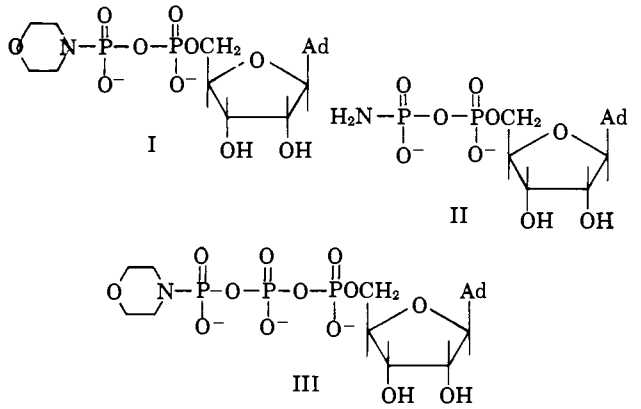
A solution of dicyclohexylcarbodiimide (5 mmoles) in *t*-butyl alcohol was added dropwise over 9 hr. to a refluxing solution of the morpholine salt of ADP (1.0 mmole) and morpholine (2.4 mmoles) in 50% aqueous *t*-butyl alcohol. After a further 12 hr. under reflux, the mixture was evaporated and partitioned between water and ether. The water phase was separated by ion-exchange chromatography on Dowex-2 (HCO_3^-) using a gradient of triethylammonium bicarbonate giving adenosine 5′-phosphoromorpholidate[4] (19%) and P¹-adenosine-5′ P²-(4-morpholino)pyrophosphate (I, 81%). Chromatographically pure I was isolated as its calcium salt. *Anal.* Calcd. for $C_{14}H_{20}N_6O_{10}P_2Ca\cdot 2H_2O$: C, 29.48; H, 4.24; N, 14.73; P, 10.86. Found: C, 30.36; H, 4.62; N, 14.50; P, 10.68.

As expected, the P–N bond formed involved exclusively the β-phosphorus of the ADP. This follows from the known reactivity of phosphoric acid monoesters, and resistance of phosphoric acid diesters,[4] toward formation of phosphoramidates and could be confirmed by enzymatic means. In a similar reaction in which morpholine was replaced by ammonium hydroxide, the interesting analog P¹-adenosine-5′ P²-aminopyrophosphate (II) was prepared from ADP in 45% yield. Direct extension of the reaction to the synthesis of a terminally activated ATP was also achieved. Thus the reaction of ATP, morpholine, and dicyclohexylcarbodiimide followed by ion-exchange chromatography gave P¹-adenosine-5′ P³-(4-morpholino)triphosphate (III) in 72% yield. *Anal.* Calcd. for $C_{14}H_{20}N_6O_{13}P_3Ca_{1.5}\cdot 4H_2O$: C, 23.84; H, 4.00; N, 11.94; total P:labile P:adenosine, 3.00:2.00:1.00. Found: C, 24.14; H, 3.97; N, 11.06; total P:labile P:adenosine, 2.96:1.94:1.00.



The synthesis of ATP-γ-P^{32} from I utilized the known reactivity of phosphoromorpholidates in pyrophosphate syntheses.[4,5] Thus, the reaction of the 4-morpholine N,N′-dicyclohexylcarboxamidine salt of I (0.1 mmole) with tributylammonium orthophosphate (0.3 mmole containing 2 mc. of P^{32}) in rigorously anhydrous dimethyl sulfoxide (2 ml.) for 45 hr. at 35° gave a mixture of products which were separated by ion-exchange chromatography on DEAE cellulose (HCO_3^-). Minor amounts of unreacted I, AMP, ADP, and AP_4 were cleanly separated from ATP-γ-P^{32} (65%) which was isolated as the chromatographically homogeneous sodium salt with a specific activity of 3 μc. per μmole. Controlled partial degradation of this product (1 μmole) with *E. coli* alkaline phosphatase (40 min. at 35° with 20 μl. of dialyzed enzyme) gave a mixture of adenosine, AMP, ADP, and unreacted ATP, which were separated on a micro-DEAE cellulose (HCO_3^-) column. While the starting material and the unreacted ATP had specific activities of 75,400 c.p.m. per optical density unit at 259 mμ, the AMP contained no isotope and the ADP fractions only 740 c.p.m. per optical density unit. This minor unidentified activity was chromatographically shown not to be due to ADP itself. At any rate, greater than 99% of the P^{32} was located in the γ-position.

The apparent generality, specificity, and good yield of these reactions makes attractive the synthesis of other labeled and unlabeled compounds which will be described shortly.

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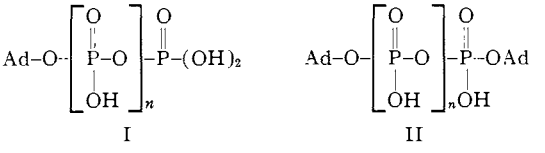
The Dismutation of Nucleoside 5′-Polyphosphates

Sir:

Several years ago it was reported that the 4-morpholine-N,N′-dicyclohexylcarboxamidine salt of adenosine 5′-triphosphate (ATP) was unstable in anhydrous pyridine at room temperature, and a mixture of the 5′-mono-, di-, tri-, and tetraphosphates was present within a few days.[1] A related degradation of the initially formed nucleoside 5′-triphosphates to the di- and monophosphates during syntheses in pyridine also has been reported by several workers.[2] We have now examined this reaction in detail and are prepared to offer a tentative mechanism.

Ion-exchange chromatography on DEAE-cellulose (HCO_3^-) of the products resulting from storage of a rigorously anhydrous solution of tetrakis(tributylammonium)adenosine 5′-triphosphate (1 mmole) in pyridine (10 ml.) for 3.5 days gave a complex pattern of eight well-resolved peaks. The main products were a homologous series of nucleoside 5′-polyphosphates (I, n = 0, 1, 2, 3, 4, 5, 6) containing from one to seven



phosphate groups.[3] Further chromatography of many of these peaks showed the presence of a second series of compounds, in much smaller amounts (5–10% of the total peak), which has been characterized by analytical and enzymatic means as α,ω-di(adenosine-5′) polyphosphates (II, n = 1, 2, 3, 4, 5, 6). These latter compounds are completely resistant to the action of *E. coli* alkaline phosphatase but are rapidly split by purified venom phosphodiesterase to AMP and an adenosine 5′-polyphosphate which then is cleaved more slowly to AMP and an inorganic polyphosphate. A homologous series of inorganic polyphosphates also is

(4) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

(5) S. Roseman, J. J. Distler, J. G. Moffatt, and H. G. Khorana, *ibid.*, **83**, 659 (1961); J. G. Moffatt and H. G. Khorana, *ibid.*, **83**, 663 (1961).

(1) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

(2) (a) M. Ikehara and E. Ohtsuka, *Chem. Pharm. Bull.* (Tokyo), **11**, 435 (1963); (b) J. Zemlicka, J. Smrt, and F. Sorm, *Collection Czech. Chem. Commun.*, **28**, 241 (1963).

(3) The following abbreviations are used: AMP, ADP, ATP, AP_4, AP_5, AP_6, and AP_7 for the adenosine 5′-mono-, di-, tri-, tetra-, penta-, hexa-, and heptaphosphates; AP_2A, AP_3A, AP_4A, AP_5A, AP_6A, and AP_7A for the appropriate α,ω-diadenosine 5′-polyphosphates; *e.g.*, AP_5A is P¹,P⁵-di-(adenosine-5′)pentaphosphate.

present in small amounts in the above dismutation reaction. Solutions of ADP or AP_4 in anhydrous pyridine also undergo rapid dismutation to products containing both longer and shorter polyphosphate chains. The reaction is not, however, unique to nucleoside 5′-polyphosphates since a similar dismutation readily occurs with *p*-nitrobenzyl triphosphate[4] in pyridine to give the corresponding mono- to pentaphosphates.

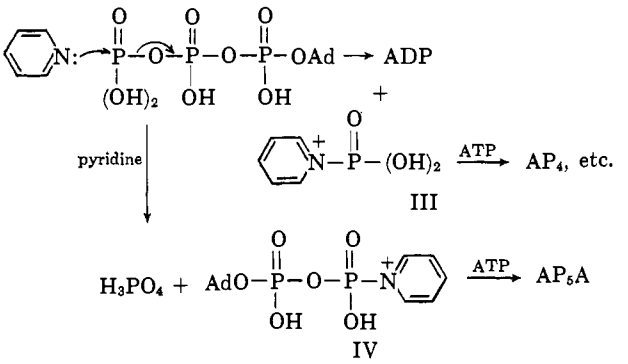
The addition of five molar equivalents of tributylammonium phosphate or pyrophosphate to the reaction mixture greatly changes the product pattern and the ATP then is converted quite rapidly into ADP and then AMP with very little formation of higher polyphosphates. This explains the degradation of initially formed product during attempted synthesis of ATP from adenosine 5′-phosphoromorpholidate and an excess of pyrophosphate in pyridine.[1,2]

The addition of small amounts of water (1–5%) to the pyridine also results in a marked decrease in the amounts of higher polyphosphates (ATP and higher) and an accumulation of ADP and AMP. A similar effect is observed on conducting the reaction in pyridine containing a large molar excess of an alcohol. In the presence of 100 molar equivalents of *p*-nitrobenzyl alcohol, for example, after 6 days, the reaction mixture contained 20% AMP, 61% ADP, 15% ATP, 3% AP_4, and one molar equivalent of *p*-nitrobenzyl phosphate. In the presence of methyl alcohol, the accumulation of methyl phosphate was observed.

Some insight into the over-all path of the reaction was achieved through studies of the dismutation of specifically P^{32}-labeled ATP. Phosphorylation of 2′,3′-O-isopropylidene adenosine with P^{32}-cyanoethyl phosphate[5,6] gave P^{32}-AMP which was converted into α-P^{32}-ATP *via* reaction of the phosphoromorpholidate[1] with pyrophosphate in anhydrous dimethyl sulfoxide.[7] Dismutation of this material in pyridine gave the usual spectrum of products all having identical specific activities (c.p.m./adenosine) and giving P^{32}-AMP and no observable labeled inorganic phosphates on degradation with purified venom phosphodiesterase. Thus, the α-phosphorus atom is not separated from the adenosine moiety during dismutation. On the other hand, exclusively γ-P^{32}-ATP[8] gives rise to a mixture of nucleoside 5′-polyphosphates which, after ion-exchange separation, contain nearly equal amounts of P^{32} in the γ-, δ-, and ϵ-positions. Roughly 10% P^{32} was found in the β-phosphorus of ADP, but no label was present in the α-position. The distribution of label in the various phosphorus atoms was determined by controlled degradation of the nucleoside 5′-polyphosphate with *E. coli* alkaline phosphatase. Thus, for example, treatment of AP_4 (0.5 μmole) with 2.2 μg. of the purified enzyme at pH 8 for 50 min. at 35° gave roughly equal amounts of adenosine, AMP, ADP, ATP, and AP_4 which were cleanly separated on a micro ion-exchange column. The relative specific activities of the last three compounds were 0.16:1.00:2.04 showing equal labeling of the γ- and δ-phosphorus atoms and relatively little in the β-position. Thus it is clear that the higher polyphosphates are built primarily by transfer of the terminal phosphate from ATP (or perhaps from other nucleoside polyphosphates).

In contrast to the ready reaction of ATP in pyridine, the γ-monomethyl ester of ATP, which was prepared in good yield by the reaction of triethylammonium ATP in methanol with dicyclohexylcarbodiimide, was largely unchanged, and independently synthesized[9] AP_3A and AP_4A were completely inert.

A definitive clue as to the mechanism of the reaction comes from the observation that whereas ATP shows very similar patterns of dismutation in pyridine and in β- and γ-picolines, it is completely stable in α-picoline. A similar, but less dramatic, stability is found in quinoline as compared with isoquinoline. Since the various picolines are of very similar basicities, these observations are taken to indicate a steric hindrance of the dismutation reaction in α-picoline. We propose a mechanism for the reaction involving nucleophilic attack of the pyridine nitrogen upon the terminal phosphorus atom of ATP (or of other polyphosphates) with formation of ADP and of the presumably very reactive phosphorylating species III which then reacts with unchanged ATP to form AP_4. In a similar way III can react with AP_4 to give AP_5, etc. The inhibition of the normal build up of higher polyphosphates upon the addition of excess inorganic orthophosphate or *p*-nitrobenzyl alcohol is explained by preferential attack on III by these species with the formation of the observed inorganic pyrophosphate and *p*-nitrobenzyl phosphate, respectively. The appearance of small amounts of AP_2A, AP_3A, AP_4A, etc., must indicate the occurrence of a less favored attack by pyridine upon the α- or β-phosphorus of ATP with release of an inorganic phosphate and formation of an activated nucleotide, *e.g.*, IV. The latter can then form diesters by reaction with a nucleoside polyphosphate.



From a synthetic point of view, it is important that the presently described dismutation reactions can be avoided by use of solvents such as dimethyl sulfoxide in which ATP is stable,[7,8] rather than the commonly used pyridine. Under these conditions many complicating features of synthetic reactions involving nucleoside polyphosphates can be obviated.

(4) Prepared by reaction of *p*-nitrobenzyl phosphoromorpholidate with pyrophosphoric acid in anhydrous dimethyl sulfoxide.

(5) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

(6) An improved synthesis of this compound will be described shortly (K. E. Pfitzner and J. G. Moffatt, in preparation).

(7) J. G. Moffatt, *Can. J. Chem.*, in press.

(8) D. L. M. Verheyden, W. E. Wehrli, and J. G. Moffatt, *J. Am. Chem. Soc.*, **85**, 1253 (1964).

(9) J. R. Reiss, D. L. M. Verheyden, and J. G. Moffatt, unpublished results.

(10) Financial support for this work from the "Stiftung für Stipendien auf dem Gebiete der Chemie" (Switzerland) is gratefully acknowledged.

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Hydrogen Bonding Effects on Triplet Energy Transfer in Solution

Sir:

We wish to report a pronounced hydrogen bonding effect on the triplet energy transfer in the benzophenone–biacetyl system. Previous authors working on