Comparison of Metal-Binding Properties of *trans*-1,2-Cyclohexanediol Diphosphate and Deacylated Phosphoinositides*

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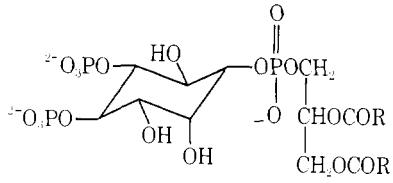
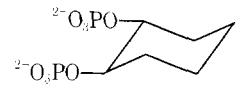
ABSTRACT: Metal-binding properties of *trans*-1,2-cyclohexanediol diphosphate were studied as a stereochemical model for metal binding by triphosphoinositide. Stability constants for the Ni(II), Mn(II), Zn(II), Ca(II), and Mg(II) complexes of *trans*-1,2-cyclohexanediol diphosphate, the Ni(II) complex of 1-(glycerylphosphoryl)-L-*myo*-inositol 4,5-diphosphate, and the Ca(II), Mg(II), and Ni(II) complexes of 1-(glycerylphosphoryl)-L-*myo*-inositol 4-phosphate were determined by pH titration. There was very little difference in the stability constants for metal complexes of *trans*-1,2-cyclohexanediol diphosphate and glycerylphosphorylinositol diphosphate, supporting the hypothesis that binding of the first metal ion by triphosphoinositide involves chelation between the two adjacent phosphate groups on the 4 and 5 positions of the inositol ring. There was no evidence for the binding of more than one metal ion by glycerylphosphorylinositol diphosphate, glycerylphosphorylinositol monophosphate, or *trans*-1,2-cyclohexanediol diphosphate, whereas it has been previously shown that triphosphoinositide micelles can bind more than one metal ion, apparently by electrostatic interactions on the micelle surface. Deacylated diphosphoinositide (glycerylphosphorylinositol monophosphate) binds metal ions less than one-tenth as strongly as does glycerylphosphorylinositol diphosphate. The possible significance of the binding site and its relation to triphosphoinositide–diphosphoinositide interconversion is discussed.

Metal binding by triphosphoinositide (TPI)[1] (structure I) may play an important role in several biological processes



I

including ion transport across membranes (Galliard and Hawthorne, 1963) and the formation and stabilization of the myelin sheath (Eichberg and Dawson, 1965; Dawson, 1966). An understanding of the mechanism of such binding would seem to be essential to the understanding of the related processes. Such a mechanism, however, has not been clearly established. The stability constants for complexes of various divalent metal ions with TPI and its deacylated analog, glycerylphosphorylinositol diphosphate, were determined by Hendrickson and Fullington (1965). They suggested that the most likely site of binding might be between the phosphate monesters at the 4 and 5 positions of the inositol ring. In order to test this hypothesis, *trans*-1,2-cyclohexanediol diphosphate (structure II) was prepared as a stereochemical model for the



II

inositol moiety of TPI. The metal-binding properties of CDP were studied and compared with those of GIP_3.

Since TPI is rapidly interconverted with diphosphoinositide (DPI) in nervous and other tissues (Galliard and Hawthorne, 1963; Thompson and Dawson, 1964; Dawson, 1966), it was of interest to determine the effect of this interconversion upon metal complexing by these phospholipids. The metal-binding properties of 1-(glycerylphosphoryl)-L-*myo*-inositol 4-phosphate (GIP_2) were thus determined and compared with those of GIP_3. The metal ions employed in these studies were Ca(II), Mg(II), Mn(II), Ni(II), and Zn(II).

Experimental Procedure

Analytical Methods. Phosphorous was determined by the method of Bartlett (1959). Phosphates were identified by paper chromatography (isopropyl alcohol–ammonia–water, 5:4:1) using the spray reagent of Hanes and Isherwood (1949).

trans-1,2-Cyclohexanediol Diphosphate. The procedure described by Brown and Usher (1965) was used for the synthesis of this ligand. The product gave a single spot upon paper chromatography and gave a nuclear magnetic resonance spectrum consistent with the structure. The nuclear magnetic resonance spectrum (D_2O) showed the following signals: an unresolved broad peak at τ 7.5–8.0 of relative area four, assigned to the eight CH_2 protons, and a peak at τ 5.4 (m) of relative area one, assigned to the two OCH protons.

Anal. Calcd for $C_6H_{10}O_8P_2Li_4 \cdot H_2O$: P, 19.5%. Found:

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[1] Abbreviations used are: TPI, triphosphoinositide; DPI, diphosphoinositide; CDP, *trans*-1,2-cyclohexanediol diphosphate; GIP_3, glycerylphosphorylinositol diphosphate; GIP_2, glycerylphosphorylinositol monophosphate.

TABLE I: Equilibrium Stability Constants.[a]

Ligand	Cation (Metal:Ligand)	Log K_{ML} (M^{-1})	Log K_{MHL} (M^{-1})
CDP	H^+	8.71 ± 0.05[b]	6.52 ± 0.05[b]
	Ca(II) (5:1)	3.74 ± 0.1	2.15 ± 0.1
	Ca(II) (10:1)	3.87	2.15
	Mg(II) (1:1)	3.76	2.34
	Mg(II) (5:1)	3.72	2.28
	Mg(II) (10:1)	3.76	2.21
	Mn(II) (1:1)	5.04	3.02
	Mn(II) (2:1)	4.89	2.76
	Ni(II) (1:1)	4.72	2.42
	Ni(II) (2:1)	4.75	2.45
	Zn(II) (1:1)	6.35	3.11
	Zn(II) (2:1)	6.31	3.08
GIP_3	H^+	8.05[b]	5.70[b]
	Ni(II) (1:1)	4.45	2.86
	Ni(II) (2:1)	4.39	2.64
GIP_2	H^+	5.99[b]	
	Ca(II) (5:1)	2.04	
	Ca(II) (10:1)	2.10	
	Mg(II) (5:1)	2.28	
	Mg(II) (10:1)	2.10	
	Ni(II) (1:1)	2.45	
	Ni(II) (2:1)	2.55	

[a] T = 20.0°, μ = 0.1 M tetrapropylammonium iodide. [b] These values are acid association constants (log K_{11} and log K_{12}).

P, 19.1%. Molecular weight (titration): Calcd: 318. Found: 322.

Glycerylphosphorylinositol Monophosphate and Diphosphate. The deacylated lipids, GIP_3 and GIP_2, were obtained from the intact lipids according to Hendrickson and Ballou (1964). Paper chromatography showed only one phosphorous-containing spot for each deacylated product. They were isolated as the lithium salts.

Reagents for pH Titrations. Stock 0.1 M Ca(II), Mg(II), and 0.01 M Mn(II), Zn(II), and Ni(II) solutions were prepared from the reagent grade nitrates and standardized by EDTA titration (Schwarzenbach, 1956). Tetramethylammonium hydroxide (10% in water) and tetrapropylammonium iodide were obtained from Eastman Organic Chemicals.

pH Titrations. Titration curves were obtained with a Radiometer pH titrator using glass and calomel electrodes. Nitrogen gas was bubbled through the solution in the titration vessel to prevent CO_2 absorption and air oxidation. The solution temperature was maintained at 20.0°. The pH meter was calibrated with buffers at pH 4.01, 7.00, and 10.00.

A measured amount of ligand (4.12–9.16 μmoles, from equivalent weight determination) was pipetted into the titration vessel, followed by 4 ml of 0.25 M tetrapropylammonium iodide, a measured amount of metal ion, and 0.4 ml of 0.1 N HCl. The volume was adjusted to 10 ml and the solution was titrated with 0.1 M tetramethylammonium hydroxide. The titration curves were automatically recorded at 0.5 pH unit/min. The pH meter readings were corrected from activities to concentrations using the relationship $(H^+) = a_{H^+}/\gamma_{H^+}$, assuming $\gamma_{H^+} = 0.799$ at 20.0° and 0.1 ionic strength (Kortüm and Bockris, 1951). The titration curves obtained were reproducible ±0.05 pH unit.

Results and Discussion

Stability constants for the CDP, GIP_3, and GIP_2 complexes of various divalent metal ions were determined by pH titration and consideration of the following equilibria.

$$H^+ + H_{J-1}L^{-N+J-1} \rightleftharpoons H_JL^{-N+J}$$

$$K_{1J} = \frac{(H_JL^{-N+J})}{(H^+)(H_{J-1}L^{-N+J-1})} \quad (1)$$

$$M^{2+} + H_JL^{-N+J} \rightleftharpoons MH_JL^{-N+2+J}$$

$$K_{MHJL} = \frac{(MH_JL^{-N+2+J})}{(M^{2+})(H_JL^{-N+J})} \quad (2)$$

$$H_2O \rightleftharpoons H^+ - OH^- \quad (3)$$

Acid association constants (K_{1J}) and metal stability constants (K_{MH_JL}) were then derived from the pH titration data by a computer method (Hendrickson, 1968). Formation of 1:1 complexes were assumed in these calculations. Theoretical and experimental titration curves are shown in Figure 1. Stability constants for metal complexes of CDP, GIP_2, and GIP_3 are listed in Table I.

The complexing agents CDP, GIP_3, and GIP_2 appear to form 1:1 complexes with all the metals studied except Mn(II), as indicated by the agreement within experimental error between stability constants calculated at different metal:ligand ratios. In the case of Mn(II), formation of a 1:2 metal–ligand complex with CDP at low concentrations of metal ions is indicated by the higher apparent stability constants at the lower metal ion concentration. The acid association constants determined for CDP agree with those determined by Usher (1966) (log K_{11} = 9.38, log K_{12} = 7.04, $\mu = 3 \times 10^{-3}$ M), when correction for the difference in ionic strength is made. As shown in Figure 1, there is some deviation of the experimental points from the theoretical curves near the second equivalence point. This deviation probably reflects some metal hydrolysis at high pH.

There is no evidence for the binding of more than one metal ion to CDP, GIP_3, or GIP_2 since the stability constants calculated for complexes, even with such strongly bound metal ions as Ni(II) or Zn(II), are the same within experimental error at high and low metal ion:ligand ratios. This is in contrast to intact TPI micelles, which can bind more than one divalent metal ion (Hendrickson and Fullington, 1965).

The comparison between CDP and GIP_3 shown in Table II indicates very little difference in metal complexing ability for these two ligands. The slight differences observed between the acid association constants and the metal stability constants can be ascribed to the electron-withdrawing effect of the hydroxyl groups on the inositol ring of GIP_3. This electron withdrawal would tend to reduce the electron-donating ability of the phosphate groups, especially in the weaker second ionization (K_{11}) of those groups. This effect on the

TABLE II: Comparison of Stability Constants.

	CDP		GIP_3		GIP_2
Cation	Log K_{ML}	Log K_{MHL}	Log K_{ML}	Log K_{MHL}	Log K_{ML}
Ca(II)	3.74	2.15	3.27[a]	2.22[a]	2.04
Mg(II)	3.72	2.28	3.45[a]	2.37[a]	2.29
Ni(II)	4.72	2.42	4.45	2.86	2.45

[a] Hendrickson and Fullington (1965).

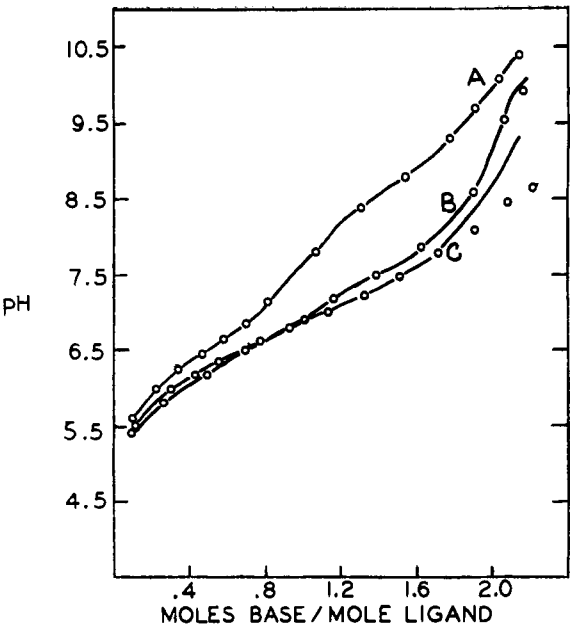

FIGURE 1: Titration curves for *trans*-1,2-cyclohexanediol diphosphate. (A) Free ligand, (B) Mg(II) (5:1), (C) Ni(II) (1:1). The solid lines represent theoretical curves and the open circles experimental points.

second ionization is reflected in the greater difference between K_{ML} for the two compounds than between K_{MHL}. Hydrogen bonding of the hydroxyl protons to the adjacent phosphate oxygens on the inositol ring could also account for some of the difference in the acid association constants for CDP and GIP_3.

Stability constants, K_{ML}, for metal complexes of GIP_2 are very similar to the constants, K_{MHL}, for the corresponding monoprotonated complexes of GIP_3 and CDP. In both cases this represents binding with essentially only one phosphate group, since the monoprotonated phosphate group in the complexes $MH(GIP_3)$ or MH(CDP) would be too weakly basic to act effectively as an electron donor to the metal ion.

There are several possible metal ion binding sites on GIP_3 which should be considered. The most likely type of binding would be chelation between the two monoesterified phosphate groups on the 4 and 5 positions of the inositol ring. Less likely sites would include (1) binding at the phosphate diester group, either monodentate or bidentate between the phosphate and an adjacent hydroxyl group; (2) binding at only one phosphate monoester group, again either monodentate or bidentate between the phosphate and an adjacent hydroxyl group; and (3) tridentate binding between the two phosphate monoester groups and an adjacent hydroxyl group. Binding at the phosphate diester site would not be likely due to the more acidic nature of the phosphate diester oxygen as compared with the more basic phosphate monoester oxygens. Binding sites of type 1 and 2 are present in both GIP_3 and GIP_2. Since GIP_2 binds metal ions less than one-tenth as strongly as does GIP_3, these binding sites are clearly not sufficient to account for the strength of binding found by GIP_3. A binding site of type 3 would also be unlikely since an additional chelate ring would make this type of chelate significantly more stable than the CDP metal chelate. The close similarity of stability constants for CDP and GIP_3 and the differences between these constants and those for GIP_2 strongly support the hypothesis that metal binding by GIP_3 involves chelation between the two phosphate monoester groups.

Metal complexing by CDP and GIP_3 can be related to binding by TPI by taking into account the electrostatic free energy factors involved in binding to the micellar structures (Hendrickson and Fullington, 1965). The comparison between CDP and TPI binding, however, must be restricted to consideration of the binding of the first metal ion by TPI, as micellar TPI has the capacity to bind more than one metal ion. The binding of additional metal ions, however, appears to involve electrostatic interactions due to the micellar structure, and is not characteristic of the TPI molecule alone. Since TPI and GIP_3 correlate closely in metal-complexing ability when the appropriate free-energy corrections are made (Hendrickson and Fullington, 1965), and CDP and GIP_3 also form similar complexes, it seems evident that metal binding by TPI involves chelation between the two adjacent phosphate groups on the 4 and 5 positions of the inositol ring.

The difference in metal-binding abilities between GIP_2 and GIP_3 (and correspondingly between DPI and TPI) provides a possible explanation for the rapid TPI–DPI interconversion observed in myelin and other tissues. Since GIP_3 binds divalent metal ions at least ten times more strongly than does GIP_2, such TPI–DPI interconversion would be expected to effect an alternate release and binding of metal ions. The implications of this for biological processes are widespread.

If the phospholipid were part of a membrane, the binding and release of Mg(II) or Ca(II) ions could alter the charge on the membrane and thus control or enhance transport of other ions such as K(I) and Na(I) across that membrane. This suggested role would be especially important in myelinated nerves at the nodes of Ranvier where very rapid ion transport occurs, and would explain the importance of TPI in myelinated nerve function as demonstrated by Eichberg and Dawson (1965). Since the nodes of Ranvier are in a sense separate from the structural myelin itself, the suggested role is consistent with Dawson's conclusion that TPI is not a structural component of myelin (Dawson, 1966). Since TPI–DPI interconversions would also effectively control the amounts of free divalent ions such as Ca(II), this interconversion could also function in muscle contraction–relaxation mechanisms in which the concentration of free Ca(II) plays an important role (Ebashi, 1958; Woolley and Campbell, 1962). Studies are presently being carried out to determine the effects of TPI–DPI

interconversion on ion permeability in artificial membrane systems.

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Use of Venom Exonuclease at Low pH for Preparation of Mononucleoside Diphosphates*

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ABSTRACT: A general method for the preparation of three classes of nucleoside diphosphates is described. In each case all four nucleoside diphosphates are obtained in good yield. The method is based on hydrolytic degradation of nucleic acids: first with micrococcal nuclease under conditions assuring high yield of dNpNp (or NpNp), then with venom exonuclease, used in high doses at low pH to degrade the dinucleotides to dN + dpNp (or N + pNp). To obtain pN>p ribonucleic acid is hydrolyzed with alkali to the stage of maximal formation of cyclic termini, then treated with venom exonuclease (low dose, high pH). The presently available preparation of venom exonuclease, originating from the North American species *Crotalus adamanteus*, was found satisfactory for this purpose. With d- and rpNp, where a large amount of exonuclease is required, the success of the method depends upon the use of low pH. At low pH the susceptibility of the substrate to exonuclease is increased and the effect of contaminating monophosphatases is decreased. Because the substrates used to prepare pN>p are susceptible, the required amount of exonuclease is low, and the effect of contaminating monophosphatase is insignificant.

We have recently found (Richards and Laskowski, 1969) that the well-known resistance to venom exonuclease displayed by deoxyribooligonucleotides bearing a 3′-monophosphoryl group (for a review, see Laskowski, 1967) is caused by a double negative charge on this group at pH 9.0. At lower values of pH the charge is reduced and the substrate becomes more susceptible, although it never becomes as susceptible as the dephosphorylated analog. One consequence of this finding is that only one-fifth the amount of enzyme required to digest such oligonucleotides at pH 9 is required at pH 6.0. Obviously, the level of contaminating phosphatase that can be tolerated is also increased by at least the same factor of 5. In reality the number may be higher because at low pH the activity of monophosphates is reduced to about 10% of their maximal activity at pH 9. Under these conditions, the presently available preparation of exonuclease from venom of *Crotalus adamanteus* (Richards *et al.*, 1967) was found to be satisfactory for identification of termini in 3′-phosphoryl oligonucleotides (Richards and Laskowski, 1969).

A second application requiring equally pure enzyme is the preparation of 3′,5′-mononucleoside diphosphates. The principle of the method is simple: DNA is digested with micrococcal nuclease to the stage of highest accumulation of dinucleotides which in turn are digested with venom exonuclease to nucleosides and nucleoside diphosphates. The efficiency of the method depends upon purity of exonuclease.

At the time when *Bothrops atrox* venom was available, an experiment of this type was performed (Sulkowski *et al.*, 1963). The reaction was carried out at pH 9, the scale was small, and only the deoxyribonucleoside diphosphates were prepared.

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