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Complexes of Bivalent Cations with Neryl and Geranyl Pyrophosphate: Their Role in Terpene Biosynthesis

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Kinetic analysis of the nonenzymic solvolysis of neryl and geranyl pyrophosphate (NPP and GPP, respectively) showed that the dissociation constants of the bis-metallic complexes with Mg^{2+} and Mn^{2+} were larger for NPP than for GPP by approximately one order of magnitude. Rate constants for reaction of the bis-metallic complexes were larger for NPP than for GPP. Qualitatively similar behavior was observed with complexes of Co^{2+}. Extents of elimination and cyclization were increased by metal ions. Carbocyclase-catalyzed formation of cyclic monoterpene hydrocarbons in the presence of Mg^{2+} involved bis-metallic complexes as the "true" substrates.

Allylic pyrophosphates are key intermediates in the biosynthesis of all isoprenoids (*1–6*), and chemical reactions of C_{10}, C_{15}, and other isoprenoid derivatives have been studied as models for the biosynthetic reactions of mono- and sesquiterpenes (*1–3, 7–12*). The reactions involve rearrangements and cyclizations of the carbon skeleton, and the intermediate carbocations often do not readily interconvert. Neryl derivatives (NX)[1] give predominantly cyclic products, but geranyl derivatives (GX) form mainly acyclic products, although in media in which carbocations are longer lived there is more cyclization (*9–11*). Linalyl derivatives (LX) give both cyclic and acyclic products.

Scheme 1 shows the major substitution products of reactions in aqueous solvents. There is extensive elimination in solvents of low polarity (*11, 12*). In this scheme, N^+, G^+, and L^+ denote intermediate carbocations or ion pairs, and their carbon skeletons or conformations are not necessarily those of their precursors.

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[1] Abbreviations used: NX, neryl derivative; GX, geranyl; LX, linalyl; NPP, neryl pyrophosphate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethane sulfonic acid.

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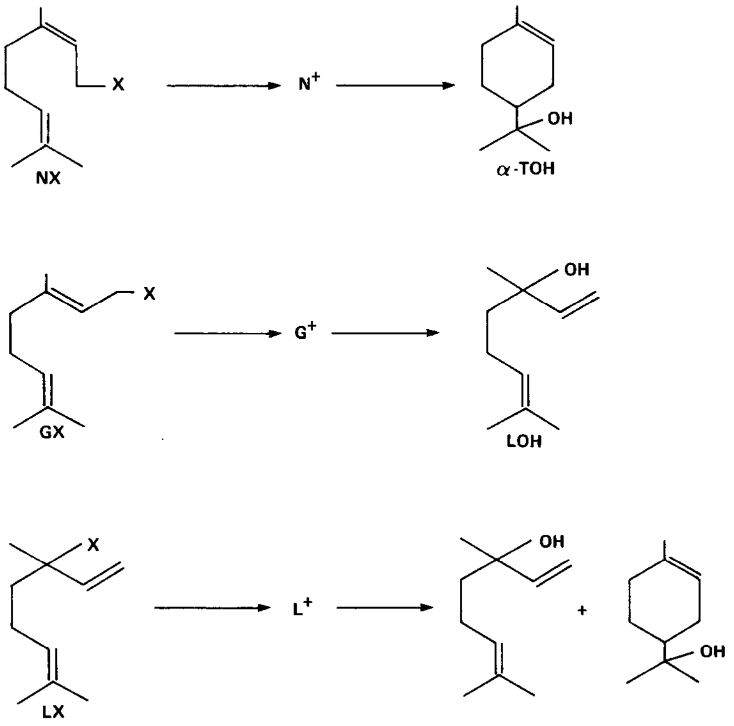

SCHEME 1

These observations suggested that neryl pyrophosphate (NPP) and not the well-known intermediate in steroid biosynthesis, geranyl pyrophosphate (GPP), should be the more efficient substrate in the biosynthesis of cyclic monoterpenes catalyzed by carbocyclases (*13, 14*).[2] This is the situation with enzyme preparations from some plant species (*16, 17*) but, with most carbocyclases, GPP or LPP (linalyl pyrophosphate) are better substrates than NPP (*3, 5, 6, 18, 19*).

Enzymatic formation of monoterpenes from GPP and related substrates catalyzed by carbocyclases is metal-ion dependent (*19, 20*), and this observation has been related to the solvolysis of allylic pyrophosphates catalyzed by bivalent metal ions. Both nonenzymatic solvolysis of allylic pyrophosphates and biosynthesis of cyclic monoterpenes seem to occur most readily through complexes of the substrate with two metal ions (*20–23*). GPP is generally the preferred substrate in the biosynthesis of cyclic monoterpenes (*5, 24*), although its nonenzymatic solvolyses generally give acyclic products (*23, 25, 26*). This apparent paradox between the ease of nonenzymatic cyclization of neryl derivatives and the greater effectiveness of GPP over NPP as substrates for carbocyclases (*3, 5, 6,*

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[2] The term "carbocyclase" is used to designate a group of enzymes which convert C_{10} allylic pyrophosphates into cyclic terpene hydrocarbons. There is not enough information on this class of enzymes to warrant a formal nomenclature such as "1-6 ligases," and the term used differentiates them from several other cyclases. This class almost certainly includes several enzymes (*15*).

20) in the absence of *E–Z* isomerization (*2*) prompted us to compare nonenzymatic metal ion-catalyzed solvolyses of NPP and GPP and the role of metal ions in enzymatic reactions of these substrates.

EXPERIMENTAL PROCEDURES

Materials and kinetics. Tritium-labeled [1-^{3}H]NPP and GPP were synthesized as described (*6, 23*), and had specific activities of 56.8 and 25 Ci mol^{-1}, respectively. The hydrolysis of NPP was carried out in glass-stoppered vials in 0.5 M Tes–HCl buffers, pH 6.75 or 7.0, at 40.0°C in the presence of varied concentrations of $MgSO_4$, $MnCl_2$, or $CoCl_2$. Substrate concentrations ranged from 10^{-5} to 2×10^{-4} M, depending upon reactivity and the tendency of the complexes to precipitate. The reaction was stopped by extracting the products (prenols and hydrocarbons) with hexane. The activity was measured by conventional β-scintillation spectrometry, and the products were analyzed by GLC (*23*). Duplicate rate constants agreed within 10%.

The pH range was restricted because acid hydrolysis is important at low pH and there is precipitation at high. Precipitation was a problem with NPP–$MnCl_2$ so the reaction was followed at pH 6.75 rather than 7.0.

Dissociation constants of the 1 : 1 complexes were estimated by competition (*27*), or were from the literature. The competition method could not be used for the NPP–Mn^{2+} complex because of precipitation in the presence of 8-hydroxyquinoline (*28*).

Enzymatic reactions. Carabocyclase, with specific activities of 3–7 nmol min^{-1} mg protein^{-1}, was a partially purified preparation from the flavedo of *Citrus limonum* (*6, 20*). It was assayed in 0.1 M Tes–HCl, pH 7.0, at 30°C in the presence of [1-^{3}H]NPP or [1-^{3}H]GPP and bivalent cations. Hydrocarbons were separated from prenols by absorbing the latter on silicic acid, and were analyzed by GLC–radioactive counting.

RESULTS AND DISCUSSION

Substrate binding to metal ions. Table I shows that the magnitude of the dissociation constants, K_1, of the 1 : 1 complexes is such that these complexes are formed quantitatively even with dilute metal ion. Dissociation constants of the bimetallic complexes were such that they had to be estimated kinetically.

The presence of buffer is a potentially complicating factor, because we had to use higher concentrations of metal ion for reactions of NPP than for GPP. Therefore, our experiments with NPP were in 0.5 M Tes, but most of our experiments with GPP were in 0.1 M Tes (*23*), and we had to consider the possible effect of this difference in [buffer].

The binding of Tes to Mg^{2+} is stated to be "negligible," (*29*) and for reactions of GPP and NPP in 0.03 M $MgSO_4$ rate constants in 0.1 and 0.5 M Tes agreed within 4%. With NPP in 0.03 M $MnSO_4$ and GPP in 0.003 M $MnSO_4$, the variations in

TABLE I

Rate and Dissociation Constants in Metal Ion-Catalyzed Solvolysis[a]

Metal	Substrate	K_1 (M)	K_2 (M)	$10^5\,k'$ (sec^{-1})	$10^5\,k''$ (sec^{-1})
Mg^{2+}	GPP	1.3×10^{-4}[b]	1.6×10^{-2}	0.055	0.26
Mn^{2+}	GPP	4×10^{-5}[b]	1.5×10^{-3}	~0	1.5
Co^{2+}[c]	GPP	1.5×10^{-5}	1.2×10^{-2}	0.06	0.9
Mg^{2+}	NPP	10^{-4}	0.2	0.19	1.0
Mn^{2+}	NPP	—	(0.9)	(1.2)	(21)
Co^{2+}[c]	NPP	Very small	0.35	0.25	9
Co^{2+}[c]	NPP		(0.65)	(0.2)	(9)

[a] At pH 7 unless specified, 25°C for the first and 40°C for the second dissociation constant. Values in parentheses are at pH 6.75.

[b] Ref. (*22*).

[c] Subject to buffer interference, see text.

going from 0.1 to 0.5 M Tes were within 3%, but buffer effects were serious with $CoCl_2$ (*30*). For GPP in 0.01 M $CoCl_2$ an increase in [Tes] from 0.1 to 0.5 M decreased the rate constant by 30%, and a similar reduction was found for reaction of NPP in 0.03 M $CoCl_2$. This difference is almost certainly due to complexation of Co^{2+} by Tes (*30*).

Metal ion catalysis of nonenzymatic hydrolysis. The variations of the first-order rate constants, k_ψ, with concentration of metal ions are shown in Figs. 1 and 2. The plots are curved, and saturation is reached with GPP (Fig. 1) but not with NPP (Fig. 2). Qualitatively, these results show that GPP is much better than NPP in binding the second metal ion, but that the weak binding of the second metal ion to NPP is offset by the higher reactivity of NPP · M_2 as compared with GPP · M_2. Thus, at low metal ion concentrations NPP and GPP have similar reactivities, but at higher metal ion concentrations NPP is more reactive than GPP.

Quantitative treatment of metal ion catalysis. The overall reactions for decomposition of substrate, S, in the presence of a bivalent metal ion, M, are shown in Scheme 2.

We can neglect spontaneous decomposition because $[M] \gg K_1$, so that there will be no free substrate, so that

$$k_\psi = k'[SM]/[S_T] + k''[SM_2]/[S_T], \qquad [1]$$

where S_T in stoichiometric substrate.

Equation [1] gives

$$k_\psi = k'K_2/(K_2 + [M]) + k''[M]/(K_2 + [M]). \qquad [2]$$

The variation of k_ψ with metal ion concentration was fitted to Eq. [2] using computer simulation, and values of k', k'', K_1, and K_2 are in Table I. The lines in Figs. 1 and 2 are predicted from these values. K_2 for NPP complexes is consistently larger than for GPP complexes, although our comparison is only qualitative for the

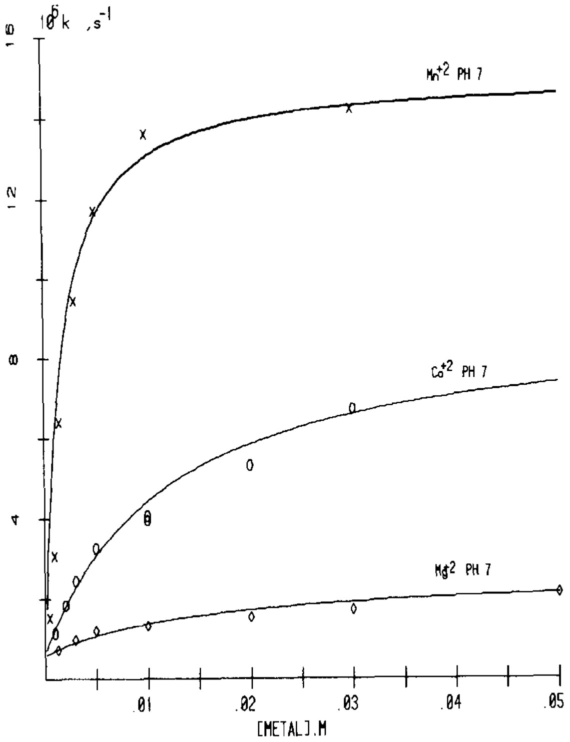


FIG. 1. Metal ion-catalyzed solvolysis of GPP. The lines are calculated.

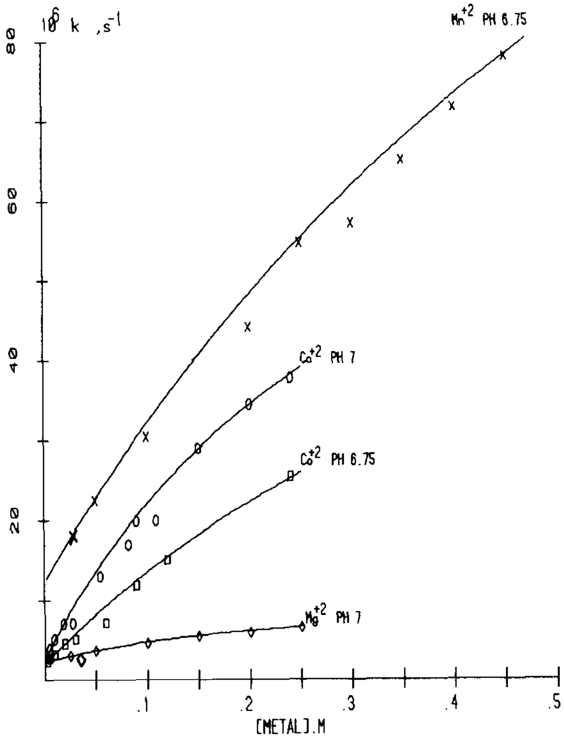
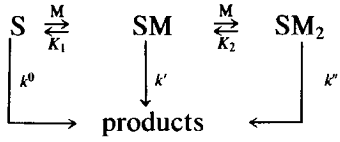


FIG. 2. Metal ion-catalyzed volvolysis of NPP. The lines are calculated



SCHEME 2

$CoCl_2$-catalyzed reactions because of buffer complexation of the metal ion (*30*). From the limited data available, values of K_1 are not very different for the NPP and GPP complexes (Table I).

There is considerable uncertainty in the rate and equilibrium constants in Table I, especially for reactions of NPP where there is little curvature in the rate plots which can be fitted by various combinations of kinetic parameters. Nonetheless, it is evident that values of K_2 are much larger for NPP than for GPP, and the observation of saturation on reactions of GPP shows that this substrate readily binds a second metal ion.

Products. The products of the metal ion-catalyzed hydrolysis of NPP are similar to those of the spontaneous or acid-catalyzed hydrolysis (*8, 23, 25, 26*) and are relatively insensitive to the nature of the metal ion (Table II). The products of hydrolysis of GPP are also not very different in the presence of metal ions, except that cyclization and elimination are increased, but not markedly so, by metal ions. The marked differences in the ratios of cyclic to noncyclic products for hydrolyses of NPP and GPP (R_{NPP} and R_{GPP}, respectively) are illustrated in Table III. For acid hydrolyses $R_{NPP}/R_{GPP} \approx 10^2$, but it is 40–50 for metal ion-catalyzed hydrolyses.

Carabocyclase reactions. Formation of cyclic monoterpenes catalyzed by carbocyclase follows the Michaelis equation based on the concentration of the bis-metal complex, e.g., GPP–Mn_2^{2+}. We could not compare these results directly with those for NPP with Mn^{2+} because of precipitation at pH 7.0. Therefore, comparison was made using the less efficient Mg^{2+} (*20*). It appears that bornyl

TABLE II

SOLVOLYSIS PRODUCTS OF NERYL PYROPHOSPHATE[a]

	Conditions			
Product	No metal	Mg^{2+}	Mn^{2+}[b]	Co^{2+}
Linalool	16	18	16	13
α-Terpineol	71	60	64	74
Nerol	5	6	10	6
Geraniol	1	0	2	0
Limonene	5	5	5	6.5
Terpinolene	2	2.5	2.5	2

[a] At 40°C, pH 7, 0.5 M Tes, and 1.9×10^{-4} M substrate unless specified.

[b] 10^{-5} M substrate.

TABLE III

CYCLIC TO NONCYCLIC PRODUCT RATIOS

	R			Hydrocarbon (mol%)	
Conditions	NPP	GPP	R_{NPP}/R_{GPP}	NPP	GPP
pH 7	3.5	0.03[a]	117	7	~0[a]
Dilute acid	2.9[b]	0.02[b]	145	3	1.7
$MnSO_4$	2.2	0.05[a]	44	7.4	6.0
$MgCl_2$	4.5	0.10[a]	45	7.5	3.2
$CoCl_2$	4.7	0.10[a]	47	8.5	5.3

[a] Ref. (*34*).
[b] Refs. (*25, 26*).

pyrophosphate synthetase activity also follows the concentration of GPP–Mg_2^{2+} (*19, 31*).

Figure 3A shows that the rate of formation of terpene hydrocarbons increases with increasing metal ion concentration while [NPP–Mg^{2+}] has become constant, but [NPP–Mg_2^{2+}] is still increasing. Experiments with either constant [NPP] or [Mg^{2+}] gave very similar values of K_M of 4×10^{-7} and 5×10^{-7} M, respectively, based on the calculated concentration of NPP–Mg_2^{2+}.

Experiments with GPP and Mg^{2+} (Fig. 3B) show similar trends. The rate of enzymatic reaction increases as the concentration of GPP–Mg_2^{2+} increases, even though that of GPP–Mg^{2+} is decreasing.

Values of K_M for reaction of the GPP complex are 5×10^{-6} and 4×10^{-6} M measured at constant GPP or metal ion concentration.

Figure 3C shows that the Michaelis plots for the calculated RPP–Mg_2^{2+} species have very different slopes, expressed as V_{max}/K_M at low [substrate], and that carbocyclase is more efficient by a factor of ca 5 with NPP–Mg_2^{2+} than with GPP–Mg_2^{2+}, which is at variance with the results expressed in terms of total [substrate] (Table IV).

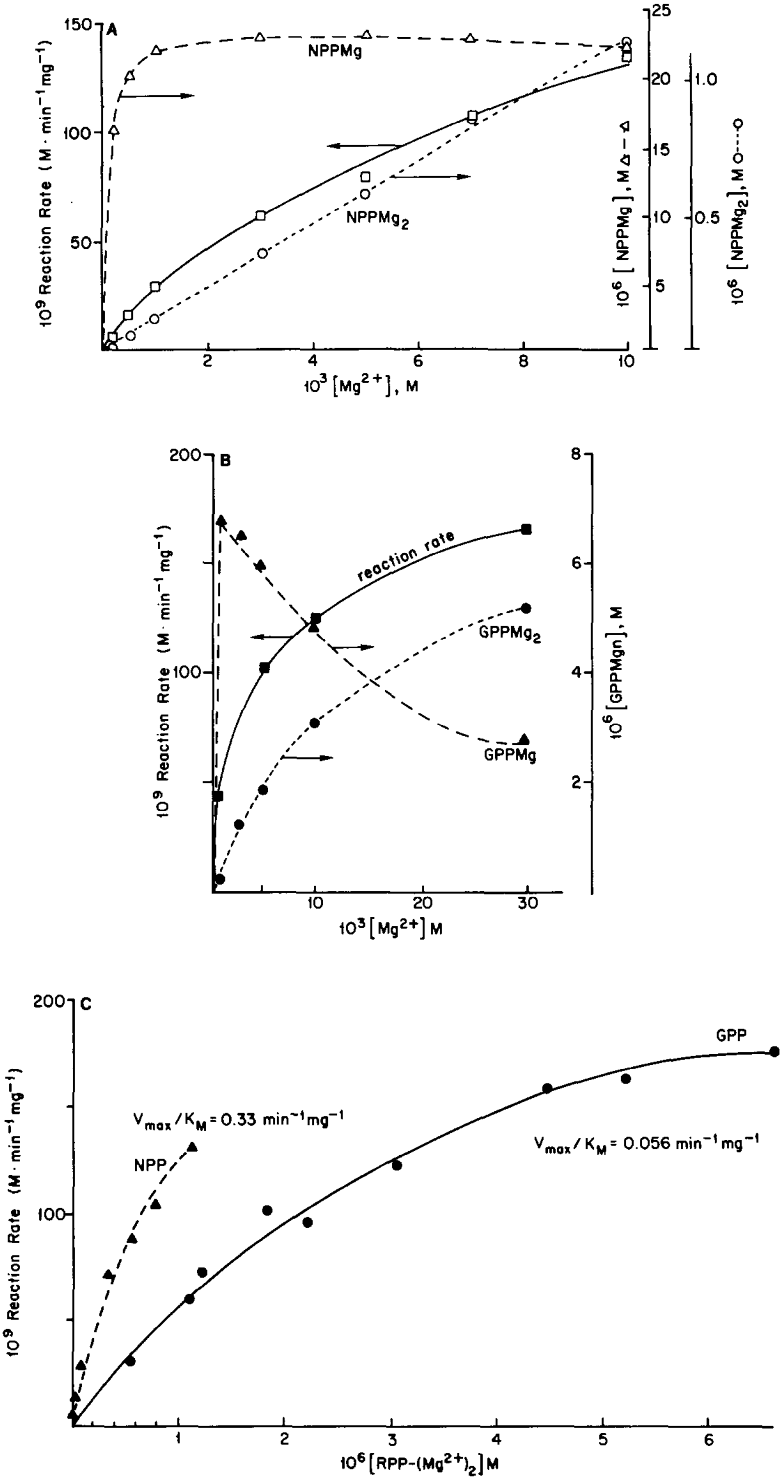
The data presented show that the dissociation constants of the monometal complexes of GPP and NPP are much smaller than those of the bis-metal com-

TABLE IV

CARBOCYCLASE-CATALYZED REACTIONS[a]

Substrate	Total substrate	bis-Metallic complex
NPP	0.015	0.32
GPP	0.03	0.056
NPP/GPP	0.5	5.7

[a] Values of V_{max}/K_m in the presence of Mg^{2+}.


A
150
100
50
10⁹ Reaction Rate (M · min⁻¹ mg⁻¹)
NPPMg
NPPMg₂
2
4
6
8
10
10³ [Mg²⁺], M
25
20
15
10
5
10⁶ [NPPMg], M △ – △
1.0
0.5
10⁶ [NPPMg₂], M ○---○
B
200
100
10⁹ Reaction Rate (M · min⁻¹ mg⁻¹)
reaction rate
GPPMg₂
GPPMg
10
20
30
10³ [Mg²⁺] M
8
6
4
2
10⁶ [GPPMgn], M
C
200
100
10⁹ Reaction Rate (M · min⁻¹ mg⁻¹)
Vmax / KM = 0.33 min⁻¹ mg⁻¹
NPP
GPP
Vmax / KM = 0.056 min⁻¹ mg⁻¹
1
2
3
4
5
6
10⁶ [RPP-(Mg²⁺)₂] M

plexes (Table I). Furthermore, there is a large difference between the dissociation constants of the bis-metal complexes of GPP and NPP. Uncertainties in the value of K_2 for NPP (Scheme 2) do not invalidate the qualitative conclusions. Electronic effects upon complex formation should be similar for these isomeric pyrophosphates, so we assume that the conformation of the *E* isomer, GPP, is more favorable to formation of bis-metal complexes than the conformation of the *Z* isomer, NPP.[3]

The lower stability of the bis-metal complex of NPP does not explain the lower effectiveness of most carbocyclases with NPP (*3, 5, 15, 19*). Figure 3A shows that the enzymatic reaction of NPP–Mg_2^{2+} exhibits a saturation-type curve. Even if the equilibrium concentration of NPP–Mg^{2+} is low, the value of K_M for carbocyclase should be low enough for observation of a saturation-type curve under our experimental conditions, as it is for GPP–Mg_2^{2+} (Fig. 3B). Generally K_M values based on total NPP are lower than for GPP, although they are not very different.

The fact that GPP is the substrate preferred by most carbocyclases, despite its apparently unfavorable conformation and the formation of acyclic products in its chemical reactions, could be explained in terms of the existence of two types of carbocyclases, specific for either NPP or GPP, which have not been separated in most enzyme preparations. Loss of NPP carbocyclase activity on aging supports this explanation (*24*). One could also speculate that enzymes specific for *E* isomers, e.g., GPP or 2,6-*E*-farnesyl pyrophosphate, evolved earlier as catalysts of biosynthesis of steroids, hopanoids (*33*), carotenoids or diterpenes, and that only some phyllae of higher plants (*34*) developed the ability to synthesize and utilize *Z* isomers such as NPP or 2-*Z*, 6*E*-farnesyl pyrophosphate. In addition, current methods of enzyme isolation mix enzymes which may be separated in different compartments in the plant cell.

The fact that carbocyclase is more efficient with NPP–Mg_2^{2+} than with GPP–Mg_2^{2+} *in vitro* may be related to the role of the *Z* and *E* isomers *in vivo*. Data on metal ion concentrations in plants and optimum concentrations for the artificial growth of plants show that the concentration of Mg^{2+} is ca 10^{-3} M, sufficient for formation of NPP–Mg_2^{2+}. The concentration of Mn^{2+} is much smaller by factors of 10–100 (*35*). Thus, despite relatively unfavorable metal binding, NPP–Mg_2^{2+} could be an effective substrate for formation of cyclic monoterpenes, whereas GPP is utilized in many other reactions in isoprenoid pathways.

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[3] The *E* and *Z* isomers will take up different preferred conformations (*32*).

FIG. 3. Enzymatic synthesis of terpene hydrocarbons. (A) Rate and estimated concentrations of mono- and bis-metallic complexes of NPP. [NPP] = 24 × 10^{-6} M. (B) As with A, but with GPP. [GPP] = 8 × 10^{-6} M. (C) Dependence of rate of hydrocarbon formation on concentration of bis-metallic complexes. Kinetic data from A and B.

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