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.

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

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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.

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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*

KOH to 5 ml of reaction mixture; the solution may then be analyzed for phosphate.[3] The apyrase here used had only 10% of the activity of the best preparation of Cori,[4] or of Molnar and Lorand.[5]

Rabbit muscle myosin, prepared by the method of Mommaerts and Parrish,[6] was a gift from Dr. D. E. Koshland. A solution of about 2.5 mg of protein per milliliter in 0.6 *M* KCl was prepared and kept in ice for each set of experiments. The hydrolyses were conducted in 0.035 *M* potassium barbital buffer with 0.01 *M* calcium chloride, 0.1 *M* potassium chloride, and a triphosphate concentration of 1–7 $\times 10^{-4}$ *M*, in 50 ml of buffer, pH 7.49 at 30°. Samples removed at timed intervals were quenched with 1 ml of trichloroacetic acid. After the protein was removed by centrifugation, the solution was analyzed for inorganic phosphate.

Crystalline inorganic pyrophosphatase (Worthington Biochemical Corp.; 2 to 20 μg) in 15 ml of succinic acid–Tris buffer, pH 6.56, was used to hydrolyze γ-phenylpropyl triphosphate at 30° in the presence of zinc ion.

A crude solution of luciferin–luciferase was prepared[7] by grinding 50 mg of firefly lanterns (Sigma Chemical Co.) with 5 ml of ice-cold 0.1 *M* solution of sodium arsenate at pH 7.4. The solution was clarified by centrifugation, and 50 mg of magnesium sulfate heptahydrate was added. The enzyme solution (0.2 ml) and water (1.0 ml) were placed in a fluorescence cell and 20 μg of ATP solution (2.5×10^{-3} *M*) was added. The intensity of the light emitted was measured with a Photovolt fluorimeter.

Results and Discussion

The rates of hydrolysis of the triphosphates in the presence of the enzymes are recorded in Tables I–V.

Table I. Hydrolysis of γ-Phenylpropyl Triphosphate and ATP Catalyzed by Crude Potato Apyrase at pH 6.56 and 30°

Compd	Concn, $M \times 10^5$	Rate, μmoles of HPO_4^{2-}/(mg of enzyme × min)
PPTP	40	5.5
PPTP	20	3.2
PPTP	10	3.1
PPTP	5	2.1
ATP	40	2.7
ATP	20	2.17
ATP	10	1.93
ATP	5	1.53

Table II. Hydrolysis of γ-Phenylpropyl Triphosphate and ATP Catalyzed by Myosin at pH 7.5 and 30°

Compd	Concn, $\times 10^4$	Rate, μmoles of HPO_4^{2-}/(mg of enzyme × min)	
PPTP	6.7	0.051	
PPTP	6.7	0.052	
PPTP	3.4	0.043	
PPTP	1.35	0.031	$K_M = 1.3 \times 10^{-4}$ *M*
PPTP	1.35	0.030	V_{max} = 0.06 μmole of HPO_4^{2-}/(mg of enzyme × min)
ATP	5.0	0.121	
ATP	2.5	0.098	$K_M = 9 \times 10^{-5}$ *M*
ATP	1.0	0.086	V_{max} = 0.13 μmole of HPO_4^{2-}/(mg of enzyme × min)

Table III. Hydrolysis of Inorganic Pyrophosphate, γ-Phenylpropyl Triphosphate, ADP, and ATP Catalyzed by Inorganic Pyrophosphatase (0.1 *M* Tris-succinate Buffer, pH 6.56 and 30°)

Compd	Concn, $M \times 10^3$	Metal Ion	Concn. $M \times 10^4$	Rate, μmoles of HPO_4^{2-}/(mg of enzyme × min)
$H_2P_2O_7^{2-}$	1.33	Zn^{2+}	6.7	300
ATP	1.17	Zn^{2+}	11.8	3.4–3.8
PPTP	1.35	Zn^{2+}	12.6	20.5–22
PPDP	1.01	Zn^{2+}	10.1	6.4
$H_2P_2O_7^{2-}$	1.33	Mg^{2+}	6.7	580
ATP	1.17	Mg^{2+}	13.3	$<10^{-3}$
PPTP	1.35	Mg^{2+}	13.3	$<10^{-3}$

Table IV. Hydrolysis of γ-Phenylpropyl Triphosphate and ATP Catalyzed by Inorganic Pyrophosphatase at Different Ratios of Zn^{2+}/Triphosphate

Compd	Concn, $M \times 10^3$	(Zn^{2+})/(triphosphate)	Rate, μmoles of HPO_4^{2-}/(mg of enzyme × min)
PPTP	1.00	1.5	37–38
PPTP	1.00	1.0	18
PPTP	1.00	0.75	13.4
PPTP	1.00	0.50	11.3
PPTP	1.00	0.25	8.9
ATP	1.00	1.5	7.3–8
ATP	1.00	1.0	3.4
ATP	1.00	0.75	0.95
ATP	1.00	0.50	0.28
ATP	1.00	0.25	0.18

Table V. Hydrolysis of γ-Phenylpropyl Triphosphate and ATP Catalyzed by Inorganic Pyrophosphatase at Various Concentrations (Z^{2+}) = (ATP) or (PPTP)

Compd	Concn, $M \times 10^4$	Rate, μmoles of HPO_4^{2-}/(mg of enzyme × min)
PPTP	10	18
PPTP	5	12.3
PPTP	1	4.4
ATP	10	3.4
ATP	5	1.02
ATP	1	0.13

The hydrolysis of γ-phenylpropyl triphosphate with crude apyrase is shown in Table I. Similar data have been obtained by Cori and his co-workers[8] with highly purified apyrase and a sample of γ-phenylpropyl triphosphate that we had supplied. Cori's values of V_{max} for ATP and PPTP are 62 and 33 μmoles of HPO_4^{2-}/(milligrams of enzyme times minutes) respectively, and his values of K_M are 1×10^{-4} and 4×10^{-4}. Our limited data do not permit an accurate determination of these parameters, but Table I shows a closer correspondence of the nucleotide and its analog; in fact, our maximum rate is almost twice as large for PPTP as for ATP. The difference between our data and those of Cori probably reflects a difference in the source or in the purification of the enzyme. However,

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for the purposes of the present discussion, it is not important whether PPTP reacts twice as fast, or only half as fast, as ATP. In either case, the two compounds have comparable maximum rates, so that the influence of the adenosine residue on the catalytic site must be minimal.

Inorganic pyrophosphatase does not attack either ATP or γ-phenylpropyl triphosphate in the presence of Mg^{2+}, although Mg^{2+} is the activating cation for its normal physiological function. However, Schlesinger and Coon[9] discovered that the enzyme rapidly attacks ATP if Zn^{2+} is substituted for Mg^{2+}. This fact might have suggested that the Zn^{2+}, in contrast to Mg^{2+}, complexes the adenine moiety of ATP, and so involves it in the enzyme action. That such is not the case is demonstrated by the rapid hydrolysis of γ-phenylpropyl triphosphate by the enzyme in the presence of Zn^{2+}; the rates at various concentrations of substrate and cation actually exceed those for ATP. The change in substrate specificity with change in cation is unexplained, but at any rate is parallel for the nucleotide and its analog.

With the luciferin–luciferase system, no light was detected when γ-phenylpropyl triphosphate was substituted for ATP, although the apparatus used was sufficiently sensitive to detect an activity $^1/_{400}$ that of ATP. Furthermore, 6×10^{-4} *M* γ-phenylpropyl triphosphate did not decrease the light emission produced by 4×10^{-5} *M* ATP.

A summary of the comparison between ATP and γ-phenylpropyl triphosphate is presented in Table VI.

Table VI. Rates of Enzyme-Catalyzed Hydrolyses

	PPTP	ATP
A. Potato Apyrase, pH 6.6, 30°, 0.002 *M* Ca^{2+}		
V_{max}, μmoles/(mg of enzyme × min)	5.6	3 (our data)
V_{max}, μmoles/(mg of enzyme × min)	33	62 (ref 8)
K_M, $M \times 10^5$	43	10 (ref 8)
B. Myosin, pH 7.5, 30°, 0.01 *M* Ca^{2+}		
V_{max}, μmoles/(mg of enzyme × min)	0.06	0.13
K_M, $M \times 10^5$	13	9

C. Inorganic Pyrophosphatase, pH 6.6, 30° (Zn^{2+} = PPDP = PPTP = ATP = 0.0012 *M*)

	PPDP	PPTP	ATP
Rate, μmoles/(mg of enzyme × min)	6.4	21	3.6

Since the three enzymes potato apyrase, muscle myosin, and inorganic pyrophosphatase (in the presence of Zn^{2+}) catalyze the hydrolysis of γ-phenylpropyl triphosphate as readily (as an average) as they catalyze the hydrolysis of ATP, the adenosine moiety obviously plays a minor role in these reactions, and the detailed structure of the nucleotide plays no role at all. These statements are very much stronger than those which previously could be made on the basis of the reactivity of other nucleoside triphosphates toward apyrase,[9] toward myosin,[10] and toward inorganic pyrophospatase and zinc ion.[8] Many of the nucleoside triphosphates are, of course, active in enzymic reactions; for example, inorganic pyrophosphatase plus Zn^{2+} readily hydrolyzes not only ATP, but GTP, ITP, CTP, and UTP as well,[9] and myosin hydrolyzes every nucleotide triphosphate tried.[10] But in all of these cases, much of the structure is preserved: the purine or pyrimidine nitrogen atoms for chelation, as well as the asymmetric ribose group. Here, however, all of these features have been removed; γ-phenylpropyl group resembles adensoine only in its large, flat aromatic ring and in blocking one of the ionizing groups of the triphosphate. The effect of structure has not been lost altogether; something (perhaps only blocking of an ionizing group in triphosphate ion) makes γ-phenylpropyl triphosphate, like ATP, susceptible to inorganic pyrophosphatase in the presence of zinc ion but not in the presence of magnesium ion. Nevertheless, the structural involvement of the adenine group is obviously minimal.

At the other extreme, γ-phenylpropyl triphosphate neither stimulates the production of light from the luciferin–luciferase system nor will it inhibit the production of light from that system plus ATP. The fact that γ-phenylpropyl triphosphate is not an inhibitor suggests that it is not bound to the enzyme. The natural (although by no means the only possible) assumption is that the adenosine group is specifically bound to the enzyme. Such binding would parallel the binding of DPN to alcohol dehydrogenase. Here adenine diphosphoribose is an especially effective inhibitor,[11,12] and is obviously specifically bound to the enzyme. It is tempting to generalize these results, and to suggest as a working hypothesis that the adenosine moiety of coenzymes is frequently involved with the specificity site on the appropriate enzymes, but is never involved in the direct chemical reactions catalyzed by these enzymes.

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