Ribonucleoside Phosphates *via* Phosphorimidazolidate Intermediates. Synthesis of Pseudoadenosine 5′-Triphosphate†

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ABSTRACT: The use of phosphorimidazolidate intermediates for the conversion of ribonucleoside 5′-monophosphates to diphosphates and 5′-diphosphates to triphosphates is described and several examples are cited. The specific phosphorylation of ribonucleoside 5′-diphosphates to afford the linear rather than branched triphosphates is demonstrated. Also reported is the successful synthesis of the branched isomer of adenosine 5′-triphosphate, ψATP, the attempted preparation of which was first reported in 1949. Certain chemical and enzymatic properties of ψATP are described, as is the conversion of ψATP to ATP *via* a presumed cyclic metaphosphate intermediate.

Current interest in the preparation of synthetic polynucleotides for physical and biochemical studies has necessitated the formulation of methods for the preparation of ribonucleoside 5′-phosphates, particularly at the diphosphate level. At least four methods have been reported for the conversion of ribonucleoside 5′-monophosphates to the corresponding diphosphates (Khorana, 1954; Michelson, 1958; Moffatt and Khorana, 1961; Cramer and Neunhoeffer, 1962; Scheit, 1968). Treatment of ribonucleoside 5′-phosphoromorpholidates with inorganic phosphate, *e.g.*, gave high yields of the corresponding diphosphates (Moffatt and Khorana, 1961). Preparation of the phosphoromorpholidate itself, however, was reported not to be convenient on a small scale (Hoard and Ott, 1965).

Hoard and Ott (1965) reported that the microscale synthesis of 5′-triphosphates from nucleoside 5′-phosphorimidazolidates proceeded in high yield and that the intermediate 5′-phosphorimidazolidates were accessible by condensation of a ribonucleoside 5′-monophosphate with 1,1′-carbonyldiimidazole. This conversion was based on the work of Cramer and Neunhoeffer (1962) and of Scheit (1968) who demonstrated the utility of phosphorodichloridates as intermediates in the synthesis of ribonucleoside 5′-di- and triphosphates. The utility of the phosphorimidazolidate moiety as the reactive group in the conversion of ribonucleoside 5′-monophosphates to diphosphates and ribonucleoside 5′-diphosphates to triphosphates is described in detail here. Also reported is the synthesis and some enzymatic properties of a branched chain adenosine 5′-triphosphate, pseudo ATP (**1**; ψATP), and its rearrangement to ATP *via* a presumed cyclic metaphosphate first postulated by Michelson and Todd (1949).

Materials and Methods

Adenylyl(3′→5′)adenosine, 1,1′-carbonyldiimidazole, ammonium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) monosodium monohydrate, and hexokinase were purchased from Sigma Chemical Co. [^{14}C]Phenylalanine (sp act. 182 Ci/mol) was purchased from International Chemical and Nuclear Corporation. The tRNA was isolated from *Escherichia coli* M72, from a tryptophan revertant of strain K12 with genotype $F^-Su^-lac^-try^+Sm^rT_1^rT_5^r$. The aminoacyl-tRNA synthetase was derived from the same strain of *E. coli* (Hecht *et al.*, 1971). Radiolabeled samples were counted on a Packard 3375 liquid scintillation spectrometer through the courtesy of Professor Daniel Kemp.

Synthesis of Nucleoside 5′-Diphosphates. (A) This method is analogous to the method utilized by Hoard and Ott (1965) for the synthesis of ribonucleoside 5′-triphosphates. To the nucleoside 5′-monophosphate (25 μmol) in 3 ml of pyridine and 0.5 ml of water was added 6.0 μl (25 μmol) of tributylamine. The solvent was evaporated under diminished pressure and the salt was rendered anhydrous by repeated evaporations of pyridine and *N*,*N*-dimethylformamide. The anhydrous salt was dissolved in 0.5 ml of dimethylformamide and 20.2 mg (125 μmol) of 1,1′-carbonyldiimidazole in 0.25 ml of dimethylformamide was added. The anhydrous reaction mixture was maintained overnight at room temperature and then treated with 8 μl (200 μmol) of methanol for 30 min to decompose the excess carbonyldiimidazole. Tributylammonium phosphate (125 μmol), prepared by analogy with tributylammonium pyrophosphate (Hoard and Ott, 1965), was added with mixing and the suspension maintained under desiccation for 24 hr. The solution was decanted, treated with methanol, and evaporated to dryness. Purification was effected by chromatography on a column of DEAE-cellulose (2.0 × 20 cm), eluting with a linear gradient of triethylammonium bicarbonate solution (2 l. total volume; 0–0.4 N; 15-ml fractions) at a rate of 175 ml/hr. The appropriate fractions were combined, desalted by repeated evaporations of portions of aqueous ethanol at 35° under diminished pressure, and then redissolved in water for a determination of spectrophotometric yield. The per cent conversions and yields are given in Table I. Each ribonucleoside 5′-diphosphate produced by this method was assayed for phosphate content by the method of Fischer (1961) and was found to have two phosphates per nucleoside (Table I).

(B) To 100 μmol of anhydrous mono(tri-*n*-butylammonium) phosphate in 0.5 ml of dimethylformamide was added 81 mg (500 μmol) of 1,1′-carbonyldiimidazole in 1 ml of dimethylformamide. The solution was maintained under anhydrous conditions overnight. Methanol (20 μl) was added and the solution was allowed to stand at room temperature for 30 min. This solution was then added, with vigorous mixing, to a solution containing 20 μmol of the anhydrous mono(tri-*n*-butylammonium) salt of the ribonucleoside 5′-monophosphate in 0.8 ml

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TABLE I: Per Cent Conversions and Yields of Ribonucleoside 5′-Diphosphates from the Corresponding Monophosphates.

Monophosphate	% Conversion[a]	% Yield[a,b]	Phosphate Analysis[c]
Method A			
AMP	80	93	2.0
UMP	82	93	1.9
GMP	69	83	2.1
CMP	78	90	2.1
IMP	80	91	1.9
dAMP	73	89	2.0
dCMP	82	90	2.0
6-Thiopurine ribonucleotide	83	91	2.1
Method B			
AMP	79	91	2.0
IMP	75	89	2.1
dAMP	71	90	2.0

[a] Determined spectrophotometrically on pure, desalted products which were redissolved in H_2O. [b] Conversion corrected for the amount of recovered starting material. [c] Number of phosphates per ribonucleoside, as determined by the method of Fischer (1961).

TABLE II: Per Cent Conversions and Yields of Ribonucleoside 5′-Triphosphates from the Corresponding Diphosphates.

Diphosphate	% Conversion[a]	% Yield[a,b]	Phosphate Analysis[c]
Method A			
ADP	65	89	3.1
IDP	65	93	2.9
dADP	63	91	3.1
Method B			
ADP	55	84	3.0
IDP	58	89	3.0
dADP	53	90	3.1

[a] Determined spectrophotometrically on pure, desalted products which were redissolved in H_2O. [b] Conversion corrected for the amount of recovered starting material. [c] Number of phosphates per ribonucleoside, as determined by the method of Fischer (1961).

of dimethylformamide. The anhydrous suspension was maintained at room temperature for 24 hr and then filtered. The filtrate was treated with an equal volume of methanol and then evaporated to dryness under diminished pressure. Chromatography on a column of DEAE-cellulose (2.0 × 20 cm), eluting with a triethylammonium bicarbonate gradient (2 l. total volume; 0–0.4 N; 15-ml fractions) at a rate of 175 ml/hr, afforded the final product after desalting by repeated evaporations of portions of aqueous ethanol at 35° under diminished pressure. The compound was redissolved in water for a determination of spectrophotometric yield. The per cent conversions and yields are given in Table I. The phosphate content of each diphosphate was verified by phosphate analysis according to the method of Fischer (1961) (Table I).

Synthesis of Ribonucleoside 5′-Triphosphates from Diphosphates. (A) To the anhydrous mono(tri-*n*-butylammonium) salt of the ribonucleoside 5′-diphosphate (20 μmol) was added 0.8 ml of dry dimethylformamide. To this was added 16.2 mg (100 μmol) of 1,1′-carbonyldiimidazole in 0.5 ml of dimethylformamide. The mixture was allowed to stand at room temperature overnight. Methanol (5 μl) was added and, after 30 min, anhydrous mono(tri-*n*-butylammonium) phosphate (100 μmol) in 0.8 ml of dimethylformamide was added, while the reaction mixture was agitated vigorously. The resulting suspension was maintained under desiccation for 24 hr and then filtered. The filtrate was treated with methanol and evaporated to dryness. Purification was effected on a DEAE-cellulose column (2.0 × 20 cm), eluting with an ammonium bicarbonate gradient (2 l. total volume; 0–0.8 N; 15-ml fractions) at a rate of 175 ml/hr. The fractions containing the desired product were combined, desalted by repeated evaporations of water at 50° under diminished pressure, and redissolved in water for a determination of spectrophotometric yield. The per cent conversions and yields are given in Table II. The presence of three phosphate groups in each molecule was verified by phosphate analysis according to the method of Fischer (1961) (Table II).

(B) To 100 μmol of anhydrous mono(tri-*n*-butylammonium) phosphate in 0.5 ml of dimethylformamide was added 81 mg (500 μmol) of 1,1′-carbonyldiimidazole in 1 ml of dimethylformamide. The anhydrous solution was maintained overnight at room temperature and was then treated with 20 μl of methanol. The solution was permitted to stand for 30 min and was then added, with vigorous mixing, to a solution containing 20 μmol of the anhydrous mono(tri-*n*-butylammonium) salt of the ribonucleoside 5′-diphosphate in 0.8 ml of dimethylformamide. The suspension was maintained under anhydrous conditions for 24 hr and then filtered. The filtrate was treated with methanol, concentrated under diminished pressure, and purified by chromatography on a DEAE-cellulose column (2.0 × 20 cm), eluting with an ammonium bicarbonate gradient (2 l. total volume; 0–0.8 N; 15-ml fractions) at a rate of 175 ml/hr. The fractions containing the desired compound were combined and desalted by repeated evaporations of water at 50° under diminished pressure. The phosphate content of each product was verified by phosphate analysis according to the method of Fischer (1961) (Table II). Per cent conversions and yields are given in Table II.

Synthesis of Pseudo-ATP (ψATP; Adenosine 5′-α,β,β′-Triphosphate) (1). To 121 mg (0.27 mmol) of adenosine 5′-phosphordiimidazolidate, prepared by analogy with the procedure of Cramer and Schaller (1961), was added 10 ml of dry dimethylformamide. This solution was treated with 1.40 g (5 mmol) of mono(tri-*n*-butylammonium) phosphate in 5 ml of dimethylformamide and the resulting mixture was stirred at room temperature for 2 days. The mixture was filtered and the filtrate was concentrated to afford a residue which was purified by chromatography on a DEAE-cellulose column (3.0 × 22 cm), eluting with an ammonium bicarbonate gradient (2 l.; 0–0.5 M; 15-ml fractions) at a rate of 225 ml/hr. The fractions containing the desired compound were combined and desalted by repeated evaporations of water at 40° under diminished pressure; yield ~11 mg (7%) of pseudo-ATP; 114 mg (89%) of adenosine 5′-diphosphate. The results of the phosphate analysis for this compound are given in Table III.

Rearrangement of Pseudo-ATP to ATP. To 20 mg (50 μmol) of ψATP, converted to the anhydrous di(tri-*n*-butylammonium) salt, was added 162 mg (1 mmol) of 1,1′-carbonyldiimidazole and 1.0 ml of dimethylformamide. The solution was maintained under anhydrous conditions for 2 days, then

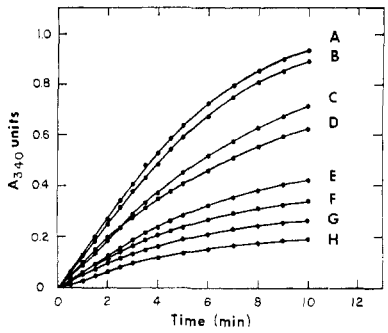


FIGURE 1: Generation of NADPH during the incubation of glucose, hexokinase, glucose-6-phosphate dehydrogenase, and variable amounts of ATP. Curves A, C, E, and G were generated in the presence of authentic ATP at final concentrations of 0.467, 0.187, 0.0935, and 0.0467 mM, respectively. Curves B, D, F, and H were generated in the presence of synthetic ATP at final concentrations of 0.37, 0.148, 0.074, and 0.037 mM, respectively.

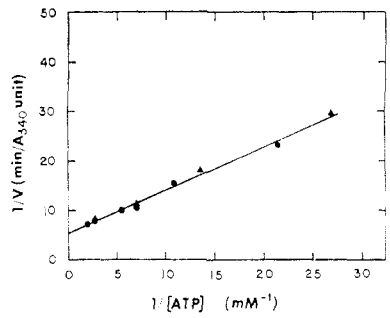


FIGURE 2: Graph of the inverse of the initial reaction velocities for the generation of NADPH (as in Figure 1) *vs.* the inverse of ATP concentration for authentic (●) and synthetic (▲) samples of ATP.

treated with water, concentrated to dryness, and assayed by chromatography on DEAE-cellulose.

Enzymatic Assays. AMINOACYLATION OF *E. coli* tRNA. The following reactions are involved.

$$E + AA + ATP \rightleftharpoons E \cdot AA \sim AMP + PP_i$$

$$E \cdot AA \sim AMP + tRNA \rightleftharpoons AA\text{-}tRNA + AMP + E$$

The buffer solution had the following composition: 0.1 M ammonium piperazine-*N*,*N*-bis(2-ethanesulfonic acid) monosodium monohydrate (pH 7.0), 0.1 M potassium chloride, 15 mM magnesium chloride, and 0.5 mM EDTA. [^{14}C]Phenylalanine (59 Ci/mol) was added to a concentration of 34 mM. To 0.6 ml of this buffer was added 0.5 ml of triphosphate solution (varying from 15 to 0.075 mM) and 0.02 ml of crude *E. coli* aminoacyl-tRNA synthetase solution (Hecht *et al.*, 1971). The reaction was initiated by the addition of 0.1 ml of tRNA solution (containing approximately 40 mg/ml of unfractionated *E. coli* tRNA). Aliquots (0.1 ml) were removed after 1, 3, 5, 10, and 20 min and applied to paper disks which had been soaked with 0.1 ml of 0.05 M cetyltrimethylammonium bromide in 1% aqueous acetic acid solution. The disks were washed extensively with 1% acetic acid, dried, placed in vials containing 5 ml of scintillation fluid, and counted in a liquid scintillation spectrometer at an efficiency of 70% with a standard deviation of 1%. Over a 200-fold variation in concentration, the synthetic ATP gave essentially identical results with those obtained for authentic ATP at each of the five time points examined.

PHOSPHORYLATION OF GLUCOSE. This assay was carried out according to the method of Secrist *et al.* (1972). Standard curves were generated using known concentrations of synthetic and authentic ATP (Figure 1). In addition, initial velocities were calculated for each of the standard curves (Figure 2).

ATTEMPTED PHOSPHORYLATION OF ADENYLYL(3′→5′)ADENOSINE. Adenylyl(3′→5′)adenosine (1 mg; 1.67 μmol) was converted to the pyridinium salt by evaporation of an aqueous pyridine solution, and the salt was rendered anhydrous by repeated evaporations of pyridine and then dimethylformamide. The anhydrous salt was dissolved in 100 μl of dimethylformamide and treated with a solution containing 10 μmol of the carbonyldiimidazole adduct of mono(tri-*n*-butylammonium) phosphate in 100 μl of dimethylformamide. The resulting suspension was allowed to stand at room temperature for 24 hr. Filtration afforded a solution which was treated with methanol and evaporated to dryness. Chromatography on a DEAE-cellulose column (2.0 × 20 cm), eluting with a triethylammonium bicarbonate gradient (2 l. total volume; 0–0.3 N; 15-ml fractions) at a rate of 175 ml/hr, afforded quantitative recovery (spectrophotometric yield) of a singly charged species, presumably ApA.

TABLE III: Phosphate Analysis of ψATP.[a]

Compd	Amount Analyzed (A_{260} Units)	OD[b] (A_{660} Units)
5′-AMP	5.0	0.14
5′-ADP	5.0	0.28
5′-ATP	5.0	0.44
ψATP	5.0	0.43

[a] Determined by the method of Fischer (1961). [b] Of resulting Molybdenum Blue solution.

Results

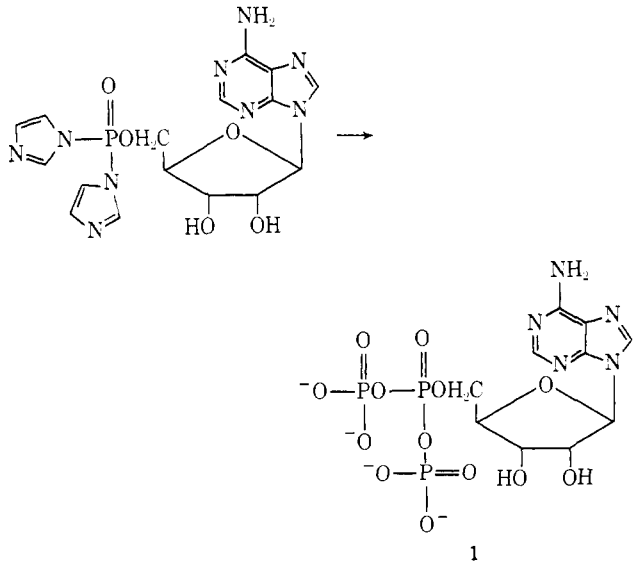
The per cent yields and conversions for various ribonucleoside 5′-monophosphates to the corresponding diphosphates are given in Table I. The yields obtained by method A, that is by the condensation of the phosphorimidazolidate of the monophosphate with the mono(tri-*n*-butylammonium) salt of phosphoric acid, were consistently about 90%, except for the slightly lower yield obtained with guanosine 5′-monophosphate. The yields obtained by condensation of the imidazolidate of phosphoric acid with the mono(tri-*n*-butylammonium) salt of the corresponding ribonucleoside 5′-monophosphates (method B) were about the same, although the per cent conversion was slightly lower. Most of the conversions were carried out on a scale which utilized 50–100 μmol of the starting nucleotides, but much smaller (~1 μmol) and larger scale conversions were also effected with similar results.

The conversion of three ribonucleoside 5′-diphosphates to the corresponding ribonucleoside 5′-triphosphates was effected in a similar fashion. Again, the yields associated with methods A and B were both about 90%, although the former method afforded a better per cent conversion. The conversion of ribonucleoside 5′-diphosphates to the corresponding 5′-triphosphates by this procedure could ostensibly afford either the straight or branched chain ribonucleoside 5′-triphosphates (*e.g.*, ATP or ψATP (**1**)). On the basis of pK_a values for known phosphate esters, a branched chain ribonucleoside 5′-tri-

phosphate would be expected to be more fully protonated at pH 7 than the corresponding linear ribonucleoside 5′-triphosphate and hence to elute more quickly from an anion exchange column in a salt gradient. At low pH, the branched 5′-triphosphate might be expected to behave similarly to adenosine 5′-diphosphate. Therefore, the finding that the synthetic ATP had the same chromatographic properties as authentic ATP at pH 3 and 7 suggested that the synthetic material consisted entirely of the straight chain isomer.

Because the straight and branched chain triphosphates might be expected to behave very differently in enzymatic conversions, the enzymatic properties of the ribonucleoside 5′-triphosphates prepared by methods A and B, relative to those of ATP, were assayed in two different systems. The first system measured the ability of the triphosphates to promote the transfer of phenylalanine to its cognate tRNA in the presence of unfractionated aminoacyl-tRNA synthetase. Adenosine 5′-triphosphate is converted to adenosine 5′-monophosphate during this process, so that utilization of a branched chain 5′-triphosphate presumably would have to involve alteration of the normal degradative process for the triphosphate. No appreciable difference was observed between synthetic and authentic ATP. The second enzyme utilized for the assay of the triphosphates was hexokinase, which uses 1 equiv of ATP in the transformation of glucose to glucose 6-phosphate. The course of the reaction was followed spectrophotometrically by the appearance of NADPH, formed by the action of glucose-6-phosphate dehydrogenase on glucose 6-phosphate. Again, as may be seen from Figures 1 and 2, no difference in activity was apparent between the synthetic and authentic samples of adenosine 5′-triphosphate. Thus both the chemical and enzymatic data suggested that the synthetic triphosphates were linear rather than branched.

To assure that the branched chain triphosphate, had it been present, could not have substituted for ATP in the enzymatic conversions, an authentic sample of the branched chain triphosphate (pseudo-ATP) was prepared. This was accomplished by treatment of the phosphorodiimidazolidate of adenosine 5′-monophosphate with an excess of the mono(tri-*n*-butylammonium) salt of phosphoric acid. The procedure



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afforded pseudo-ATP in addition to much larger amounts of adenosine 5′-diphosphate. The identity of the former, obtained in 7% yield, was verified by phosphate analysis and

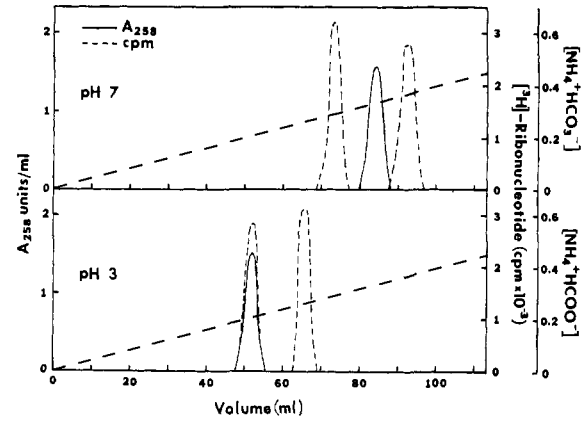


FIGURE 3: Chromatography of ψATP (—) on DEAE-cellulose in the presence of [^{3}H]ADP and ATP (- - -) at pH 7 and 3.

by its chromatographic and enzymatic properties. Specifically, phosphate analysis according to the method of Fischer (1961) indicated the presence of 3 equiv of phosphate/equiv of adenosine (Table III). Treatment of ψATP with crude snake venom (Tris-HCl, pH 8.6) resulted in complete degradation to adenosine, indicating attachment of the phosphates through C-5′. As anticipated chromatography on DEAE-cellulose (Figure 3) indicated pseudo-ATP to be intermediate in charge between ADP and ATP at pH 7, but essentially identical with ADP at pH 3. In terms of the behavior of ψATP in enzymatic conversions, its utilization as an energy source in the presence of tRNA, crude aminoacyl-tRNA synthetase, and amino acid resulted in very low levels of tRNA aminoacylation, relative to that promoted by ATP. It should be noted that in the presence of the crude synthetase solution, AMP was equally as effective as ψATP in promoting aminoacylation. and both were much less active than ADP. This suggested that aminoacylation due to ψATP probably resulted from its enzymatic conversion to ATP in low yield. Incubation of glucose, hexokinase, and glucose-6-phosphate dehydrogenase in the presence of ψATP did not afford detectable levels of NADPH. However, pseudo-ATP did act as a weak inhibitor of ATP utilization in this system. An attempt was made to convert ψATP to ATP *via* the cyclic metatriphosphate intermediate **2**, postulated by Michelson and Todd (1949) and Smith and Khorana (1958) as a precursor to ATP in the synthesis of the triphosphate from AMP. Treatment of ψATP with an excess of 1,1′-carbonyldiimidazole, followed by hydrolytic work-up, afforded ATP as one of the reaction products. ψATP itself was found to be stable in neutral, aqueous solution at temperatures up to 40°; decomposition to a mixture of ADP and AMP was obtained upon treatment with water at 100° for 2 hr (Figure 4). A more complete description of the enzymatic properties of ψATP will be published elsewhere (J. W. Kozarich *et al.*, manuscript in preparation).

To assess the utility of phosphorimidazolidate intermediates in the synthesis of 5′-di- and triphosphates using oligonucleotides as substrates, the synthesis of the branched chain isomer of adenylyl(3′→5′)adenosine was attempted. Treatment of the presumed phosphorimidazolidate of ApA with 5 equiv of tributylammonium phosphate afforded only starting material. This was consistent with the work of Hoard and Ott (1965), which indicated the formation of pppTpT, d-pppTpC, pppTpTpT, and d-pppApT from the respective 5′-monophosphates, to the apparent exclusion of branched chain products, and suggests the general applicability of this approach to conversions involving oligonucleotides.

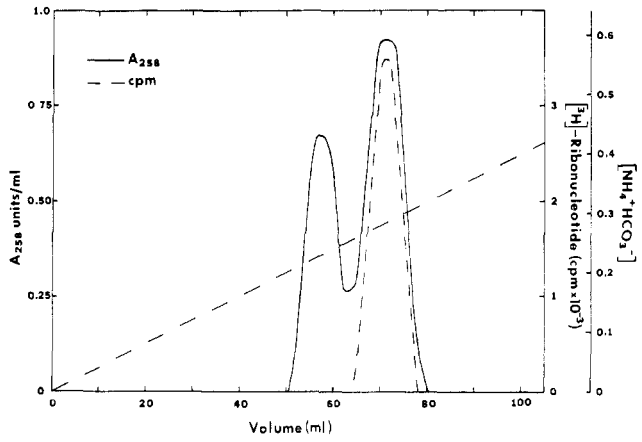
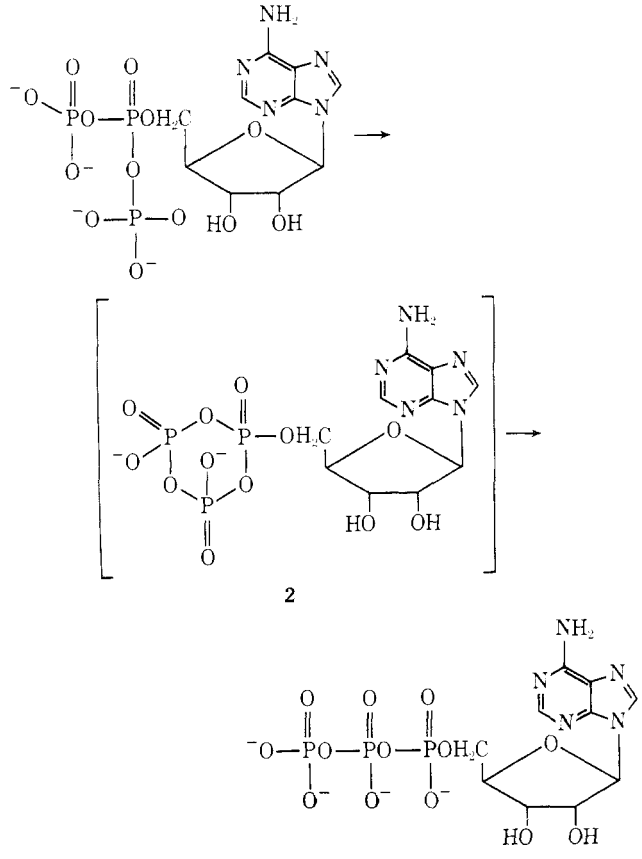


FIGURE 4: Chromatography of ψATP on DEAE-cellulose (pH 7.0) after treatment with boiling water for 2 hr. A portion of [^{3}H]ADP (- - -) was added as a marker immediately prior to the chromatography.



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Discussion

The use of di- and triphosphates as substrates for enzymatic conversions and for the synthesis of polynucleotides suggests a need for an efficient chemical method of nucleoside 5′-phosphate synthesis, particularly one which may be applicable to work on a microscale. This is especially true for the preparation of phosphate esters of unusual modified ribonucleosides, which may not be accessible *via* enzymatic synthesis. The use of phosphorimidazolidates as intermediates in a generalized method of ribonucleoside 5′-phosphate synthesis was suggested by the work of Cramer and Neunhoeffer (1962). Additional evidence, as presented here and by Hoard and Ott (1965), indicates that this method of synthesis is efficient and applicable to work on a small scale.

The direct conversion between adenosine 5′-diphosphate and adenosine 5′-triphosphate parallels the conversion mediated by several enzymes, *e.g.*, hexokinase, creatine phosphokinase, and pyruvate kinase, and might well be useful in connection with studies of the interaction of nucleotide analogs with these enzymes. The conversion also affords a chemical method for the synthesis of γ-^{32}P-labeled nucleoside 5′-triphosphates (S. M. Hecht and J. W. Kozarich, manuscript in preparation), which supplements the method described by Wehrli *et al.* (1965) and may prove to be a useful alternative to enzymatic methods (Penefsky, 1967; Post and Sen, 1967), particularly for the production of radiolabeled ribonucleoside 5′-phosphate analogs (Barrio *et al.*, 1973).

The synthesis of ψATP was first attempted by Michelson and Todd in 1949. They condensed the disilver salt of adenosine 5′-monophosphate with an excess of dibenzyl chlorophosphonate and obtained only ATP, rather than ψATP. They concluded that the initially formed ψATP had isomerized to ATP *via* cyclic metaphosphate **2**. Smith and Khorana (1958) postulated the intermediacy of a cyclic metaphosphate in the phosphorylation of UMP to explain the accumulation of UTP at longer reaction times at the expense of other ribonucleoside phosphates.

In agreement with the work of Michelson and Todd (1949), we found that the conversion of ribonucleoside 5′-diphosphates to the corresponding 5′-triphosphates proceeded without the formation of branched chain triphosphates, suggesting the more difficult formation of these isomers. Indeed, the deliberate synthesis of ψATP was accompanied by the formation of substantial amounts of adenosine 5′-diphosphate. However, contrary to the suggestion of Michelson and Todd (1949), ψATP was found to be reasonably stable, reflecting no decomposition upon standing in neutral, aqueous solution for extended periods of time. Decomposition of ψATP to a mixture of ADP and AMP was effected by treatment with boiling water for 2 hr (Figure 4). Consistent with the suggestions of Michelson and Todd (1949) and of Smith and Khorana (1958), hydrolytic work-up of the cyclic metatriphosphate **2**, presumably formed by the action of 1,1′-carbonyldiimidazole on ψATP, resulted in the formation of ATP.

Acknowledgment

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Identification of the Product Excited States During the Chemiluminescent and Bioluminescent Oxidation of *Renilla* (Sea Pansy) Luciferin and Certain of Its Analogs†

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ABSTRACT: We have synthesized a *Renilla* luciferin [3,7-dihydro-2-benzyl-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*a*]-pyrazin-3-one] which has an activity equal to that of native luciferin in producing light with *Renilla* luciferase. It will react with luciferase to produce the same color of light with the same quantum yield as that produced by native luciferin. Both the synthetic and the native compounds produce a bluish chemiluminescence when dissolved in dimethylformamide. Oxygen is required for this chemiluminescence, and carbon dioxide and oxyluciferin are the products. The structure of the latter compound has been confirmed by synthesis. Detailed examination of the chemiluminescence and fluorescence emission data of synthetic luciferin, synthetic oxyluciferin and a number of synthetic analogs of each has provided evidence that the monoanion of oxyluciferin represents the electronic excited state responsible for the emission during chemiluminescence. We also find that just as in chemiluminescence the bioluminescent oxidation of luciferin by luciferase and oxygen leads to the formation of carbon dioxide and oxyluciferin. The data also suggest that bioluminescence emission arises from the electronic excited state of the oxyluciferin monoanion. There is sufficient evidence to suggest that *Renilla* luciferin or a structure very similar to it is involved in the bioluminescence of all coelenterates that have been carefully examined including the jellyfish *Aequorea*. The data also suggest that the products of those bioluminescent reactions are analogous to if not identical with those reported here.

The structure and chemical synthesis of a biologically active form of *Renilla* (Sea Pansy) luciferin [3,7-dihydro-2-methyl-6-(*p*-hydrophenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one] has recently been reported (Hori and Cormier, 1973a). This compound (I in Figure 1) was found to be 10% as active as native luciferin in producing light with *Renilla* luciferase. Furthermore, the spectral characteristics of I were found to be identical with those of native luciferin as judged by their absorption, fluorescence, and bioluminescence emission characteristics. According to mass spectral data, native luciferin (II in Figure 1) differs from the synthetic compound (I) by the replacement of methyl with a rather bulky group designated R (Hori and Cormier, 1973b). From the mass data we deduced that replacement of the methyl group of I by benzyl would result in a compound similar to but not identical with native luciferin. That is, native luciferin contains a group of 107 mass units attached to the benzyl moiety. However, the synthetic benzyl compound (III in Figure 1) is reported here to be fully active in the bioluminescence assay. Like I it is also active in producing light with luciferases isolated from a variety of bioluminescent coelenterates (Hori and Cormier, 1973b).

We recently observed that *Renilla* luciferin will produce a brilliant bluish luminescence when dissolved in aprotic solvents such as dimethylformamide. Using the biologically active synthetic luciferins we have studied this chemiluminescence and provided evidence for the nature of the products formed and for the identification of the electronic excited state responsible for this emission. Using this information we also provide evidence that the same species is responsible for emission during bioluminescence using *Renilla* luciferase. A preliminary report of some of these data has been published (Hori *et al.*, 1973).

Materials and Methods

Materials. All reagents used for synthetic and purification procedures were the best commercial grades available.

Luciferase Preparation. We have noted recently that the amount of enzymatically active luciferase can vary from preparation to preparation depending on the isolation techniques employed. We have thus developed a new isolation procedure, superior to the one reported previously (Karkhanis and Cormier, 1971), and which results in a higher specific activity luciferase. The specific activity of the current preparations are about 50×10^{12} $h\nu$ sec^{-1} mg^{-1}. The enzyme appears homogeneous by various criteria including chromatographic, disc gel electrophoretic, and sedimentation equilibrium measure-

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