Adenylyl Imidodiphosphate, an Adenosine Triphosphate Analog Containing a P–N–P Linkage*

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ABSTRACT: An analog of adenosine triphosphate, adenylyl imidodiphosphate (AMP-PNP), in which a NH-grouping replaces the terminal bridge oxygen of the triphosphate chain has been synthesized by the reaction of imidodiphosphate with P^1-adenosine P^2-diphenyl pyrophosphate (Michelson, A. (1964), *Biochim. Biophys. Acta 91*, 1). AMP-PNP is stable (<3% hydrolysis) for months at −20° as the tetrasodium salt and in solution at 25° at neutral pH for at least 16 hr. The principal nonenzymic breakdown products are inorganic phosphate and adenylyl phosphoramidate. Alkaline phosphatase (*Escherichia coli*) cleaves the terminal phosphate of the analog, a finding which extends the substrate specificity of this enzyme to include P–N-type bonds. Snake venom phosphodiesterase cleaves AMP-PNP to adenosine 5′-monophosphate and imidodiphosphate. AMP-PNP is not a substrate for hexokinase (glucose phosphorylation) or for myokinase (phosphorylation of adenosine monophosphate). AMP-PNP binds Ca^{2+}, Mg^{2+}, and Mn^{2+} at pH 8.5 more tightly than ATP. Similar results were found with the comparable methylene analog, adenylyl methylenediphosphonate, at pH 7.4 and 9.2 with Ca^{2+} and Mg^{2+}. An improved method of synthesis of the methylene analog is described and the structural properties of the P–C–P and P–N–P analogs are compared to adenosine triphosphate based on recent X-ray structural data of imidodiphosphate, pyrophosphate, and methylenediphosphonic acid.

ATP is perhaps the most important small molecular weight compound in biological systems. Yet our understanding of its interactions with enzymes as both a substrate and as an allosteric effector is incomplete and unsatisfactory. A similar situation exists with our understanding of the interaction of ATP with most biological transducers, such as the contractile system in muscle and the membranes of nerves and other cells.

To better understand these interactions we have been synthesizing analogs of ATP in which the triphosphate chain has been modified (Yount *et al.*, 1966a,b). Recently an increasing number of such analogs have been synthesized (see Cook, 1970, and references therein for a partial list). One of the earliest and most useful of such compounds was AMP-PCP[1] (Myers *et al.*, 1963) an analog in which a CH_2 group replaced the terminal bridge oxygen of the triphosphate chain of ATP. Because of the inherent stability of the P–C–P bonds in this compound, AMP-PCP offered the opportunity to study the interaction of an ATP-like molecule with various enzymes without the possibility of cleavage between the β,γ-phosphates.

In many cases, however, AMP-PCP has had little effect on the system under study. For example, AMP-PCP was shown by Moos *et al.* (1960) not to support contraction of glycinerated muscle fibers, not to dissociate actomyosin at high ionic strength, or even to inhibit the ATPase activity if excess Mg^{2+} was present. Since we were particularly interested in such interactions, we reasoned that an ATP analog with P–N–P bond, if sufficiently stable, would be structurally and chemically more similar to ATP and hence would mimic ATP more effectively. Such a compound, AMP-PNP, has been synthesized (Figure 1). This paper reports on the synthesis and properties of AMP-PNP in comparison to those of AMP–PCP and ATP.

During the course of this work the crystal structure of the tetrasodium salt of imidodiphosphate was solved (Larsen *et al.*, 1969) and it was shown to be remarkably similar to the previously known structure of sodium pyrophosphate. This close structural similarity rationalizes the overall similarity of ATP and AMP-PNP in their interactions with a number of enzyme systems. The more detailed studies of the interaction of AMP-PNP and AMP–PCP with myosin, heavy meromyosin, and actomyosin is given in the following paper (Yount *et al.*, 1971).

Experimental Section

Materials. Sodium imidodiphosphate was prepared by the hydrolysis of diphenylimidodiphosphoric acid (Kirsanov and Zhmurova, 1958) with the following modification of method B of Neilsen *et al.* (1961). Diphenylimidodiphosphoric acid (14.5 g, 0.045 mole) was mixed with 40 g (1 mole) of sodium hydroxide, 39 g (0.45 mole) of phenol, and 108 ml of water in a 1-l. flask and heated as rapidly as possible with magnetic stirring to 140° in an oil bath preheated to 170–180°. After 10 min at 140–145°, the hot solution was transferred to a beaker where on cooling, crystals of sodium imidodiphosphate formed. After filtering the product was dissolved in 250 ml of water and ethanol added to incipient cloudiness and the solution cooled at 4° overnight. The crystals were filtered and tested for the presence of phenols (Feigl and Jungreis, 1959) and recrystallized as before until a negative phenol test was obtained. Phosphate analysis gave

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[1] The following abbreviations have been used: AMP-PCP, adenylyl methylenediphosphate; AMP-PNP, adenylyl imidodiphosphate; PNP_i, inorganic imidodiphosphate; PCP_i, methylenediphosphonate; ADP·NH_2, adenylyl phosphoramidate.

a formula weight of 444 corresponding to sodium imidodiphosphate·decahydrate. Lower degrees of hydration were obtained depending on the mode of drying the crystals and molecular weights were determined for each preparation before using them in a synthetic reaction. Crystals were stored in capped vials at −20°.

Methylenediphosphonic acid was prepared by the hydrolysis of tetraethyl methylenediphosphonate (Cade, 1959) with 6 N HCl under reflux for 72 hr. After removing HCl and ethanol under reduced pressure, the methylenediphosphonic acid was recrystallized twice from hot glacial acetic acid containing small amounts of water. Phosphorus analysis (Ames and Dubin, 1960) for $C_1H_6O_6P_2$ gave a molecular weight of 173; theory 176.

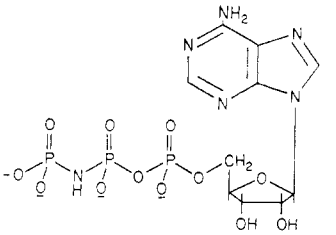
Pyridine was refluxed over calcium hydride or sodium hydroxide pellets, distilled through a short column, and stored over calcium hydride or molecular sieves (Linde Division, Union Carbide Corp., Tonawanda, N. Y., Type 4A, $^1/_{16}$-in. pellets). Dimethylformamide and chloroform were commercial preparations dried over molecular sieves for at least 10 days. Trioctylamine (Eastman) and tributylamine (Aldrich) were redistilled under vacuum and stored in dark bottles at 4°. Triethylamine (Aldrich) was refluxed with a few grams of *p*-toluenesulfonyl chloride added to react with any secondary amines, distilled at atmospheric pressure, and stored in the dark at 4°. Triethylammonium bicarbonate solutions (1 M) were prepared by bubbling CO_2 through a sintered glass diffuser into triethylamine solutions in an ice bath until the pH fell to 7.5. Diphenyl chlorophosphate (Aldrich) was used from freshly opened bottles or from freshly distilled preparations. Chromatographic columns used were either from Pharmacia (Piscataway, N. J.) or Chromatronix Inc. (Berkeley, Calif.).

Analytical Methods. Acid-labile phosphate and acid-labile ammonia were determined on P–N–P compounds after heating 30 min at 100° in 1 N HCl. P_i was measured as the reduced phosphomolybdate complex (Fiske–Subbarow, 1925) and ammonia with Nessler's reagent (Umbreit *et al.*, 1962). Total phosphate was determined as P_i after ashing with $Mg(NO_3)_2$ and subsequent hydrolysis with 1 N HCl (Ames and Dubin, 1960). When triethylammonium bicarbonate was present, it was removed by taking the solution to dryness before doing phosphate analyses since phosphomolybdate precipitates with trialkylamines.

Compounds were detected on paper chromatograms and electrophoretograms by ultraviolet quenching using a Trans-illuminator and Chromato-Vue cabinet (Ultra-violet Products, San Gabriel, Calif.) and by a phosphate spray reagent (Kolloff, 1961) after acid hydrolysis. This latter step was accomplished by suspending the dried, developed chromatogram or electrophoretogram with thin Teflon tubing in a jar over 4 N HCl. The jar was covered with Saran Wrap and heated 30–45 min in an 85–90° oven. If the paper was not noticeably weakened, an additional 10 min of heating was used. The above spray reagents will detect less than 0.01 μmole of P_i.

The per cent composition (based on adenine) of various nucleotide preparations was determined by measuring the absorbance at 260 mμ after eluting the appropriate ultraviolet-absorbing compounds from paper chromatograms with 0.05 N HCl. This method routinely gave values within ±1% for duplicate samples if 0.5–1 μmole of total adenine compounds were initially chromatographed.

Elemental analyses for the methylene analog were by A. Bernhardt (Mülheim, West Germany).



Adenylyl - imidodiphosphate
(AMP - PNP)

FIGURE 1: Structure of adenylyl imidodiphosphate.

Results

Preparation of Tetrasodium Adenylyl Imidodiphosphate. The general anion-exchange method of Michelson (1964) was used. Sodium imidodiphosphate (1.2 mmoles) was dissolved in 10 ml of cold water and the resulting solution was added to a 2-cm diameter column containing 60 mequiv of freshly regenerated and washed Dowex 50 H^+ (X8, 50–100 mesh) at 0–2°. The free acid of PNP_i was eluted with cold water collecting all acidic fractions (pH paper) in a stirred solution of excess tributylamine and water in an ice bath. This solution was stripped to a gum on a rotatory evaporator using a Dry Ice–2-propanol cooled trap, a mechanical vacuum pump and a bath temperature of 25–30°. The residue was dissolved in 10–20 ml of dry chloroform and stripped to dryness. This process was repeated twice and the resulting dried tributylammonium salt stored in a stoppered flask at −20° until used.

AMP·H_2O (1 mmole, free acid, Sigma grade) was dissolved in several milliliters of absolute methanol containing 1 mmole of trioctylamine (0.353 g) with stirring under reflux. The methanol was removed under reduced pressure as before and the residue dried by repeated addition and evaporation of three 5-ml portions of dry dimethylformamide. The residue was again dissolved in dimethylformamide (5 ml) and 0.3 ml of diphenyl chlorophosphate (1.5 mmoles) and 0.47 ml of tributylamine (2.0 mmoles) were added and the solution was allowed to stand at room temperature for 2 hr protected from moisture. If a precipitate formed, it was dissolved by adding tributylamine dropwise. Solvents were removed under reduced pressure and dry ether (50 ml) was added to the residue with swirling. The flask was cooled in an ice bath for 20 min and the ether decanted. Remaining ether was removed under pressure and the residue dissolved in 5 ml of dimethylformamide. This solution was added dropwise over a 10-min period to a stirred solution of the tributylammonium salt of PNP_i dissolved in 5 ml of dimethylformamide and to which 15 ml of pyridine was added. (Adding the solution in reverse order or all at once led to greatly increased yields of a by-product containing 2 moles of AMP linked to 1 mole of PNP_i.) Solvents were removed under reduced pressure after the reaction mixture was allowed to stand at room temperature for 30 min. The residue was extracted with ether (50 ml) and the ether removed as before. The resulting gum was dissolved in 50 ml of cold water containing 0.4 ml of concentrated ammonium hydroxide. The final pH was near 9.0. If the solution was cloudy, it was extracted with ether and the clear water layer was applied to a 2.5 × 75 cm DEAE-cellulose (HCO_3^-) column at 2°. Purification followed the general procedure of Moffatt (1964). The results of a typical separation are shown in

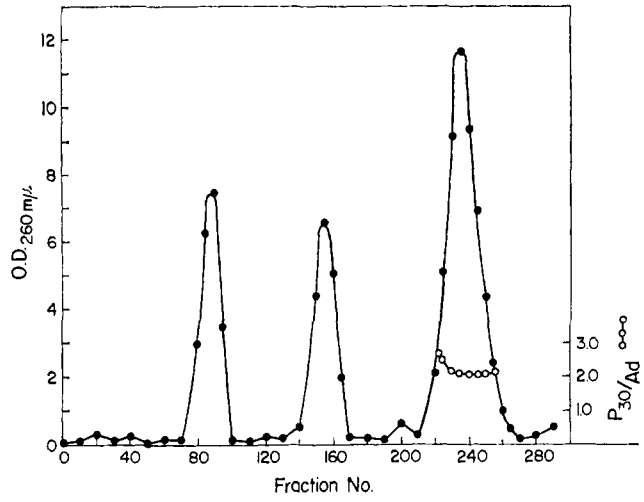


FIGURE 2: Chromatographic purification of AMP-PNP on DEAE-cellulose. Flow rate 5 ml/min, 25-ml fractions collected, linear gradient of 0–0.4 M triethylammonium bicarbonate, 8-l. total volume. Temperature = 2°. The solutions in tubes 230–260 whose P_{30}/Ad ratio was 2.0 ± 0.1 were pooled and the AMP-PNP (0.324 mmole) isolated as described in the text. P_{30} is the P_i resulting from treatment at 100°, 30 min in 1 N HCl. Ad is the adenine content determined from the $OD_{260\ m\mu}$ and molar absorbancy index of 1.54×10^4.

Figure 2. AMP-PNP was located by determining acid-labile phosphate/adenine ratios on representative tubes and by the chromatographic behavior of aliquots of peak tubes. The tubes containing the AMP-PNP peak were pooled and evaporated to dryness with a bath temperature of 25°. Residual triethylammonium bicarbonate was removed by repeated evaporations with 25-ml portions of methanol. The residue was transferred to a 40-ml glass centrifuge tube with several rinses of small amounts of methanol. After concentrating the solution to 4–5 ml with an air stream, a 1 M sodium iodide in acetone solution was added (6–8 equiv relative to the equivalents of adenine present) to precipitate the sodium salt of AMP-PNP. Additional cold acetone was added and the precipitate was collected by centrifugation in the cold. The precipitate was washed three times with cold acetone, twice with cold ether, and then dried briefly with a gentle air stream. After drying over anhydrous magnesium perchlorate *in vacuo* the free-flowing white powder was stored desiccated at −20°. Less than 3% decomposition in 8 months was observed with two different preparations as judged using the two solvent systems and the electrophoresis system given in Table I. Alternately the nucleotide was stored frozen in liquid nitrogen as 50 mM solutions at pH 9.0. Again less than 2% decomposition in 2 months was observed. Overall isolated yields in eight preparations were 20–35% based on AMP. The major impurity, if any, was adenylyl phosphoramidate resulting from the hydrolysis of the terminal phosphate of AMP-PNP. Hydrolysis to yield ADP and inorganic phosphoramidate appears to be much less favored. Breakdown of AMP-PNP was reduced by minimizing the time it was kept in the triethylammonium bicarbonate solutions.

Anal. Calcd for $C_{10}H_{13}N_6Na_4O_{12}P_3 \cdot 4H_2O$ (666); P, 14.0; acid-labile ammonia, 1.0; acid-labile phosphate, 2.0. Found: mol wt 660 (from $OD_{260\ m\mu}$ assuming a molar absorbancy index of 15,400); P, 14.1; acid-labile ammonia, 1.06; acid-labile phosphate, 1.97.

TABLE I: Characterization of Adenylyl Imidodiphosphate by Paper Chromatography and Paper Electrophoresis.[a]

Compound	R_F A	R_F B	Mobility (cm), *I*
AMP-PNP	0.39	0.34	12.0
ATP	0.50	0.37	14.0
ADP	0.54	0.42	11.0
ADP-NH_2	0.69	0.55	9.5
PNP_i	0.20	0.29	19.0
P_i	0.30, 0.45 (two spots)	0.35	15.0

[a] Ascending chromatography with Whatman No. 31ET paper was used with solvent systems (A) 2-propanol–dimethylformamide–methyl ethyl ketone–water–concentrated ammonia (20:20:20:39:1, v/v) (Biberacher, 1956) and (B) 1-propanol–concentrated ammonia–water (6:3:1, v/v). Whatman No. 31ET paper was used for electrophoresis on a water-cooled flat-plate apparatus using 0.05 M sodium citrate (I) (pH 6.8), 35 V/cm for 45 min.

Tetrasodium Adenylyl Methylenediphosphonate. AMP (1 mmole) was converted into P^1-adenosine P^2-diphenyl pyrophosphate and dissolved in 5 ml of dimethylformamide as outlined before. This solution was added dropwise over a 10-min period to a magnetically stirred solution of methylenediphosphonic acid (2 mmoles, 0.352 g) and tributylamine (4 mmoles, 0.94 ml) in 10 ml of pyridine. After reacting for 3 hr at room temperature, the solvents were removed under reduced pressure and the resulting AMP-PCP was worked up and purified as outlined for AMP-PNP. Yields (three preparations) were 52, 48, and 40% based on AMP. This is to be compared to yields of 16 and 22% of AMP-PCP prepared using dicyclohexylcarbodiimide coupling or the reaction of adenosine 5′-phosphoramidate with PCP_i (Myers *et al.*, 1963). The AMP-PCP obtained was homogeneous in solvent systems A and B (Table I) as well as in an acidic solvent (isobutyric acid–water–concentrated ammonia (66:33:1, v/v) and R_F values were identical with those of an authentic sample of AMP-PCP (Miles Laboratories).

Anal. Calcd for $C_{11}H_{14}N_5Na_4O_{12}P_3 \cdot 3H_2O$ (647): C, 20.41; H, 3.10; N, 10.82; P, 14.37. Found: C, 20.23; H, 3.05; N, 10.73; P, 14.01.

Formation constants were determined by the resin competition method of Schubert (1956) as used by Walaas (1958) for nucleotides. The resin (Bio-Rad AG-1, X-8, 100–200 mesh) was treated as described previously (Yount *et al.*, 1966a) and contained 12% water.

The basic equation for this method is

$$\frac{1}{K_d} = \frac{K_f}{K_d^0}[M^{2+}] + \frac{1}{K_d^0}$$

where K_d is the distribution coefficient of nucleotide between the resin and solution, K_d^0 is the distribution coefficient at zero concentration of divalent metal ion, and K_f is the affinity constant. K_d was determined at different M^{2+} concentrations and K_f was obtained from the slope and intercept of a plot of $1/K_d$ *vs.* $[M^{2+}]$.

The procedure employed was as follows. Varying samples of resin large enough to absorb 73–77% of the nucleotide from solution (*i.e.*, 15–40 mg depending on the pH) were weighed to within ±0.2 mg into 25-ml glass-stoppered erlenmeyer flasks. Solution (10 ml) as given in Table II was added and the mixture equilibrated on a rocking arm shaker in a constant-temperature room for 1.5–2 hr. Duplicate samples of three to five different metal ion concentrations were run along with the metal-free blank solutions. With the possible exception of AMP-PCP with higher concentrations of Mn^{2+}, there was no evidence of other than one to one complexes being formed; *i.e.*, all the $1/K_d$ *vs.* M^{2+} plots were linear, intercepting the ordinate at $1/K_d^0$.

The data given in Table II indicate that both AMP-PCP and AMP-PNP bind Ca^{2+}, Mg^{2+}, Mn^{2+}, with affinities equal to or greater than ATP. This is true even for AMP-PCP at pH 7.4 where protons might be expected to compete effectively with divalent metals for the weaker acid group of the phosphonate analog. At the pH values where all three analogs are principally in the −4 net charge form, *i.e.*, pH 8.5 for ATP and AMP-PNP and pH 9.2 for AMP-PCP, each divalent metal binds to AMP-PCP and AMP-PNP with almost equal affinity but only half as strongly to ATP.

Acid Dissociation Constants. Potentiometric titrations of AMP-PNP were run using a Corning Model 12 pH meter equipped with a 0–14 pH combination microelectrode (A. H. Thomas). Standard NaOH (0.322 N) or standard HCl (0.200 N), made up in boiled deionized water, was added *via* a Manopet microburet (E. Greiner) to a 3-ml solution of 0.015 M AMP-PNP in 0.1 M tetramethylammonium chloride in a plastic beaker. The solution was kept under nitrogen gas and mixed by rotation of the beaker. The temperature was 23°. Titration of crystalline H_2Na_2ATP (Sigma) under similar conditions with NaOH gave pK_a values of 4.5 (purine ring proton) and 7.1 (terminal phosphate proton) corresponding well with values from the literature (Phillips *et al.*, 1963). Titration of two different preparations of Na_4AMP-PNP gave a pK_a value of 7.7 ± 0.1 for the terminal phosphate grouping by titration, first with HCl and then back-titration with NaOH.

Enzyme Studies. E. coli alkaline phosphatase (0.1 mg, Worthington) or snake venom phosphodiesterase (0.02 mg, Worthington) was incubated with 0.5 ml of 0.01 M AMP-PNP in 0.025 M Tris·Cl buffer (pH 8.0) at 25°. Aliquots (50 μl) were taken at 0, 0.5, 1, 2, and 16 hr and chromatographed with solvents A and B (Table I). With alkaline phosphatase only spots corresponding to AMP-PNP and increasing amounts of $ADP \cdot NH_2$ were observed by ultraviolet quenching. Similarly phosphodiesterase action yielded only AMP and PNP_i, the latter being detected with phosphate spray reagents after acid hydrolysis. With crude snake venom (*Crotalus atrox*, Calbiochem), AMP-PNP was degraded under similar reaction conditions, to adenosine, P_i, and PNP_i. In all the above experiments no nonenzymic breakdown of AMP-PNP was observed.

Myokinase and hexokinase were tested to see if they would catalyze the transfer of the terminal phosphate of AMP-PNP to AMP and glucose, respectively. Myokinase (Boehringer, 0.1 mg) was incubated with 0.01 M AMP-PNP, 0.01 M AMP, 0.02 M $MgCl_2$ in 0.025 M Tris·Cl (pH 7.4) buffer at 25° for periods up to 8 hr. Aliquots (50 μl) were taken at 1-hr intervals and electrophoresed as described in Table I. No ADP was seen, though as little as a 2% conversion of AMP into ADP could have been detected. Control experiments with ATP in place of AMP-PNP gave equilibrium quantities of AMP, ADP, and ATP in 1 hr.

TABLE II: Affinity Constants of AMP-PNP, AMP-PCP, and ATP for Ca^{2+}, Mg^{2+}, and Mn^{2+}.[a]

Nucleotide	pH	K_a		
		Ca^{2+} ($M^{-1} \times 10^{-3}$)	Mg^{2+} ($M^{-1} \times 10^{-3}$)	Mn^{2+} ($M^{-1} \times 10^{-3}$)
AMP-PNP	8.5	11.8 ± 0.2	38.2 ± 0.4	85 ± 3
ATP	8.5	5.4 ± 0.4	14.9 ± 0.2	32 ± 3
AMP-PCP	7.4	4.8 ± 0.5	12.9 ± 0.1	
ATP	7.4	1.8 ± 0.1	4.5 ± 0.1	
AMP-PCP	9.2	13.0 ± 0.5	38.1 ± 0.4	83 ± 4

[a] Conditions: 0.1 M KCl, 0.025 M Tris·Cl, and 0.06–0.07 mM nucleotide, 25°. Values are given plus and/or minus standard error of the mean.

AMP-PNP was similarly ineffective as a substrate for hexokinase using the assay of Kaji and Colowick (1965). Solutions (3 ml) containing 1 μmole of nucleotide (ATP or AMP-PNP), 1.1 μmoles of $MgCl_2$, 0.75 μmole of TPN (Sigma), 22 μmoles of glucose, 0.10 mmole of Tris·Cl (pH 8.0), 0.01 ml (1.7 units) of glucose 6-phosphate dehydrogenase (Boehringer), and 0.5 unit of hexokinase (crystalline, Sigma) were monitored at 340 mμ and 25°. With ATP this system gave $\Delta A_{340\,m\mu}$ of 0.60/min. With AMP-PNP, the absorbancy at 340 mμ increased to 0.130 in 2 hr and to 0.20 in 4 hr. This apparent low activity was, however, the result of the slow oxidation of glucose alone by G-6-P-dehydrogenase since the nucleotide free control gave identical absorbancy changes, even after 22-hr reaction time. Similar results with glucose alone and this enzyme have been observed by Beutler and Morrison (1967). These results indicate that AMP-PNP is ineffective as a phosphate donor for glucose with hexokinase or for AMP with myokinase.

Discussion

Enough is now known to make a comparison of the chemical and physical properties of PCP_i, PNP_i, and PP_i with respect to what is known about AMP-PCP, AMP-PNP, and ATP. As pointed out by Irani and Callis (1961), as the bridging atom of the simple diphosphate compounds becomes more electronegative, *i.e.*, CH_2 to NH to O, the attached phosphate group becomes a stronger acid. Likewise, the last pK_a of AMP-PCP, AMP-PNP, and ATP decreases from 8.4 to 7.7 to 7.1, respectively. Although these latter pK_a values were not determined under exactly the same conditions, the overall trend is as expected. Thus, in cases where similar net charge is important and the pH must be near neutrality, AMP-PNP should be favored over AMP-PCP as an analog.

The affinity constants of AMP-PNP and AMP-PCP for Ca^{2+}, Mg^{2+}, and Mn^{2+} are, in general, a factor of 2–2.5 times larger than the corresponding values for ATP at the same pH. Thus, when AMP-PCP and AMP-PNP do not react with a given enzyme, this lack of reactivity is not due to the inability of the analogs to bind divalent metal ions. It remains to be seen if the predominant complex formed for each metal ion is the same for each analog as it is with

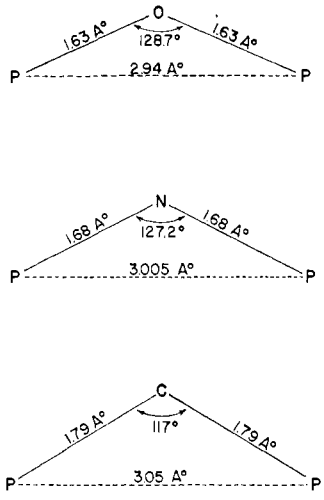


FIGURE 3: Comparison of the P–X–P bond angles and P–X bond distances for PP_i, PNP_i, and PCP_i taken from Larsen *et al.* (1969). The data for PCP_i are from F. Lovell (personal communication, 1967) and Lovell (1964).

ATP. For this reason investigation of the effects of divalent metals on the phosphorus nuclear magnetic resonance of these analogs should be informative. Such studies with ATP (Cohn and Hughes, 1962) have revealed that Mg^{2+} and Ca^{2+} interact principally with the β- and γ-phosphates of ATP while Mn^{2+} interacts with all three phosphate groups.

Figure 3 shows the P–X–P bond angles and P–X bond distances of pyrophosphate, imidodiphosphate, and methylenediphosphonic acid taken from Larsen *et al.* (1969). These values show the remarkable similarity of P–N–P and P–O–P bond angles and distances. They also show that although the P–C–P bond angle is more acute, 117° *vs.* 127° and 129° for PNP_i and PP_i, respectively, because of the longer P–C bond[2] the P–P distance is essentially the same for all three compounds. Thus it would be expected that the oxygens on adjacent phosphate groups would be essentially the same distance apart and X-ray analysis (Larsen *et al.*, 1969) verifies this. It is reasonable, therefore, to assume that the geometry and stability of the metal ion complexes would be similar. At least in the case of the stability of the divalent metal ion complexes with AMP-PCP, AMP-PNP, and ATP, this is true.

The acid stability of various PNP compounds including PNP_i has been studied by Quimby *et al.* (1960). They found that, in general, imido links are less stable than oxygen links in acid solutions but are equally, or more stable, in basic solutions. Interestingly, the quarter-lifetime of PNP_i at pH 1 and 60° was twice that at pH 3.5 and 60°. We have found PNP_i is more stable than PP_i in 1 N HCl and hydrolysis times three to four times longer than with PP_i are needed to determine acid-labile phosphate. This difference in acid stability led to the discovery of P–N–P bonds in "polyphosphate" from algal sources (Correll, 1966). We have found paper chromatography in pH 3.7 isobutric acid buffers causes AMP-PNP to break down partially and that neutral and basic chromatographic solvents were preferred. In general, once AMP-PNP was isolated as the sodium salt it was stable for several months if kept desiccated at −20°. What decomposition did occur was believed to have originated during the work-up of the column fractions, possibly due to triethylamine-catalyzed hydrolysis. AMP-PNP solutions near neutral pH in the presence and absence of Ca^{2+} and Mg^{2+} were stable (<2% decomposition) for periods of several hours at 25° (Yount *et al.*, 1971) and as shown by the control reactions for the alkaline phosphatase reactions in this paper.

So far, AMP-PNP will not replace ATP with any of the kinases tested. These include myokinase and hexokinase. In general, with the exception of *E. coli* alkaline phosphatase (see below) enzymes which cleave the β,γ-phosphate linkage of ATP are without effect on AMP-PNP. As shown in the accompanying paper (Yount *et al.*, 1971) neither heavy meromyosin nor myosin will detectably cleave (<2%) this analog in 17 hr under conditions in which comparable concentrations of ATP are 90% cleaved in 10 min. AMP-PNP is also not cleaved by plasma membrane ATPases (M. Rodbell, 1970, personal communication) or by mitochondrial ATPases. It is, however, translocated by mitochondria some 40% as well as ATP (M. Klingenberg, 1970, personal communication). Thus the replacing of the terminal bridge oxygen with an NH group allows AMP-PNP to retain the structural characteristics of ATP but prevents enzymic cleavage by a variety of enzymes.[3]

So far, the only enzyme found which will cleave the β,γ-imido bond of AMP-PNP is *E. coli* alkaline phosphatase. This enzyme is known to hydrolyze ATP completely to adenosine and P_i (Heppel *et al.*, 1962). With AMP-PNP, however, it stops after cleaving only the terminal phosphate group. The cleavage of a P–N bond extends the substrate specificity of this enzyme in that previously it was found not to be active against phosphoramidate-type substrates (Lazdunski and Lazdunski, 1969). Generally, any substitution for the oxygens of the phosphate group prevent enzymic attack. For example, thiophosphate esters (Eckstein, 1966) and a γ-fluoro analog of ATP (B. Haley and R. Yount, 1970, unpublished results) are not substrates for this enzyme.

Some enzymes which cleave ATP between the α- and β-phosphates, however, attack AMP-PNP. Snake venom phosphodiesterase and certain of the amino acid activating enzymes (Papas and Case, 1970) utilize AMP-PNP as a substrate. Adenylyl cyclase from plasma membranes also

[2] The longer P–C bond of PCP_i *vs.* the P–O bond distance of POP_i has been used to explain the unusual tight binding of AMP-PCP to formyl tetrahydrofolate synthetase (Curthoys and Rabinowitz, 1970). AMP-PCP binds ten times more tightly than ATP to this enzyme, a finding contrary to most previous results with this analog. This tight binding is explained by suggesting that since the C–P bond is 10% longer than the O–P bond that part of the binding energy contributed by the γ-phosphate of ATP may be used to stretch the O–P bond into the transition state required for bond cleavage and reaction with formate.

[3] The use of AMP-PNP to reveal the allosteric effects of ATP should not be overlooked. For example, the enzyme CTP synthetase catalyzes the reaction (eq 1) and can use either glutamine or ammonia as a nitrogen source.

$$UTP + ATP + Gln\ (or\ NH_3) \xrightarrow[GTP]{Mg^{2+}} CTP + ADP + P_i + Glu \quad (1)$$

The question was whether the enzyme utilized glutamine directly or first hydrolyzed it to glutamate and ammonia. By replacing ATP with AMP-PNP which was not a substrate it was shown that glutamine was hydrolyzed to ammonia before being utilized (A. Levitski and D. E. Koshland, Jr., 1970, personal communication). Since for this partial reaction to occur all the substrates and cofactors had to be present, only an ATP analog incapable of reacting could be used. AMP-PCP had little effect on this reaction.

converts AMP-PNP (but not AMP-PCP) to cyclic AMP and PNP_i (M. Rodbell, 1970, personal communication). With other of the amino acid activating enzymes, AMP-PNP is a competitive inhibitor but not a substrate. AMP-PNP is less than 1% as effective as ATP in the $[^{32}P]PP_i$-exchange assay of the mixed amino acid activating enzymes from *Bacillus brevis* (B. Haley and R. Yount, unpublished results). The reason for this low activity remains obscure.

Berman and Cohn (1970) have also found that AMP-PNP and AMP-PCP will not replace ATP with the enzyme phosphoenolpyruvate synthetase. In this case, a pyrophosphoryl-enzyme is believed to be formed by attack of the enzyme on the β-phosphate of ATP. Presumably this reaction will not take place if the bridge atom of the terminal pyrophosphoryl group of ATP is altered in any way.

Finally, the stability and properties of AMP-PNP means that other closely related nucleotides can be made; *e.g.*, those with different purine or pyrimidine bases or possibly those with α-β-imido linkages. The availability of both methylene and imido compounds of this type should greatly aid the enzymologist interested in defining ATP interactions in one of the many enzyme systems requiring, or affected by, nucleotide di- or triphosphates.

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