Dismutation Reactions of Nucleoside Polyphosphates. IV. A Mechanism for the Dismutation Reaction[1]

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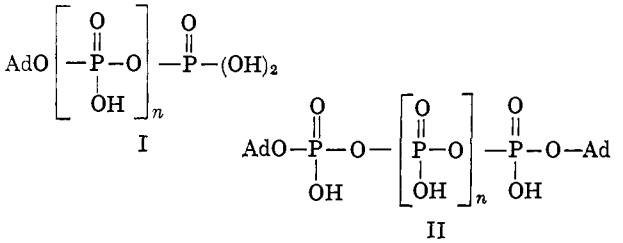
Further experiments designed to cast light upon the mechanism of the dismutation reactions of nucleoside polyphosphates in pyridine are described in this paper. Dismutation of α-^{32}P-labeled ATP gave a series of adenosine polyphosphates containing isotope in only the α-positions. The reaction of γ-^{32}P-ATP in pyridine, however, gave a similar series of polyphosphates in which the γ- and all more remote phosphate groups were roughly equally labeled. A small amount of isotope was also present in the β-position of these products. Reaction of ATP in pyridine in the presence of a large excess of p-nitrobenzyl alcohol led to degradation of the ATP to ADP and AMP accompanied by the accumulation of an almost stoichiometric amount of p-nitrobenzyl phosphate. The γ-monomethyl ester of ATP, unlike the parent compound, was almost completely stable in anhydrous pyridine. While ATP underwent typical dismutation in β- and γ-picolines, it was virtually unaffected in α-picoline, 2,6-lutidine, and 2,4,6-collidine. On the basis of these observations a mechanism is presented for the dismutation reaction involving nucleophilic attack by pyridine at the γ-phosphorus of ATP, giving ADP and the covalently bonded N-phosphoryl-pyridinium ion V. The latter species is a reactive phosphorylating agent and condenses with ATP to form AP$_4$, etc. A modified mechanism is necessary to explain the formation of minor amounts of diadenosine polyphosphates.

In part I of this series[3] we described the general characteristics of the "dismutation" reaction which nucleoside 5′-polyphosphates undergo when dissolved in anhydrous pyridine. Primarily, the reaction consists of the transformation of the starting material into a variety of products containing both longer and shorter polyphosphate chains. Thus, the tributylamine salt of ATP[4] (I, n = 2) is rapidly converted into a series of adenosine 5′-polyphosphates of type I containing from 1 to 7 phosphate groups (I, n = 0–6) and also, to a minor extent, into a series of diadenosine polyphosphates of type II with n = 0–5.



I

II

This type of reaction was found to be characteristic of monoesterified polyphosphates in general and occurred equally well with ADP and AP$_4$ as well as with *p*-nitrobenzyl triphosphate. The reaction was, however, inhibited by the presence of excess ortho- or pyrophosphate anions or of moderate amounts (5–10%) of water. Under these conditions extensive degradation of ATP occurred, but was characterized by the formation of only products containing shorter polyphosphate chains. In this paper we will describe further studies designed to shed light upon the mechanism of the dismutation reaction. A preliminary account of some of these studies has already appeared.[5]

We have recently described the development of completely chemical syntheses of nucleoside 5′-triphosphates labeled with ^{32}P in exclusively the α-, β-, or γ-phosphate groups.[6] The availability of these materials has allowed us to study the fate of the various specific phosphate groups during the dismutation of ATP. Thus, the tributylamine salt of α-^{32}P-ATP was dissolved in anhydrous pyridine and allowed to stand at room temperature for 4 days. Paper chromatography then revealed the presence of ^{32}P-labeled AMP, ADP, ATP, AP$_4$, and AP$_5$, as well as a trace amount (0.3% of the total isotope) of ^{32}P-orthophosphate. The latter compound was conveniently separated from the nucleotides by preparative paper chromatography in solvent E, and the five nucleoside polyphosphates were then separated by chromatography in solvent A.[7] The specific activities of the various nucleotides are shown in Table I.

Table I. Specific Activities of the Products from the Dismutation of α-^{32}P-ATP

Compd.	%	Specific activity, c.p.m./μmole[a]
AMP	9	2.05×10^5
ADP	25	2.08×10^5
ATP	52	2.06×10^5
AP$_4$	12	2.04×10^5
AP$_5$	2	2.09×10^5

[a] Based upon ultraviolet absorption in 0.1 *N* HCl and using ϵ_{max} 15,100. Each compound gave a true adenosine spectrum.

(1) For part III see J. R. Reiss and J. G. Moffatt, *J. Org. Chem.*, in press.

(2) Syntex Postdoctoral Fellow, 1963–1964. Supported in part by the "Stiftung für Stipendien auf dem Gebiete der Chemie" (Switzerland), whose aid is gratefully acknowledged.

(3) D. L. M. Verheyden, W. E. Wehrli, and J. G. Moffatt, *J. Am. Chem. Soc.*, 87, 2257 (1965).

(4) The following abbreviations are used: AMP, ADP, ATP, AP$_4$, AP$_5$, etc., refer to the homologous series of adenosine 5′-mono-, di-, tri-, tetra- and pentaphosphates. AP$_2$A, AP$_3$A, AP$_4$A, and AP$_5$A refer to the homologous series of α,ω-di(adenosine-5′) polyphosphates. Thus, *e.g.*, AP$_5$A refers to P^1,P^5-di(adenosine-5′) pentaphosphate.

(5) W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *J. Am. Chem. Soc.*, 86, 1254 (1964).

(6) W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *ibid.*, 87, 2265 (1965).

(7) Subsequently we have found that ion-exchange chromatography on DEAE cellulose (HCO_3^-) provides a more convenient separation of orthophosphate from the adenosine polyphosphates even on less than a μmole scale.[6]

Table II. Chromatography of the Products from Dismutation of γ-^{32}P-ATP

Fraction no.	Total O.D.$_{260}$	% of total O.D.	Total c.p.m.[a]	% ^{32}P	C.p.m./μmole of adenosine[b]	Identification
40–51	34	10.0	3.84×10^5	2.9	...[c]	AMP + HPO_4^{2-}
72–86	83	24.4	5.60×10^5	4.3	1.04×10^5	ADP + some pyrophosphate
87–96	6	1.8	5.53×10^4	0.42	1.41×10^5	AP_3A
103–117	100	29.4	3.78×10^6	28.8	5.82×10^5	ATP
121–125	4	1.2	3.43×10^4	0.26	1.32×10^5	AP_4A
128–142	100	29.4	7.04×10^6	53.8	1.08×10^6	AP_4
153–163	12	3.5	1.28×10^6	9.7	1.64×10^6	AP_5

[a] Total ^{32}P content and specific activities were all determined during 1 day. [b] Specific activities were based upon adenosine content as determined by ultraviolet absorption in water using ϵ_{259} 15,400. [c] Autoradiography showed that none of the ^{32}P was in the AMP.

As can be seen from Table I the specific activities of all the nucleotides were identical within experimental error. This clearly indicates that at no time did the α-phosphate become separated from its covalently bound adenosine moiety and incorporated into a higher nucleoside polyphosphate. Had the α-phosphorus been involved in polyphosphate chain elongation then the specific activity of the AP_4 and AP_5 would have been distinctly higher. Confirmation that all the isotope in each product was still located exclusively in the α-position was obtained by enzymatic degradation. Thus, an aliquot of each product containing 1–2 $\times$ 10^4 c.p.m. was exhaustively degraded with phosphodiesterase-I from *Crotalus adamanteus* venom. In each case the product was completely degraded to AMP and an inorganic phosphate or polyphosphate, all of the isotope being found in the mononucleotide. Once again, had there been any isotope in other than the α-position, it would have appeared in the inorganic phosphate or polyphosphate following enzymatic cleavage. In this experiment we have ignored the trace amounts of α,ω-diadenosine polyphosphates which we know run together with the various adenosine polyphosphates in solvent A.[3] Their presence will have no effect upon the specific activity measurements, and upon complete degradation with phosphodiesterase-I they also give AMP and an inorganic polyphosphate.

The dismutation of γ-^{32}P-ATP was also studied in both pyridine and in a mixture of pyridine and dimethylformamide (1:1).[3] The products were separated both by paper chromatography (as in the case of α-^{32}P-ATP, above) and by ion-exchange chromatography on DEAE cellulose (HCO_3^-). The latter method proved to be more convenient, and the results of such a separation applied to the products from the dismutation of 22 μmoles of γ-^{32}P-ATP with an initial specific activity of 1 μcurie/μmole are shown in Figure 1. Quantitative data on the specific activities of the various compounds are given in Table II.

From Figure 1 and Table II it can be seen that less than 3% of the total isotope is released as orthophosphate during the dismutation reaction. The AMP produced was, as expected, completely free of isotope and only a very small amount of radioactivity was found in the ADP. Autoradiography of the pooled ADP peak showed that roughly half of the ^{32}P was actually present as ^{32}P-pyrophosphate, leaving only 2–3% of the total radioactivity in ADP itself. The ATP, AP_4, and AP_5, however, were strongly radioactive and, assuming that the ATP contains only one radioactive phosphate group, the AP_4 and AP_5 were respectively shown to contain 1.86 and 2.83 isotopic phosphorus atoms. The distribution of the isotope in the various phosphorus atoms of the ATP, AP_4, and AP_5 was then determined, using the analytical method we have previously described.[6] Thus, approximately 0.5 μmole of each compound was partially hydrolyzed with *E. coli* alkaline phosphatase, giving a series of degradation products containing fewer phosphate groups. These hydrolysis products were then separated on microcolumns of DEAE cellulose (HCO_3^-), and the specific activity of each product was determined.

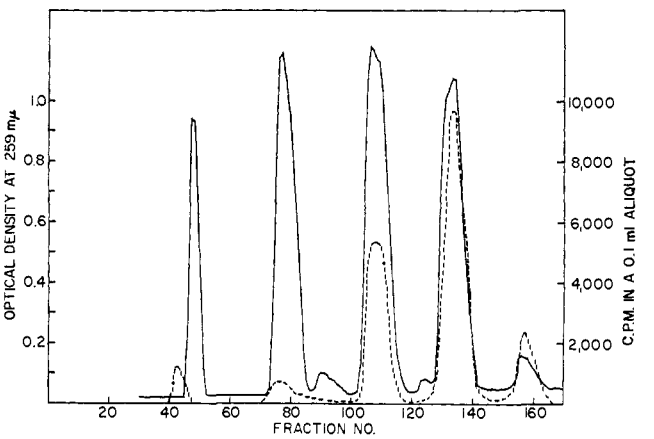


Figure 1. Ion-exchange chromatography of the products from the dismutation of γ-^{32}P-ATP in pyridine–dimethylformamide (1:1). ———, optical density at 259 mμ; ---------, c.p.m. in a 0.1-ml. aliquot. See Experimental for details of the chromatography.

Under these conditions orthophosphate was cleanly separated from the nucleotides, and the specific activity of the resulting AMP gave a direct measure of the ^{32}P in the α-phosphate of the original adenosine polyphosphate. Similarly, the specific activity of the ADP minus that of the AMP gave the specific activity of the original β-phosphate, etc. The results of these calculations are given in Table III, the distribution of the ^{32}P in each phosphate group being expressed as a percentage of the total isotope content.

From Table III it can be seen that a consistently small amount (less than 10%) of isotope was present in the β-phosphate of each dismutation product. The remaining radioactivity was, however, fairly equally distributed among the γ-, α-, and ϵ-phosphate groups present in the particular species. These experiments quite clearly demonstrate that the formation of AP_4 occurs, to a large degree, through transfer of the

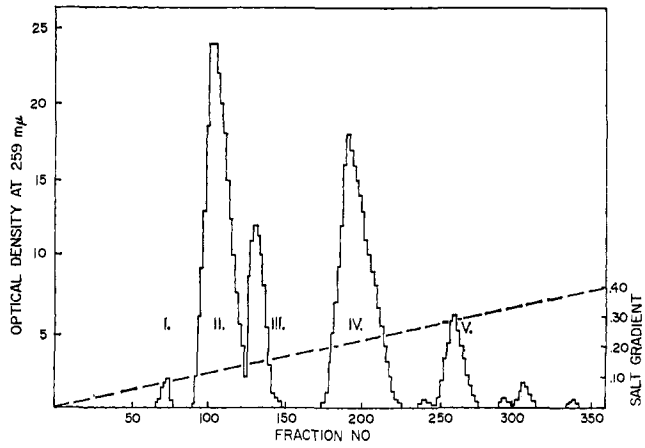


Figure 2. Ion-exchange chromatography of the products from dismutation of ATP in the presence of 100 equiv. of *p*-nitrobenzyl alcohol. See Experimental for details of the chromatography and identification of the various peaks.

terminal phosphate of the original ATP onto another molecule of ATP giving AP_4 and ADP. Thus

$$APPP^* + APPP^* \longrightarrow APPP^*P^* + APP$$

Similarly, AP_5 must arise through reaction of AP_4 with either ATP or a second molecule of AP_4. Thus

$$APPP^*P^* + APPP^* \longrightarrow APPP^*P^*P^* + APP$$

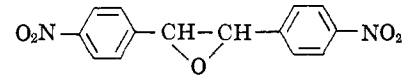
This suggests that in anhydrous pyridine the terminal phosphate of a nucleoside polyphosphate becomes activated in such a way as to permit its transfer to the end of another polyphosphate chain. We have already shown[3] that the addition of 5 molar equiv. of ortho- or pyrophosphate relative to the ATP in a dismutation reaction almost completely blocks the formation of AP_4 and higher adenosine polyphosphates and leads only to extensive degradation to ADP and AMP. A similar effect is produced by the addition of 5–10% water to the pyridine.[3] Since the presence of excess phosphate anions leads to products containing only shorter polyphosphate chains, it is suggested that the activated terminal phosphate of the ATP is no longer covalently bonded to its parent molecule. If the activation were present in the intact ATP molecule, then in the presence of excess phosphate anions we would expect to see increased amounts of AP_4, AP_5, etc. The same conclusion can be reached from the fact that only 5% of the total products in a dismutation mixture are α,ω-di(adenosine-5′) polyphosphates.[1,3] Considerably larger amounts of these compounds would be expected if a terminally activated ATP was permitted to react with a mixture of excess adenosine polyphosphates.

Table III. Isotope Distribution in the Major Products from Dismutation of γ-^{32}P-ATP

Product	% total ^{32}P in each phosphate group				
	α	β	γ	δ	ϵ
ATP	...	9.5	90.5	...	...
AP_4	...	8.7	49.6	41.7	...
AP_5	...	3.3[a]	43.4	25.3	28.0

[a] This figure is probably somewhat low since very little ADP accumulated and the error in determination of the adenosine content was considerable.

In an attempt to trap the activated phosphate fragment we examined the dismutation of ATP in anhydrous pyridine containing 100 molar equiv. of recrystallized *p*-nitrobenzyl alcohol. After 7 days at room temperature the pyridine solution was filtered from a small amount of crystalline product (see below), and after ether extraction of the *p*-nitrobenzyl alcohol the nucleotides were separated by ion-exchange chromatography as shown in Figure 2. Identification of the peaks showed that the ATP (peak V) had largely disappeared and had been converted primarily to ADP (peak IV). Some AMP (peak III), but almost no AP_4 and other higher polyphosphates, was also present. The compound in peak II was isolated in crystalline form and shown to be *p*-nitrobenzyl phosphate which was identical with a synthetic sample.[3] A fairly precise stoichiometry could be demonstrated since the production of AMP and ADP corresponding to the removal of 0.85 mmole of phosphate was accompanied by the formation of 0.89 mmole of *p*-nitrobenzyl phosphate. A small amount of *p*-nitrobenzyl diphosphate[6] was also present in peak IV. The highly insoluble crystalline material that separated during the dismutation reaction was identified by elemental analysis, nuclear magnetic resonance spectra, and physical constants as *trans*-4,4′-dinitrostilbene oxide (III).[8] This compound was also produced in low yield upon storage



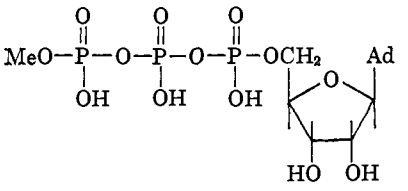
III

of a solution of *p*-nitrobenzyl alcohol in pyridine alone. It has previously been obtained by alkaline treatment of *p*-nitrobenzyl chloride in the presence of *p*-nitrobenzaldehyde[8a] or by alkaline treatment of *p*-nitrobenzylsulfonium ion.[8b] The mechanism of its formation in the present case is not clear since the *p*-nitrobenzyl alcohol was recrystallized prior to use and was free of impurities such as *p*-nitrobenzaldehyde as determined by thin layer chromatography. The yield of III from *p*-nitrobenzyl alcohol was 2%, and this level of impurities would have been readily detected in the starting material.

A similar dismutation of ATP in pyridine containing 10% methanol was shown to lead to the accumulation of monomethyl phosphate which was identified chromatographically. Also, dismutation of ATP in the presence of 5 molar equiv. of ^{32}P-labeled orthophosphate led to the accumulation of nearly 20% of the total radioactivity as ^{32}P-pyrophosphate. An appreciable amount of ^{32}P-tripolyphosphate was also formed, and smaller amounts of isotope became incorporated into ADP and ATP. These experiments all confirm the presence of an activated phosphate fragment originating with the terminal phosphate of ATP and capable of being attacked by suitable nucleophilic species in the reaction.

Since the original attack appears to be located at the terminal phosphorus of ATP, we have examined the

(8) (a) S. B. Hanna, Y. Iskander, and Y. Riad, *J. Chem. Soc.*, 217 (1961); (b) I. Rothberg and E. R. Thornton, *J. Am. Chem. Soc.*, **86**, 3302 (1964).



IV

reaction of the closely related γ-monomethyl ester of ATP[6] (IV) in anhydrous pyridine.

After 3 days at room temperature, ion-exchange chromatography showed that 95% of the starting material was unchanged, the remaining 5% being principally AMP. Hence, substitution of the terminal phosphate group effectively blocks the dismutation reaction. A similar stability of the dinucleoside polyphosphates AP_3A and AP_4A has also been reported.[1]

Definitive information as to the mechanism of the dismutation reaction comes from studies on the stability of ATP in solvents closely related to pyridine. The tributylamine and 4-morpholine N,N′-dicyclohexylcarboxamidine[9] salts of ATP were found to have very little solubility in α-, β-, and γ-picoline. The addition of 10% dimethyl sulfoxide, however, led to homogeneous solutions, and we have previously shown that ATP is stable in this solvent alone. As a control, however, we also studied the stability of ATP in pyridine containing 10% dimethyl sulfoxide and found that the dismutation reaction was considerably repressed, less starting material having reacted and less AP_4 being produced. Interestingly, it was found that no AMP was produced under these conditions. The dismutation pattern was nevertheless clear and the results of ion-exchange separation of the products following 3-days storage of ATP in pyridine and α- and β-picoline (each containing 10% dimethyl sulfoxide) are shown in Figure 3. The patterns in pyridine and β-picoline are clearly similar (γ-picoline behaves identically), but almost no reaction was observed in α-picoline. Paper chromatographic examination of similar reactions in 2,6-lutidine and 2,4,6-collidine showed that no reaction had occurred in these solvents either. The base strengths of α- and β-picolines (pK values of 5.97 and 5.68, respectively[10]) are quite similar, and since pyridine[10] (pK = 5.17) and γ-picoline[10] (pK = 6.02) behave similarly to β-picoline but have pK values with considerable variance, we cannot directly relate the efficacies of these various solvents in promoting dismutation to their basicities. Since only those pyridine derivatives having methyl groups adjacent to the heterocyclic nitrogen (α-picoline, 2,6-lutidine, and 2,4,6-collidine) fail to produce dismutation, we attribute their failure to steric hindrance of the nitrogen atom. A similar, although less pronounced, effect was noted in comparing the stabilities of ATP in quinoline and isoquinoline. After 3-days reaction in quinoline, 50% of the ATP was recovered unchanged while under the same conditions in isoquinoline only 24% remained. Once again the basic nitrogen is more sterically hindered in quinoline than in isoquinoline.

The various effects noted above can be largely rationalized by a reaction mechanism involving nucleo-

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

(10) H. C. Brown and X. R. Michm, *ibid.*, 77, 1723 (1955).

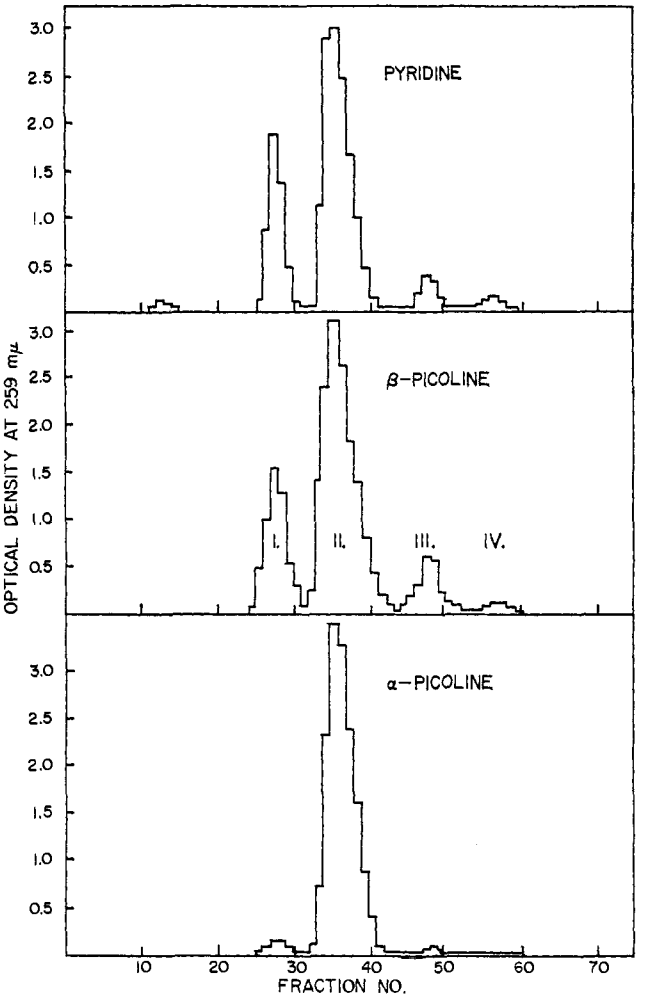
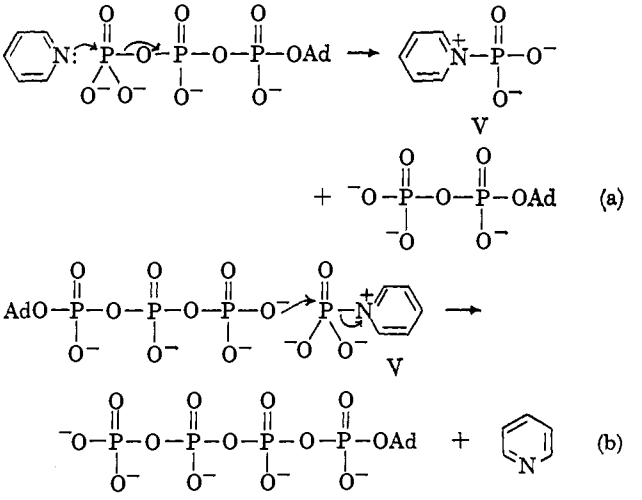


Figure 3. Ion-exchange chromatography of ATP in pyridine and in α- and β-picolines containing 10% dimethyl sulfoxide. See Experimental for details of the chromatography.

philic attack by the pyridine nitrogen upon the terminal phosphate of ATP giving ADP and the covalently bonded N-phosphorylpyridinium ion V. Thus

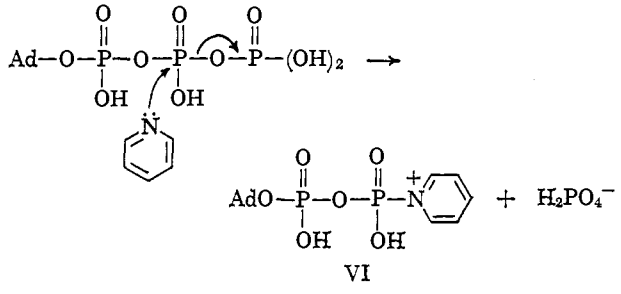


Electronically V is similar to the protonated species that has been proposed as the active intermediate in pyrophosphate syntheses *via* the phosphoramidate method.[9,11] Possessing a full positive charge, how-

(11) V. M. Clark and S. G. Warren, *Proc. Chem. Soc.*, 178 (1963).

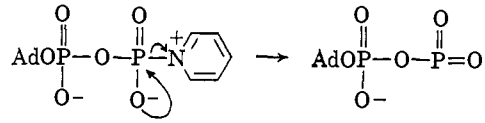
ever, V would be expected to be an extremely active phosphorylating agent that would readily react with monosubstituted phosphate derivatives to form new pyrophosphate bonds. During the initial stages of the dismutation reaction the most abundant reactive nucleophile present would be the terminal phosphate of another ATP molecule, and reaction between these two species would proceed as in (b) to give adenosine tetraphosphate and pyridine. Thus, by summation of reactions a and b, we see the net conversion of 2 molecules of ATP into 1 each of ADP and AP_4. Similar terminal attacks by pyridine upon ADP and AP_4 can also occur, and the nucleophilic species in reaction b will be largely determined by the relative abundances of the various nucleoside polyphosphates present. Thus, as AP_4 accumulates it too can react with V to form AP_5, etc. The observed inhibition of the formation of products of increased polyphosphate chain length and the accumulation of AMP and ADP brought about by the addition of excess phosphate anions, alcohols, water, etc., can be readily explained by competitive attack by these species upon V with the formation of pyrophosphates, esters, and orthophosphate, respectively. The retardation of the dismutation reaction in the presence of 10% dimethyl sulfoxide can probably be attributed to solvation of the ATP which partially inhibits attack by pyridine. As seen in Figure 3 the presence of 10% dimethyl sulfoxide also leads to the production of decreased amounts of AP_4 and AP_5 relative to ADP. This probably can be explained by a relatively inefficient competitive nucleophilic attack by dimethyl sulfoxide upon the intermediate V, thereby partially blocking the synthesis of new pyrophosphate bonds.

The mechanism above adequately explains the formation of simple nucleoside polyphosphates but cannot lead to the observed small amounts of α,ω-diadenosine polyphosphates. The formation of these compounds must require the activation of the terminal phosphate in an intact nucleoside polyphosphate. This must indicate that to at least a minor extent[12] pyridine is capable of attacking the β-phosphorus of ATP with expulsion of orthophosphate and formation of β-activated ADP (VI). Thus



VI

The species VI can then be attacked by the various nucleoside polyphosphates present in the reaction to give a series of diadenosine polyphosphates. Thus, AMP will react with VI to give AP_3A while ADP will give AP_4A, etc. We cannot rule out the possibility that VI might collapse to a pyrophosphate derivative of monomeric metaphosphoric acid VII which could be the active phosphorylating species. Kinetic evidence has, however, been presented[11] suggesting that the analogous monomeric metaphosphate ester is not the active species during pyrophosphate formation from phosphoramidates.



VI VII

The only evidence that cannot be immediately reconciled with the above general mechanisms is the inertness of the γ-methyl ester of ATP in pyridine. In the presence of sufficient tributylamine to neutralize all the phosphate groups present, we would expect ATP to be completely ionized in pyridine. The γ-phosphate group in the γ-methyl ester of ATP, having only a single negative charge, might be expected to be more susceptible to nucleophilic attack than the corresponding phosphorus atom in ATP which bears two charges. It seems unlikely that this pronounced difference should be solely due to steric effects, and an elaboration of this point must await further experiments.

Finally, we have taken advantage of the information we have gained on the mode of phosphate transfer to demonstrate dismutation of tripolyphosphoric acid. Storage of an anhydrous pyridine solution of tributylammonium tripolyphosphate could be shown chromatographically to lead to the formation of orthophosphate, pyrophosphate, and a diffuse smear of higher polyphosphates. In view of the varying color yields with the molybdate spray, however, it was difficult to get any quantitative idea of the extent of dismutation. By conducting the above experiment in the presence of 10 molar equiv. of AMP it was possible to demonstrate the formation of ADP and ATP. These products were separated by ion-exchange chromatography, and by ultraviolet measurements it was shown that for each equivalent of tripolyphosphate used 0.84 equiv. of phosphate was incorporated into nucleotides. Adenosine 5′-phosphate itself remains completely unchanged in pyridine and, hence, the formation of ADP and ATP is a demonstration of the trapping of the activated fragment V originating from tripolyphosphate by AMP.

Recently another reaction involving selective chemical attack on the terminal phosphorus of ATP has been described. Acid-catalyzed treatment of ATP dissolved in methanol was shown to convert 30% of the γ-phosphorus into methyl phosphate.[13] On the other hand, ADP was quite inert under the same conditions.

Smith and Khorana[14] have referred to the conversion of ADP into AMP and ATP upon prolonged treatment with dicyclohexylcarbodiimide in aqueous pyridine and have suggested that AP_4A might be an intermediate in this reaction. The present study does not permit us to interpret readily this interesting result since the presence of a powerful dehydrating agent such as dicyclohexylcarbodiimide can be expected to lead to many types of reaction. It is quite reasonable to assume that AP_4A should be an initial product in this reaction but, since we have shown that AP_4A is itself

(12) Roughly 5% of the total products from dismutation of ATP in pyridine are diadenosine polyphosphates.

(13) J. Eichberg and R. M. C. Dawson, *Biochim. Biophys. Acta.*, 93, 425 (1964).

(14) M. Smith and H. G. Khorana, *J. Am. Chem. Soc.*, 80, 1141 (1958).

completely stable in pyridine, further carbodiimide-promoted activation steps must be involved prior to hydrolysis leading to AMP and ATP. Without further evidence it is premature to postulate structures for these more highly activated intermediates.

Experimental

General Methods. The chromatographic, electrophoretic, analytical, and enzymatic methods used are described in a preceding paper.[1] In addition to the paper chromatographic solvent systems A–D listed there, we have used solvent E, 1-butanol–acetic acid–water (5:3:2), to separate orthophosphate from adenosine nucleotides. Thin layer chromatography was done on Merck Silica-G containing Radelin type P-1 phosphor as previously described.[15] Measurements of ^{32}P were made on aluminum planchets using a Nuclear Chicago gas flow counter, and radioactivity on paper chromatograms was detected by autoradiography on Kodak medical X-ray film or by use of a Nuclear Chicago strip counter.

Dismutation of α-^{32}P-Adenosine 5′-Triphosphate. α-^{32}P-Labeled ATP[6] (3 μmoles, specific activity 3×10^5 c.p.m./μmole) was converted into the tetrakis(tri-*n*-butylammonium) salt as previously described[3] and dried by three evaporations with 2-ml. portions of anhydrous pyridine. It was finally dissolved in anhydrous pyridine (0.2 ml.) and stored at room temperature for 4 days. The solvent was then evaporated and residual pyridine was removed by several evaporations with 0.25-ml. portions of water. The products were then chromatographed as a 7-cm.-long streak in solvent E which separated some orthophosphate containing a total of 2180 c.p.m. (0.30% of the total ^{32}P) from the slower moving nucleotides. The combined nucleotides were eluted and rechromatographed as a 6-cm. streak in solvent A for 24 hr. The five well-resolved radioactive and ultraviolet absorbing bands were quantitatively eluted in small volumes of water, and the specific activity of each was determined. The very minor band corresponding to AP_5 was first rechromatographed in solvent C since, as directly obtained, its ultraviolet spectrum was not identical with that of an adenosine nucleotide. The results of the specific activity determinations are in Table I.

An aliquot of each product containing $1–2 \times 10^4$ c.p.m. (0.75–1.5 optical density units) was incubated with 20 μl. of purified phosphodiesterase-I from *Crotalus adamanteus* venom[16] and 5 μl. of 1 *M* Tris buffer (pH 8.5) at 37° for 7 hr. The entire sample was then chromatographed in solvent A and examined in a Nuclear Chicago strip scanner. In each case the radioactivity was located only in the AMP and no isotope could be detected in the various inorganic polyphosphates.

Dismutation of γ-^{32}P-Adenosine 5′-Triphosphate. The tetrakis(tri-*n*-butylammonium) salt of γ-^{32}P-ATP[6] (22 μmoles) with an initial specific activity of 1 μcurie/μmole and at least 99% of the isotope in the γ-position was prepared in the usual way.[3] After three evaporations with pyridine the residue was dissolved in anhydrous pyridine (0.6 ml.) and stored at room temperature for 3 days. An identical experiment was carried out using 0.6 ml. of anhydrous pyridine–dimethylformamide (1:1) as the final solvent. The solvent was evaporated to dryness *in vacuo* and the nucleotides were applied to a 1×65 cm. column of DEAE cellulose (HCO_3^-). After a thorough water wash elution was effected with a linear gradient of 3 l. of triethylammonium bicarbonate (0.005–0.40 *M*). The various fractions were followed by ultraviolet absorption and by radioactivity in 0.1-ml. aliquots, giving the results shown in Figure 1 and Table II. The various pooled fractions were carefully evaporated to dryness *in vacuo* and freed from residual salt by several evaporations with methanol. They were then dissolved in water and stored at pH 7 in the freezer. The products were identified by comparison with authentic standards in several chromatographic systems.[3]

Determination of the ^{32}P Distribution in Adenosine Polyphosphates. Aliquots (0.5 μmole) of the ATP, AP_4, and AP_5 peaks above were incubated at 37° with 50 μl. of 0.05 *M* Tris acetate buffer, pH 9.0, and 20 μl. of *E. coli* alkaline phosphatase for 30, 60, and 60 min., respectively. Water (1 ml.) and chloroform (1 ml.) were then added and the mixture was vigorously agitated for 5–10 min. and then centrifuged. The clear aqueous solution was directly applied to a 0.4×18 cm. column of DEAE cellulose (HCO_3^-), and after a thorough water wash elution was effected with a linear gradient of 160 ml. of triethylammonium bicarbonate[17] (0.005–0.40 *M*). The specific activity of the various peaks was then determined and from these figures the ^{32}P distributions shown in Table III were calculated as described in the text.

Dismutation of ATP in the Presence of p-Nitrobenzyl Alcohol. Tetrakis(tri-*n*-butylammonium)ATP (1 mmole) was prepared and rendered anhydrous in the usual way. It was then dissolved in pyridine (12 ml.) and mixed with 15.3 g. (100 mmoles) of freshly recrystallized (chloroform) *p*-nitrobenzyl alcohol. The clear solution was kept at room temperature for 7 days. A small crystalline deposit (220 mg., see below) was removed by filtration and after evaporation of most of the pyridine the residue was partitioned between water and ether. A further 94 mg. of the crystalline material above separated from the ether. The water layer was applied to a 3×38 cm. column of DEAE cellulose (HCO_3^-) and after a water wash the column was eluted with a linear gradient of 8 l. of triethyl ammonium bicarbonate (0.005–0.40 *M*). The elution pattern is shown in Figure 2. The most important peaks were as follows: peak II, 8000 optical density units at 275 mμ (0.86 mmole), was *p*-nitrobenzyl phosphate. After conversion to the free acid this gave 190 mg. of crystalline product, m.p. 150–155°, which had chromatographic and spectral properties identical with an authentic sample[3]; peak III (784 optical density units at 259 mμ) was mainly AMP contaminated with some *p*-nitrobenzyl phosphate; peak IV (7800 O.D.U. at 259 mμ) was ADP containing a trace of *p*-nitrobenzyl diphosphate[6]; peak V (1910

(15) K. E. Pfitzner and J. G. Moffatt, *J. Org. Chem.*, **29**, 1508 (1964).

(16) This enzyme, with a specific activity of 620 μmoles/hr. ml.$^{-1}$ toward *p*-nitrophenylthymidine 5′-phosphate, was provided by Dr. W. E. Razzell.

(17) For maximum accuracy it is advisable to distil carefully the triethylamine prior to formation of the bicarbonate solution. This reduces the background optical density to about 0.02 at 259 mμ and reduces the errors with peaks of low optical density.

O.D.U. at 259 mμ) was ATP; peak VI (353 O.D.U. at 259 mμ) was AP_4.

The crystalline material that separated from the original reaction was recrystallized from dioxane as pale yellow rhombohedrons, m.p. 202–203°, λ_{max}^{MeOH} 286 mμ. Its nuclear magnetic resonance spectrum in deuterated dimethyl sulfoxide[18] showed a benzylic singlet at 262 c.p.s. and two doublets (J = 9 c.p.s. each) centered at 463 and 496 c.p.s., typical of *para*-disubstituted benzenes (four aromatic protons per benzylic proton). This material was identified as *trans*-4,4′-dinitrostilbene oxide (III), lit. m.p.[8] 200–201°.

Anal. Calcd. for $C_{14}H_{10}N_2O_5$: C, 58.74; H, 3.52; N, 9.79; O, 27.95. Found: C, 58.72; H, 3.61; H, 3.61; N, 10.36; O, 28.33.

The Stability of the γ-Methyl Ester of ATP in Pyridine. Chromatographically pure γ-methyl ATP (3 μmoles) was converted into its tributylamine salt and dried by four evaporations with anhydrous pyridine. The final residue was dissolved in dry pyridine (0.5 ml.) and kept at room temperature for 3 days. The solvent was then evaporated and the products were chromatographed on a 0.5 × 20 cm. column of DEAE cellulose (HCO_3^-) using a linear gradient of 200 ml. of triethylammonium bicarbonate (0.005 to 0.40 *M*). Two peaks were obtained, the first being AMP (5%) and the second unreacted starting material (95%). Both products were characterized by paper chromatography in several solvents.

The Stability of ATP in α- and β-Picolines. Three samples of the 4-morpholine N,N′-dicyclohexylcarboxamidine salt of ATP[19] (3 μmoles each) were carefully dried by repeated evaporation with pyridine, and residual pyridine was removed by two evaporations with benzene. The final residues were separately dissolved in anhydrous dimethyl sulfoxide (0.01 ml. each), and pyridine, α-picoline, and β-picoline (0.09 ml.) were added, respectively. The clear solutions were kept at room temperature for 3 days and then separated by ion-exchange chromatography on 1 × 25 cm. columns of DEAE cellulose (HCO_3^-) using linear gradients of 200 ml. of triethylammonium bicarbonate (0.005 to 0.40 *M*). The results are shown in Figure 3. The peaks numbered I, II, and III are ADP, ATP, and AP_4, respectively, as determined by paper chromatography in solvents A, B, and C.

The Reaction of Tripolyphosphate and Excess AMP in Pyridine. The pentasodium salt of tripolyphosphoric acid[20] (3.7 mg., 10 μmoles) was converted into the pyridine salt with Dowex 50 (pyridinium) resin and dissolved in 80% pyridine together with AMP (35 mg., 100 μmoles). Tri-*n*-butylamine (0.1 ml., 0.42 mmole) was added and the clear solution was evaporated to dryness. After four evaporations with 5-ml. portions of pyridine the final residue was dissolved in 0.5 ml. of anhydrous pyridine and kept at room temperature for 3 days. The solvent then was evaporated and the products were chromatographed on a 1.0 × 40 cm. column of DEAE cellulose (HCO_3^-) using a linear gradient of triethylammonium bicarbonate (0.005 to 0.5 *M*). Three ultraviolet absorbing peaks were obtained. Peak I (95 μmoles) was AMP, peak II (6.0 μmoles) ADP, and peak III (1.2 μmoles) ATP. Each peak was identified chromatographically by comparison with standards.

(18) Obtained on a Varian A-60 spectrometer. Chemical shifts are measured in c.p.s. downfield from an internal standard of tetramethylsilane.

(19) This salt was prepared by addition of excess free base 4-morpholine N,N′-dicyclohexylcarboxamidine[9] to a solution of pyridinium ATP in methanol and precipitation with ether. We have shown separately that this material behaves identically with the tributylamine salt in its dismutation behavior in pyridine alone.

(20) A generous gift of the Victor Chemical Works, Chicago Ill.

Communications to the Editor

Cage Recombination of *t*-Butoxy Radicals

Sir:

The bimolecular interaction of alkoxy radicals is of fundamental importance in autoxidation and other free radical reactions.[1-3] For example, termination of autoxidation and hydroperoxide decomposition reactions have been written alternatively as the sequence[1b,4,5]

$$2ROO\cdot \longrightarrow 2RO\cdot + O_2 \quad (1)$$

$$2RO\cdot \longrightarrow ROOR \quad (2)$$

or as a single step[2b,3,6]

$$2ROO\cdot \longrightarrow ROOR + O_2 \quad (3)$$

in which eq. 3 may occur through a cyclic process[6]

$$\begin{array}{l} R{-}O\cdots O{-}R \\ \quad\;|\qquad\;| \\ \quad O{-}{-}O \end{array} \longrightarrow ROOR + O_2 \quad (4)$$

or a cage collapse[2b,5,7]

$$ROOOOR \longrightarrow [RO\cdot O_2\cdot OR]^{cage} \longrightarrow ROOR + O_2 \quad (5)$$

Although dialkyl peroxides have been detected in the products of hydroperoxide decompositions[8,9] and

(1) (a) C. Walling, "Free Radicals in Solution," John Wiley and Sons, Inc., New York, N. Y., 1957, Chapters 9 and 10; (b) p. 505 of ref. 1a.

(2) (a) P. D. Bartlett and T. G. Traylor, *J. Am. Chem. Soc.*, 85, 2407 (1963); (b) *Tetrahedron Letters*, No. 24, 30 (1960).

(3) (a) R. Hiatt, J. Clipsham, and T. Visser, *Can. J. Chem.*, 42, 2754 (1964); (b) R. Hiatt, unpublished data.

(4) E. R. Bell, J. H. Raley, F. F. Rust, F. H. Seubold, and W. E. Vaughan, *Discussions Faraday Soc.*, 10, 242 (1951).

(5) H. S. Blanchard, *J. Am. Chem. Soc.*, 81, 4548 (1959).

(6) C. A. McDowell and S. Sifniades, *Can. J. Chem.*, 41, 300 (1963).

(7) W. H. Richardson, *J. Am. Chem. Soc.*, 87, 1096 (1965).

(8) M. H. Dean and G. Skirrow, *Trans. Faraday Soc.*, 54, 849 (1956).

(9) C. Walling and L. Heaton, *J. Am. Chem. Soc.*, 87, 38 (1965).