Interaction of γ-Phenylpropyl Triphosphate with Cations

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Abstract: The stability constants for the complexes of Mg^{2+} and Cd^{2+} with γ-phenylpropyl triphosphate are nearly identical with those for the complexes with ATP. Furthermore, Ca^{2+} and Cd^{2+} catalyze the phosphorylation of inorganic phosphate by γ-phenylpropyl triphosphate at rates close to those for the corresponding reactions of ATP. However, the hydrolysis of γ-phenylpropyl triphosphate is less strongly catalyzed by polyvalent cations than is that of ATP; the largest differences occur with Cu^{2+} rather than with those ions (Mg^{2+}, Ca^{2+}) which are essential to enzymic reactions of ATP.

In the previous papers of this series, we outlined the synthesis of γ-phenylpropyl triphosphate[1] and showed that it is hydrolyzed enzymically[2] and nonenzymically[1] at rates very similar to those for ATP. In this paper, we examine the reactions of γ-phenylpropyl triphosphate with cations. The work is divided into three parts: (A) the determination of the binding constants of cations for γ-phenylpropyl triphosphate and for ATP, (B) metal ion promoted hydrolyses of these same triphosphates, and (C) the metal ion catalyzed reaction between the triphosphates and inorganic phosphate, to generate diphosphates and inorganic pyrophosphate. This last reaction, discovered by Lowenstein[3] for ATP, proceeds according to the equation

$$ROPO_2^-—OPO_2^-—OPO_3^{2-} + HPO_4^{2-} \longrightarrow ROPO_2^-—OPO_3^{2-} + P_2O_7H^{3-} \quad (1)$$

This reaction might be considered to be an analog of the transphosphorylation catalyzed by myokinase.

$$ATP + AMP \longrightarrow 2ADP$$

The objective of these studies is to compare the reactions of the coenzyme ATP with those of its analog, γ-phenylpropyl triphosphate, and so delineate the role of the adenosine moiety in the enzymic and nonenzymic reactions of ATP.

Experimental Section

Materials. γ-Phenylpropyl diphosphate (PPDP) and triphosphate (PPTP) were synthesized by the methods previously outlined.[1,4] Other chemicals were reagent grade. Solutions of tetrapropylammonium hydroxide were prepared by passing Eastman's tetrapropylammonium bromide through Dowex-1 in the hydroxide form. Eastman's reagent grade 8-hydroxyquinoline was recrystallized from water and then from ligroine.

Other Materials. Tris, 2-amino-2-(hydroxymethyl)-1,2-propanediol, was purchased from Sigma Chemical Co. The ATP, disodium salt, was also obtained from Sigma. The cadmium metal used to prepare the amalgam was a highly pure material obtained from Professor J. J. Lingane. The other chemicals were common reagent grade products.

Dissociation Constants. Dissociation constants were determined by potentiometric titration with a Radiometer pH meter (Model 4B) with a combined glass electrode and calomel electrode joined by an asbestos fiber salt bridge. Carbonate-free tetrapropylammonium hydroxide was stored under nitrogen, and the titrations were performed under nitrogen with magnetic stirring, using calibrated microburets. The third dissociation constants of γ-phenylpropyl triphosphate and ATP were measured at 25° in the presence of enough tetrapropylammonium bromide to raise the ionic strength to 0.10; the pK values were found to be 7.17 ± 0.03 and 7.04 ± 0.03, respectively.

Stability Constants. Stability constants were determined by two methods: (a) spectrophotometric and (b) electrochemical.

(a) The spectrophotometric method of Burton[5] was applied to magnesium complexes using 8-hydroxyquinoline (oxine) in N-ethylmorpholine buffers.[6] The pH was maintained at 7.93 at 25° (*ca.* 7.81 at 30°) and the ionic strength at 0.1 with tetrapropylammonium bromide.

Measurements were performed by placing 5 ml of a solution containing 8×10^{-5} M oxine, 5 to 10×10^{-4} M γ-phenylpropyl triphosphate, and 0.06–0.08 M N-ethylmorpholine buffer in each of two 5-cm quartz cells thermostated at 30° in a Zeiss PMQ III spectrophotometer. Successive 20-μl portions of 0.0125 M magnesium sulfate were added to the working cell, while corresponding 20-μl portions of water were added to the reference cell. About 15 additions were made for each determination of a stability constant. The calculations for the determination of the stability constants require a knowledge of the molar extinction coefficient and stability constant for the Mg^{2+}–oxine complex. A value of 2320 ± 10 at 355 mμ was determined from a solution containing 0.02 M $MgSO_4$ in a borate buffer at pH 9.03. Published values[7] of the stability constant show that oxine is bound essentially completely under these conditions. In our investigation the stability constant, 250 M^{-1}, for the Mg^{2+}–oxine complex at pH 7.81 was determined from the absorbance of the complex as a function of the Mg^{2+} concentration. With this constant, absorbance readings at various concentrations could be corrected; the value of 2320 for the extinction coefficient was confirmed.

As a first approximation, the stability constant for a Mg^{2+}–triphosphate complex can be obtained from the optical density of solutions of known composition. The constant K_1 = (Mg–oxine)/(Mg^{2+})(oxine) is known, the total Mg^{2+}, the total oxine, and the total triphosphate concentrations are known from the compositions of the solutions, and the concentration of the Mg–oxine complex is measured spectrophotometrically. These suffice to determine the concentrations of Mg^{2+}, free oxine, free triphosphate, and Mg–triphosphate complex. The constant K_2 = (Mg–triphosphate)/(Mg^{2+})(free triphosphate) can then be calculated.

The value of K_2 so obtained is however slightly inaccurate because of the formation of ternary complexes, such as the ATP–Mg–oxine complex that Burton[5] postulated to account for the dependence of K_2 on the concentration of ATP. In order properly to evaluate the stability constant of a complex, the observed values of K_2 must be extrapolated to infinite dilution.

Finally, the value of the extrapolated constant, K_2', must be corrected for the incomplete ionization of the triphosphate. The true constant K_2^0 is that for the fully ionized triphosphate; at pH 7.81, the acid is only 75% ionized. The correct value of K_2^0 is then given by the equation

$$K_2^0 = K_2'(1 + (H^+)/K_a)$$

where $K_a = 10^{-7.17}$, the third ionization constant of γ-phenylpropyltriphosphoric acid.

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(b) The spectrophotometric method failed for Cd^{2+}, because this ion forms an insoluble complex with oxine; other complications cause failure with bipyridyl.[8] A successful electrochemical method was based on a calomel reference electrode[9] and a working cell with cadmium amalgam. The working cell was a thermostated 25 × 150 mm tube, with socket joint for the salt bridge (made from a solution of 4 g of agar, 40 g of KNO_3, and 80 ml of water) which connected the half-cells. The amalgam was stirred with a magnetic stirrer electrically shielded by a grounded brass cup. A polyethylene disk, placed just above the level of the solution, prevented loss of solution and delayed the diffusion of oxygen into the cell. Solutions were introduced into the cell through a capillary tip that passed through the polyethylene disk, as did a small filter stick through which nitrogen was bubbled. A $CdSO_4$ solution was made by dissolving dried reagent in 0.1 *M* tetramethylammonium methanesulfonate solution. The buffer was morpholine–morpholinium methanesulfonate, μ = 0.1, pH 8.00 at 30°. The methanesulfonate anion was chosen because it neither complexes Cd^{2+} nor corrodes the electrodes.

The emf values were measured with a Leeds and Northrup Model K-3 potentiometer combined with an L and N Model 2430 galvanometer and a 6-v storage battery. Within the range of concentration of Cd^{2+} from 0.6 to 7.5 × 10^{-4} *M*, the measured value of $E°$ was 579.5 + 0.2 mv *vs.* the calomel electrode. The data for the calculation of the stability constants were obtained by adding a solution of the triphosphate to the cell, and taking emf measurements following successive additions of $CdSO_4$.

The electrochemical method leads to a determination of the concentration of free Cd^{2+} in the solution. Since the total concentration of complexed and free Cd^{2+} is known, and the total concentrations of triphosphate is known, simple stoichiometry allows a calculation of the concentrations needed to evaluate the approximate complexing constant. In order to obtain the true

$$K_2 = (\text{PPTP–Cd})/(\text{Cd}^{2+})(\text{PPTP})$$

binding constant, however, the value of K_2 must be corrected, first to take account of the incomplete ionization of the polyphosphoric acid, as explained above, and second to take account of the apparent "binding" of alkali metal ions to the triphosphate (or more precisely the large effect of salts on activity coefficients). Alberty[10] has evaluated the binding "constants" for alkali metals with ATP; his values are 38 M^{-1} for lithium ion, and 14 M^{-1} for sodium ion, both at an ionic strength of 0.2 at 25°. Since the corrections introduced by these weak bindings is small, we have assumed that the same constants apply to γ-phenylpropyl triphosphate at an ionic strength of 0.1 at 30°. The constants, corrected to take into account both the incomplete ionization of the acid and the effect of alkali metal ions, are about 25% greater than the values of K_2 originally observed; the algebraic method of correction is straightforward.[8]

Hydrolyses. The cupric ion catalyzed hydrolyses of ATP and of γ-phenylpropyl triphosphate were studied at 59.7° in 0.1 *M* acetate buffer (pH of 5.3 at 25°). At timed intervals, aliquots were withdrawn from the thermostated reaction mixture and quenched by pipetting them into a cold solution of EDTA. The mixtures were analyzed for phosphate by the method of Fiske and Subbarow.[11] The hydrolyses catalyzed by calcium and cadmium ions were studied at 40° in 0.09 *M* Tris buffer, pH 8.6 at 40°, or 0.085 *M* Tris buffer, pH 8.1 at 40°, or 0.03 *M* morpholine–morpholinium methanesulfonate buffer, pH 8.3 at 40°. The rates of hydrolysis were determined by measuring the phosphate concentration, by the Lowry–Lopez method,[12] of aliquots withdrawn from the reaction mixture and frozen until they could be analyzed. The reaction (eq 1) between inorganic phosphate and ATP, to yield inorganic pyrophosphate, is catalyzed by calcium and cadmium ions,[8] but it is so slow that errors in the rate constants amount to less than 10%.

Phosphorylation of Phosphate Ion. The reaction of eq 1 can be demonstrated by paper chromatography, and the extent of reaction followed by a radiochemical method,[3] based on the observation that inorganic phosphate forms a butanol-soluble molybdate complex, whereas pyrophosphate does not. Reactions were conducted in 0.2 ml of 0.1 *M* morpholine buffer containing about 5 μcuries of ^{32}P as inorganic phosphate. At timed intervals, 20-μl aliquots, which contained about 0.5 μcurie of ^{32}P, were pipetted into a 1-ml volumetric flask with 1 ml of 1.7% ammonium molybdate–1 *N* H_2SO_4, and the solution was extracted with ten 1-ml portions of water-saturated isobutyl alcohol. The 1-ml water layer was washed into a scintillation counting tube along with 15 ml of a "polyether" scintillation fluid (1500 ml of dioxane, 250 ml of anisole, 250 ml of diethylene glycol diethyl ether, 8 g of 2,5-diphenyloxazole, and 0.2 g of *p*-bis-2-(5-phenyloxazolyl)benzene (Pilot Chemical Co.). The samples were counted with a Nuclear Chicage liquid scintillation system (720 series), and the results were corrected for variable degrees of quenching by the channels-ratio method.[13]

The formation of pyrophosphate was confirmed by an isotope dilution method. A mixture of 0.025 *M* γ-phenylpropyl triphosphate, 0.021 *M* $CdSO_4$, and 5 μcuries of ^{32}P as inorganic phosphate was allowed to react in a pH 8.2 buffer for 24 hr at 40°. An aliquot of the solution was treated with acid molybdate and extracted with isobutyl alcohol to remove labeled inorganic phosphate. The excess molybdate was removed by adding a little phosphoric acid and reextracting with isobutyl alcohol. A weighed sample of tetrasodium pyrophosphate was dissolved with heating in a measured volume of the remaining solution, and then the salt was allowed to crystallize from the solution. The tetrasodium pyrophosphate was recrystallized three times from 4-ml portions of water. Part was counted, and part was hydrolyzed and analyzed for inorganic phosphate. The results showed that at least 90% of the radioactivity that was incorporated into nonextractable compounds was present as inorganic pyrophosphate.

Another aliquot of the same reaction mixture was chromatographed on paper with Ebel's[14] acidic solvent. The ratio of the radioactivity in the pyrophosphate spot to the activity not extractable as phosphomolybdate into isobutyl alcohol was 1.02. Some of the data for product analysis are shown in Table I.

Table I. Product Analysis. Reaction of γ-Phenylpropyl Triphosphate with HPO_4^{2-} Catalyzed by Cd^{2+} (0.021 *M* $CdSO_4$, 0.025 *M* PPTP, 5 μcuries of $H^{32}PO_4^{2-}$, 24 hr)

Samples treated by	Counts/min — Sample	Counts/min — Blank	^{32}P in $P_2O_7^{4-}$, %
Extraction	14,271	...	4.7
Paper chromatography			
$P_2O_7^{4-}$ region	8,814	728	4.8
PPDP and PPTP region	251	115	

Results

A. Complexing Constants. The values determined for the stability constants of the complexes of γ-phenylpropyl triphosphate with magnesium ion and for the complexes of both γ-phenylpropyl triphosphate and of ATP with cadmium ion are recorded in Tables II and III. The various corrections, outlined in the Experimental Section (to take into account both incomplete ionization of the triphosphate and the in-

Table II. Stability Constants for γ-Phenylpropyl Triphosphate–Mg^{2+} at 30°, μ = 0.1

PPTP × 10^4, *M*	K_2 × 10^{-4} M^{-1}	K_2' × 10^{-4}, M^{-1}	K_2^0 × 10^{-4}, M^{-1}
10	4.0		
5.0	5.9		
5.0	5.2	7.0 ± 0.9	8.0 ± 0.9

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Table III. Stability Constants of ATP–Cd^{2+} and of γ-Phenylpropyl Triphosphate–Cd^{2+} at 30°, $\mu = 0.1$

Compd	Concn × 10^4, M	$K_2 \times 10^{-5}$, M^{-1}	$K_2^0 \times 10^{-5}$, M^{-1}	Ratio of complex to (free anion)
ATP	9.0	1.42 ± 0.02	1.57 ± 0.04	0.1–10[a]
ATP	17.0	1.55	1.78	0.1–1.5[a]
PPTP	9.0	1.21	1.55	0.1–4[a]
PPTP	9.0	1.14 ± 0.04	1.47	0.1–2[a]
PPTP	17.0	1.20	1.65	0.1–2[a]

[a] Range over which measurements were performed.

fluence of alkali metal ions on the complexing), have been incorporated into the values of K_2^0.

The corresponding value of $K_2^0 \times 10^{-4}$ for ATP–Mg^{2+}, determined by the same method,[6] is 7.3 M^{-1}. Widely divergent values, however, which differ by a factor of almost 50, have been reported for determinations by other methods; these prior values have previously been summarized and discussed.[6]

B. Metal Ion Catalyzed Hydrolysis of ATP and of γ-Phenylpropyl Triphosphate. The rate constants for the hydrolyses of PPTP at 40°, catalyzed by cadmium ion, are recorded in Table IV, and the corresponding data for catalysis by calcium ion in Table V. The rate constants for the hydrolyses of PPTP at 60°, catalyzed by cupric ion, are presented in Table VI.

Table IV. Cadmium Ion Catalyzed Hydrolysis of ATP and γ-Phenylpropyl Triphosphate at 40° in 0.09 M Tris Buffers

Compd	Concn, M	Cd/ATP or Cd/PPTP	pH	k_1, $sec^{-1} \times 10^7$
ATP	0.0071	1.25	8.1	15.7
ATP	0.0067	1.0	8.1	12.0
ATP	0.0071	0.75	8.1	4.7
ATP	0.0067	0	8.1	0.9[a]
ATP	0.0071	1.0	8.3	14[b]
PPTP	0.0071	1.25	8.1	3.9[b]
PPTP	0.0067	1.0	8.1	1.5
PPTP	0.0071	0.75	8.1	1.4
PPTP	0.0067	0	8.1	0.2[a]
PPTP	0.0071	1.0	8.3	3.5[b]

[a] Too slow to measure accurately. [b] Precipitate formed during the reaction (0.03 M morpholine buffer).

Table V. Calcium Ion Catalyzed Hydrolysis of ATP and γ-Phenylpropyl Triphosphate at 40° in 0.09 M Tris Buffers

Compd	Concn, M	Ca/ATP or Ca/PPTP	pH	k_1, $sec^{-1} \times 10^7$
ATP	0.0071	1.25	8.1	10.4[a]
ATP	0.0067	1.0	8.6	6.7
ATP	0.0071	0.75	8.1	3.25
PPTP	0.0071	1.25	8.1	3.28
PPTP	0.0067	1.0	8.6	2.5
PPTP	0.0071	0.75	8.1	1.4

[a] Precipitate formed during the reaction.

C. Transfer of Phosphate from PPTP to HPO_4^{2-}. The (approximate) rates of reaction 1 are shown in Tables VII–XI. Table VII shows that at pH values above 8.5 precipitation slows down the reaction markedly after a 5-hr reaction time. For this reason the rate of incorporation of PO_4^{3-} into $P_2O_7^{4-}$ was determined during a 5-hr reaction period. The

Table VI. The Cu^{2+} Catalyzed Hydrolysis of ATP and γ-Phenylpropyl Triphosphate at 59.7° in 0.1 M Acetate Buffer

Compd	Concn, M	Cu^{2+}, M	pH[a]	$k_1 \times 10^6$, sec^{-1}
ATP	0.002	...	5.33	5.6
ATP	0.002	0.002	5.30	19.8
ATP	0.020	0.020	5.05	220
PPTP	0.002	...	5.36	4.6
PPTP	0.002	0.002	5.34	8.2
PPTP	0.020	0.020	5.07	10.0

[a] Determined at 25°.

Table VII. Amount of ^{32}P Incorporated into $H_2P_2O_7^{2-}$ at Various Reaction Times (0.025 M PPTP, 0.021 M $CaCl_2$, 5 μcuries of $H^{32}PO_4^{2-}$, 40°)

pH	^{32}P Incorporated, % 0 hr	5 hr	10 hr
8.18	0.13	1.0	3.9
8.64[a]	0.22	9.2	13.8
8.94[b]	0.35	11.0	13.2

[a] Precipitation of calcium pyrophosphate began after 3 hr. [b] Precipitation began after 1 hr.

Table VIII. Rate of Incorporation of ^{32}P into $H_2P_2O_7^{2-}$ as a Function of pH (0.025 M PPTP, 0.021 M, $CaCl_2$ or $CdSO_4$, 40°)

pH	Metal ion	^{32}P Incorporated/hr,[a] %
8.18	Ca^{2+}	0.38
8.64	Ca^{2+}	1.8
8.94	Ca^{2+}	2.2
9.22	Ca^{2+}	2.1
8.18	Cd^{2+}	0.61
8.64	Cd^{2+}	0.25[b]

[a] Rates calculated from reaction time of 5 hr, except for first entry which was based on a 10-hr determination. [b] Precipitation and hydroxide formation lower the rate as the pH is raised.

Table IX. Rate of Incorporation of ^{32}P into $H_2P_2O_7^{2-}$ as a Function of the Ratio of the Concentrations of Metal Ion and Triphosphate (0.01 M γ-Phenylpropyl Triphosphate, pH 8.2, 40°)

Ratio of $(M^{2+})/(PPTP)$	^{32}P Incorporated/hr, % Cd^{2+} catalysis	Ca^{2+} catalysis
0.83	0.25	0.10–0.13
0.67	0.05–0.06	0.016
0.50	0.01	0.004
0.33	0.001	0.000

Table X. Rate of Incorporation of ^{32}P into $H_2P_2O_7^{2-}$ as a Function of the Concentration of γ-Phenylpropyl Triphosphate (M^{2+}/PPTP = 0.83, pH 8.2, 40°)

PPTP, M	^{32}P Incorporated/hr, % Cd^{2+} catalysis	Ca^{2+} catalysis
0.25	0.61	0.38
0.10	0.25	0.10–0.13
0.0077	0.23	0.06
0.0032	0.10–0.15	0.07

Table XI. Comparison of the Reaction Rates of PPTP and ATP ($(M^{2+})/(PPTP) = (M^{2+})/(ATP) = 5/6$; pH 8.2, 40°)

Compd	Concn, M	^{32}P Incorporated/hr, %: Cd^{2+} catalysis	Ca^{2+} catalysis
ATP	0.025	0.54	0.2–0.3
PPTP	0.025	0.61	0.38
ATP	0.003	0.18	
PPTP	0.003	0.14	

reaction between a triphosphate (either ATP or γ-phenylpropyl triphosphate) and inorganic phosphate is catalyzed by various metal ions. The data here presented for catalysis by Ca^{2+} and Cd^{2+} permit a comparison with the data of Lowenstein for the corresponding catalyzed reaction of ATP.

Discussion

The association constants of the complexes of γ-phenylpropyl triphosphate with magnesium and cadmium ions are almost precisely the same as those for the corresponding complexes with ATP. Therefore adenosine does not effectively stabilize these complexes.

Furthermore, the rates of the phosphorylation of inorganic phosphate by γ-phenylpropyl triphosphate catalyzed by calcium and cadmium ions are approximately equal to those for the corresponding reactions of ATP. In these reactions, therefore, the adenosine residue cannot play an important role. Presumably the metal ion complexes both the triphosphate and the inorganic phosphate group, and brings them together for the reaction shown in eq 1. There is some evidence that two metal ions are required. The details of the reaction are, however, difficult to evaluate, and insufficient quantitative data are available to allow the formulation of a complete mechanism. In particular, the reaction is complicated both by the complexing of the metal ions with the components of the buffer solution and by the precipitation or incipient precipitation of metal ion–phosphate compounds. (In general, precipitation occurs in alkaline solution unless the buffer complexes the excess cation.)

The metal ion catalyzed hydrolyses of ATP are, however, faster than those of γ-phenylpropyl triphosphate. When the hydrolytic reaction is catalyzed by calcium or cadmium ions, the reaction with ATP is faster by factors of 2 to 4; with cupric ion, the hydrolysis of ATP is faster by a factor of 20 or more, depending upon the concentration of Cu^{2+}. An interaction of the adenosine moiety at least with cupric ion is therefore strongly indicated. Again, problems of complexing with the buffer and of precipitation complicate the detailed interpretation of the system. However, since none of the enzyme systems that require ATP are dependent on cupric ion, the Cu^{2+}-catalyzed system, although interesting in its own right, is unrelated to enzymology. The data on the complexing of ATP and γ-phenylpropyl triphosphate with metal ions, and the reactions of the compounds catalyzed by metal ions, reinforce the conclusions of the previous papers of this series[1,2] to the effect that the adenosine residue of ATP, although important for the specificity of enzymic reactions, is not involved either in catalysis or in special binding of cations.

Lowenstein and Tetas[15] discovered that cupric ion is unusually effective in promoting the hydrolysis of ATP, while Schneider and Brintzinger[16] found that copper is relatively ineffective with methyl triphosphate. The work with cupric ion described here is worth noting because, unlike that in the previous report,[16] it was done with a well-characterized triphosphate.

The identity of the reactive species in the metal ion catalyzed hydrolyses is uncertain. Schneider and Brintzinger concluded from their data that the transition state contains a single metal ion, which is bound to the β-phosphoryl oxygen,[16] an idea similar to proposals for the acid-catalyzed hydrolyses of phosphates and pyrophosphates.[4,17–22] Although this type of mechanism appears well established in acid-catalyzed reactions, the data from metal ion catalyzed reactions are equivocal. A complex containing two metal ions per triphosphate may be the reactive species, which would resemble a tetrasubstituted pyrophosphate.

This ambiguity also enshrouds the enzymic hydrolyses, and it prevents the assignment of a role in the reaction to water at this time.

The primary findings of this series of papers are that adenosine is not involved in the common hydrolytic reactions of ATP and that the proton-catalyzed reactions probably proceed, to the extent that any reactions do, by a "monomeric metaphosphate" mechanism.

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