletons **1**–**14** are shown in Scheme 1 and many oxygenated compounds found in plant tissues [5, 6] derive from these fundamental structures.

The building blocks of all isoprenoid compounds are two isomeric C_5 molecules, [6] derived from mevalonic acid; isopentenyl pyrophosphate (Scheme 2) (**16**) and dimethylallyl pyrophosphate (Scheme 2) (**15**). Their chemical properties are complementary: DMAPP loses its POP group and generates an allylic carbocation or an ion pair which alkylates the terminal (C-4) methylene group of IPP, generating a new allylic pyrophosphate (Scheme 2A, **10c**), a 'prenologue'† of DMAPP. This 1′,4 condensation reaction, is catalysed by prenyltransferases or prenylsynthetases‡ which may form isoprenoids ranging in different taxa from two to several thousands [5] of

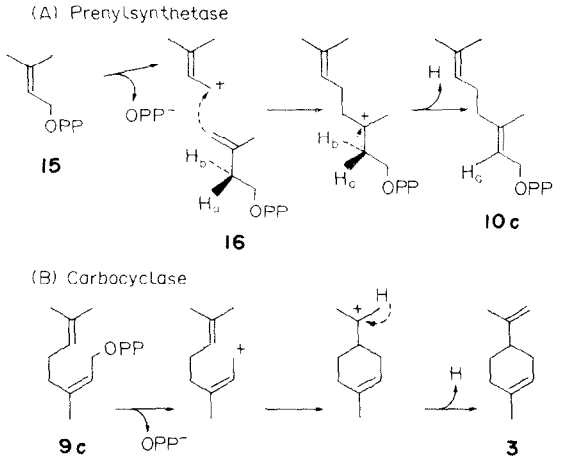


Scheme 1. Mono and sesquiterpenoid skeletons: (**1**) α-pinene, (**2**) β-pinene, (**3**) limonene, (**4**) camphene, (**5**) thujene, (**6**) γ-terpinene, (**7**) sabinene, (**8**) car-3-ene, (**9**) geranyl, (**10**) neryl, (**11**) linalyl, (**12**) α-terpinyl, (**13**) 2*E*,6*E*-farnesyl, (**14**) 2*Z*,6*E* farnesyl.

Abbreviations*: IPP, Isopentenyl pyrophosphate (16**); DMAPP, Dimethylallyl pyrophosphate (**15**); GPP, Geranyl pyrophosphate (2*E*, C_{10}) (**10c**); NPP, Neryl pyrophosphate (2*Z*, C_{10}) (**9c**); LPP, Linalyl pyrophosphate (**11c**); 2*E*,6*E*-FPP, 2*E*,6*E*-Farnesyl pyrophosphate (**13c**); 2*Z*,6*E*-FPP, 2*Z*,6*E*-Farnesyl pyrophosphate (**14c**); POP, Inorganic pyrophosphate; DTNB, Dithiobis(nitrobenzoic) acid.

†The terms 'prenologue' or 'isoprenologue' have been used [7] to characterize isoprenoids which have the same chemical function but differ by one or more C_5 units.

‡The Enzyme Commission name of 'Prenyltransferase' (EC 2.5.1.1) will be used preferentially. However, in some cases when it may be ambiguous, the name 'synthetase' will be used, i.e. *Z*-prenylsynthetase or 2*E*,6*E*-farnesyl pyrophosphate synthetase [63].



Scheme 2. Analogy between prenylsynthetase and carbocyclase mechanisms: (**15**) dimethylallyl pyrophosphate, (**16**) isopentenyl pyrophosphate.

isoprene units (Scheme 2A). There are other mechanisms for chain lengthening leading to the formation of lavandulyl or chrysanthemyl (C_{10}) skeletons, or to steroids and carotenes, which will not be discussed in the review [8].

Many cyclic terpenoids are formed through an intramolecular alkylation of double bonds of allylic pyrophosphates [6] in a reaction which may be mechanistically very similar to the growth of the allylic terpene chain (Scheme 2B). Product specific enzymes called cyclases or carbocyclases* catalyse this reaction.

The formation of six-membered rings presents a steric problem connected with the geometry of the immediate precursor of the cyclic monoterpenes, which must adopt at some stage a *Z* or a *syn* conformation in order to allow a C-1–C-6 ring closure.

It has been recognized that biosynthetic pathways occur according to patterns known to the organic chemist, and that 'there is no magic' in enzymes [9]. The formation of multiple isoprenoid molecules from two C_5 isomers is a good example of this principle [4] and exploration of chemical models has furnished much information [4, 5, 8, 10]. As with the synthetic organic chemist, the plant cell faces two types of structural problems in the biosynthesis of isoprenoids: the carbon skeleton and the introduction of functional groups. The former is characterized by the number of carbon atoms, the number of double bonds and the resulting conformations,† the presence of single or multiple rings and the aromatization of cyclohexenoid structures [10, 11]. Introduction of functional groups leads to the formation of diols, ketones, esters and glycosides [6, 12].

The present review attempts to analyse the role of prenyl transferases and carbocyclases in the formation of the carbon skeleton of the simplest isoprenoids, the cyclic monoterpenes, and to correlate it with pertinent chemical evidence. Conclusions must often be based on analogous studies in the biosynthesis of sesquiterpenoids (Scheme 1, **13**, **14**) or higher prenologues, and on evidence obtained with enzymes of animal origin or micro-organisms. The reader is referred to several comprehensive reviews [2, 5–7, 8, 10, 12–16] for the metabolic steps from acetylCoA to IPP, to the problems of further derivatization of the carbon skeleton [5, 6, 10, 12, 16] as well as for the formation of 'irregular monoterpenes' [5, 12].

DISTRIBUTION OF MONOTERPENES

The simplest terpenoids (C_5–C_{25}) are mainly restricted to Tracheophitae [5]. Monoterpene hydrocarbons and their oxygenated derivatives have been reported in 46 families of the class Dicotyledones [5], in the 94 species of *Pinus* [17] and generally in most conifers [18]. Volatile monoterpenes have been reported in Ascomycetes [19] and algae [20]. They have been found in several phyllae of invertebrates [21–23], although in some cases they could be of dietary and not of biosynthetic origin [20]. Generally, plants are the main sources of monoterpene hydrocarbons. Their distribution has been considered of taxonomic value [10, 17, 24, 25] in plants and it has even been proposed as a criterion to establish metabolic patterns [26]. Composition and biosynthetic activity vary within different tissues of the same organ [27].

TECHNICAL PROBLEMS

It appears from the available data that the study of monoterpene biosynthesis is restricted to higher plants. Information obtained by infusion of radioactive precursors of monoterpenes into whole plants or organs is limited by permeability barriers and competing pathways which decrease the incorporation of radioactive precursors to a few hundredths of a percent [3, 10, 28]. The obvious choice is the use of cell free systems or purified enzymes, but this requires several technical difficulties to be overcome [5, 6, 8, 10].

Proteins are found in low concentration in plant cells, and are frequently bound to the cellulose cell wall. The vigorous grinding required to bring them into solution tends to denature them [29]. This process is further accelerated by the flavonoids, tannins and other phenolics which are liberated from the cell vacuoles. Polyvinylpyrrolidone, polystyrene resins [30, 31] or detergents [32] protect some enzymes.

Acetone powders [33] have been used as a source of prenyltransferases from *Citrus* flavedo but carbocyclases are denatured by this treatment. The activity of interfering phosphatases may be decreased at pH values above 7 or by the addition of bivalent metals [33], or it may be eliminated in the purification procedure [5].

Phenol oxidases may also inactivate enzymes directly or through the formation of oxidized catechols [34]. They also interfere with the accurate determination of proteins. Treatment of bovine serum albumin with mushroom tyrosinase in the presence of catechols increased the apparent values of protein concentration [35] by 800% if

* The term 'carbocyclase' will be used to distinguish a group of enzymes forming cyclic terpene hydrocarbons from other 'cyclases' which form ethers, such as cineole through C–O–C bond formation or form C–C bonds, but add a hydroxyl group to generate cyclic prenols, such as α-terpineol. The term '1–6-ligases, pyrophosphate forming' is still too ambiguous regarding products and substrates. Unless explicitly discussed, no assumption is made regarding the existence of one or more carbocyclase [35].

† As in previous publications [35, 63] *E*- and *Z*-isomers will be referred to as 'diastereomers', i.e. stereoisomers which are not enantiomers differing in 'conformation', as recommended by IUPAC.

determined through its *A* at 280 nm, by 320% according to the Biuret method, by 70% according to the phosphomolybdate method and by 40% if determined with the rather insensitive turbidimetry method [Traverso, A., Loomis, W. D. and Cori, O., unpublished results]. The effect of tyrosinase may be abolished by extensive dialysis against 10 mM potassium cyanide at pH 4.0 [34], but the enzymes of isoprenoid biosynthesis often do not tolerate this treatment.

Some procedures, not frequently used, e.g. precipitation with polyethylene glycol [36] or dissociative gel filtration [33, 36, 37] have been helpful in the purification of carbocyclases and prenyltransferases or in the elimination of associated pigments. Elimination of non-protein components must also be considered a purification process [33, 36], but enzymologists tend to view an increase of activity referred to protein concentration as the sole criterion of purification.

Another difficulty is the fact that the possible direct precursors of monoterpenes are not commercially available, and must be synthesized biochemically or through chemical reactions of low yield to obtain high radiochemical purities.

There are many reports of formation of secondary products in tissue cultures [38] but the stress has been on alkaloids, phenolics and steroids [39]. Formation of the lower terpenoids has been explored to a much lesser extent [5, 10, 40] and studies with enzymes isolated from tissue cultures are very scarce [41]. Studies of carbocyclases obtained from tissue cultures could offer a breakthrough in our understanding of monoterpene biosynthesis.

The plant physiologist follows the biological behaviour of a given taxon or group of taxa. In contrast, those interested in the chemistry of a given biosynthetic process should explore as many sources of plant tissue as feasible, and sometimes a rather unexpected material may help to a breakthrough, as was the case for kaurene biosynthesis in *Echynocystis macrocarpa* [42].

LENGTH AND CONFORMATION OF THE ISOPRENOID CHAIN

The formation of a carbon skeleton of a given chain length presupposes a number of 1′,4 condensations of C_5 units [8] (Scheme 2A). The specificity of prenyltransferases varies with the species [8] or cell compartment [43]. Prenyltransferases from pig or avian liver form 2*E*,6*E*-FPP (**13**) from DMAPP (**15**) plus two IPPs (**16**) without accumulation of C_{10} pyrophosphates [44–46]. Conversely, the specificity spread in the enzymes from micro-organisms is very large [47–49], some form C_{35}–C_{55} chains without accumulating shorter prenologues, while other transferases, even from the same organism accumulate C_{10}–C_{20} pyrophosphates [50–52]. In some systems the different condensing C_5 moieties may originate from different metabolic pools [5, 10].

Conversion of mevalonic acid or IPP into C_{10} or higher isoprenoids, is evidence for the presence of prenyltransferases [53], but direct demonstration in enzymes from plants of the 1′,4 condensation reaction is rather scarce [8].

A prenyltransferase from pumpkin (*Cucurbita pepo*) converts C_5 units into FPP (C_{15}), but not into geranylgeranyl pyrophosphate (C_{20}), and another enzyme from the same source forms C_{20} from C_5 units without accumulation of intermediate prenologues [54]. The enzyme from *Ricinus communis* [55] forms geranylgeranyl pyrophosphate (C_{20}) from C_5 or C_{10}, but not from C_{15} allylic pyrophosphates. FPP synthetases have been demonstrated in *Gossypium hirsutum* [56] and in *Pisum sativum* [57, 58].

Monoterpenes must originate from a C_{10} precursor, either GPP (**10c**) [3] or NPP (**9c**) [59]. These products or the corresponding alcohols geraniol and nerol (**9a**, **10a**) have been identified in cell free enzyme preparations from *Pinus radiata* seedlings or *Citrus sinensis* flavedo which utilized [2-^{14}C]mevalonic acid or [4-^{14}C]IPP [60–62]. Cell free extracts from *Citrus paradisii* contain C_{10} and C_{15} prenylsynthetases [Pérez, L. M., Lozada, R. and Cori, O., unpublished results]. These activities have been partially purified, but not dissociated, from the flavedo of *Citrus sinensis* [63]. Flower heads of *Rosa dilecta* transform [2-^{14}C]mevalonic acid into the glucosides of nerol (**9a**) and geraniol, (**10a**) with an efficiency unparalleled in other plant tissues [64]. With few exceptions [51, 52, 65] only plant enzymes accumulate free neryl or geranyl derivatives, as opposed to most prenyltransferases which proceed non-stop to the higher prenologues. A systematic search for the presence and cellular compartmentation of prenyltransferases forming C_{10} final products would be desirable [27].

The mechanism of prenyltransferase has been elucidated for the enzymes from pig or avian liver [8, 44–46, 66] (Schemes 2B and 3A). IPP is alkylated by C_1 of DMAPP with POP as the leaving group and with inversion mode at C_1 of the allylic substrate [66].

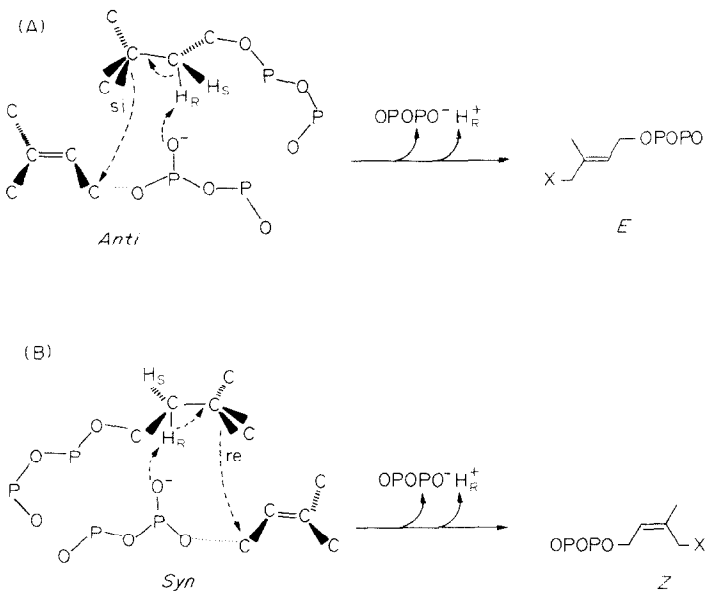
Comparison of non-enzymic model reactions with prenyltransferase has shown that the mechanism is ionic, and that racemization at C-1, which occurs in a non-enzymic model [67], is prevented through the binding of the allylic substrate to the enzyme.

Catalysis requires the binding of two atoms of Mg^{2+} or Mn^{2+} at the active site [45, 46]. The reaction proceeds through a tightly structured ion pair, formed by the carbocation derived from the allylic substrate and the POP leaving group. The assistance of the π electrons from IPP is not required for ion pair formation [8, 68, 69]. A new allylic 2,3-double bond is formed through elimination of a proton from C-2. Since other bases would be sterically hindered, the POP leaving group has been assigned the role of assisting stereospecific proton elimination. This mechanism has been demonstrated for the avian liver enzyme, which forms exclusively *E* double bonds [8, 45, 46].

A complex between metal and GPP is probably the substrate for the enzyme from *Citrus sinensis*, as supported by the fact that inactivation of this prenyltransferase by DTNB is retarded by the presence of the Mg^{2+} complex of the allylic substrate or its analogues, while inactivation is accelerated by uncomplexed ligands [63].

Most of the known prenyltransferases form products with *E* conformation around the double bonds [8]. The obvious exception is the enzyme from *Hevea brasiliensis* latex [70, 71] where the rubber chain exhibits an all *Z* conformation. Prenyltransferases involved in gossypol biosynthesis [56] also form C_{15} pyrophosphates of *Z* conformation. Cell free preparations from *Pinus radiata* and from *Citrus sinensis* [60, 63] form phosphorylated neryl or 2*Z*,6*Z*-farnesyl derivatives (**9c**, **14c**) in addition to the *E*-isomers (**10c**, **13c**).

There are still some yet unsettled problems in the stereochemistry of chain lengthening. The elegant studies



Scheme 3. Stereochemistry of *E*- and *Z*-prenylsynthetases. The conformation around the 2,3 bond of IPP and the face of its olefinic bond attacked by C-1 of the allylic substrate define the geometry of the new 2,3 double bond and the stereochemistry of the proton eliminated from C-2 of IPP.

of Popják and Cornforth [66] with stereospecifically labelled mevalonic acid led to the formulation of a biogenetic rule which states that isoprenoids with *E* conformation are formed through elimination of the pro-*R* proton from C-2 of IPP,* (Scheme 2A) and that loss of the pro-*S* proton is necessarily coupled to the formation of a *Z* double bond [7, 16, 49, 66, 70–72]. However, exceptions to this rule have been reported in plant tissues.

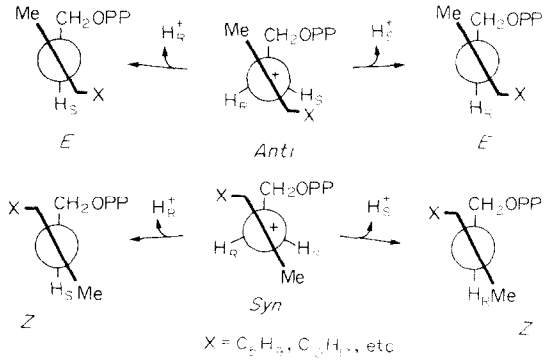
The formation of abscisic acid, which has a 2*Z* double bond occurs in *Persea gratissima* with loss of the 2-pro-*R* proton from IPP [73]. A similar stereochemistry has been found in the formation of geranyl and neryl glucosides in *Rosa* [64]. The pro-*R* proton is lost in the formation of both *E* and *Z* monoterpene glucosides. Cell free extracts from *Pinus radiata* seedlings or orange flavedo eliminate the pro-*R* proton of IPP in the biosynthesis of both *E* and *Z* allylic pyrophosphates [62].

The formation of *Z* prenylpyrophosphates and the reported exceptions to the biogenetic rule have been interpreted as due to the formation of an *E* isomer followed by the isomerization to the *Z* pyrophosphate catalysed by an isomerase [8, 14, 15, 74, 75]. It must be stressed that whenever this *E*–*Z* isomerization of allylic pyrophosphates has been searched for, the evidence has been conclusively negative [36, 61, 76–80].

Inspection of molecular models shows that there is no obligatory correlation between the stereochemistry (pro-*R* or pro-*S*) of the leaving proton and the formation of an *E* or *Z* substituted double bond [64, 80, 81, 82]. The latter is a consequence of the *syn* or *anti* conformation of the 2, 3 single bond (Scheme 4). The orienting influence of the enzyme on the way the substrates bind before catalysis defines the geometry of the new double bond, but there is no *a priori* reason to assume that all prenylsynthetases must have the same stereochemistry [82].

Scheme 3A shows the accepted stereochemical relationships for prenyltransferases which form *E* substituted double bonds with loss of the 2-pro-*R* proton from IPP [8, 45, 46]. The allylic pyrophosphate alkylates C-4 from the 3-*si* face of IPP, which has been bound in the *anti* conformation. In this case, only the 2 pro-*R* proton is accessible to the leaving POP group. In the 'rubber stereochemistry' (not shown) it may be assumed that the same face is attacked, that IPP has adopted a *syn* conformation, the 2-pro-*S* proton leaves with the POP group [8, 45, 46] and that the final *Z* double bond is formed.

The situation depicted in Scheme 3B is consistent with the observations reported for several *Z* prenyltransferases



Scheme 4. Stereochemistry of proton elimination and double bond formation.

*The sequence rule for C-4 of mevalonic acid is reversed when this atom becomes C-2 of IPP. Thus the 4-pro-*S* proton of mevalonic acid becomes the 2-pro-*R* proton of IPP without inversion mode. The prochiral protons will be referred to IPP.

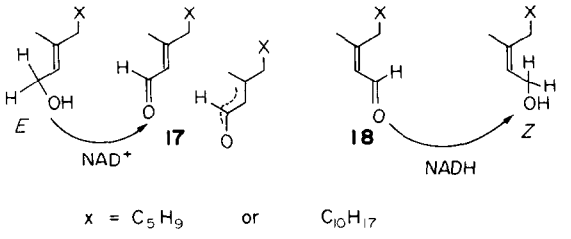
from plants [62, 64, 73]. C_4 of IPP, bound in this case in the *syn* conformation, would be attacked from its 3-*re* face by the allylic substrate, while the 2-pro-*R* proton contacts the leaving POP group. This stereochemical alternative explains the observed loss of 2-pro-*R* protons coupled to the formation of a *Z* substituted double bond without resorting to the undemonstrated *E*–*Z* isomerization of the pyrophosphates.

Enzymes with opposed stereospecificities have been described in different and even in the same species [72, 83]. The presence of opposite enantiomers in different species of *Pinus* [17] and even of opposed routes to enantiomeric pinane skeletons [82] in the same species indicate that although stereochemistry is a powerful tool to understand enzymic processes, it is not possible *a priori* to generalize about the steric relationships of an enzyme from one taxon to another. It is even conceivable that other prenyltransferases may form *E* substituted double bonds with loss of the 2-pro-*S* proton.

Except for rubber prenyltransferase [70, 71] it has not been possible to isolate prenylsynthetases specific for *Z* products from plant sources, but enrichment or inactivation of the *E* or *Z* synthetase, as well as seasonal variations [76] point to the existence of more than one enzyme for the biosynthesis of a given pair of isomeric prenologues [56, 63, 76, 80].

REDOX ISOMERIZATION OF ALLYLIC PRENOLS

In contrast to the absence of *E*–*Z* isomerization of allylic pyrophosphates, an interconversion mechanism for the corresponding alcohols (Scheme 5) has been described for C_{10} and C_{15} prenols in *Citrus*, *Rosa*, *Tanacetum* and *Andrographis* [6, 84–91] with the participation of aldehydes (**17** and **18**) as intermediates [84, 90]. Conjugation of the carbonyl group with the allylic double bond facilitates a non-enzymic isomerization step even under very mild conditions [85, 92].



Scheme 5. Redox isomerization of allylic prenols: (**17**) geranial or 2*E*,6*E*-farnesal, (**18**) neral or 2*Z*,6*E*-farnesal.

Oxidation of the carbinol group entails the loss of a proton [86–88, 90]. This occurs with opposite stereospecificities when proceeding in the *E*–*Z* or in the *Z*–*E* direction [75, 87]. The two processes could be catalysed by different dehydrogenases [92, 93].

This redox isomerization of prenols coexists with stereospecific biosynthesis of *E* and *Z* pyrophosphates [63]. Extracts from *C. sinensis* flavedo form 2*E*,6*E*- and 2*Z*,6*E*-farnesol (**13a** and **14a**) from GPP plus [4-^{14}C]IPP in the order shown in Scheme 5, with a time lag in the appearance of the latter (**14a**). Trapping aldehydes with aniline completely blocks the interconversion [63, 84]. More highly purified preparations from the same source form simultaneously both 2*Z*,6*E*- and 2*E*, 6*E*-FPP (**14c** and **13c**) without a lag period and this is not affected by aniline [63, 80].

Conversion of (3*R*,5*S*)-[5-^{3}H]mevalonate into 2*Z*,6*E*-farnesol (**14a**) by enzymes from *Andrographis* without loss of the tritium label [89] excludes the participation of the redox isomerization. In the light of later results, that is most simply interpreted as being due to a direct isomerization of 2*E*,6*E*-FPP into its 2*Z*-isomer and subsequent hydrolysis [94]. However, since no such *E*–*Z* isomerization of pyrophosphates has been found [84], these results may be as validly interpreted as due to stereospecific synthesis of 2*Z*,6*E*-FPP followed by hydrolysis.

Formation of cyclohexanoid rings from 2*E*,6*E*-FPP or from GPP [94, 95] without loss of tritium from C-1 of the substrate also excludes redox isomerization in these cases. However, these findings should not be generalized to the point of denying it a role in the whole plant.

Probably one should take the following eclectic view: the existence of the redox isomerization of alcohols in cell free systems has been sufficiently proved [84–91], but it is also clear that it is not the source of NPP or of 2*Z*,6*E*-FPP (**9c**, **14c**) in these or other preparations [5, 14, 15, 63, 94, 95]. Its role may be important in the whole plant, starting with hydrolysis of *E*-allylic pyrophosphates [33, 96] followed by redox isomerization to form the *Z*-prenols which could be phosphorylated with ATP by prenyl kinases [16, 97, 98]. This would explain the incorporation of radioactive prenols into final products [10, 86, 99] in the whole plant. Evolution may have preserved this mechanism in the green plant, where ATP required for rephosphorylation of the prenols is not at a premium. There are no reports of a systematic search of the redox process in different organisms.

CARBOCYCLASES AND CYCLIC MONOTERPENE BIOSYNTHESIS

The formation of the cyclohexanoid ring from an acyclic precursor requires a *syn* or *Z* conformation either in the ground state or in the activated complex. In the *E* or *anti* geometry, the distance between C-1 and C-6 is too large to form a bond. In 1934 Horiuchi [100] provided proof for a possible participation of nerol (**9a**) in monoterpene biosynthesis *in vivo* but this evidence went unnoticed.

On the basis of structural limitations and of chemical analogies, [28, 59, 101] it was thought that NPP, (**9c**) the *Z*-isomer of GPP (**10c**) could be the more plausible precursor of cyclic monoterpenes. In non-enzymic reactions, neryl derivatives (**9a**–**9c**) form cyclic products at higher rates and in higher proportions than geranyl derivatives which yield predominantly acyclic products [101–107] (Scheme 5). The role of NPP as the sole precursor of α- and β-pinene (**1**, **2**) was first demonstrated with enzymes from *Pinus radiata* seedlings and GPP was not a substrate [79]. This agreed with the fact that the same enzyme preparation formed 100% more NPP and nerol (**9a**) than GPP or geraniol (**10a**) from mevalonic acid [60]. Peppermint or *Salvia* enzymes form α-terpineol (**12a**) 1,8-cineole, or limonene (**3**) preferentially or exclusively from NPP [108, 109].

This chemically rational picture was complicated by the fact that enzymes from other sources form cyclic monoterpenes from GPP. As Gleizes comments, [110] the view that NPP is the obligate precursor of cyclic monoterpenes

must become "beaucoup plus nuancée", i.e. it must be reconsidered.

Cell free extracts from *Citrus sinensis* flavedo form limonene (**3**) both from [2-^{14}C]NPP or GPP, whereas α-pinene (**1**) is formed only from NPP [61]. An extract from leaves of *Thymus vulgaris* forms γ-terpinene (**6**) both from GPP and NPP in the absence of interconversion of acyclic precursors [11]. Bornyl pyrophosphate, the cyclic precursor of camphene (**4**), is formed by enzymes from *Salvia officinalis* both from GPP and NPP [78, 111]. A partially purified enzyme from *Citrus limonum* flavedo forms α-pinene, β-pinene, sabinene (**7**) and limonene from both GPP and NPP at comparable rates [35]. Conversion of GPP and NPP into α-pinene (**1**), β-pinene (**2**), camphene (**4**), and monocyclic terpenes by cyclases from *Salvia* has been reported [112].

The simplest way to explain the transformation of GPP into cyclic monoterpenes in spite of the steric limitations would be to assume an *E–Z* isomerization of GPP [74] followed by cyclization of NPP. This mechanism has been ruled out completely, because no such enzymic isomerization of GPP or NPP has been obtained in a number of different enzyme preparations [11, 35, 61, 76–80, 95, 113]. Any mechanism proposed for carbocyclase must account for three facts: the utilization of both GPP and NPP; the absence of *E–Z* isomerization; and the *syn* geometry required for the activated complex [5, 14, 36, 80, 81, 96].

Carbocyclases, and generally cyclases, have been reported or partially purified from *Salvia* [95, 96, 112], *Foeniculum* [113], *Pinus* [79], *Mentha* [108], *Citrus sinensis* [61] and *C. limonum* [36] but no homogenous enzyme has been obtained. The highest final specific activity reported at initial rates are 7–10 pkat/mg protein [36] and the enzyme has been further purified by a factor of 10 by ion exchange chromatography [Rojas, M. C., Chayet, L., Portilla, G. and Cori, O., unpublished results]. Carbocyclase preparations form more than one hydrocarbon and they frequently utilize more than one substrate.

The amount of cyclic monoterpene hydrocarbons formed by a given cell free preparation may or may not agree with the composition of the essential oil of the original plant [36, 112]. This may be due to problems in the extraction of carbocyclases or to differences in stability. It is likely that a carbocyclase preparation contains a mixture of enzymes from different parts of the cell or tissue used [27].

Dissociation of activities during the purification of carbocyclase [36, 113] supports the view that enzymes with different substrate and product specificity are involved in monoterpene biosynthesis. Aging does not affect the ability of carbocyclase from *C. limonum* to utilize GPP, but it affects the product pattern and abolishes the utilization of NPP [80]. This again counters the sequential pattern of GPP–NPP isomerization: GPP → NPP → hydrocarbons; whereas it is consistent with a convergent mechanism of hydrocarbon formation: NPP → hydrocarbons ← GPP.

The well-known fact that distribution of monoterpenes is genetically conditioned [24, 25] fits in with the assumption that different products are formed by distinct enzymes. Plant tissues contain opposite enantiomers of cyclic monoterpenes [17, 82]. It is thus conceivable to imagine carbocyclases with enantiomeric product specificity. Attention has been paid recently to configurational problems in biosynthesis [11, 114].

Fractionation of subcellular components and experiments with different labelled precursors in *Pinus pinaster* [115, 116] shows that the biosynthesis of mono- and sesquiterpene hydrocarbons appears to be connected with the membranes of the endoplasmic reticulum. The extraction procedures used in the preparation of cyclases release them from the particulate fractions, and for this reason they appear as 'soluble' enzymes [36, 116]. The use of more gentle fractionation procedures in the early steps of enzyme purification may indicate whether cyclases are soluble or weakly bound membrane enzymes.

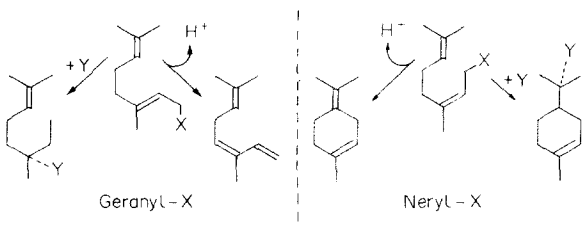
A number of phosphatases found in partially purified carbocyclase preparations reduce the concentration of pyrophosphorylated substrates. They may have different activities with GPP or NPP [96] and this may be the explanation for the fact that utilization of the two precursors differs from one preparation to another [36, 79, 96]. Selective elimination of phosphatases during the isolation procedure should be one of the aims in the purification of carbocyclase [33].

Inflections in the pH curves and reversible inactivation by DTNB and *para*-chloromercuribenzoate suggest the participation of cysteine in the *Citrus limonum* carbocyclase reaction [36, 117]. Benzyl bromide, which reacts with methionyl groups four times faster than with sulfhydryl groups at pH 6.0 also inactivates the enzyme. Protection by the Mn^{2+} complexes of NPP or GPP and titration of reacted sulfhydryl groups suggests the participation of both cysteinyl and methionyl groups in the carbocyclase reaction [117].

COMPARISON OF CARBOCYCLASE WITH NON-ENZYMIC MODEL REACTIONS

The study of non-enzymic reactions as guidelines for the understanding of terpene biosynthesis is as old as the present century [102]. Chemical evidence has stressed in the past the retention of the geometry of the substrate in solvolysis or elimination reactions of geranyl or neryl derivatives (Scheme 6). Equilibrium and rates favour the formation of cyclic compounds from neryl derivatives and of open chain products from geranyl derivatives [80, 102, 107, 118]. This was the rationale to look for NPP as the direct precursor of cyclic monoterpenes [28, 59, 79].

Carbocyclases form cyclohexanoid rings from GPP in spite of its sterically unfavourable *E* conformation and require the presence of bivalent metals such as Mn^{2+}. It was thus pertinent to investigate models for the less predominant process, i.e. the formation of cyclic products from geranyl (**10**) or 2*E* farnesyl (**13**) derivatives. This occurs under conditions which stabilize an ion pair or carbocation originating from a substrate of *E* conformation and permits its conversion into the *syn* geometry



Scheme 6. Formation of products from geranyl or neryl derivatives.

required for cyclization. The conditions which favour cyclization of *E* substrates should find their counterparts in the properties of carbocyclase, and the participation of metals in enzymic and non-enzymic reactions should be taken into account.

Cyclic products like γ-bisabolene are formed to the extent of 41% in elimination reactions of the 2*E*,6*E*-isomer of farnesyl biphenylphosphate (**13e**) in non-aqueous solvents [119]. Rearrangement of sesquiterpene alcohols in 100% formic acid occurs with formation of *ca* 45% of cyclic product (γ-bisabolol). This is independent of the *E* or *Z* conformation of the substrates and is also the same for the tertiary alcohol nerolidol. The rates are similar for all three substrates [120].

The elimination products of geranyl diphenylphosphate (**10e**) were 33% cyclic hydrocarbons, as compared with 76% from the neryl isomer (**9e**) [107]. The differences between *E* and *Z* derivatives are much less marked than those observed in the solvolysis of the pyrophosphates in 0.1 N acid, where the amount of cyclic hydrocarbons formed from NPP exceeds by a factor of nine those formed from GPP [101–103]. In the solvolysis of geranyl chloride (**10d**), the addition of ClO_4^- as a counter ion increases the proportion of cyclic products by a factor of five–seven, [104] and in concentrated acid, geranyl derivatives may rearrange to 72% of cyclic products [121, 122]. Rearrangement of allylic phenyl phosphonates which occurs in strong acids is replaced by allylic cleavage in nucleophilic solvents [123].

It may be concluded that conditions which stabilize a carbocation or ion pair, such as a non-polar environment, absence of nucleophilic solvents, or a large counter ion, will permit or facilitate the formation of cyclic products from a precursor with *E* conformation, i.e. geranyl or 2*E*-farnesyl derivatives. Carbocyclases may furnish this sort of environment at their active site [114] facilitating elimination of the POP group, stabilizing and steering the resulting ionic species into the adequate conformation and excluding water to favour the formation of hydrocarbons (Scheme 8). Bivalent metals and nucleophiles such as cysteine and methionine contribute to these functions [117].

It has been observed that Mn^{2+} and Mg^{2+} promote the solvolysis of FPP to farnesol and nerolidol even at pH 8.0 [124] and similar effects were observed for GPP and chrysanthemyl pyrophosphate [125].

The presence of 3 mM Mn^{2+} produces a 50-fold increase of the uncatalysed solvolysis rate of GPP at neutral pH. Only 20% of the products are primary alcohols, suggesting the predominance of C–O fission [126]. The reaction catalysed by Mn^{2+} forms more cyclic products and hydrocarbons than acid-catalysed solvolysis [81, 103, 105], although open-chain products still predominate.

A plot of the solvolysis rate as a function of the molar fraction of Mn^{2+},

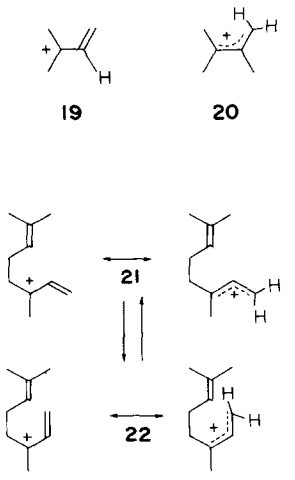
$$\frac{[Mn^{2+}]}{[Mn^{2+}]+[GPP]},$$

maintaining constant the sum of Mn plus GPP concentrations [127], shows that the highest reaction rate is attained with the GPP–$(Mn^{2+})_2$ complex [126]. Comparable results were obtained for the NPP–Mn complex. If kinetic curves for substrate and Mn^{2+} requirements of *Citrus* carbocyclase were constructed as functions of the calculated concentrations of the different metal–substrate complexes a good Michaelis–Menten correlation occurred between the concentration of substrate–Mn_2 and reaction rate. The kinetic parameters thus calculated agreed irrespective of whether substrate or metal concentration was the variable [Rojas, M. C., Chayet, L., Portilla, G. and Cori, O., unpublished results].

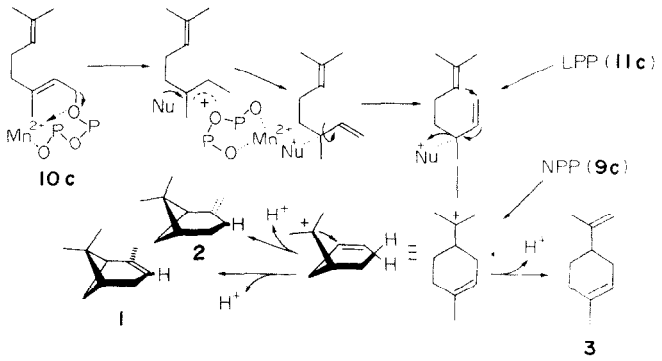
The solvolysis of citronellyl pyrophosphate, the 2,3-saturated analogue of GPP, is increased by Mn^{2+} only by a factor of five. It is not a substrate for carbocyclase [80, 109], but a moderately active inhibitor [80]. Geranyl monophosphate (**10b**) whose solvolysis rate is decreased by Mn^{2+} [126] is not a substrate and is a very ineffective inhibitor of carbocyclase [80]. Enzyme specificity and Mn^{2+}-catalysed solvolysis contrast with the acid-catalysed reaction, where the nature of the leaving group is unimportant [103–105]. This and the difference in product distribution suggest that metal-catalysed solvolysis is more closely related to the enzymic process than acid catalysis. Mn^{2+} may act not only in facilitating the leaving of the POP group, but also by interacting with the organic moiety and moderately favouring cyclization and elimination over substitution reactions [126].

If the carbocation or ion pair derived from GPP is stabilized by one or more of the factors analysed, then rotation around the delocalized 2,3 double bond could take place. It has been established, both experimentally and through calculations [128–130], that the barrier to rotation is of the order of 11–13 kcal/mol for model carbocations (Scheme 7, **19**, **20**). However carbocations derived from GPP, with unequal contributions of the tertiary and primary structures (**21**, **22**) could have a lower hindrance. The activation energies for hydrocarbon formation by *Citrus limonum* carbocyclase are 12.6 ± kcal/mol for NPP and 15.8 ± 2 kcal/mol for GPP. This suggests that rotation around the C-2–C-3 bond in the enzyme-bound ion pairs or carbocations is not out of the question [80, 126].

The fact that there is no ^{18}O scrambling in GPP in the similar prenyltransferase reaction [68, 69] could argue against a facile rotation of an enzyme-bound carbocation. The geometric conditions in carbocyclase may be completely different from those of prenyltransferase, which has to weld two molecules, while carbocyclase closes a cyclohexanoid ring. Participation of the π electrons seems



Scheme 7. Carbocations.



Scheme 8. Formation of cyclic monoterpene hydrocarbons from different substrates.

to have a role in model cyclization reactions [104, 105, 126]. Participation of nucleophiles, such as cysteine or methionine [117], may bind to C-3 of the carbocation and by displacing electrons towards C-2 and C-1 facilitate a change from *anti* to *syn* conformation (Scheme 8).

THE ROLE OF LINALYL DERIVATIVES IN MONOTERPENE BIOSYNTHESIS

Some cyclic terpenoid skeletons are formed from rearranged pyrophosphate esters different from the primary product of the prenyltransferase reaction [112, 131, 132] and a pyrophosphorylated derivative of linalol may be a plausible intermediate between GPP or NPP and the cyclohexanoid skeleton (Scheme 8). The 2,3 single bond of LPP (**11c**) permits the *syn* conformation required for cyclization, and it could be formed through suprafacial migration of the POP group of GPP or NPP from C-3 to C-3 [14, 15, 61, 95, 131].

The time course of prenol rearrangements [133] supports the participation of linalyl derivatives in the formation of cyclic products from geraniol. When geraniol rearrangement of citric acid to α-terpineol (**12a**) occurs, the first product is linalol (**11a**) and the cyclic alcohol appears later. Conversely, α-terpineol is the first rearrangement product of nerol (**10c**), suggesting direct cyclization and a slower and independent formation of linalol [105, 133].

There are several reports of linalol formation from mevalonic acid, or of incorporation of radioactivity from linalyl derivatives into cyclic monoterpenoids [11, 61, 91, 134], but there are also views against its participation [6, 78, 135, 79]. A phosphorylated linalyl derivative is formed from [4-^{14}C]IPP by enzymes from *Citrus sinensis*, and its concentration exceeds that of the other C_{10} allylic phosphates [61]. This observation was not considered conclusive because Mn^{2+} was not removed from the incubation mixtures during the analytical process and the observed linalol could have been a solvolysis product [124]. However, later reports [77, 95, 112, 135] point again to LPP (**11c**) as a possible precursor of cyclic monoterpenes.

Carbocyclases from *Salvia* utilize LPP, GPP or NPP to form cyclic hydrocarbons [112]. A partially purified carbocyclase from *C. limonum* forms α-pinene (**1**), β-pinene (**2**) and limonene (**3**) from [1-^{3}H]LPP which proved to contain no detectable GPP or NPP [136]. Under initial rate conditions, the efficiency V_{max}/K_m (min^{-1} mg protein^{-1}) was 2.75, as compared with 0.10 for NPP and 0.25 for GPP, which acted as inhibitors [136]. LPP could not be isolated from these enzyme systems while forming cyclic hydrocarbons from GPP [112, 136]. Some enzyme preparations even lose their ability to utilize LPP, but remain active with GPP [80, 136].

LPP solvolyses in the presence of Mn^{2+} 40 times faster than NPP or GPP. It also rearranges spontaneously in basic solutions to the extent of several per cent per month [136]. This reactivity may explain why it has not been detected in the enzymic experiments. The acyclic hydrocarbons observed in the carbocyclase reaction [112] could be accounted for by the rapid non-enzymic solvolysis catalysed by Mn^{2+} [136].

It is not possible to project conclusions from the formation of cyclic sesquiterpenoids from 2*E*,6*E*-FPP, mediated by the tertiary nerolidyl pyrophosphate through the effect of an isomerase-cyclase [14, 15, 131], to the role of LPP in cyclic monoterpene biosynthesis. In the latter case, the free tertiary isomer has not been isolated. The fact that some aged enzyme preparations do not utilize LPP, while retaining their activity with GPP, argues against its role as an obligatory intermediate [5, 6, 136]. LPP is a good substrate, probably because of a structural resemblance with an enzyme-bound intermediate of carbocyclase. It could eventually be considered as an 'active complex analogue' which is not an inhibitor, but a substrate.

OVERVIEW

Monoterpene hydrocarbons are formed in plant tissues from C_5 units through the operation of two mechanistically similar enzymic reactions: prenyltransferases, which provide the adequate C_{10} chain [60, 61]; and carbocyclases, which form the basic ring structures (Scheme 2). The two reactions have some common mechanistic features. The prenyltransferase reaction is an intermolecular alkylation of an olefine, while carbocyclase catalyses the intramolecular equivalent [80, 111]. A carbocation or ion pair is formed from the allylic substrate by metal assisted POP elimination. The positive charge thus developed adds to a double bond, forming a new carbocation, and the final product is formed through regio- and stereospecific elimination of a proton (Schemes 2, 3 and 8). Other intramolecular additions, hydride shifts and skeletal rearrangements must occur before the final proton elimination in the biosynthesis of bicyclic monoterpenes [1, 2, 7, 8, 12].

The isomerization of IPP to DMAPP, catalysed by IPP isomerase, also bears mechanistic resemblance to the other two reactions, as it entails the addition of a positive

charge to a double bond and elimination of the 2 pro-*R* proton of IPP [62, 66, 72]. There are, however, some stereochemical differences between FPP synthetase and IPP isomerase [8].

The extent and stereochemistry of chain lengthening is defined by the geometry of interaction of the enzymes involved with the substrates (Scheme 3). Product stereospecificity has no chemically predictable link with the chiralty of the leaving proton (Scheme 4).

Cyclic monoterpenes are formed from several substrates, and although non-enzymic evidence suggests a *Z*-pyrophosphate as the chemically plausible precursor of cyclic monoterpenes, enzymic evidence does not support this view. Carbocyclases, from several plant species, do not exhibit absolute substrate specificity and utilize GPP in the absence of *E–Z* isomerization. The formation of cyclic products may be understood through those chemical models in which the products are formed from geranyl derivatives under circumstances that tend to stabilize ionic species to permit conformational changes. The interpretation of models does not extend to the formation of bicyclic hydrocarbons, which have not been observed in non-enzymic model reactions [81, 104, 105]. These skeletons are formed under rather drastic synthesis conditions [137, 138], while in the cell the enzyme orientates the substrate to adopt the most adequate conformation.

Utilization of LPP as the preferred substrate of carbocyclase has been proved experimentally but this compound has not been identified as a free intermediate. Rotation of an enzyme-bound geranyl carbocation to form a neryl- or linalyl-like isomer, is by exclusion the most plausible mechanism for carbocyclase, but it does not have direct experimental support (Scheme 8).

Uncertainties comparable to those in carbocyclases exist for the stereochemistry of formation of short isoprenoid chains of *Z* conformation in plants. There is overwhelming experimental evidence against the assumption that NPP or the *Z*-isomers of FPP may be formed by isomerization of GPP or 2*E*,6*E*-FPP. Confirmation of a separate synthesis of *Z*- and *E*-isomers, based on the dissociation of two different prenyltransferases, requires a complete separation of the two enzymes which would allow the investigation of stereochemical differences between *Z*-prenylsynthetases from different species.

It would appear that at this stage there are more questions than answers regarding the mechanism of chain lengthening and cyclization reactions in higher plants. The answer to these two basic problems must await the isolation of enzymes specific for a single substrate and product. Meanwhile, we have to struggle with the indirect and sometimes conflicting evidence available.

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