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ENHANCEMENT OF THE HYDROLYSIS OF GERANYL PYROPHOSPHATE BY BIVALENT METAL IONS. A MODEL FOR ENZYMIC BIOSYNTHESIS OF CYCLIC MONOTERPENES

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Abstract—Hydrolysis of geranyl pyrophosphate is catalyzed by salts of Mn^{2+} and involves C–O bond cleavage. The first order rate constants reach limiting values with $[Mn^{2+}] > 10^{-2}$ M, and the most reactive species is $GPP(Mn^{2+})_2$ at the optimum pH of 6.5–7. The products are similar to those from acid hydrolysis except that more cyclic hydrocarbons are formed in the presence of metal ions. Hydrolysis of geranyl phosphate is inhibited, and that of citronnellyl pyrophosphate is weakly catalyzed by Mn^{2+}. Other divalent metal cations catalyze the hydrolysis of geranyl pyrophosphate and the sequence of effectiveness is $Cu^{2+} > Mn^{2+} > Zn^{2+} > Co^{2+} < Mg^{2+} \sim Ca^{2+}$.

Geranyl pyrophosphate (GPP) is a postulated precursor in the biosynthesis of cyclic monoterpenes such as limonene, α-pinene, β-pinene,[1,2] although the *E*-configuration of GPP precludes direct cyclization;[3] the *Z*-isomer, neryl pyrophosphate (NPP), is a precursor of bicyclic monoterpenes in *Pinus radiata*.[4] The original postulate of the role of NPP was based in part on the product distribution in nonenzymic solvolyses and rearrangements of GPP, NPP and related compounds,[5–7] where the *Z*-isomer yielded mainly cyclic products. Recent enzymic studies with carbocyclase from *Citrus limonum*[8] and *Salvia officinalis*[9] showed that GPP is also a precursor of cyclic monoterpenes, but because enzymic isomerization of GPP to NPP was excluded,[8,9] rotation or interconversion of an enzyme bound carbocationic species derived from GPP was postulated.[3,8]

Carbocyclase is a Mn^{2+} dependent enzyme[8] and consistently nonenzymic hydrolyses of farnesyl,[10] geranyl and chrysanthemyl pyrophosphate[11] are speeded, at neutral pH, by Mg^{2+} or Mn^{2+} ions.

These observations suggest that metal catalyzed solvolysis of GPP could be a model for the enzymic reaction. The present work describes the effect of Mn^{2+} on the hydrolysis of GPP and geranyl monophosphate (GP), in an attempt to understand the role of Mn^{2+}, or other divalent metal ions, in the biosynthesis of cyclic monoterpene hydrocarbons in plant tissues.

RESULTS AND DISCUSSION

Rates and products

In the presence of excess Mn^{2+} at pH 7.0 and 40° GPP is transformed into a mixture of prenyl alcohols and terpene hydrocarbons (Scheme 1) and the overall reaction is first order with respect to GPP. Figure 1 shows the dependence of the initial rate of formation of products upon [GPP], with a large excess of Mn^{2+}. If

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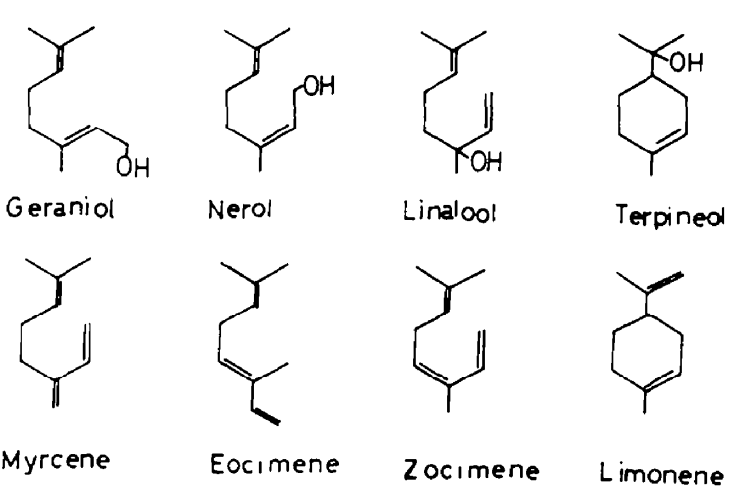


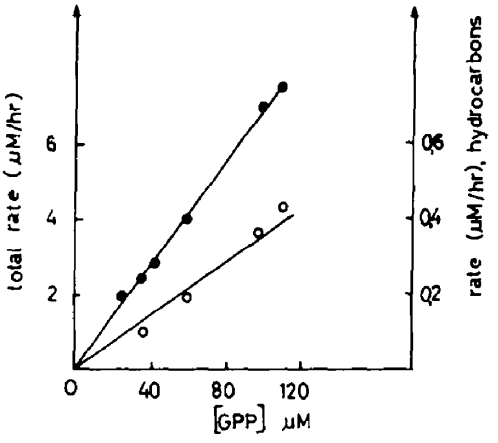


Fig. 1. Effect of [GPP] on the rate of Mn^{2+} catalyzed hydrolysis at 40° and 0.1 M TES, pH 7 and 0.03 M $MnCl_2$. ●—●, Formation of total products; O—O, Formation of hydrocarbons. For further details, see text.

log (dp/dt) (where p is product) is plotted as a function of log GPP concentration, a straight line of unit slope is obtained.

The rate of hydrolysis of 10^{-4} M GPP at 40° and pH 7.0 is increased by a factor of up to 50 by Mn^{2+}. The first order rate constants (k obs) are 3.4×10^{-7} s^{-1}, in the absence of metal and 1.8×10^{-5} s^{-1}, in 10^{-2} M Mn^{2+}. Complexing of Mn^{2+} with EDTA eliminates the catalysis.

Added acetone or dioxane has little effect on the spontaneous hydrolysis, but with 0.005 M Mn^{2+}, organic solvent at a mole fraction of 0.2 reduces the rate by approximately 80%. These effects were not investigated further.

The products at pH 7 are markedly affected by Mn^{2+}. The spontaneous hydrolysis probably occurs largely with P–O rather than C–O bond fission, or by concerted nucleophilic substitution, and therefore gives only geraniol. Hydrolysis of GPP in dilute acid occurs with C–O bond fission,[5,6] and the products are qualitatively similar to those of the Mn^{2+} catalyzed hydrolysis, except that Mn^{2+} favors hydrocarbon formation (Table 1).

No buffer effects were observed on products or rates either in the presence or absence of metal. This is to be expected, because potentiometric[12] and EPR binding[13] measurements have shown that there is no significant interaction between TES buffer base and Mn^{2+}. Increase of ionic strength with 0.3 M KCl decreases the rate of reaction by 20% in the presence of 10^{-4} M Mn^{2+} and by 50% in its absence at pH 7.0 and 40°.

Analysis at partial reaction in the presence of Mn^{2+} showed that no geranyl monophosphate is formed, and solvolysis did not proceed stepwise as with the enzymic hydrolysis.[14] Control experiments with nerol geraniol, linalool and α-terpineol showed that Mn^{2+} did not catalyze their interconversions or dehydrations. No other prenyl pyrophosphate was found in the reaction mixture, thus excluding isomerization of the substrate.

Effect of [Mn^{2+}] *on hydrolysis rate*

The dependence of the rate of hydrolysis of GPP on [Mn^{2+}] is shown in Fig. 2 for reaction using either $MnCl_2$ or $MnSO_4$. At high [Mn^{2+}] the rate constants reach limiting values so that GPP must be converted completely into a reactive species, $xGPP - yMn^{2+}$. Because the association constant for formation of the 1:1 complex is 2.5×10^4 M^{-1} at 25°,[11] at 5×10^{-4} M Mn^{2+} and 10^{-4} M GPP over 90% of the GPP is present as the 1:1 GPP-Mn complex. Figure 2 shows that the maximum rate of hydrolysis is obtained at a much higher concentration of Mn^{2+}, so that the reactive species is a complex with a Mn^{2+}/GPP ratio > 1.

Prenyl pyrophosphates and phosphates behave similarly with respect to hydrogen ion catalysis,[6,15] but quite differently with respect to metal ion catalysis. At pH 7.0 Mn^{2+} retards the hydrolysis of GP (Fig. 3). No hydrocarbons are formed and geraniol (94%) is the major product, and the products are similar to those from spontaneous hydrolysis of the monoanion, via metaphosphate ion. Complexing of Mn^{2+} to the phos-

Table 1. Products of hydrolysis of GPP[a]

Products	10^{-2}M Mn^{2+}	0.42M H_2SO_4[b]
alcohols	94 (100)	98.3
hydrocarbons	6[c]	1.7
linalool	71.0 (29.0)	77.7
geraniol	19.0 (65.0)	17.1
nerol	0.6 (2.0)	0.9
α-terpineol	3.3 (3.3)	2.6
myrcene	2.7	0.3
ocimene (E+Z)	1.7	1.2
limonene	1.2	0.07
unidentified	0.3	

a) Moles % of products at 40°C with 10^{-4}M GPP and TES buffer, pH 7.0, unless specified. Values in parentheses are in the absence of Mn^{2+};

b) at 20°C[6]; c) no hydrocarbons were detected in the absence of Mn^{2+}.

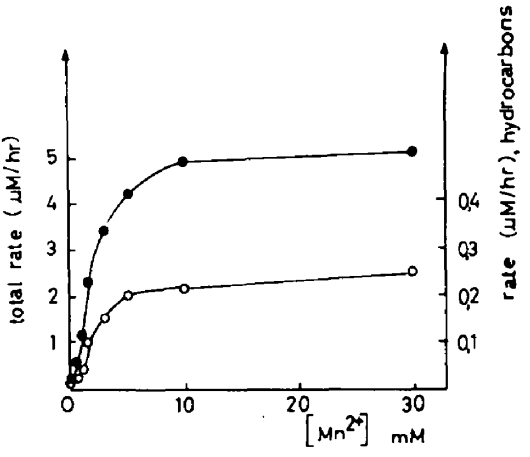


Fig. 2. Effect of Mn^{2+} on GPP hydrolysis at 40°, 0.1 M TES, pH 7.0 and 10^{-4} M GPP. ●—●, Formation of total products; ○—○, Formation of hydrocarbons.

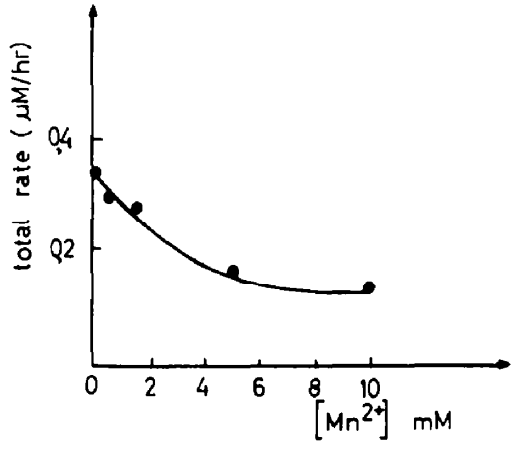


Fig. 3. Effect of Mn^{2+} on GP hydrolysis (conditions as in Fig. 2).

phate moiety will inhibit elimination of metaphosphate ion. The differences between GPP and GP as substrates are readily understandable because phosphate monoesters cannot readily chelate with a metal ion.

The rate of hydrolysis of the 2,3-dihydro analog of GPP, citronellyl pyrophosphate (CiPP), is increased by only a factor of five by 10^{-2} M Mn^{2+} at pH 7.0 and 40°, and no hydrocarbons are formed. The allylic double bond in GPP favors formation of a carbocationic species and may also help to stabilize a reactive metal-substrate complex.

Effect of pH

Figure 4 shows that a change in pH from 6.5 to 7.0 has little effect on the rate of solvolysis, and a further change to pH 7.6 reduces the rate by only one third, over a wide range of $[Mn^{2+}]$. A wider range of pH could not be used, because at pH 7.6 a visible precipitate was formed, and at pH < 6.5 the spontaneous hydrolysis is fast, relative to the Mn^{2+} catalyzed reaction.

Stoichiometry of the reactive complex

The data in Fig. 2 show that more than one mole of Mn^{2+} is bound per mole of GPP and Job's method[16] was used to obtain the stoichiometry of the reactive complex. Initial rates were plotted as a function of the mole

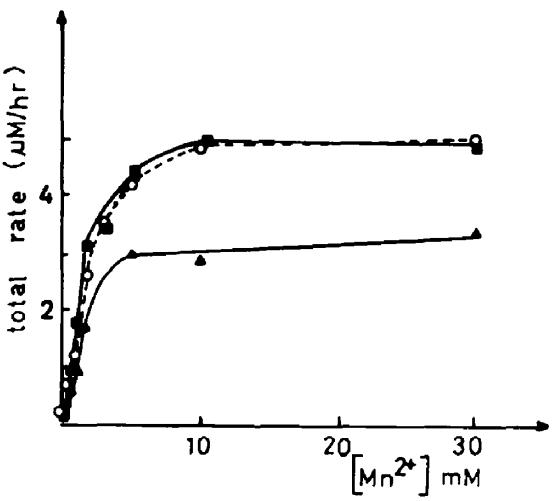


Fig. 4. Effect of pH and $[Mn^{2+}]$ on GPP hydrolysis (conditions as in Fig. 2). ■—■, pH = 6.5; ○—○, pH = 7.0; ▲—▲, pH = 7.6.

fraction of Mn^{2+}, at constant $[Mn^{2+}]+[GPP]$, (Fig. 5). Maximum rates of product or hydrocarbon formation were observed at a mole fraction of 0.67, corresponding to a Mn^{2+}:GPP ratio in the reactive species of 2:1. (Allowance was made for the contribution of the uncatalyzed reaction.) The similarity of the two Job plots in Fig. 5 suggests that alcohols and hydrocarbons are formed from the same GPP $(Mn^{2+})_2$ complex. This experiment shows that this complex is the most reactive species, but there is probably a contribution of hydrolysis of the 1:1 complex, because the rate follows the concentration of GPP $(Mn^{2+})_2$ when the mole fraction of Mn^{2+} is relatively high; but at a low mole fraction of Mn^{2+} the

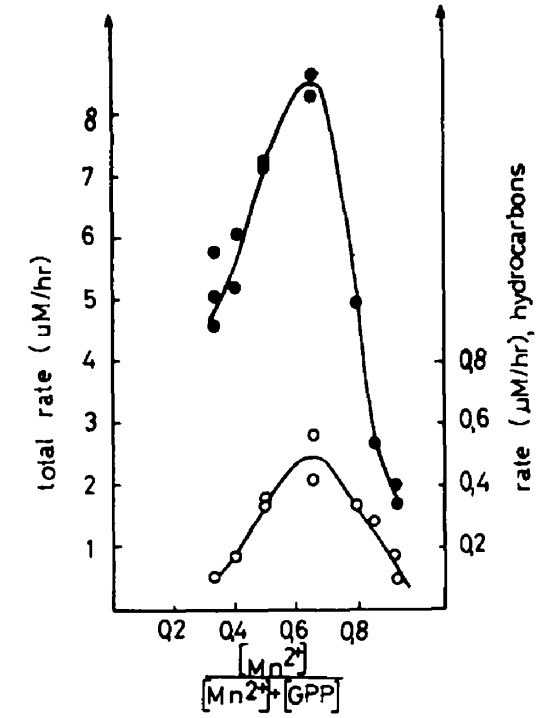


Fig. 5. Effect of mole fraction of Mn^{2+} on rates of formation of alcohols and hydrocarbons from GPP, at 40°C, 0.1 M TES, pH 7.0, with $[GPP]+[Mn^{2+}] = 1.5 \times 10^{-3}$ m. ●—●, Formation of total products; ○—○, Formation of hydrocarbons.

rate is higher than predicted from the concentration of $GPP(Mn^{2+})_2$ (Fig. 5). In addition the points of inflection in plots of rate as function of $[Mn^{2+}]$ (Fig. 2) suggest that the dimanganese complex is the more, but not the only, reactive, species. The experiments were in 0.1 M TES buffer, and similar results were found with 0.025 M TES, pH 7.0, except that precipitation sometimes occurred.

Our results agree with those for several hydrolyses of pyro- and triphosphates in which ligand-$metal_2$ complexes appear to be the reactive species.[11, 17, 18]

Overall kinetic scheme

For a reaction in which $GPP(Mn^{2+})_2$ is the most reactive species the kinetic scheme for the hydrolysis of GPP catalyzed by Mn^{2+} may be summarized as follows:

$$GPP + Mn^{2+} \overset{K_1}{\rightleftarrows} GPP(Mn^{2+})$$

$$GPP(Mn^{2+}) + Mn^{2+} \overset{K_2}{\rightleftarrows} GPP(Mn^{2+})_2 \overset{k}{\rightarrow} \text{Products}$$

The stability constant K_1 for the 1:1 complex is $2.5 \times 10^4\ M^{-1}$.[11] From $[Mn^{2+}]$ at half of the limiting rate (Fig. 2) an estimate can be made of:

$$K_2 = \frac{[GPP(Mn^{2+})]}{[GPP(Mn^{2+})][Mn^{2+}]} = 4 \times 10^2\ M^{-1}.$$

This value of K_2 differs from that of *ca.* $1.4\ M^{-1}$ reported by Brems and Rilling.[11] This difference, however, may be due to the differences in experimental conditions. These authors performed their experiments at 55°, at an ionic strength of 4.8 to 12, with 10^{-6} M substrate and pH 7.6 in TRIS buffer, which may form mixed complexes with metal and substrate.[19] Furthermore, in the figure in Ref. 11 there seems to be no levelling off of reaction rate even up to concentrations of metal ion of 2 M.

The highest rates observed by Brems and Rilling were greater than ours in the presence of Mn^{2+} by approximately two orders of magnitude, which is much larger than expected from the temperature difference, but they were observed at relatively high concentration of metal ion. Divalent manganese tends to precipitate as hydroxide at pH close to neutrality, and it may be that under the conditions used by Brems and Rilling submicroscopic colloidal particles were present. Colloidal aggregates of heavy metal ions are very effective catalysts of hydrolysis of phosphate esters,[20] and their existence would explain the high rates of hydrolysis observed by Brems and Rilling, as well as the apparent absence of any levelling off of the rate with increasing metal ion concentration.

Temperature dependence

Table 2 compares the temperature dependence of the reaction rate in spontaneous and metal catalyzed hydrolyses of GPP, under conditions in which the predominant species is GPP $(Mn^{2+})_2$. The Arrhenius plots are linear. The activation parameters for the metal ion catalyzed hydrolysis include terms for complex formation and breakdown, including alcohol and hydrocarbon formation, and the catalysis is due to an increase in log A (more positive $\Delta S^{\ddagger}$) which offsets the higher activation energy (Table 2). The percentage of hydrocarbon formation increases with increasing temperature, as is generally found in elimination-substitution reactions and the difference in activation energies is *ca.* 4 kcal mol^{-1}, which agrees with the difference observed between elimination and substitution reactions.[21]

Effects of other metal ions

Figure 6 shows that other metal ions catalyze hydrolysis of GPP. Transition metal ions are the most effective in the sequence: $Cu^{2+} > Mn^{2+} > Zn^{2+} > Co^{2+}$, but Ca^{2+} nor Mg^{2+} are relatively ineffective. There is no obvious relation between Lewis acidity[22] and catalytic effectiveness of the metal ion. (The experiments with Cu^{2+} were in HEPES buffer to avoid complexation.)

The apparent high reactivity of Cu^{2+} may be due to catalysis by colloidal aggregates because a precipitate was formed above 0.002 M Cu^{2+}. However the sequence of effectiveness depends on temperature, for example at 30° the sequence of catalytic effectiveness is: $Mn^{2+} > Cu^{2+} > Co^{2+}$. This question was not explored in detail.

The product compositions are similar for reactions in the presence of the various metal ions (Table 3). However, the amounts of cyclic products were largest with Co^{2+}, Zn^{2+} and Mg^{2+}. Although Ca^{2+} and Mg^{2+} give little overall rate enhancement (Fig. 6) the products are derived by C–O bond cleavage.

Table 2. Temperature effects upon overall reaction and hydrocarbon formation from GPP[a]

	$10^7 k_{obs}, s^{-1}$	
T°C	no Mn^{2+}	Mn^{2+}
15.0	0.20	2.21 (2.2)
25.0	1.44	7.4 (3.2)
37.8	4.0	76.0
46.5	19.0	258.0 (6.0)
54.0		683.0 (6.0)
E, kcal. $mole^{-1}$	25	31
log A	11,3	16,7

a) At pH 7, 0.1 M TES buffer and 0.005 M Mn^{2+}. Values in parentheses are moles % hydrocarbon.

Table 3. Effects of metal ions on the products[a]

Product, moles %	Metal Ion				
	3mM Cu^{2+}[b]	10mM Mn^{2+}	10mM Co^{2+}	10mM Zn^{2+}	30mM Mg^{2+}
alcohols	93.3	94.3	94.7	96.7	96.8
hydrocarbons	6.7	5.9	5.3	3.3	3.2
linalool	69.5	71.0	65.4	71.0	66.5
geraniol	17.1	19.9	19.9	18.0	21.3
nerol	--	0.6	1.0	0.5	--
α-terpineol	3.3	3.3	8.4	7.2	9.0
myrcene	3.6	2.7	1.9	1.6	1.9
ocimene (E+Z)	3.1	1.7	1.9	1.2	1.4
limonene	--	1.3	0.6	0.5	--

a) At 40°C, pH 7.0, TES buffer, except where specified; 10^{-4}M GPP.

b) HEPES buffer.

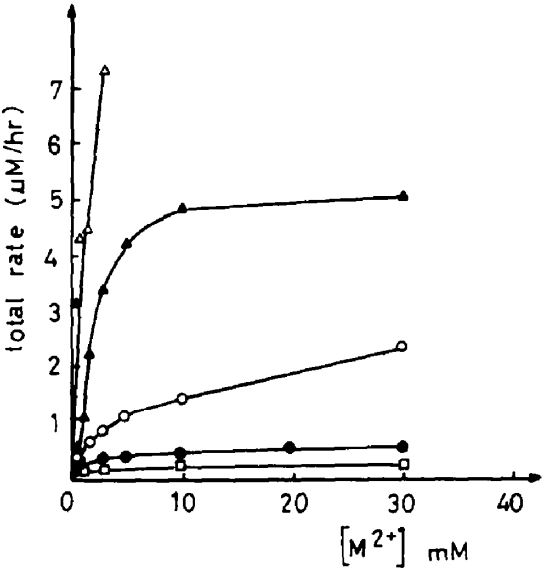


Fig. 6. Effect of concentration of metal ions on GPP hydrolysis at 40°, 0.1 M TES, pH 7.0 and 10^{-4} M GPP. HEPES buffer was used. Δ—Δ, Cu^{2+}; ▲—▲, Mn^{2+}; ○—○, Co^{2+}; ●—●, Mg^{2+}; □—□, Ca^{2+}.

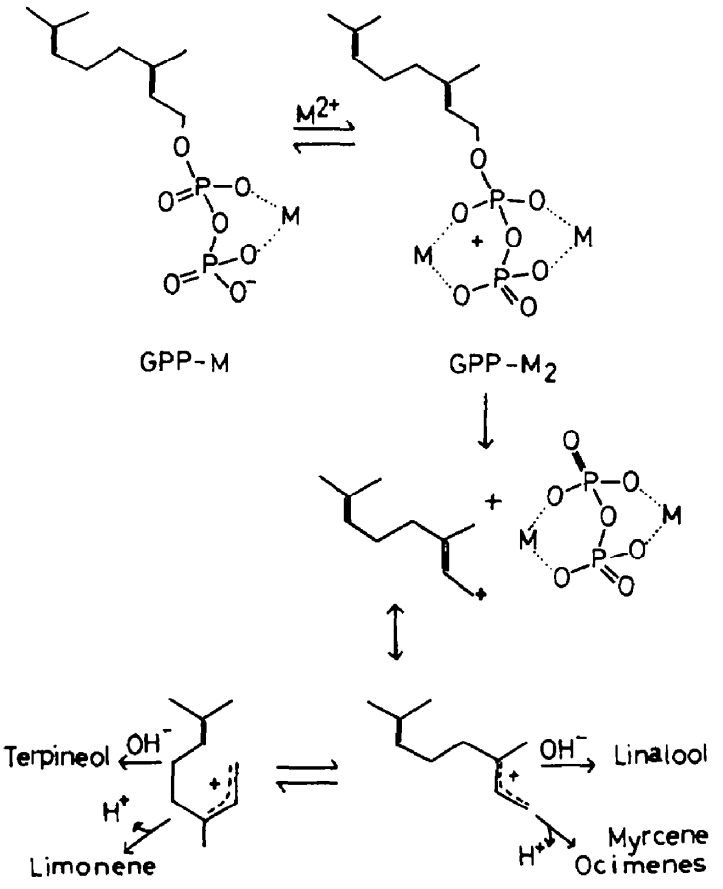
Nature of the metal ion catalyzed hydrolysis

Both hydrogen and metal ions catalyze the formation of carbocationic intermediates from GPP by neutralizing the negative charge of the phosphate moiety.[23] But divalent metal ions also increase the amounts of elimination, and cyclization, relative to the hydrogen ion catalyzed reaction. (Tables 1 and 3).

The fact that more limonene was formed in metal ion rather than in acid catalyzed hydrolysis deserves comment. The transformation of GPP, with its *E*-conformation, into cyclic products requires rotation about the delocalized 2,3-double bond of the intermediate carbocation. This interconversion is not rapid with other allylic carbocations in an aprotic solvent.[24-26] We found no evidence of *E-Z* interconversion of the pyrophosphates *via* the tertiary linalyl pyrophosphate although it may be too reactive under our conditions to be detected. In addition Cramer found no isotopic scrambling in the hydrolysis of ^{18}O neryl monophosphate.[27] Another difference between hydrogen ion and Mn^{2+} catalyzed hydrolyses of prenyl substrates is that for the hydrogen ion catalyzed reactions, product distribution and rates are essentially independent of the leaving group,[6,15] which is clearly not so for hydrolyses of GPP and GP in the presence of Mn^{2+}. Metal ions can participate in enzymic reactions of phosphorylated substrates by shielding negative charges in the phosphate moiety.[28] But the absence of a relationship between Lewis acidity[22] and catalytic effectiveness of metal ions, and the differences between mono- and pyrophosphate hydrolyses suggest that the metal ion may play a more complicated role. Two metal ions could coordinate with a geranyl pyrophosphate trianion, giving a cationic complex which should readily break a C–O bond (Scheme 2). Formation of the neutral PP_i–Mn_2 complex by breakdown of a cationic complex should provide much of the driving force for the reaction. Therefore $GPP(Mn^{2+})_2^+$ makes the major contribution to reaction, although the equilibrium is in favor of $GPP(Mn^{2+})$, (*cf* Ref. 11). Such complexes cannot be formed from monophosphate esters, which is consistent with the marked differences in behavior of the mono- and pyrophosphate esters.

The increased formation of cyclic products in metal ion, relative to the hydrogen ion, catalyzed hydrolyses of GPP, suggests that the carbocationic species formed from the metal ion complex has a longer life than from the acid catalyzed reaction. At the same time interactions between the double bonds and the metal ions may favor a conformation compatible with cyclization.

Bond cleavage of a $GPP(Mn^{2+})_2^+$ complex will give the carbocation and the formally neutral PP_i complex (Scheme 2). This neutral complex should however be strongly hydrated and therefore deactivate adjacent water molecules, so that the cation has a longer lifetime, and can more easily change its conformation from *anti* to *syn* and then lose a proton, forming an alkene, rather than undergo addition of water. There are, however, examples of high nucleophilicity of metal bound OH^-.[29,30]



Therefore metal ion catalysis seems to be better than acid hydrolysis as a model for enzymic reactions. Although enzymic reactions occur in aqueous media, the active sites of enzymes are typically in hydrophobic regions and shielded from the solvent, a purpose which might be served by the PP_i–Mn leaving group.

EXPERIMENTAL

Synthesis of radioactive substrates

1-^{3}H Ortho- and pyrophosphates of geraniol and citronellol were prepared from the corresponding labelled alcohols. Geraniol (Aldrich) and citronellol (Chemical Procurement Labs) were distilled in a spinning band column at 5–10 torr. Less than 1% of contaminants were detected by GLC of the distilled alcohols. Geraniol was oxidized with activated MnO_2[31] and citronellol with CrO_3-pyridine in CH_2Cl_2.[32,33] The aldehydes thus obtained were reduced with a slight excess of NaB^3H_4 (New England Nuclear) of 293 Ci/mol.[8] The 1-^{3}H alcohols thus obtained were diluted to final specific radioactivities of 2.9 to 5.2×10^{13} dpm mol^{-1} and were phosphorylated with di-(triethylammonium) phosphate.[34] The ortho- and pyrophosphates were separated by ion exchange chromatography on DEAF-Sephadex A 25[35] using a linear gradient of triethylammonium bicarbonate, pH 7.5,[36] between 0.05 M and 0.5 M, controlled by conductivity measurement. The samples were freeze dried, to remove the buffer.

The fractions containing phosphate or pyrophosphate esters were extracted from the freeze dried sample with n-propanol-conc. NH_4OH 2:1 (*v/v*), which does not dissolve inorganic ortho- and pyrophosphate. The solvent was evaporated, the samples were dissolved in dil. NH_3 pH 7.0, and were stored at 4°. They were less stable when stored as frozen solutions or as dried powders at −18°.

Identification of the radioactive substrates

Radioactive pyro- and monophosphates were identified by TLC on silica gel-H in n-PrOH-conc NH_4OH–H_2O (6:3:1, *v/v*) or silica gel-H ammonium phosphate plates.[37] The alcohol in the phosphorylated substrate was identified after its liberation by hydrolysis with apyrase plus phosphomonoesterase from *E.coli*,[38] followed by extraction with hexane, by tlc on Adsorbosil-5 plates (Applied Science Laboratories) with twofold development in EtOAc-hexane 15:85 (*v/v*) or by glc.[5]

The phosphorus:alcohol ratio was determined by measuring the liberation of orthophosphate and hexane soluble radioactivity either directly in acid molybdovanadate,[39] which splits only the C–O allylic bond, or after hydrolysis in 1 M HCl for 10 min at 100°,[40] which also splits the P–O–P bond. The ratio was 1:1 for GP and 1:2 for GPP. These two products liberated 95% of their radioactivity in 20 min in 0.1 M HCl at 37°, whereas the citronellyl derivatives were acid stable under these conditions.

Measurement of the rate of hydrolysis

The total reaction rate was measured by the liberation of hexane soluble radioactivity from a given labelled substrate. Duplicate incubations were performed, if not otherwise stated, in sealed ampoules at 40° in a total volume of 1 ml of 0.1 M TES-NH_4OH buffer, pH 7.0. Substrate concentrations were 0.5–5×10^{-4} M. Metal concentrations are indicated for each experiment.

Reaction was quenched by cooling the ampoules to 0° and extracting the aqueous phase with 2 ml of hexane (b.p. 40–60°). Radioactivity in one aliquot of the hexane phase was measured by conventional beta liquid scintillation spectrometry.[38] Another portion of the hexane extract was treated with silicic acid to adsorb alcohols,[8] and radioactive hydrocarbons were measured in an aliquot of the supernatant hexane.

Initial rates were measured for up to 7% of total reaction and

observed first order rate constants were calculated from averaged duplicate values by the method of least squares.

In some experiments, the unreacted substrate in the aqueous phase was identified after hexane extraction. The aqueous phase was added to EDTA of a final concentration of 0.02 M. The sample was applied to a 0.8 × 15 cm column (8 ml) of DEAE-Sephadex A-25[35] and eluted with a linear gradient of NH_4HCO_3. No radioactive GP emerged from this column. The only radioactive peak, corresponding to GPP, was hydrolyzed with potato apyrase plus phosphomonoesterase from *E.coli*.[38] The resulting alcohol was extracted with hexane and identified as geraniol by glc.[8]

Identification of reaction products

Radioactive products were identified qualitatively by tlc and glc. The tlc of alcohols was performed as described in the presence of appropriate carriers, which were detected by exposure to I_2 vapour. The plates were scraped in 0.5 cm portions into scintillation vials for counting. The glc was performed in an instrument with a thermal conductivity detector, whose outflow was connected to a gas phase Geiger counter (Biospan 4998, Nuclear Chicago). Radioactivity and carrier peaks were simultaneously recorded in a two channel instrument.[8] The products were identified by co-chromatography of the radioactivity with the carrier peak of known standards. Alcohols were separated at 100° on a Chromosorb G AW DMCS column coated with 0.8% polyethyleneglycol adipate; hydrocarbons were separated on the same column at 60°, and were further identified on Apiezon L[41] and $\beta\beta'$-oxydipropionitrile[4] columns.

Radioactivity peak areas were determined by weighing copies of the tracings.

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Abbreviations: GPP, geranyl pyrophosphate; GP, geranyl monophosphate; CiP and CiPP, citronellyl mono- and pyrophosphate; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TES, N-tris (hydroxymethyl)methyl-2-amino ethanesulfonic acid.

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