The Hydrolysis of γ-Phenylpropyl Di- and Triphosphates[1]

David L. Miller and F. H. Westheimer

Contribution from the James Bryant Conant Laboratory of Harvard University, Cambridge, Massachusetts. *Received November 12, 1965*

Abstract: The nucleotide analogs, γ-phenylpropyl di- and triphosphates, have been synthesized and characterized, and their rates of hydrolysis have been measured. The rates in buffer solution at 95°, over a pH range from 0.3 to 10, are similar to those of adenosine diphosphate (ADP) and adenosine triphosphate (ATP). However, the rate for monoprotonated γ-phenylpropyl diphosphate is 2000 times as great as that for P,P′-di-γ-phenylpropyl pyrophosphate. Furthermore, the hydrolysis of γ-phenylpropyl diphosphate does not show strong amine catalysis. These facts are interpreted in terms of a "monomeric metaphosphate" mechanism for the hydrolytic reactions.

The objective of the present research is to help elucidate the role of the adenosine residue in the chemical and enzymic reactions of adenosine triphosphate (ATP). The adenosine moiety has been ascribed direct catalytic functions[2] and various auxiliary effects concerned with binding metal ions at the nitrogen atoms of the adenine.[3] In the enzymic reactions, various nucleoside triphosphates sometimes will substitute[4] for ATP, but the requirement for the adenosine group has not been clearly defined.

In order to examine the role of adenine in enzymic and nonenzymic reactions of ATP, γ-phenylpropyl diphosphate (PPDP) and γ-phenylpropyl triphosphate (PPTP) have been synthesized, and their reactions have been studied. The phenylpropyl residue has none of the

$[C_6H_5CH_2CH_2CH_2OPO_2OPO_3]^{-3}$
PPDP

$[C_6H_5CH_2CH_2CH_2OPO_2OPO_2OPO_3]^{-4}$
PPTP

functional groups of adenosine, and so cannot participate (*e.g.*, as an internal nucleophile) in any hydrolytic or phosphorylation reactions; it cannot serve as a group for binding metal ions, although in enzymic systems it might be attracted to a hydrophobic binding site. Recently, Schneider, Brintzinger, and Erlenmeyer have reported the synthesis[5] of methyl triphosphate, and Schneider and Brintzinger have published the rates for its metal ion promoted hydrolyses.[6] That work is discussed in the third paper of this series.[7]

This paper describes the hydrolysis of γ-phenylpropyl di- and triphosphates in neutral and acid solutions. The accompanying papers describe the enzymic hydrolyses of these compounds and the metal promoted hydrolyses of ATP and γ-phenylpropyl triphosphate.

(1) Preliminary communication: D. L. Miller and F. H. Westheimer, *Science*, **148**, 667 (1965).
(2) A. Szent-Györgyi, "Bioenergetics," Academic Press Inc., New York, N. Y., 1957, p 70.
(3) A. E. Martell and G. Schwartzenbach, *Helv. Chim. Acta*, **39**, 653 (1956); M. Morales and K. Hotta, *J. Am. Chem. Soc.*, **83**, 997 (1961).
(4) R. Martenez, *Arch. Biochem. Biophys.*, **93**, 508 (1961); P. D. Boyer in "The Enzymes," Vol. 6, 2nd ed, Academic Press Inc., New York, N. Y., 1962, p 101; C. Liebecq, A. Lallemand, and M. Degueldre-Guillaume, *Arch. Biochem. Biophys.* **97**, 609 (1962); W. Kielley, H. Kalckar, and L. Bradley, *J. Biol. Chem.*, **219**, 95 (1956).
(5) P. W. Schneider, H. Brintzinger, and H. Erlenmeyer, *Helv. Chim. Acta*, **47**, 992 (1964).
(6) P. W. Schneider and H. Brintzinger, *ibid.*, **47**, 1717 (1964).
(7) D. L. Miller and F. H. Westheimer, *J. Am. Chem. Soc.*, **88**, 1511 (1966).

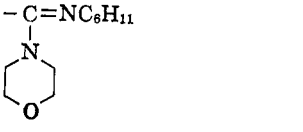
Experimental Section

Materials. γ-Phenylpropyl monophosphate was prepared by Cramer's general method.[8] Crystalline phosphoric acid was prepared by drying Fisher reagent grade 85% acid on a rotary evaporator at 1 mm for 1 hr at 60°. γ-Phenylpropanol was purified by vacuum distillation from Eastman White Label material; triethylamine was purified by refluxing over sodium. A mixture of 0.05 mole of Aldrich trichloroacetonitrile, 0.10 mole of γ-phenylpropanol, 0.02 mole of triethylamine, and 0.01 mole of phosphoric acid was heated for 3 hr at 70°. The mixture was mixed with an equal volume of ether and extracted with four 100-ml portions of water and the water was reextracted with ether. The aqueous solution was reduced to 100 ml on a rotary evaporator, and filtered, and 0.01 mole of barium chloride in water was added at pH 9. After barium phosphate had been removed by filtration, addition of an equal volume of ethanol caused the slow crystallization of the barium salt of γ-phenylpropyl monophosphate, in 60% yield. A slurry of this barium salt and an equivalent quantity of washed Dowex-50 (H^+) was poured onto a column containing 2 to 5 additional equiv of Dowex-50 (H^+) and the γ-phenylpropyl dihydrogen phosphate was washed through the column. It was then titrated to pH 10 with carbonate-free lithium hydroxide. The aqueous solution was evaporated to saturation and clarified by centrifugation, and the salt was precipitated with four volumes of ethanol.

Anal. Calcd for $C_9H_{11}PO_4Li_2$: C, 47.39; H, 4.87; P, 13.59. Found: C, 47.10; H, 4.68; P, 13.90.

A weighed sample of the barium salt was converted to the acid form with Dowex-50 (H^+). The acid gave two end points on electrometric titration, with exactly equal amounts of strong and weak acid, and a molecular weight was determined from the titration of 357 (calcd 352). The ultraviolet spectrum is qualitatively and nearly quantitatively identical with that of γ-phenylpropanol. The principal infrared bands of the lithium salt are 8.9, 9.2, and 9.7 μ. The lithium salt gave a single spot on paper chromatography with a solvent of 1-propanol, water, and concentrated ammonia in ratios 7:1:2 or 7:2:1. The phosphate and other phosphates were detected by spraying the paper with an acid molybdate solution[9] and irradiating the paper with ultraviolet or sunlight.[10] Table I shows the R_f values for the various phosphates and polyphosphates synthesized in this work.

γ-Phenylpropyl Diphosphate. A. Phenylpropyl phosphoromorpholidate was synthesized by the general procedure[11] of Moffatt and Khorana. The acid form of γ-phenylpropyl monophosphate, prepared as shown above from the barium salt, was allowed to react with morpholine, using dicyclohexyl carbodimide (DCC) in refluxing *t*-butyl alcohol–water (4:1) as condensing agent. The substituted guanidine



(8) F. Cramer and G. Weimann, *Chem. Ind.* (London), 46 (1960).
(9) C. S. Hanes and F. Isherwood, *Nature*, **164**, 1107 (1949).
(10) R. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(11) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

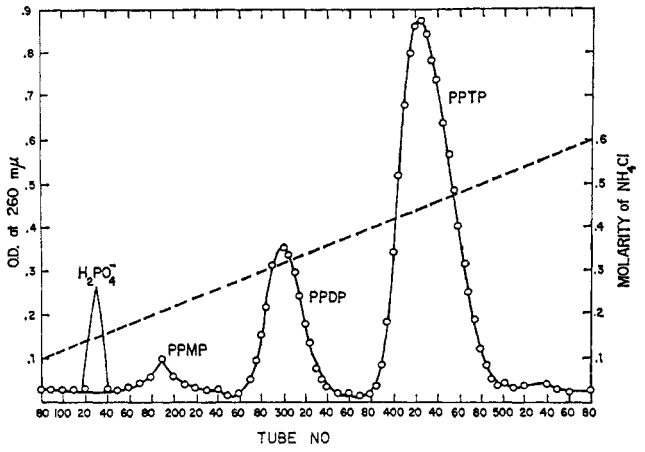


Figure 1. Chromatographic separation of inorganic phosphate, γ-phenylpropyl phosphate, γ-phenylpropyl diphosphate, and γ-phenylpropyl triphosphate: column, 180 ml of Dowex-1 (Cl^-), 36 cm; eluent, linear gradient of ammonium chloride in 0.004 *M* HCl and 30% ethanol; flow rate, 7 ml/min. Each tube contained 13.7 ml.

is formed by the addition of morpholine to DCC, and the phenylpropyl phosphoromorpholidate was isolated as the salt of this substituted guanidine. The crude salt was purified by extraction with ether and recrystallized from dimethoxyethane in large prisms, mp 162–163°.

Anal. Calcd for $C_{30}H_{51}N_4O_5P$: C, 62.26; H, 8.88; N, 9.68; P, 5.36. Found: C, 62.36; H, 8.98; N, 9.53; P, 5.35.

Table I. R_f Values

Compd	Solvent[a] 1	2	3
γ-Phenylpropyl monophosphate	0.40	0.44	0.90
γ-Phenylpropyl phosphoromorpholidate		0.90	
γ-Phenylpropyl diphosphate	0.25	0.36	0.71
γ-Phenylpropyl triphosphate	0.21	0.32	0.54
P,P′-Di-γ-phenylpropyl pyrophosphate	0.80		
PO_4^{3-}	0.05		0.66
$P_2O_7^{4-}$			0.37

[a] Solvent 1, 1-propanol–ammonia–water (7:2:1); solvent 2, 1-propanol–ammonia–water (7:2:2); solvent 3, isopropyl alcohol–water–trichloroacetic acid–ammonia (75 ml/25 ml/5 g/0.2 ml).

The compound gave a single spot on paper chromatography.

B. The morpholidate was allowed to react with the monotri-*n*-butylammonium salt of phosphoric acid in anhydrous pyridine, following the procedure of Khorana and Moffatt for cytidine dephosphate,[11] but with ten times the quantities.

The γ-phenylpropyl diphosphate was successfully purified by column chromatography over Baker's Dowex-1 or -2. These resins had to be boiled with 2 *M* sodium hydroxide, separated from the fines by decantation, washed, boiled with 2 *M* hydrochloric acid in water, and then with 2 *M* hydrochloric acid in 75% ethanol to remove impurities absorbing at 260 mμ. The washed resins were stored in 95% ethanol. For preparative chromatography, about 1 g of the mixed phosphates from the phosphoromorpholidate step was dissolved in a minimum amount of water and placed on a column 40–50 cm long containing 100–200 ml of resin. Best separations were obtained with a column heated to 50°. The phosphates were obtained by gradient elution, using a solvent of either 0.5 *M* sodium chloride or 0.5 *M* ammonium chloride in 0.004 *M* HCl and 40% ethanol as the second component. A Buchler dual-action micropump pumped the salt solution out of the mixing bottle onto the column at twice the rate that it was pumped from the concentrated salt solution into the mixing bottle. The flow rate was about 0.03 ml/min/ml of resin.

The effluent from the column was monitored by measuring its ultraviolet absorption at 260 mμ. The fractions containing γ-phenylpropyl diphosphate in 40% ethanol and salt solution were combined, and the barium salt precipitated at pH 10. This salt was converted to the lithium salt by the same procedure as that already outlined for γ-phenylpropyl monophosphate.

Anal. Calcd for $C_9H_{11}O_7P_2Li_3 \cdot H_2O$: C, 32.56; H, 3.95; P, 18.66; H_2O, 5.42. Found: C, 32.76 ± 0.44; H, 3.78 ± 0.16; P, 19.30; H_2O, 5.22.

(The carbon and hydrogen analyses are the average of four determinations. The range in the analyses is due to different degrees of hydration of the samples.) The compound gave a single spot on paper chromatography, but analysis for inorganic phosphate showed about 1% present as an impurity; perhaps 1% γ-phenylpropyl monophosphate was also present. Titration of the acid (prepared with Dowex-50 (H^+) as in previous cases) showed the ionizable protons in the ratio of 2.002 (strong acid) to 1 (weak acid). The ultraviolet spectrum was again similar to that of γ-phenylpropanol; the major infrared peaks for the lithium salt are at 8.3, 8.5, 8.9, 9.7, and 10.4 μ.

γ-Phenylpropyl Triphosphate. The triphosphate was prepared by allowing γ-phenylpropyl phosphoromorpholidate to react in dimethyl sulfoxide as solvent with the ditri-*n*-butylammonium salt of pyrophosphoric acid, according to the method previously developed by Moffatt and Khorana[11–13] for other triphosphates. The progress of the reaction was followed by paper chromatography, with Ebel's[14] acidic solvent, to estimate the amount of unreacted morpholidate. Dimethyl sulfoxide gives a spurious test for phosphate, and so must be removed by washing the paper with ether.

After the reaction had gone to completion, the solvent was removed by rotary evaporation. The remaining solid was dissolved in a minimal amount of water and chromatographed on Dowex-1 using the same techniques as for the purification of γ-phenylpropyl diphosphate. A typical chromatogram is shown in Figure 1.

γ-Phenylpropyl triphosphate was isolated from the effluent of the column as the barium salt by procedures similar to those for the barium salt of the diphosphate, and was again converted to the acid with Dowex-50 (H^+), Both the sodium and lithium salts were prepared from the acid. The sodium salt is particularly useful because it precipitates from aqueous ethanol, as does the disodium salt of ATP;[15] the precipitation occurs under conditions where no corresponding salt of the diphosphate or inorganic phosphate coprecipitates. The crystalline salt is hygroscopic and readily develops electrostatic charge; after equilibration in a moist atmosphere for 0.5 hr, it was easily handled, and analyzed for the monohydrate. The compound showed only 0.1% inorganic phosphate.

Anal. Calcd for $C_9H_{11}O_{10}P_3Na \cdot H_2O$: C, 24.67; H, 3.45; P, 21.21; mol wt, 438. Found: C, 24.58; H, 3.12; P, 21.51; mol wt, 441. Calcd for $C_9H_{11}O_{10}P_3Li_4 \cdot H_2O$: C, 25.86; H, 3.15; P, 22.23; mol wt, 418. Found: C, 25.44; H, 3.39; P, 21.81; mol wt, 425. Calcd for $C_9H_{11}O_{10}P_3Ba_2 \cdot 2H_2O$: C, 15.83; H, 2.21; P, 13.61; mol wt, 683. Found: C, 15.77; H, 2.30; P, 14.08; mol wt, 686.

Although the salts showed only a single spot on paper chromatography in the Ebel acidic solvent, the lithium salt contained 2–3% inorganic phosphate as impurity. The molecular weights, determined by titration, confirm the degree of hydration of the salts indicated by the analyses. The ratio of strongly acidic to weakly acidic protons, found on titration, was 2.99:1. A sample of the lithium salt was hydrolyzed in 0.1 *N* HCl to determine the amount of "labile" phosphate.[16] It showed 2.00 μmoles/μmole of salt, in agreement with theory. The ultraviolet absorption spectrum is qualitatively and quantitatively similar to that of γ-phenylpropanol. The principal infrared peaks for the barium salt are at 8.0, 8.9, 9.7, 10.1, and 10.8 μ.

The sodium and barium salts showed X-ray powder photographs demonstrating their crystallinity. The lithium salt gave only faint lines, and is presumably amorphous.

P,P′-Di-γ-phenylpropyl pyrophosphate was synthesized from γ-phenylpropyl monophosphate and dicyclohexylcarbodiimide in ether as solvent, following the method of Todd and Khorana.[17]

(12) J. G. Moffatt, *Can. J. Chem.*, **42**, 599 (1964).
(13) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, pp 82–89.
(14) J. P. Ebel, *Bull. Soc. Chim. France*, 991 (1953).
(15) L. Berger, *Biochim. Biophys. Acta*, **20**, 23 (1956).
(16) L. Leloir and C. Cardini in "Methods of Enzymology," Vol. III, S. Colowick and N. Kaplan, Ed., Academic Press Inc., New York, N. Y., 1957, p 840.
(17) A. Todd and H. G. Khorana, *J. Chem. Soc.*, 2260 (1953).

The crude material was purified by precipitating the barium salt from 0.05 *N* hydrochloric acid. The acid was prepared from the barium salt with Dowex-50 (H^+) as previously described for other salts, and the sodium salt was prepared from the acid by titration. After the aqueous solution had been evaporated to dryness, the sodium salt was precipitated from water–ethanol–dimethoxyethane. It showed a single, fast-moving spot on chromatography in Ebel's acidic solvent.

Anal. Calcd for $C_{15}H_{22}O_7P_2Na_2 \cdot H_2O$: C, 45.40; H, 5.07; P, 13.01; mol wt, 476. Found: C, 44.75; H, 5.03; P, 13.17; mol wt, 482.

On titration, the compound showed no weakly acidic protons. Its major infrared peaks are at 8.2, 8.8, 9.7, and 10.6 μ.

Reaction Products. The phosphomolybdate analysis was used to determine inorganic phosphate according to the procedure of Lowry and Lopez,[18] with low concentrations of molybdate.[19] Other products (including pyrophosphate) were determined by paper chromatography, by the method of Usher.[20]

Buffers. Since hydrolyses were carried out at 95 and 99°, the pH values of buffers at these temperatures were needed. The pH values at 95° were determined colorimetrically by comparison with buffers calibrated at this temperature by the National Bureau of Standards.[21] The pH values at 99° were determined by measuring pH values at 25, 60, 70, 80, 90, and 95°, and extrapolating to 99°. The indicators used were Eastman White Label brom thymol blue ($pK \sim 7$), brom cresol purple ($pK \sim 6$), brom cresol green ($pK \sim 4$), and 2,4-dinitrophenol ($pK \sim 3$). The dinitrophenol was recrystallized from water, and the sulfonephthaleins from benzene–ligroin. The buffers used in this work are shown in Table II.

Table II. Buffers Used in the Hydrolysis Study at 95°, Ionic Strength = 0.10

Buffer no.	Compn, *M*	pH at 25°	pH measured at 95°
1	0.12 KOH	12.96	10.23[b]
2	0.10 KOH, H_3BO_3	10.22[a]	9.87[a,c]
3	0.10 KOH, H_3BO_3	9.38[a]	9.03[a,c]
4	0.10 KOH, H_3BO_3	8.75[a]	8.40[a]
5	0.10 HCl, Tris	9.02[a]	7.48[a]
6	0.10 HCl, Tris	8.55[a]	7.01[a]
7	0.10 HCl, Tris	8.02[a]	6.48[a]
8	0.10 HCl, Tris	7.56[a]	6.02[a]
9	0.10 KOH, HOAc	4.81[a]	5.04[a]
10	0.10 KOH, HOAc	3.95[a]	4.18[a]
11	0.05 KOH, phthalic acid	3.30[a]	3.38[a]
12	0.01 HCl, 0.09 KCl	2.10[a]	
13	0.10 HCl	1.10	
14	1.0 HCl		

[a] When diluted to $\mu = 0.05$. [b] Extrapolated from the data of R. Bates, "Electrometric pH Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954, p 87. [c] See ref 21.

Kinetic Method. Samples of phosphate and buffer were sealed in Pyrex tubes (for acidic solution) or were held in brass-jacketed Teflon tubes (for alkaline solutions). The latter were closed with Teflon plugs, and the pressure fit from the brass jacket prevented leakage. Tubes were placed in thermostats at appropriate temperatures, and removed serially for phosphate analysis.

The hydrolysis of P,P′-di-γ-phenylpropyl pyrophosphate was followed by precipitating the starting material from the reaction mixture with 0.02 *M* barium chloride in 0.02 *M* hydrochloric acid solution, and determining the ultraviolet absorbance of the supernatant. Control experiments showed that the method is quantitative.

Results

The hydrolysis of γ-phenylpropyl diphosphate gave good first-order rate constants to about 50% reaction, but then they began to increase. This increase was traced to the slow hydrolysis of γ-phenylpropyl monophosphate, which increased the amount of phosphate produced in a given time; when allowance was made for this hydrolysis[22] the rate data could be fully accounted for.

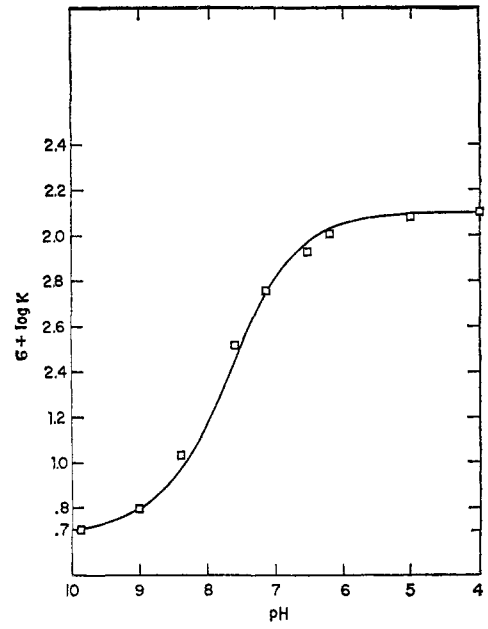


Figure 2. Rates of hydrolysis of γ-phenylpropyl diphosphate at 95°.

The data for the hydrolyses at 95° are presented in Table III and in Figure 2. The results of the hydrolyses at 99° confirm these data. In these experiments, the ionic strength was 0.05. The hydrolyses are insensitive to ionic strength; an increase in tetramethylammonium bromide concentration from 0.025 to 2.53 *M* decreased the rate of hydrolysis of γ-phenylpropyl diphosphate at pH 5 by only 15%. The hydrolyses of γ-phenylpropyl triphosphate and of the other compounds here considered undergo cleavage principally to phosphate, with only small amounts (up to 10%) of pyrophosphate as determined by paper chromatography.[22]

An attempt was made to detect amine catalysis in the hydrolysis of γ-phenylpropyl diphosphate. Such catalysis could result from nucleophilic attack of the amine on phosphorus[23,24] or by general base catalyzed[25] attack of water. An increase in the concentration of a buffer composed of three parts Tris–HCl and two parts Tris (pH 6.5 at 99.1°) from 0.004 *M* to 0.08 *M* caused a decrease in rate of nearly 20%. An increase in the concentration of a buffer composed of equal parts of imidazole and imidazole hydrochloride (pH 5.9 at 99.1°) caused an increase in rate of only 10–20% (2.5 *M* tetramethylammonium chloride and 2.5 *M* tetrahydrofuran scarcely affected the rate).

Discussion

The rate of hydrolysis of γ-phenylpropyl diphosphate follows the rate law

(18) O. Lowry and J. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).
(19) V. Potter, *ibid.*, **169**, 17 (1947).
(20) D. Usher, *J. Chromatog.*, **12**, 262 (1963).
(21) V. Bower and R. Bates, *J. Res. Natl. Bur. Std.*, **59**, 261 (1957).
(22) D. L. Miller, Thesis, Harvard University, 1965.
(23) W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *J. Am. Chem. Soc.*, **86**, 1254 (1964).
(24) R. L. Blakeley, F. Kerst, and F. H. Westheimer, *ibid.*, **88**, 121 (1966).
(25) G. Dudek and F. H. Westheimer, *ibid.*, **81**, 2641 (1959).

Table III. Rate Constants for Hydrolysis of Di- and Triphosphates at 95°, $\mu = 0.05$

Buffer no.	pH at 95°	k_1, sec^{-1} × 10^5 PPDP	PPTP	$P_2O_7^{4-}$	ATP	ADP	Di-PPDP
1	(0.12 *M* KOH)	0.50					<0.01
2	9.87	0.51		0.084		1.0	
3	9.02	0.63	2.1		3.2		
4	8.40	1.15				1.8	
5	7.60	3.23					
6	7.17	5.65	6.93	2.67	7.2	5.54	
7	6.56	8.35					
8	6.22	10.1					
9	5.04	12.1	14.9	17.8	17.3	15.1	0.007
10	4.18	12.6	15.5	16.8	18.2	15.0	
11	3.38	12.9		18.0		15.5	
12	2.12	21.9					
13	1.12	41.7		88.8		63.5	16.8
14	0.3	155					

$$d(P_i)dt = k_1(PPDP^{3-}) + k_2(PPDPH^{2-}) + k_3(PPDPH_2^{-}) + k_4(PPDPH_3) \quad (1)$$

where the concentrations of the various ionic species may be calculated from the total concentration and the ionization constants. The pK values were all estimated except pK_3 at 95°, which was measured; they are reasonable in relation to those of other polyphosphates. The constants k_1 and k_2 may be observed directly at pH 9 and 4, respectively, but k_3 and k_4 cannot easily be separated, and are calculated by applying eq 1 to the data of the tables. The constants chosen are presented in Table IV. The calculated pH–rate profile, together with the experimental points, is presented in Figure 2. The rates of hydrolysis of γ-phenylpropyl diphosphate and of ADP are similar at all pH values. Furthermore, the rate of hydrolysis of γ-phenylpropyl triphosphate is similar to that of ATP at all pH values investigated (see Table V). On the other hand, the rate of hydrolysis of P,P′-di-γ-phenylpropyl pyrophosphate is only $^1/_{2000}$ of that of monoprotonated γ-phenylpropyl diphosphate.

Table IV. Kinetic Constants for Hydrolysis of γ-Phenylpropyl Diphosphate

	Rate constants, sec^{-1} × 10^4				pK values		
	k_1	k_2	k_3	k_4	pK_a'''	pK_a''	pK_a'
99.1°	0.14	2.0	2.4	14	6.75	2.7	1.3
95°	0.05	1.25	1.4	5	7.0	2.7	1.6

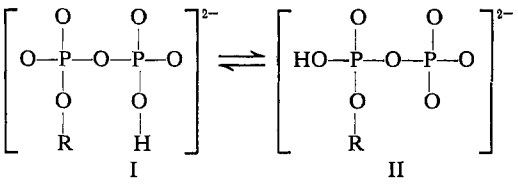
Table V. A Comparison of the Rates of Hydrolysis of γ-Phenylpropyl Di- and Triphosphates and ADP and ATP at 95°

	$PPDP^{3-}$	$PPTP^{4-}$	ADP^{3-}	ATP^{4-}
k, sec^{-1} × 10^5	0.5	2.1	1.0	3.2
	$PPDPH^{2-}$	$PPTPH^{3-}$	$ADPH^{2-}$	$ATPH^{3-}$
k, sec^{-1} × 10^5	12.5	15.0	15.0	17.5

These data suggest that the hydrolysis of monoprotonated γ-phenylpropyl diphosphate proceeds at pH 3–6 by a "monomeric metaphosphate" mechanism.[26–30]

(26) W. W. Butcher and F. H. Westheimer, *J. Am. Chem. Soc.*, 77, 2420 (1955).

The argument from the present data is the following. The rate of hydrolysis of $(ROPO_2–OPO_3H)^{2-}$ is 2000 times as fast as that of $(ROPO_2–O–PO_2–OR)^{2-}$ where R is γ-phenylpropyl. Why? A reasonable possibility rests on the equilibrium



(2)

The equilibrium between I and II will be displaced far to the side of I because of the difference in basicity of the two positions to which the proton may be attached. A reasonable estimate of the ionization constants of I and II suggests that only 1 part in 10,000 to 100,000 of II will be present in the equilibrium mixture. Nevertheless, II would presumably decompose very rapidly and we postulate that the reaction is funneled through this ion as intermediate. Brown and Hamer[31] and Samuel and Silver[32] have shown that unsymmetrical diethyl pyrophosphate is formed in the phosphate-catalyzed hydrolysis of tetraethyl pyrophosphate; the position of phosphorus–oxygen cleavage is consistent with the reaction (R = ethyl)

$$(RO)_2PO–O–PO_3^{2-} \longrightarrow (RO)_2PO_2^{-} + [PO_3^{-}] \quad (3)$$

Although the rate constant for reaction 3 was not determined, it is obviously large. Thus a compound with the structure II would be expected to hydrolyze rapidly. If the hydrolysis of γ-phenylpropyl diphosphate proceeds by way of the ion II, then the rate constant for the decomposition of II is 10^4–10^5 as great as k_2, the rate constant for the equilibrium mixture of I and II, since this mixture is predominately I. The rate constant for the hydrolysis of II is large enough to be consistent with the data of Brown and Hamer, on the basis of the assumption that all unsymmetrical disubstituted pyrophosphates react at more or less the

(27) F. H. Westheimer, Special Publication No. 8, The Chemical Society, London, 1957, p 1.
(28) P. W. C. Barnard, C. A. Bunton, D. R. Llewellyn, K. G. Oldham, B. L. Silver, and C. A. Vernon, *Chem. Ind.* (London), 760 (1955); C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3574 (1958).
(29) A. Todd, *Proc. Natl. Acad. Sci. U. S.*, **45**, 1389 (1959).
(30) W. Jencks, *Brookhaven Symp. Biol.*, **15**, 134 (1962).
(31) D. M. Brown and N. Hamer, *J. Chem. Soc.*, 1155 (1961).
(32) D. Samuel and B. Silver, *ibid.*, 4321 (1961).

same rate. The mechanism for the hydrolysis of monoprotonated phenylpropyl diphosphate then presumably follows eq 3, where one of the R groups represents the phenylpropyl group, and the other R represents a proton. This scheme would explain the large rate difference between mono-γ-phenylpropyl diphosphate and the corresponding symmetrical di-γ-phenylpropyl diphosphate. The mechanism would further explain our observation that the hydrolysis is insensitive to (or independent of) nucleophilic or general base catalysis in the pH region around 5. The idea that scission of a P–O bond is rate limiting does not quite imply the formation of a fully independent monomeric metaphosphate ion; the mechanistic ambiguity in phosphate hydrolyses, as noted earlier,[33] parallels that which has plagued the detailed interpretation of the solvolysis of certain alkyl halides.[34] When ATP is dissolved in pyridine, the amine acts as a nucleophile upon the ionized triphosphate;[23] perhaps this base might provide nucleophilic assistance for the hydrolysis of pyro- and triphosphates even in aqueous solution. Such an effect of pyridine bases has been observed in the hydrolysis of *p*-nitrophenyl phosphate.[35] But our data suggest that the primary driving force in the reactions here studied is provided, as in the example of Brown and Hamer,[31] by the cleavage of the P–O bond.

Of course, the mechanism postulated for the pH region 3–6 (for the monoprotonated species I and II) need not be extended to the more acid solutions; there a nucleophilic attack on phosphorus by water may well prove the preferred pathway.

The similarities in rates of hydrolysis for γ-phenylpropyl di- and triphosphates with those of ADP and ATP are shown in Table V. Since ADP and ATP hydrolyze at rates essentially the same as those of γ-phenylpropyl di- and triphosphates, the mechanisms for these hydrolyses are presumably the same. Thus the data show that the adenosine residue plays no role in the acid-catalyzed hydrolysis of ADP and ATP.

Acknowledgment. The work was financed by the National Science Foundation under Grant No. GP-2098. One of us (D. L. M.) wishes further to thank the National Science Foundation for predoctoral fellowships, 1961–1964, and the National Institutes of Health for support under a training grant, 1964–1965.

(33) P. S. Traylor and F. H. Westheimer, *J. Am. Chem. Soc.*, **87**, 553 (1965).

(34) E. R. Thornton, "Solvolysis Mechanisms," The Ronald Press, New York, N. Y., 1964, p 95 ff.

(35) A. J. Kirby and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 3209 (1965).

The Enzymic Hydrolysis of γ-Phenylpropyl Di- and Triphosphates[1]

David L. Miller and F. H. Westheimer

Contribution from the James Bryant Conant Laboratory of Harvard University, Cambridge, Massachusetts. *Received November 12, 1965*

Abstract: γ-Phenylpropyl triphosphate, an analog of ATP, is readily hydrolyzed by crude potato apyrase, by muscle myosin, and by inorganic pyrophosphatase in the presence of Zn^{2+}. The rates for these reactions are comparable to those for ATP. On the other hand, γ-phenylpropyl triphosphate generates no light with the luciferin–luciferase system, and does not inhibit the evolution of light from this system with ATP. Presumably the adenosine residue of ATP is not involved in the catalytic function of apyrase, myosin, or inorganic pyrophosphatase, but is essential to the specificity of luciferase.

In the previous paper[2] we outlined the synthesis of γ-phenylpropyl di- and triphosphates, and compared their rates of hydrolysis in neutral and acid solutions with those of ADP and ATP. In this paper we have presented the results of the hydrolysis of these same compounds with some enzymes that hydrolyze ATP. The objective of the research is to delineate the role of the adenosine residue in ADP and ATP by comparing the reactions of these coenzymes with those of the analogs that do not contain any of the reactive groups of the adenosine molecule.

The present work shows that the rates of hydrolysis of γ-phenylpropyl triphosphate with crude potato apyrase, with myosin, and with inorganic pyrophosphatase plus Zn^{2+} are about the same as those of ATP, that the rates of hydrolysis of γ-phenylpropyl diphosphate with apyrase and with inorganic pyrophosphatase are about the same as those of ADP, but that γ-phenylpropyl triphosphate is without effect in the luciferin–luciferase system. The conclusions from these experiments are recorded in the Discussion.

(1) Preliminary communication: D. L. Miller and F. H. Westheimer, *Science*, **148**, 667 (1965).

(2) D. L. Miller and F. H. Westheimer, *J. Am. Chem. Soc.*, **88**, 1507 (1966).

Experimental Section

Materials. The synthesis, purification, and identification of γ-phenylpropyl diphosphate (PPDP) and of γ-phenylpropyl triphosphate (PPTP) have been presented in an accompanying paper.[2]

Enzymes. Crude potato apyrase was purchased from Sigma Chemical Co. (lot No. A41B-57) and used without further purification. A stock solution of 3.0 mg in 100 ml of a succinate buffer, pH 6.56, was stable for months at 4°. The hydrolyses were studied at 30° in 0.1 *M* potassium succinate buffer, pH 6.56, with 2×10^{-3} *M* calcium chloride and 0.3 μg/ml of crude apyrase. The triphosphate concentration was varied from 5×10^{-5} *M* to 4×10^{-4} *M*. The enzyme can be denatured by adding 0.5 ml of 0.5 *M*