Hydrolysis of Bismetallic Complexes of Linalyldiphosphate and their Participation in the Biosynthesis of Cyclic Monoterpenes

Gloria Portilla, M. Cecilia Rojas, Evaristo Chiong, and Osvaldo Cori*
Departamento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

The allylic diphosphate ester linalyldiphosphate forms mono- and bis-metallic complexes with Mg^{2+} and Mn^{2+}. Complexes of the trianion of the ligand are 50 to 100 times more stable than those of the dianion. Mg^{2+} and Mn^{2+} are bound in the monometallic complex 10^3 and 10^4 times more strongly than in the bismetallic species.

The rate constant of the uncatalysed hydrolysis of linalyldiphosphate at pH 7.0 is $1.2 \times 10^4 s^{-1}$, which is higher than reported values for the primary diphosphates. Mg^{2+} and Mn^{2+} affect its rate of hydrolysis and it is shown that the rate enhancement observed is a function of the concentration of the bismetallic complexes.

The rate of cyclic terpene hydrocarbon biosynthesis catalysed by carbocyclase from *Citrus limonum* also correlates with the concentration of the bismetallic complexes.

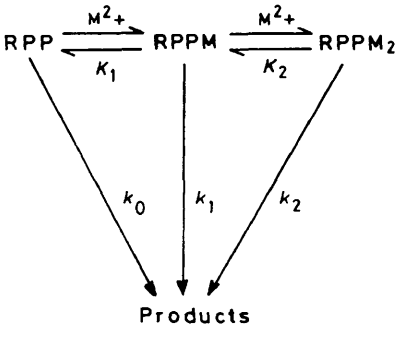
Linalyl monophosphate, which is not a substrate for carbocyclase hydrolyses at a rate proportional to the concentration of the monometallic complex of Mn^{2+}. The neutral charge of this complex, as opposed to the positively charged bismetallic complex of linalyl diphosphate may be an explanation of the absence of utilization of this complex as a substrate.

It appears from the data presented and from previous evidence that bismetallic complexes of allylic diphosphates are the most reactive species in their hydrolysis and the only reactive species in the enzymic cyclization of these precursors of monoterpenes.

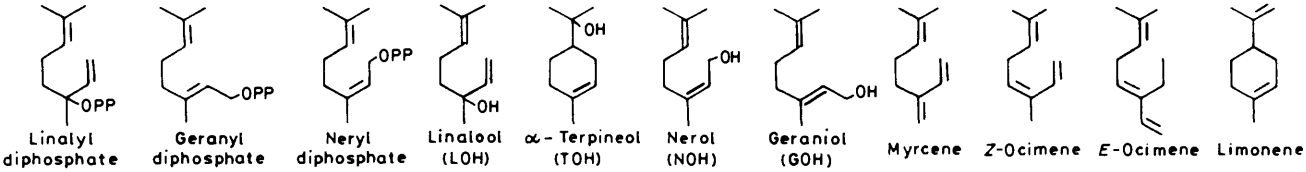
Cyclic monoterpenes such as limonene, α-pinene and β-pinene are formed in plant tissues from geranyldiphosphate (GPP)† and neryldiphosphate (NPP) by enzymes called carbocyclases.[1,2] As opposed to NPP whose structure permits cyclization, direct participation of GPP is not obvious[3] due to its *E* conformation.‡ In view of this steric restriction and as *E-Z* isomerization of GPP has been discarded,[4] the tertiary isomer, linalyl diphosphate (LPP), has been postulated as a stereochemically plausible intermediate,[4-7] and it has been found to be an efficient substrate for carbocyclases.[8-10] However, evidence for its formation in plant tissues is not conclusive. Thus, although linalool has been obtained in some biosynthetic experiments,[4,6] this could be an artifact due to the non-enzymic solvolysis catalysed by Mn^{2+} or Mg^{2+} of GPP newly formed through prenyltransferase activity.[11,12] Furthermore, as opposed to the primary diphosphates GPP and NPP which are formed in plant tissues[13-15] the role of LPP as a free intermediate in the formation of cyclic hydrocarbons has not been confirmed. When GPP was incubated with carbocyclase from *C. limonum*[8] or with enzymes from *Salvia*,[16] LPP was not detected in the reaction media, suggesting that LPP if formed, could be a tightly enzyme-bound intermediate.

The mechanism of these cyclizations has been visualized as an intramolecular alkylation of remote double bonds by allylic diphosphate esters[17-19] with allylic carbocations or possibly ion pairs as intermediates.[20]

The electrophile studied in model reactions has been most frequently the hydronium ion,[21,22] but it has also been shown that bivalent metals catalyse the solvolysis of NPP or GPP at neutral pH[11,12] to form cyclic and non-cyclic alcohols and hydrocarbons. It was established that the most reactive complex is the bismetallic complex $RPPM_2^+$, and thus solvolysis may occur according to Scheme 1.



Scheme 1. k_0, k_1 and k_2 Are kinetic constants for the solvolysis of the allylic diphosphate RPP and its mono- and bis-metallic complexes RPPM and $RPPM_2$ respectively. K_1 and K_2 Are dissociation constants for the species involved.



† TES = *N*-Tris(hydroxyethyl)-2-aminoethanesulphonic acid; DMAP = dimethylallyl monophosphate; IP = isopentenyl monophosphate; LP = linanyl monophosphate; FP = farnesyl monophosphate; RPP = free allylic diphosphate ($RPPH^{2-}$ + RPP^{3-}); RPPM = monometallic complex (RPPHM + $RPPM^-$) of allylic diphosphates; $RPPM_2$ = bismetallic complex of allylic diphosphate ($RPPM_2^+$); K_1, K_2 = dissociation constants of the mono- and bis-metallic complex respectively.

‡ *E* and *Z* isomers shall be referred to as diastereoisomers differing in 'conformation' according to IUPAC specifications (*J. Org. Chem.*, 1970, **35**, 2849).

All carbocyclases require bivalent cations and their true substrates are also the bis-metallic complexes of NPP and GPP.[23,24]

In several reports enzymic hydrocarbon formation from total LPP is comparable and often better than from GPP or NPP.[8-10]

In order to extend the picture of the enzymic formation of cyclic monoterpenes, it was deemed necessary to compare the participation of bivalent cations in the solvolytic reaction of LPP with its participation in the biosynthetic process. It seemed also important to study the hydrolysis of the monophosphate LP, which is not a substrate and inhibits carbocyclase very ineffectively.[18]

The present communication reports dissociation and rate constants of metal complexes of allylic ligands obtained by potentiometric and kinetic procedures and evaluates the possible role of LPP-metal complexes in hydrocarbon biosynthesis by carbocyclases from *Citrus limonum*.

Experimental Procedures

Synthesis of Substrates and Solvolysis.—[1-^{3}H]-LPP, [1-^{3}H]-LP, [1-^{3}H]-NPP, and [1-^{3}H]-GPP (specific activity 1.7×10^7, 3.5×10^7, 12.5×10^7 and 6.7×10^7 d.p.m./μmol respectively) were prepared by phosphorylation of the corresponding labelled alcohols as described previously.[8,25] Unlabelled LPP was prepared from commercial(±)-linalool (Dragoco). It was first purified from the phosphorylation mixture in a silica gel 60 column with a linear gradient of propanol–ammonia–water from 12:3:0 to 6:3:1 (v/v). Then it was crystallized with LiCl and the precipitate was washed 4 times with acetone and diethyl ether at −20 °C. After this procedure the sparely soluble LPP-Li salt was resuspended in water, and the Li^+ exchanged by Na^+ in Chellex column with distilled water elution. LPP Na was stored at 4 °C in NaOH at pH 10 or as a dried powder at −20 °C. 60 MHz N.m.r. shows the signals expected for LPP.[26] The enzymic hydrolysis with *E. Coli* alkaline phosphatase and apyrase yielded only linalool as product, as assayed by g.l.c. The linalool: phosphorus ratio was 2.05. Unlabelled primary substrates were prepared in a similar fashion.

[1-^{3}H]-LPP and [1-^{3}H]-LP hydrolysis was performed in glass-stoppered tubes at 30 and 40 °C respectively in a total volume of 1 ml of 0.1M TES-HCl buffer pH 7.0. Incubations were carried out in the presence of variable concentrations of $MnSO_4$ or $MgSO_4$. Unless otherwise stated, [^{3}H]-LPP and [^{3}H]-LP concentrations were 15×10^{-6}M or 38×10^{-6}M respectively.

The reaction was stopped by cooling the tubes to O °C and vigorous shaking with light petroleum (b.p. 40—60 °C; 2 ml). This procedure extracts more than 99% of hydrocarbons and alcohols.[1] Radioactivity of this hexane phase was measured by conventional β-scintillation spectrometry and the products were analysed by radio-g.l.c.[8]

Determination of Rate and Dissociation Constants.—(a) *Potentiometric measurements.* The pK_a of allylic phosphates and the $K_{diss.}$ of the monometallic complexes RPPHM, $RPPM^-$, $RPHM^+$, RPM were determined by potentiometric titration at 25 °C in 0.1M KCl and under nitrogen. The amount of substrate hydrolysed was controlled and in the range 3—10% at the end of the experiment. $K_{diss.}$ Of the metallic complexes were calculated from pH differences in the titration curves of LPP obtained in the absence and in the presence of equimolar concentrations of metal. A rise of pH above 7.0 in the presence of Mn^{2+} must be avoided, since hydroxocomplexes interfere with the titration.[27]

An apparent $K_{diss.}(K_1)$ for pH 7.0 was calculated with $K_{diss.}$ values (K_{RPPM}, K_{RPPHM}) obtained for the different ionic species of the complexes, according to equations (1) and (2).

$$K_1 = \frac{([RPPH^{2-}] + [RPP^{3-}])([M^{2+}])}{[RPPHM] + [RPPM^-]} \quad (1)$$

$$K_1 = \frac{[H^+]/K_a + 1}{K_{RPPM} + \frac{K_{PPHM}[H^+]}{K_a}} \quad (2)$$

Dissociation constants were compared in some cases with those obtained by hydroxyquinoline titration.[28]

(b) *Kinetic determinations.*—The observed rate constants k_ψ are expressed as $k_\psi = k_0\ [LPP] + k_1\ [LPPM] + k_2\ [LPPM_2]$. The rate constant k_1 was determined at metal concentrations where all substrate was complexed as the monometallic species.

The kinetic rate constant k_2 for the bismetallic complexes $LPPM_2$ and the dissociation constant K_2 were obtained by computer simulation of the rate data according to equation (3)[24] which may be linearized to equation (4).

$$k_\psi = \frac{k_1K_2 + k_2[M^{2+}]}{K_2 + [M^{2+}]} \quad (3)$$

$$(k_\psi - k_1) = \frac{(k_2 - k_1)\cdot[M^{2+}]}{K_2 + [M^{2+}]} \quad (4)$$

The contribution of free LPP to the observed rate can be neglected due to the low value of K_1, which makes free LPP negligible even at low metal concentrations.

Enzymic Assay.—LPP carbocyclase was assayed in 0.1M TES (pH 7.0) at 30 °C, in the presence of variable concentrations of [1-^{3}H]-LPP and of Mn^{2+} or Mg^{2+}. Incubations were carried out for 3 to 5 min to avoid non-enzymic solvolysis of the substrate, in the presence of 0.1 units of partially purified carbocyclase, obtained from the flavedo of *Citrus limonum*.[23] One unit is defined as the amount of enzyme which forms 1 nmol of terpene hydrocarbons per minute at saturating concentrations of substrate.

The reaction was stopped by cooling the tubes to 0 °C and the aqueous phase was extracted with hexane. Radioactive hydrocarbons were determined in this fraction by scintillation spectrometry after adsorption of the radioactive alcohols on silicic acid.[1]

All enzymic initial rates were determined under conditions in which less than 5% of the substrate had been transformed to total products (LOH, LP, and hydrocarbons). This minimizes substrate drainage by phosphatase.

Results and Discussion

Ionization Constants.—The pK_a value of $LPPH^{2-}$ as determined by potentiometric titration in 0.1M KCl was 6.75. It extrapolated to 6.85 at zero ionic strength. As reported for adenosine phosphate esters,[29] the pK_a of $LPPH^{2-}$ levels off to 6.2 at 0.6M ionic strength. The pK_a for the primary diphosphates NPP and GPP were also determined and compared with the values for other prenyl phosphates (Table 1). The results show that the organic moiety has little influence on the ionization of the uncomplexed phosphate and diphosphate groups. Metal binding increases the acidity of the last ionizable proton and also introduces marked differences in the pK_a values of the complexes of isomeric diphosphates. Mn^{2+} has a greater affect than Mg^{2+}.

Dissociation Constants.—Scheme 2 represents the binding of bivalent cations to differently protonated ligand species. The

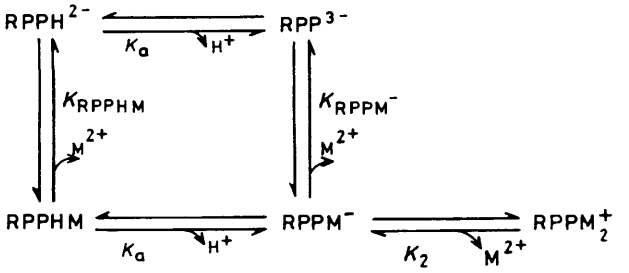
Table 1. Acid dissociation constants (25 °C, 0.1M KCl) of $RPPH^{2-}$: $RPPH^{2-} \rightleftharpoons RPP^{3-} + H^+$ and RPPHM: $RPPHM \rightleftharpoons RPPM^- + H^+$

	pK_a		
		Mg^{2+}	Mn^{2+}
LPP	6.75	5.01	4.7
GPP	6.55	5.8	4.4
NPP	6.35	4.6	3.6
LP	6.75		5.7
IP[a]	6.8		
DMAP[a]	6.5		

[a] From B. K. Tidd, *J. Chem. Soc. B*, 1971, 1168.

Table 2. Dissociation constants for RPPHM, $RPPM^-$ and RPM

$RPPM^{2+}$	$K_{RPPHM} \times 10^5$/M	$K_{RPPM^-} \times 10^5$/M
$LPPMg^{2+}$	470	8.5
$LPPMn^{2+}$	370	3.3
$GPPMg^{2+}$	2 000	36
$GPPMn^{2+}$	570	4.1
$NPPMg^{2+}$	910	17.5
$NPPMn^{2+}$	1 110	1.9
$LPMn^{2+}$	9 100	910



Scheme 2.

values of the dissociation constants of RPPHM to the dianion $RPP\text{-}H^{2-}$ and $RPPM^-$ to the trianion RPP^{3-} are presented in Table 2. As would be expected, binding of metals by the unprotonated ligands is 50 to 500 times stronger than that of the protonated species. This is within the range reported for ADP complexes.[27]

The binding of Mn^{2+} to LP is 27 times weaker than to LPP. There was no evidence for the formation of an LPMg complex within the sensitivity of the potentiometric or hydroxyquinoline methods.

In order to compare the dissociation constants K_2 of the bismetallic complexes obtained kinetically at pH 7.0 with the dissociation constants of the monometallic complexes, it was necessary to correct the pH independent values of Table 2. This was done *via* equation (2) (Experimental procedures) and the values thus obtained for pH 7.0 are shown in Table 3. From the pK_a values (Table 1) it may be estimated that at this pH *ca.* 36% of the ligand exists in the form of the dianion $RPPH^{2-}$, which binds only weakly to metal. For this reason, the values of K_1 shown in Table 3 are higher than the dissociation constants of $RPPM^-$ shown in Table 2.

In all cases studied, the dissociation constants of the Mg^{2+} complexes were higher than those of the Mn^{2+} complexes and the bismetallic complexes were much more dissociated than the monometallic complexes.

Uncatalysed Solvolysis of LPP.—[1-^{3}H]-LPP hydrolysed spontaneously at pH 7.0 and 30 °C with a rate constant k_0 =

Table 3. Apparent dissociation constants at pH 7.0

	$K_1 \times 10^5$/M	K_2/M
$LPPMg^{2+}$	13	5
$LPPMn^{2+}$	5.2	0.25
$GPPMg^{2+}$	48[a]	1.6×10^{-2}[b]
$GPPMn^{2+}$	5.5[a]	1.5×10^{-3}[b]
$NPPMg^{2+}$	21[a]	0.2
$NPPMn^{2+}$	2.3	0.9
$LPMn^{2+}$	1 350	

[a] These values for GPP agree with those determined by titration with 8-hydroxyquinoline (ref. 28). [b] From refs. 11 and 24.

Table 4. Rate constants for the hydrolysis of free and complexed allylic phosphate and diphosphate esters. All the kinetic data were obtained at 40 °C and those corresponding to LPP at 30 °C due to the higher reactivity of the latter.

	k_0/s^{-1}	k_1/s^{-1}	k_2/s^{-1}
$LPPMg^{2+}$	1.2×10^{-4}	4.3×10^{-5}	7.7×10^{-4}
$LPPMn^{2+}$	1.2×10^{-4}	1.1×10^{-4}	7.3×10^{-4}
$GPPMg^{2+}$	3.4×10^{-7}[a]	5.5×10^{-7}[a]	2.6×10^{-6}[a]
$GPPMn^{2+}$	3.4×10^{-7}[a]	3.4×10^{-7}[a]	1.5×10^{-5}[a]
$NPPMg^{2+}$	1.0×10^{-6}	1.9×10^{-6}[a]	1×10^{-5}[a]
$NPPMn^{2+}$	1.0×10^{-6}	1.2×10^{-5}[a]	2.1×10^{-4}[a]
$LPMn^{2+}$	6.3×10^{-6}	1.7×10^{-5}	

[a] From refs. 11 and 24

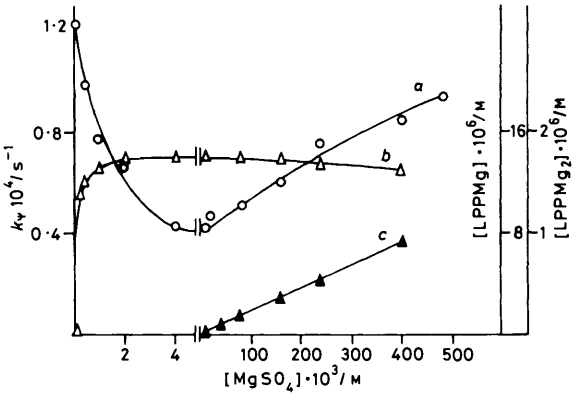


Figure 1. Relationship between the concentration of Mg^{2+} complexes of LPP and the rate of hydrolysis: [LPP] = 14.4×10^{-6}M; 30 °C; *a* rate constant, k_ψ ○—○, *b* [LPPMg] △—△, and *c* [LPPMg$_2$] ▲—▲

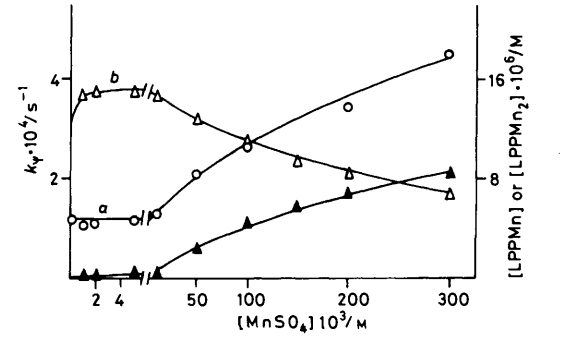


Figure 2. Relationship between the concentration of Mn^{2+} complexes of LPP and the rate of hydrolysis: [LPP] = 15×10^{-6}M; 30 °C; *a* rate constant, k_ψ ○—○, *b* [LPPMn] △—△, and *c* [LPPMn$_2$] ▲—▲

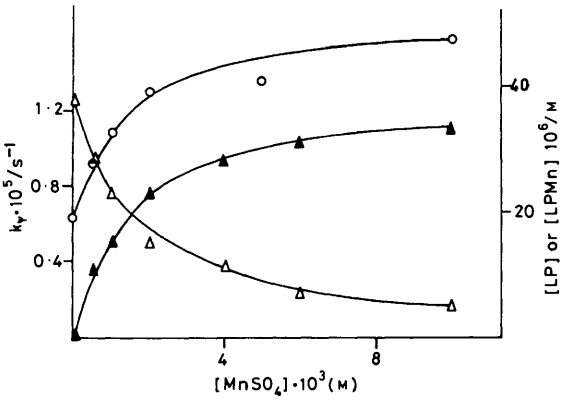


Figure 3. Effect of Mn^{2+} on LP hydrolysis: $[LP]_{TOT} = 38 \times 10^{-6}$M; 40 °C; rate constant, k_ψ ○—○, [LP] △—△, and [LPMn] ▲—▲

1.2×10^{-4} s^{-1} which exceeded the rate constant for its primary isomer GPP by a factor of 500.[11]

Effect of Mg^{2+} *and* Mn^{2+} *on the Solvolysis of LPP.*—The addition of Mg^{2+} or Mn^{2+} modified in different ways the rate of solvolysis of LPP. This is shown in Figures 1 and 2 (left-hand ordinates, curves *a*). The concentration of the mono- and bis-metallic species LPPM and $LPPM_2$ (right-hand ordinates curves *b* and *c*), were calculated from the apparent dissociation constants listed on Table 3. At Mg^{2+} concentrations close to 4 mM, the LPPMg complex is the predominant species (Figure 1, curve *b*). Its solvolysis rate constant $k_1 = 4 \times 10^{-5}$ s^{-1} corresponded to the minimum value of k_ψ of curve *a*. Above 10 mM Mg^{2+} the rate increased with the concentration of the $LPPMg_2$ (curve *c*).

The LPPMn complex hydrolysed at the same rate as free LPP and it was thus not detected kinetically (Figure 2). Changes in Mn^{2+} concentration between 0 and 5 mM had no effect on LP hydrolysis although at the latter concentration all the substrate is present as the monometallic species. Rising concentrations of $LPPMn_2$ was parallelled by an increase in the rate of solvolysis.

Kinetic constants for the solvolysis of free LPP, GPP and NPP and their metallic complexes with Mg^{2+} and Mn^{2+} are summarized in Table 4. The constant for the bismetallic complexes of all three ligands are higher than those for the monometallic complexes, but LPP differs from the primary diphosphates since the rate constants for the monometallic species are lower for LPPM than for the uncomplexed substrate.

Solvolysis of LP.—The effect of metal ions on the hydrolysis of LP was different from their effect on the hydrolysis of LPP. The addition of Mg^{2+} does not alter the rate of reaction of LP. This agrees with the lack of interaction between this ligand and Mg^{2+}. Manganese formed a complex with LP which was 2.7 times more reactive than the free substrate (Figure 3, Table 4). The rate of reaction correlated with the concentration of the complex calculated from K_1. Since this constant was obtained by potentiometric titration at equimolar concentrations of Mn^{2+} and ligand it reflects the dissociation of LPMn. There is no kinetic evidence for the participation of a bismetallic complex of LP, whose formation would be structurally very unlikely.

The results described, complete the picture for the metal-catalysed hydrolysis of allylic phosphate esters. In this reaction the nature of the leaving group has an influence on the rate, in contrast with the acid catalysed process.[21]

Product Distribution.—LPP and LP were transformed into a mixture of alcohols and hydrocarbons at pH 7.0 in both the presence and absence of added Mg^{2+} or Mn^{2+} ions. (Table 5). Under uncatalysed conditions, substitution products exceeded elimination products by a factor of 4, and non-cyclic products predominated. This differs from the acid-catalysed solvolysis of these substrates where alcohols exceed hydrocarbons by a factor of *ca.* 40 (Table 5).

In the uncatalysed process at pH 7.0 the amount of cyclic products almost doubled the amount reported for acid hydrolysis, the Mn^{2+} further increased this proportion (Table 5). The amount of hydrocarbons formed from LPP at pH 7.0 (22.0%) was much higher than the percentage formed from NPP of GPP which were 7 and 6% respectively.[24]

The more stable tertiary ion pair has a longer half-life and thus elimination becomes more significant.

The solvolysis products of LPMn, on the other hand were very similar to those obtained in the absence of metal (Table 5). This points to a greater stabilization of the linalyl cation by the uncharged $PPMn_2$ leaving group as opposed to $H_2P_2O_7^{2-}$ or P-Mn^-. Stabilization of the ion pair due to the different polarity of the leaving group as well as to interactions of metal with double bonds would permit the necessary conformational changes to form cyclic products as well as to prevent water from adding to the cationic moiety of the ion pair.

The results described in this communication and those reported for the metal-catalysed solvolysis of GPP and NPP[11,24] show a dissociation between the effect of metal on rate of reaction and product distribution. The effect of Mn^{2+} observed on the rate of solvolysis of LPP is smaller than for the primary substrates. The ratios of k_2/k_0 are 6.0, 44 and 233 for LPP, GPP and NPP respectively.[11,24] On the other hand, the effect of Mn^{2+} on products is similar for the three substrates, and Mn^{2+} favours the formation of the cyclic hydrocarbon limonene as compared with the acid-catalysed reaction by a factor which ranges from 4 for NPP to 18 for GPP and LPP. This could be explained by an interaction of the C-6 double bond with the metal[30] leading to a stabilization of intermediates and to the formation of cyclic hydrocarbons.

Metal Specificity of LPP Carbocyclase.—Carbocyclases catalyse the formation of limonene and α- and β-pinene from various non-cyclic allylic diphosphates in the presence of bivalent cations.[1,2,8]

Reaction rate was compared with the concentration of LPPM and $LPPM_2$ calculated from K_1 and K_2 at different LPP and metal concentrations. Figures 4 and 5 show that $LPPM_2$ is the complex utilized by carbocyclase, since the observed enzymic reaction rate correlates only with the concentration of this species.

Rate data obtained for variable LPP concentrations at 3 mM Mn^{2+} and rate data shown in Figure 4 are plotted as a function of the calculated bismetallic complex $LPPMn_2$. All the data fit a single hyperbolic curve from which the K_m value was obtained. Free metal and LPPMn concentrations are very different for both sets of data suggesting that under these conditions these species are not utlized as substrates nor bound by carbocyclase.

Table 6 shows that the efficiency of carbocyclase ($V_{max.}/K_m$) was highest for the $LPPMn_2$ complex. $LPPMg_2$ was utilized with a much lower catalytic efficiency.

The differences in enzymic efficiency between $RPPM_2$ complexes and total substrate are very large for NPP and LPP (120/1.3 and 11800/81 respectively). On the other hand, it differed only by a factor of 2 for GPP (Table 6). This may be due to the lower K_2 value for the latter substrate, which is mainly in the form of $GPPMn_2$ in the assay conditions, whereas the more dissociated $NPPM_2$ and $LPPM_2$ are present in a much lower proportion.

It is worth stressing that whereas catalytic efficiency ($V_{max.}/K_m$) for GPP is larger than for NPP when referred to

Table 5. Product distribution in LPP and LP hydrolysis

	LPP				LP		
	pH 7.0				pH 7.0		
	Uncatalysed	Mg^{2+}	Mn^{2+}	pH 0.4[a]	Uncatalysed	Mn^{2+}	pH 0.4[a]
LOH	37.1	44.9	27.8	59.7	44	41	63.4
TOH	38	41.2	49	23.0	28	36	17.7
GOH	1.9		0.6	11.3	2.5	2.4	11.6
NOH	1.8		0.4	3.3	5.7	4.4	3.9
Myrcene	11	13.8	5.7	0.6	9.8	8.8	0.7
Limonene	1.6		6.2	0.34	1		0.6
(*Z*) + (*E*)-Ocimenes	8.6		10.3	1.2	8.7	7.9	1.5
% Alcohols	79	86	77	97.3	80.2	83.8	96.6
% Hydrocarbons	21.3	13.8	22	2.3	19.5	16.7	3.3
% Cyclic products	39.6	41.2	55	23.3	29	36	18.3

[a] From ref. 21.

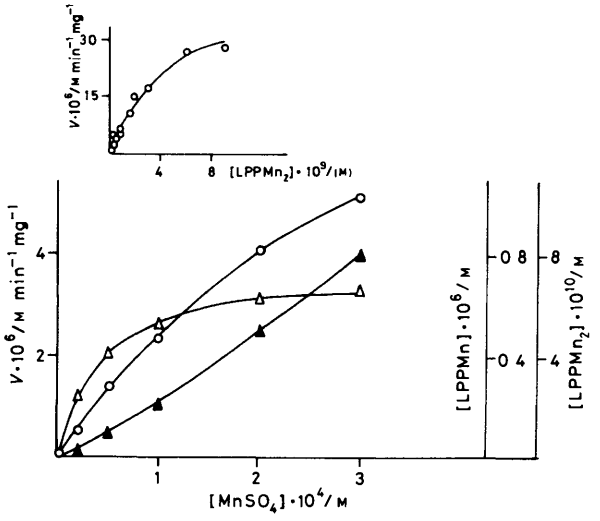


Figure 4. Relationship between the concentration of Mn^{2+} complexes of LPP and the rate of LPP carbocyclase reaction: [LPP] = 0.77 × 10^{-6}M; enzymic reaction rate ○—○; [LPPMn] △—△; [LPPMn$_2$] ▲—▲. Insert: reaction rate as a function of the calculated concentration of the bismetallic complex LPPMn$_2$. Rate data obtained at variable LPP concentrations are included.

Table 6. Kinetic parameters for RPPM$_2$ and RPP$_{TOT}$ in the carbocyclase reaction

	K_m/M	10^6 $V_{max.}$/ M min^{-1} mg^{-1}	$\frac{V_{max.}}{K_m}$ / min^{-1} mg^{-1}
LPPMn$_2$	2.75 × 10^{-9}	32.5	11.800
LPPMg$_2$	4.8 × 10^{-8}	7.6	160
NPPMn$_2$	1.08 × 10^{-8}	1.3	120
GPPMn$_2$	1.4 × 10^{-6}	7.0	5
LPP$_{TOT}$(Mn^{2+})	4 × 10^{-7}	32.5	81
NPP$_{TOT}$(Mn^{2+})	1 × 10^{-6}	1.3	1.3
GPP$_{TOT}$(Mn^{2+})	2.9 × 10^{-6}	7.0	2.4

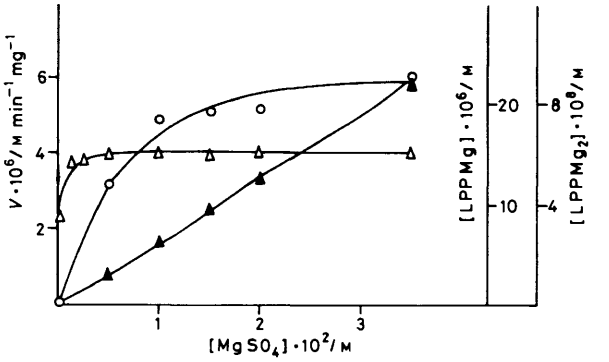


Figure 5. Relationship between the concentration of Mg^{2+} complexes of LPP and the rate of LPP carbocyclase reaction: [LPP] = 15 × 10^{-6}M; enzymic reaction rate ○—○, [LPPMg] △—△, and [LPPMg$_2$] ▲—▲

total substrate, the enzyme is more efficient with the NPPM^{2+} complexes than with their *E* isomers. This may have some relevance as to the physiological role of the two isomers *in vivo*.[24]

It is interesting to point out the extremely low value of K_m of carbocyclase for the LPPMn$_2$ complex (2.75 × 10^{-9}M). This very high affinity may be an explanation for the lack of experimental evidence of its formation as a free intermediate from GPPMn$_2$ to cyclic hydrocarbons.

The data presented show that carbocyclase from *Citrus limonum* utilizes LPP in the form of a bismetallic complex. This stoicheiometry seems to be the rule for the enzymic cyclizations and non-enzymic solvolysis reactions of allylic diphosphates. It may also be calculated that bornylpyrophosphate synthetase and α-pinenocyclase from *Salvia officinalis* utilize the same complex.[2,3] This stoicheiometry probably favours the stabilization of an ionic intermediate with an uncharged $M_2P_2O_7$ leaving group more plausibly than a diphosphate anion. In addition to this higher reactivity of the bismetallic complex, the low K_m of this species points to a tight binding to the enzyme. It could be visualized to occur through adequately positioned electron donor groups like SH or methionine[23] co-ordinating with both metal atoms. These groups of the enzyme could not efficiently bind the free substrates.

An equivalent interpretation might be that LP-Mn, the monometallic complex, is not utilized as a substrate by these enzymes, although it is chemically more reactive than the uncomplexed species.

A study of metal-catalysed solvolysis of allylic phosphates and diphosphates suggests a rationale for an understanding of carbocyclase mechanisms.

Acknowledgements

This work was submitted by M. E. Chiong as partial fulfillment of the requirements for the title of Químico—Farmacéutico, Universidad de Chile.

This work was partially financed by Grants from D.I.B., Universidad de Chile and FONDECYT, Chile. Mr. Victor Calvo, Facultad de Odontología, Universidad de Chile participated in the early phases of this work.

We thank Dr. M. de la Luz Cárdenas (Facultad de Ciencias, Universidad de Chile) and Dr. Luz M. Pérez (Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile) for valuable suggestions in preparing the manuscript.

References

1 L. Chayet, C. Rojas, E. Cardemil, A. M. Jabalquinto, and O. Cori, *Arch. Biochem. Biophys.*, 1977, **180**, 318.
2 R. Croteau and F. Karp, *Arch. Biochem. Biophys.*, 1976, **176**, 734; H. Gambliel and R. Croteau, *J. Biol. Chem.*, 1984, **259**, 740.
3 O. Cori, *Arch. Biochem. Biophys.*, 1969, **135**, 416; R. Croteau and F. Karp, *Arch. Biochem. Biophys.*, 1979, 198, 512. R. Croteau, M. Felton, and R. C. Ronald, *Arch. Biochem. Biophys.*, 1980, **200**, 534.
4 C. George-Nascimento and O. Cori, *Phytochemistry*, 1971, **10**, 1803.
5 J. A. Attaway, A. P. Piereinger, and L. J. Barabos, *Phytochemistry*, 1967, **6**, 25.
6 V. M. Potty and J. H. Bruemmer, *Phytochem.*, 1970, **9**, 1229.
7 D. E. Cane, A. Saito, M. Felton, J. Shaskus, and R. Croteau, *J. Am. Chem. Soc.*, 1982, **104**, 5831.
8 G. Portilla, M. C. Rojas, L. Chayet, and O. Cori, *Arch. Biochem. Biophys.*, 1982, **218**, 614.
9 D. V. Banthorpe, P. N. Christon, C. R. Pink, and D. G. Watson, *Phytochem.*, 1983, **22**, 2465.
10 R. Croteau and F. Karp, *Arch. Biochem. Biophys.*, 1977, **179**, 257.
11 M. V. Vial, C. Rojas, G. Portilla, L. Chayet, L. M. Pérez, and O. Cori, *Tetrahedron*, 1981, **37**, 2351.
12 D. N. Brems and H. Rilling, *J. Am. Chem. Soc.*, 1977, **99**, 8351.
13 D. Banthorpe, D. Long, and C. Pinck, *Phytochem.*, 1983, **22**, 2459.
14 L. M. Pérez, R. Lozada, and O. Cori, *Phytochem.*, 1983, **22**, 431.
15 E. Beytia, P. Valenzuela, and O. Cori, *Arch. Biochem. Biophys.*, 1969, **129**, 346.
16 H. Gambliel and R. Croteau, *J. Biol. Chem.*, 1982, **257**, 2335.
17 C. A. Bunton, J. P. Leresche, and D. Hachey, *Tetrahedron Lett.*, 1972, **24**, 2431.
18 O. Cori, L. Chayet, M. De la Fuente, L. A. Fernández, U. Hashagen, L. M. Pérez, G. Portilla, M. C. Rojas, G. Sánchez, and M. V. Vial, *Mol. Biol. Biochem. Biophys.*, 1980, **32**, 97.
19 C. D. Poulter and C. R. King, *J. Am. Chem. Soc.*, 1982, **104**, 1420; V. Jo, Davisson, R. T. Neal, and C. D. Poulter, *ibid.*, 1985, **107**, 5277.
20 C. D. Poulter and C. R. King, *J. Am. Chem. Soc.*, 1982, **104**, 1422.
21 F. Cramer and W. Rittersdorf, *Tetrahedron*, 1967, 3015.
22 P. Valenzuela and O. Cori, *Tetrahedron Lett.*, 1967, **32**, 3089.
23 M. C. Rojas, L. Chayet, G. Portilla, and O. Cori, *Arch. Biochem. Biophys.*, 1983, **222**, 389; O. Cori, G. Portilla, and L. Chayet, *Arch. Biol. Med. Exp.*, 1982, **15**, 357.
24 L. Chayet, M. C. Rojas, O. Cori, D. McKenzie, and C. A. Bunton, *Bioorg. Chem.*, 1984, **12**, 329.
25 O. Cori and M. C. Rojas, *Methods Enzymol.*, 1984, **110 A**, 406.
26 Bates *et al.*, in 'NMR Data Tables for Organic Compounds,' ed. Frank Bovey, John Wiley and Sons, New York 1967, vol. I., p. 292 and 608.
27 M. H. Taqui Khan and A. E. Martell, *J. Am. Chem. Soc.*, 1962, **84**, 3037; M. H. Taqui Khan and A. E. Martell, *J. Phys. Chem.*, 1962, **66**, 10.
28 K. Burton, *Biochem. J.*, 1959, **71**, 388.
29 R. Phillips, P. George, and R. Tutman, *Biochemistry*, 1963, **2**, 501.
30 R. Benn and A. Rufinske, *Organometallics*, 1985, **4**, 209.

Received 3rd *June* 1986; *Paper* 6/1106