Dismutation Reactions of Nucleoside Polyphosphates. III. The Synthesis of α,ω-Dinucleoside 5'-Polyphosphates[1]

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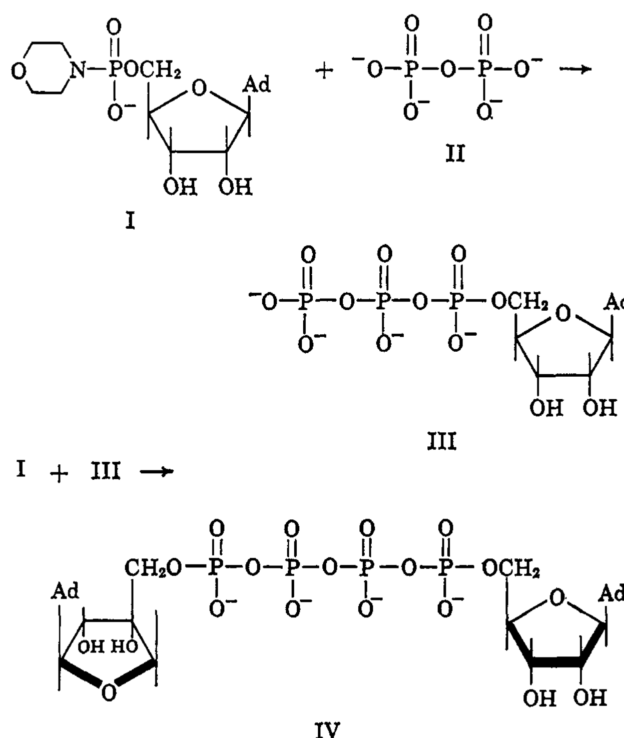
The reaction of adenosine 5'-phosphoromorpholidate and tributylammonium pyrophosphate (2:1) in anhydrous pyridine gives, as its major products, a series of α,ω-diadenosine polyphosphates. Of these P^1,P^3-di(adenosine-5')triphosphate, P^1,P^4-di(adenosine-5')tetraphosphate, and P^1,P^5-di(adenosine-5')pentaphosphate have been isolated and characterized by analytical and enzymatic methods. Smaller amounts of diadenosine pyrophosphate and of adenosine 5'-mono-, di-, tri-, tetra-, and pentaphosphates are also produced as well as a novel compound characterized enzymatically as P^1-(3'-phosphoryl adenosine-5')-P^4-adenosine-5'-tetraphosphate (VI). P^1,P^3-Di(adenosine-5')triphosphate was also synthesized in 37% yield from adenosine 5'-phosphoromorpholidate and orthophosphate (2:1). A similar reaction between thymidine 5'-phosphoromorpholidate and pyrophosphate (2:1) gave the analogous P^1,P^3-di(thymidine-5')triphosphate and P^1,P^4-di(thymidine-5')-tetraphosphate which were isolated and characterized. The formation of these various products can be explained in terms of the dismutation of the initially formed nucleoside 5'-triphosphate prior to coupling with a second mole of morpholidate.

When this work commenced we were concerned with finding methods for the direct synthesis of nucleoside 5'-triphosphates through condensation of a nucleoside 5'-phosphoromorpholidate with inorganic pyrophosphate. Early experiments[2] had shown that such a condensation of adenosine 5'-phosphoromorpholidate (I) with five molar equivalents of tributylammonium pyrophosphate (II) in anhydrous pyridine quite rapidly gave a moderate yield of adenosine 5'-triphosphate (ATP,[3] III), but prolonged reaction led only to degradation of this initial product to ADP and AMP. In part I of this series[4] we have shown that this degradation to products with shorter polyphosphate chains is a special case of the phenomenon of "dismutation" of nucleoside polyphosphates brought about by the presence of a large excess of inorganic pyrophosphate anions. Thus, while the tributylamine salt of ATP alone in pyridine rapidly disproportionates into products with both longer and shorter polyphosphate chains, only ADP and AMP are produced in the presence of an excess of ortho- or pyrophosphate.[4] In these synthetic experiments we used a fivefold excess of pyrophosphate in order to reduce the probability that initially formed ATP would effectively compete with pyrophosphate for reaction with the morpholidate. If such a competitive reaction were to occur, the product would be P^1,P^4-di(adenosine-5')tetraphosphate (AP_4A, IV; Ad = adenosine-5'), a type of nucleotide that had not previously been described. The closely related compound P^1,P^4-di(guanosine-5')tetraphosphate has, however, recently been isolated in quite substantial quantities from brine shrimp eggs by Finamore and Warner.[5] Smith and Khorana[6] have also postulated IV as an intermediate in the unusual conversion of ADP into AMP and ATP brought about by the action of dicyclohexylcarbodiimide in aqueous pyridine.



Since compounds such as IV might logically be expected to arise as by-products during syntheses of nucleoside polyphosphates by routes such as I → III, it was desirable to have reference compounds available. With this in mind we attempted the condensation of AMP-morpholidate (I)[2] with tributylammonium pyrophosphate (II) in a molar ratio of 2:1, conditions which should favor the formation of diesters such as IV. Such a reaction was carried out in anhydrous pyridine, and aliquots were removed and examined by ion-exchange chromatography after 2, 5, and 24 hr. The elution patterns are shown in Figure 1 and the identification of the various peaks is given in Table I. Peak numbers in each case refer to the same compounds. Characterization of the various compounds was made, in this case, solely by paper chromatographic comparison in several solvent systems with authentic materials obtained from preparative reactions described in this paper. The initial product of this reaction was, as expected, ATP, which was present in 32% yield after

(1) For part II, see W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *J. Am. Chem. Soc.*, **87**, 2265 (1965).

(2) J. G. Moffatt and H. G. Khorana, *ibid.*, **83**, 649 (1961).

(3) The following abbreviations are used: AMP, ADP, ATP, AP_4, and AP_5 refer to the homologous series of adenosine 5'-mono-, di-, tri-, tetra-, and pentaphosphates, respectively. AP_2A, AP_3A, AP_4A, AP_5A, and AP_6A refer to the homologous series of α,ω-di(adenosine-5') polyphosphates. Thus, *e.g.*, AP_4A is P^1,P^4-di(adenosine-5')tetraphosphate, etc. Similar abbreviations are used for the analogous thymidine compounds.

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

(5) F. J. Finamore and A. H. Warner, *J. Biol. Chem.*, **238**, 344 (1963).

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

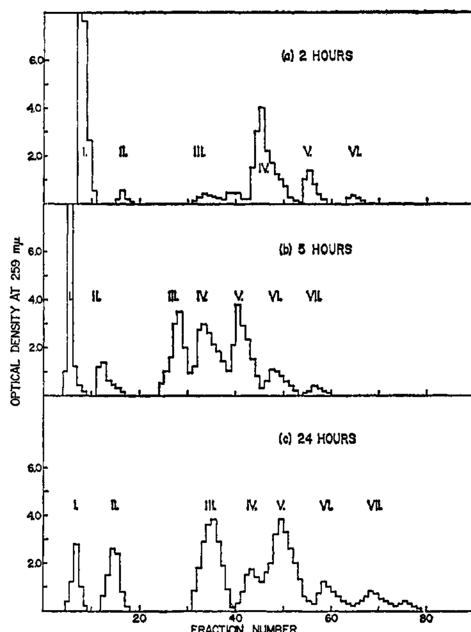


Figure 1.—Ion-exchange chromatography of the products from the reaction of AMP-morpholidate and pyrophosphate (2:1) after (a) 2 hr., (b) 5 hr., and (c) 24 hr. The same compounds are numbered identically in each case.

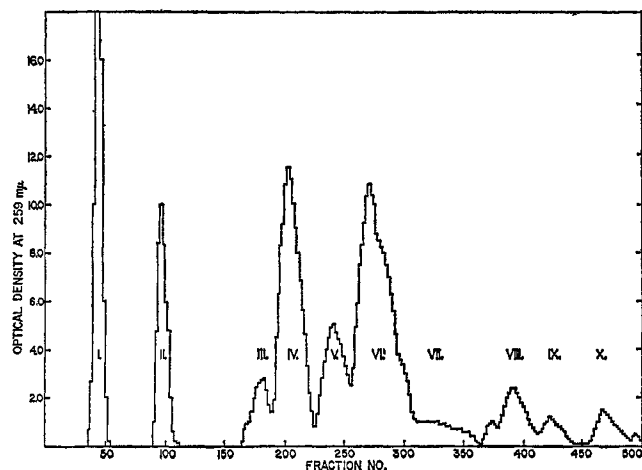


Figure 2.—Ion-exchange chromatography of the preparative reaction between AMP-morpholidate (2 mmoles) and pyrophosphate (1 mmole). See Experimental Section for details.

2 hr. In subsequent aliquots, however, the amount of ATP diminished and two principal new products (peaks III and V) were formed. This pattern is reminiscent of that obtained when ATP alone is dissolved in pyridine,[2,4] but peaks III and V can be readily shown not to consist of adenosine 5′-di- and tetraphosphates.

In order to obtain adequate quantities of these various compounds for characterization, etc., a similar reaction was carried out using 2 mmoles of AMP-morpholidate and 1 mmole of tributylammonium pyrophosphate in anhydrous pyridine at room temperature for 2 days. Separation of the reaction products by ion-exchange chromatography on a DEAE cellulose (HCO_3^-) column gave a pattern similar to that in Figure 1 (c) but included several additional minor components. The pattern is shown in Figure 2. Several of the peaks shown in Figure 2 were shown chromatographically and enzymatically to consist of simple, well-known adenosine nucleotides that were not of interest in

TABLE I

PRODUCTS FROM THE REACTION OF AMP-MORPHOLIDATE AND PYROPHOSPHATE (2:1) IN PYRIDINE (SEE FIGURE 1)

Peak no.	2 hr.	5 hr.	24 hr.	Compound[a]
I	62[b]	33	18	AMP-morpholidate
II	1	4	13	AP_2A + some AMP
III	1	16	22	AP_3A[c] + some ADP
IV	32	21	7	ATP
V	3	17	24	AP_4A[c]
VI	1	6	8	AP_4
VII	...	2	4	AP_5A[c]

[a] Since in many cases the peaks are not completely resolved under these conditions, the characterization given is that for the major product and the amount of material refers to the entire peak as pooled. [b] Yields are based upon ultraviolet absorption at 259 mμ. [c] Adequate characterization of these compounds will be found later in this paper.

the present study. Thus, peak I (17%) was found to be excess AMP-morpholidate; peak II (9%) was 90% diadenosine pyrophosphate (AP_2A) and 10% AMP; peak III (6%) was ADP; and peak V (9%) was ATP. The ADP and ATP were identified both by paper chromatography in several solvent systems and by specific enzymatic assays using coupled pyruvate kinase[7] and hexokinase[8] systems, respectively. Similarly the very diffuse peak VII (pooled from tubes 295–382 and containing a total of 8% of the total nucleotides) was chromatographically shown to consist of 86% adenosine 5′-tetraphosphate (AP_4)[1,4] contaminated with 7% each of peaks VI and VIII. The compounds in the remaining peaks (IV, VI, VIII, IX, and X) were chromatographically distinct from common adenosine 5′-polyphosphates and were isolated as their calcium or sodium salts for further study. In all cases the isolated compounds were chromatographically homogeneous with respect to ultraviolet absorption, but in several instances traces of inorganic polyphosphates were also present, and, as will be shown, could be quantitatively measured. Whenever necessary a portion of the material was first purified from such contaminants by preparative paper chromatography prior to total and labile phosphorus determinations. The analytical results obtained for the pertinent peaks are given in Table II.

TABLE II

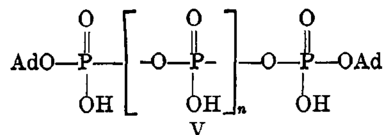
ANALYSES OF SIGNIFICANT COMPOUNDS

Peak no.	Yield, %	P-adenosine ratios: P, total	P, labile	Adenosine	Structure
IV	18	1.61	0.61	1.00	AP_3A
VI	23	1.97	0.97	1.00	AP_4A
VIII	4	2.44	1.48	1.00	AP_5A
IX	1.5	4.96	4.20	1.00	AP_6
X	1.8	2.89	1.95	1.00	AP_4AP + AP_6A

The analytical data presented in Table II together with the results of enzymatic degradations described in this paper show that the products in peaks II, IV, VI, VIII, and X (totaling 55% of the total nucleotides in the reaction) constitute an homologous series of α,ω-di(adenosine-5′)polyphosphates of structure V. Only the first member of this series, diadenosine pyrophosphate (V, n = 0), has been described prior to this

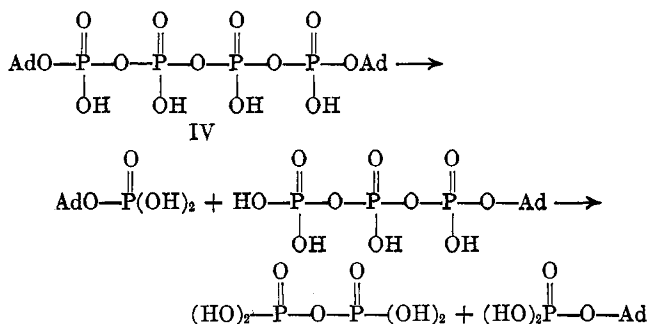
(7) "Methods of Enzymatic Analysis," H. V. Bergman, Ed., Academic Press Inc., New York, N. Y., 1963, p. 573.
(8) See ref. 7, p. 543.

work. We have, however, recently found compounds of type V ($n = 0$–5) to be present in minor amounts amongst the products from the dismutation of adenosine triphosphate in anhydrous pyridine.[4] α,ω-Diesters of tri- and higher polyphosphoric acids in general have seldom been described, notable exceptions being P^1,P^3-diphenyl and P^1,P^3-dithymyl tripolyphosphate which have been synthesized by Schaller, *et al.*,[9] through the reaction of diimidazolyl phosphinate with monophenyl and monothymyl phosphates, respectively.



In addition to the analytical data in Table II the compounds were characterized by specific enzymatic reactions. The dinucleoside polyphosphates were most clearly distinguished from simple nucleoside polyphosphates by their complete resistance to the action of *E. coli* alkaline phosphatase. This enzyme has previously been shown[10] to attack most compounds containing monoesterified phosphate groups at a roughly equivalent rate. Nucleoside polyphosphates, such as ATP and its higher homologs, are suitable substrates and are degraded in a stepwise fashion with consecutive removal of the phosphate groups from the end of the chain.[1,4,10] Of the compounds included in Table II only that in peak IX was degraded to an homologous series of lower adenosine polyphosphates and ultimately to adenosine itself when a high enzyme to substrate ratio was used.[11] From its phosphorus analysis and chromatographic behavior the compound in peak IX was clearly adenosine 5′-pentaphosphate. Prolonged incubation of the compounds in peaks IV, VI, and VIII with *E. coli* alkaline phosphatase, however, led to no change in the ultraviolet-absorbing products. By quantitative measurement, however, it was shown that, respectively, 3.1, 2.2, and 4.9% of the total phosphorus was released as orthophosphate during phosphatase treatment of these three compounds. Since on the scale upon which these studies were made such quantities of nucleotides would be readily detected, these minor amounts of phosphatase-sensitive impurities can be identified as inorganic polyphosphates.

Verification of the structures of these compounds was obtained by selective cleavage with phosphodiesterase-I from *Crotalus adamanteus* venom. This enzyme, which was completely free of 5′-nucleotidase activity, is known to hydrolyze a wide variety of 5′-nucleotide esters and pyrophosphates with release of the parent nucleoside 5′-phosphate.[12] Evidence has been presented that the cleavage of both nucleotide esters and nucleoside polyphosphates is brought about by a single enzyme.[13] The action of this enzyme upon a compound such as IV would initially cleave the polyphosphate chain between the α- and β-phosphorus atoms at one end or the other to give AMP and ATP. The latter compound is still a good substrate for the enzyme and is further cleaved to AMP and pyrophosphate.



By examining the hydrolysis mixture after both a brief exposure to the enzyme and after complete reaction it is possible to chromatographically identify both the initially produced nucleoside polyphosphate and the ultimate inorganic phosphate (ATP and pyrophosphate in the case above). Thus, two independent confirmations of the structure of the initial dinucleoside polyphosphate can be obtained from a single enzymatic treatment. Applied to the case of AP_3A (V, $n = 1$) from peak IV it was found that the initial cleavage to AMP and ADP was very rapid, but the subsequent conversion of ADP to AMP was quite slow. This is in agreement with the observation that ADP is a poor substrate for this enzyme relative to ATP, higher adenosine 5′-polyphosphates or diadenosine polyphosphates.[12,14] The ultimate inorganic phosphate was, as expected, orthophosphate. Similarly AP_4A (IV) from peak VI was rapidly cleaved to AMP and ATP, but the latter compound, being itself a good substrate, was quickly further hydrolyzed giving pyrophosphate as the sole inorganic phosphate. The product distribution from equivalent treatments of AP_3A and AP_4A with venom phosphodiesterase for various times is shown in Table III.

Table III

Products from the Treatment of AP_3A and AP_4A (0.5 μmole) with Venom Phosphodiesterase-I (50 μl.)

Time, hr.	AP_3A		AP_4A	
	AMP	ADP	AMP	ATP
1[a]	59	40	73	27
3	74	26	94	6
4.5	80	20	100	..
24	100	..		

[a] No starting material remained after 1 hr.

Under the same conditions the hydrolysis of AP_5A (V, $n = 3$) led completely to AMP within 1 hr., and in order to identify AP_4 as an intermediate it was necessary to use much shorter exposures to the enzyme. Examination of aliquots after 5, 10, and 15 min. clearly showed the initial cleavage to produce AMP and AP_4 and after complete hydrolysis the only products were AMP and tripolyphosphate. The quantitative results of this experiment are shown in Table IV.

The above degradations provide positive characterization of AP_3A, AP_4A, and AP_5A, and leave only the product in peak X to be identified. This material ran as a single spot in several solvent systems but gave

(9) H. Schaller, H. A. Staab, and F. Cramer, *Chem. Ber.*, **94**, 1621 (1961).

(10) L. A. Heppel, D. R. Harkness, and R. J. Hilmoe, *J. Biol. Chem.*, **237**, 841 (1962).

(11) Since *E. coli* alkaline phosphatase is quite strongly inhibited by orthophosphate, the degradation of higher nucleoside polyphosphates frequently stops before complete conversion to adenosine if a large excess of enzyme is not used.[4,10]

(12) W. E. Razzell, "Methods in Enzymology," Vol. VI, Academic Press Inc., New York, N. Y., 1963, p. 236.

(13) G. Pfleiderer and F. Ortanderl, *Biochem. Z.*, **337**, 431 (1963).

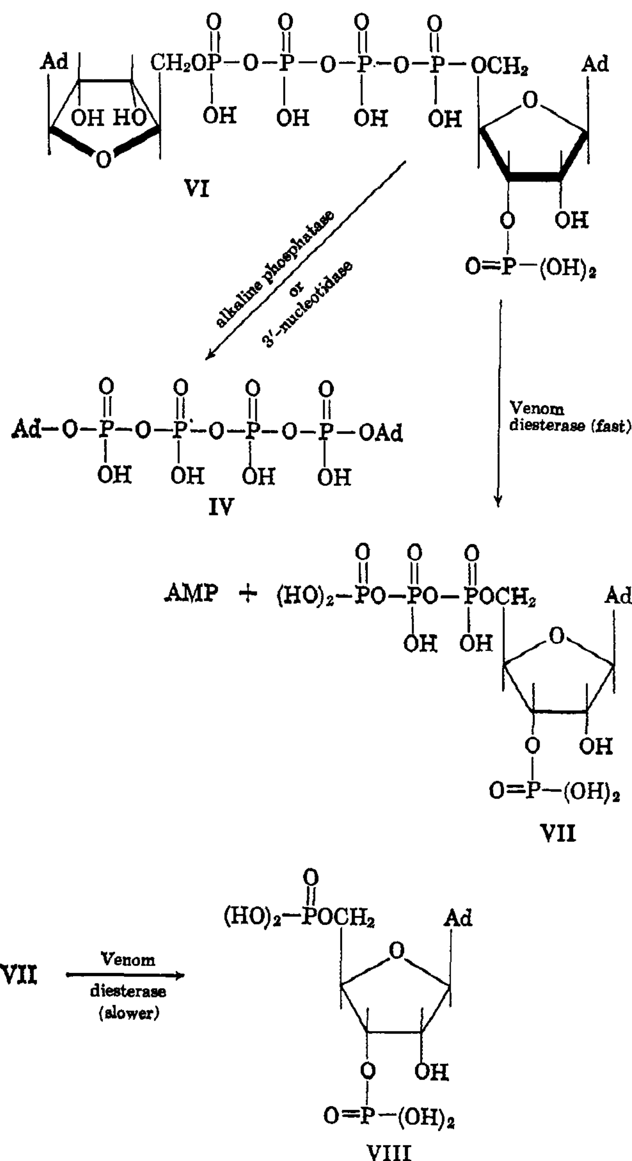
(14) Unpublished experiments by Dr. W. E. Razzell at this institute.

TABLE IV

HYDROLYSIS OF AP_6A (0.5 μMOLE) WITH VENOM PHOSPHODIESTERASE (50 μl.)

Time	AP_6A	AP_4	AMP
5 min.	75	8	17
10 min.	45	17	38
15 min.	17	22	61
4 hr.	..	..	100

phosphorus analyses difficult to reconcile with any nucleoside polyphosphate or dinucleoside polyphosphate emerging from the column later than adenosine pentaphosphate. Incubation with *E. coli* alkaline phosphatase released 15.3% of the total phosphorus as orthophosphate and converted 80% of the starting material into a compound moving with the same R_f as AP_4A in several solvent systems. The remaining 20% was chromatographically identical with the starting compound and was not further changed upon retreatment with the enzyme. Peak X therefore consisted of two compounds, 20% being presumably AP_6A (V, $n = 4$) and 80% a compound containing roughly 15% of its phosphorus in a monoesterified form. No chromatographic evidence for contamination by an inorganic polyphosphate could be found. Incubation of the crude product with venom diesterase resulted in complete disappearance of the starting material within 15 min. giving AMP and a predominant nucleotide moving with the same R_f as AP_4 in solvent A (acidic) and the same R_f as AP_5 in solvent B (alkaline). Rechromatography of these spots failed to change this picture. Prolonged incubation of the crude product with unfractionated *Crotalus adamanteus* venom led to complete hydrolysis to AMP and a periodate negative spot moving similar to ADP in solvent A but considerably slower in solvent B. The accumulation of a nucleotide following incubation with crude venom can most readily be explained by substitution on the 2'- or 3'-hydroxyl groups of the ribose. A very active 5'-nucleotidase is present in venom but requires that these positions be unsubstituted.[15] The stable nucleotide resulting from the action of crude venom was examined by paper chromatography in solvent D and shown to be identical with adenosine 3',5'-diphosphate (VIII).[16] No sign of the isomeric 2',5'-diphosphate was to be seen. Final confirmation of the presence of a 3'-phospho monoester group in the peak X product came from studies on the action of purified rye grass 3'-nucleotidase. This enzyme closely paralleled *E. coli* alkaline phosphatase in its action, converting 80% of the starting material into a compound chromatographically identical with AP_4A in three solvent systems within 1 hr. and then giving no further change. The remaining 20% was, once again, unaffected by this enzyme. The AP_4A produced by the action of 3'-nucleotidase was, in turn, characterized by degradation with venom phosphodiesterase-I initially to AMP and ATP and finally to AMP and pyrophosphate as described above. These results can all be rationalized if peak X contains 20% AP_6A and 80% 3'-phosphoryl AP_4A (VI).



The action of either alkaline phosphatase or 3'-nucleotidase would then convert VI to AP_4A (IV) leaving the 20% AP_6A unaltered. The latter compound was present in such small quantities that it was not further characterized. The presence of a 3'-phosphate group is known to greatly reduce the rate of cleavage of 5'-phospho diester[17] and 5'-pyrophosphate[16,18] groups by venom phosphodiesterase-I. Hence, the reaction of this enzyme with V is expected to be exclusively at the end of the molecule not bearing the 3'-phospho monoester group and leading to AMP and 3'-phosphoryl ATP (VII) which would, in turn, be cleaved only very slowly to adenosine 3',5'-diphosphate (VIII).

The origin of all these compounds can be readily explained by consideration of the dismutation reactions known to occur in pyridine.[4] The original condensation reaction between AMP-morpholidate (I) and pyrophosphate quite rapidly leads to the formation of ATP as is shown in Figure 1. As it is formed, however, the latter compound will undergo the previously described dismutation reaction leading principally to ADP, ATP, and AP_4 as well as lesser amounts of AMP,

(15) (a) A. Kornberg and W. E. Pricer, *J. Biol. Chem.*, **186**, 557 (1950); (b) R. W. Chambers, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 3747 (1957).

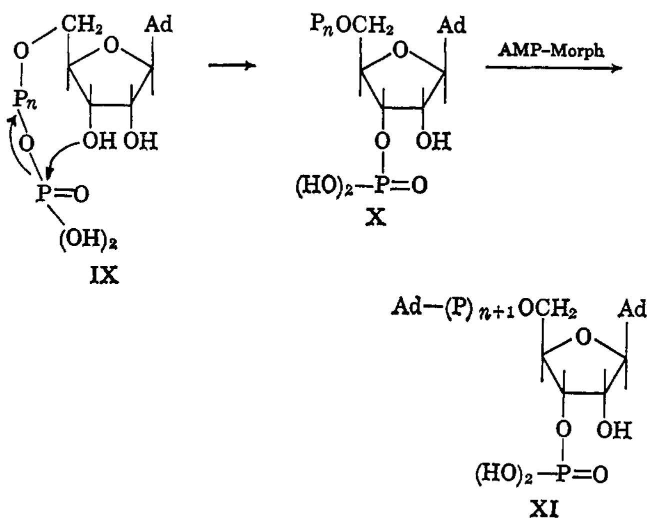
(16) J. G. Moffatt and H. G. Khorana, *ibid.*, **83**, 663 (1961).

(17) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

(18) J. P. H. Verheyden and J. G. Moffatt, *ibid.*, **86**, 1236 (1964).

AP_5, and other compounds.[4] Each of these nucleoside polyphosphates is then capable of reaction with the excess AMP-morpholidate leading to the observed series of diadenosine polyphosphates. From Table I it can be seen that, while the amount of ATP present in the reaction decreases with time, the amounts of the various dinucleotide polyphosphates continually increase. This suggests that the diesters are not themselves susceptible to the dismutation reaction in pyridine. This was confirmed by examining the stability of pure AP_3A and AP_4A dissolved in anhydrous pyridine as their tributylammonium salts. Even after 10 days at room temperature there were only trace amounts of decomposition products detectable by paper chromatography. Thus, there is a remarkable difference between the stability of the dinucleoside polyphosphates and the extensive dismutation that has occurred with nucleoside polyphosphates under comparable conditions in less than 1 day.[4]

Compound VI is quite unique in that it is the only product detected in either the present study, or in our studies on the dismutation of ATP,[4] that contains a phosphate group bonded to the nucleoside in other than the 5' position. Since it has been shown by both paper chromatography of crude venom digests and by use of the specific 3'-nucleotidase that the extraneous phosphate group is exclusively located at the 3' position, we are led to believe that its introduction must be by way of a sterically restricted intramolecular phosphorylation by a 5'-polyphosphate. If phosphorylation were to occur *via* some free activated derivative of orthophosphate, then random attack at the 2'- and 3'-hydroxyl groups would be expected. Thus



where P_n is a polyphosphate chain of undetermined length. For such a mechanism to give rise to compound VI with such apparent selectivity it would be most reasonable if $n = 3$ and IX was AP_4. It is, however, most difficult to explain why such a reaction should occur even to such a limited extent.

We have also prepared AP_3A (V, $n = 1$) *via* a somewhat different route. Thus, condensation of AMP-morpholidate with tributylammonium orthophosphate in a ratio of 2:1 gave a 37% yield of AP_3A which was isolated by ion-exchange chromatography and shown to be chromatographically, electrophoretically, and analytically identical with the same compound described earlier. An independent synthesis of AP_4A has also been recently described.[1]

Finally, while we have not made an exhaustive study of the other reaction products, we have prepared and isolated P^1,P^3-di(thymidine-5')triphosphate (TP_3T) and P^1,P^4-di(thymidine-5')tetraphosphate (TP_4T) from the condensation of thymidine 5'-phosphoromorpholidate[19] with tributylammonium pyrophosphate (2:1) in anhydrous pyridine. Ion-exchange chromatography of this reaction mixture gave a simpler picture than that from the analogous adenosine reaction (Figure 2). Four principal peaks were obtained from the DEAE cellulose (HCO_3^-) column together with a number of lesser ones. Peak I (21%) was unreacted TMP-morpholidate and peak II (10%) was largely the well-known P^1,P^2-di(thymidine-5')pyrophosphate[20] and a small amount of thymidine 5'-phosphate. Peaks III and IV (26% each) were largely the desired TP_3T and TP_4T but, unlike the results from the adenosine series, these products were admixed with lesser amounts of thymidine 5'-di- and triphosphates, respectively. In each case these mixed products could be readily separated by rechromatography on a column of DEAE cellulose (acetate) at pH 5.0. The isolated diesters gave the expected analyses (see Experimental Section) and were characterized by their resistance toward the action of *E. coli* alkaline phosphatase and by cleavage with venom phosphodiesterase-I to the expected products. Thus, TP_3T was rapidly attacked by the enzyme giving TMP and TDP, the latter being then slowly converted to TMP and orthophosphate. In a similar way TP_4T initially gave TMP + TTP and ultimately only TMP and pyrophosphate. It therefore appears that the reaction of an inorganic phosphate or polyphosphate with an excess of a nucleoside 5'-phosphoromorpholidate in anhydrous pyridine provides a versatile route to α,ω-dinucleoside polyphosphates. We are currently investigating the synthesis of the naturally occurring guanosine compounds[5] by this and other routes.

Finally, it is to be noted that the direct synthesis of nucleoside 5'-triphosphates *via* the condensation of a nucleoside phosphoromorpholidate and pyrophosphate can be efficiently accomplished if dimethyl sulfoxide is used as the reaction solvent rather than pyridine.[21]

Experimental Section

General Methods.—Paper chromatography was carried out by the descending technique on sheets of Schleicher and Schuell No. 589 orange ribbon paper using the following systems: solvent A, isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* disodium ethylenediaminetetraacetic acid (100:60:1.6); solvent B, 1-propanol–29% ammonium hydroxide–water (6:3:1); solvent C, ethanol–0.5 *M* ammonium acetate buffer, pH 3.8 (5:2); solvent D, 2-propanol–water–trichloroacetic acid–29% ammonium hydroxide (75 ml.:25 ml.:5.0 g.:0.25 ml.). Paper electrophoresis was done on the same paper impregnated with either 0.05 *M* ammonium bicarbonate, pH 7.6, or with 1 *M* acetic acid and using 1000–2000 v. Phosphorus-containing compounds were visualized on paper chromatograms using the molybdate spray of Hanes and Isherwood[22] followed by ultraviolet irradiation.[23] Vicinal glycols were detected by the peri-

(19) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).
(20) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *ibid.*, **80**, 6223 (1958).
(21) J. G. Moffatt, *Can. J. Chem.*, **42**, 599 (1964).
(22) C. A. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).
(23) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

odate–benzidine spray of Viscontini, *et al.*[24] R_f values of most of the pertinent compound in solvents A and B are reported in a previous paper.[4] Total phosphorus analyses were obtained by the method of King,[25] and labile phosphorus was determined in the same way except that the digestion with perchloric acid was replaced by treatment with 1 N HCl at 100° for 8 min. Determination of orthophosphate was done by a scaled-down modification of the method of Lowry and Lopez.[26] Ultraviolet measurements were made on Cary Model 15 and Zeiss PMQ-II spectrophotometers. All evaporations were carried out at a vacuum of roughly 1 mm. and a bath temperature of 30° using a Buchler flash evaporator, on which the condensing flask was cooled to −15° with circulating refrigerated glycol.

Enzymes.—(a) *E. coli* alkaline phosphatase obtained from the Worthington Biochemical Co., Freehold, N. J., was made up to a concentration of 100 μg./ml. in 0.5 M Tris buffer, pH 9.

(b) Phosphodiesterase-I was a highly purified preparation from *Crotalus adamanteus* venom that was free of 5′-nucleotidase activity. It was made up in 0.2 M Tris buffer and had a specific activity of 620 μmoles/hr./ml. toward *p*-nitrophenyl thymidine 5′-phosphate. This preparation was kindly provided by Dr. W. E. Razzell.

(c) Rye grass 3′-nucleotidase was made up in 0.1 M Tris buffer pH 7.6 and had a specific activity of 1.4 μmoles/hr./μl. toward adenosine 3′-phosphate. Its activity toward adenosine 2′- and 5′-phosphates was less than 0.1% of this. We are very grateful to Dr. R. J. Hilmoe of the National Institutes of Health for a generous gift of this enzyme.

The Reaction of Adenosine 5′-Phosphoromorpholidate and Pyrophosphate (2:1).—Tetrasodium pyrophosphate·$10H_2O$ (446 mg., 1 mmole) was dissolved in water and passed through a column containing 20 ml. of Dowex 50 (pyridinium) resin. The eluate and water wash were evaporated to a volume of 10 ml. and diluted with pyridine (30 ml.). Distilled tributylamine (0.71 ml., 3 mmoles) was added, and the clear solution was evaporated to dryness. The residue was then dried by four evaporations with 20-ml. portions of pyridine.[27] Separately the 4-morpholine N,N′-dicyclohexylcarboxamidine salt of adenosine 5′-phosphoromorpholidate (2 mmoles) was dried by three evaporations with 10-ml. portions of pyridine and added to the pyrophosphate. The mixture was then evaporated twice with pyridine and then stored in pyridine (50 ml.) for 2 days at room temperature. The solvent was then evaporated, and residual pyridine was removed by several evaporations with water. The final residue was dissolved in water (100 ml.), adjusted to pH 7 with ammonium hydroxide, and applied to a 3.5 × 32 cm. column of DEAE cellulose (HCO_3^-). After a thorough water wash elution was effected with a linear gradient of triethylammonium bicarbonate (pH 7.6, 12 l. from 0.005 to 0.4 M). Fractions of roughly 20 ml. were collected and followed by ultraviolet absorption as shown in Figure 2. Where necessary the individual fractions were examined by paper chromatography so as to pool only homogeneous products. The products in peaks I, II, III, V, and VII were identified chromatographically and enzymatically as AMP-morpholidate, diadenosine pyrophosphate (containing 10% AMP), ADP, ATP, and AP_4 (total P–labile P–adenosine = 4.03:3.01:1.00), respectively. The remaining peaks were evaporated to dryness and freed from residual bicarbonate by repeated evaporations with methanol. The products were then dissolved in ethanol and isolated as their calcium salts by addition of a 1 M ethanolic solution of calcium chloride. Some of the compounds were isolated as their sodium salts by addition of 1 M sodium iodide in acetone to a methanol solution of the triethylammonium nucleotide. The precipitates were washed free of halide ions with ethanol or acetone and dried *in vacuo* giving the following compounds.

(a) Calcium P^1,P^3-di(adenosine-5′)triphosphate, 164 mg. *Anal.*[28] Calcd. for $C_{20}H_{24}N_{10}O_{16}P_3Ca_{1.5}\cdot3H_2O$: C, 27.90; H, 3.51; N, 16.27; total P–labile P–adenosine = 1.50:0.50:1.00. Found: C, 28.12; H, 4.52; N, 16.18; total P–labile P–adenosine = 1.61:0.61:1.00.

(b) Calcium P^1,P^4-di(adenosine-5′)tetraphosphate, 207 mg. *Anal.* Calcd. for $C_{20}H_{24}N_{10}O_{19}P_4Ca_2\cdot8H_2O$: P, 11.72; total P–labile P–adenosine = 2.00:1.00:1.00. Found: P, 11.85; total P–labile P–adenosine = 1.97:0.97:1.00.

(c) Sodium P^1,P^5-di(adenosine-5′)pentaphosphate, 28 mg. *Anal.* Calcd.: total P–labile P–adenosine = 2.50:1.50:1.00. Found: total P–labile P–adenosine = 2.44:1.48:1.00.

(d) Sodium adenosine 5′-pentaphosphate, 19 mg. *Anal.* Calcd.: total P–labile P–adenosine = 5.00:4.00:1.00. Found: total P–labile P–adenosine = 4.96:4.20:1.00.

A similar reaction was used to determine the product distribution at various times as shown in Figure 1 except that 0.3 mmole of AMP-morpholidate and 0.15 mmoles of pyrophosphate were used.

Enzyme Degradations. (a) ***E. coli* Alkaline Phosphatase.**—An aliquot of the compound corresponding to approximately 1 μmole of adenosine content was incubated at 37° with 50 μl. of the enzyme made up in 0.5 M Tris buffer, pH 9. After 1- and 3-hr. incubation an aliquot was withdrawn and examined by paper chromatography in solvent A. For quantitative results orthophosphate released by the enzyme was measured by a scaled-down modification of the method of Lowry and Lopez.[26]

(b) **Venom Phosphodiesterase-I.**—Approximately 15 optical density units of the compound as its ammonium salt was incubated at 37° with 25 μl. of 0.2 M Tris buffer (pH 9) and 50 μl. of phosphodiesterase-I. Aliquots were removed after 1, 3, 4, 5, and 24 hr. in the case of AP_3A and AP_4A and after 5, 10, and 15 min., and 4 hr. in the case of AP_5A and directly chromatographed in solvent A. The results are given in Tables III and IV.

(c) **Rye Grass 3′-Nucleotidase.**—Roughly 10 optical density units of peak X were incubated at 37° with 25 μl. of 0.2 M Tris buffer pH 7.8 and 20 μl. of 3′-nucleotidase (see above). Aliquots were removed after 1 and 2 hr. and directly chromatographed in solvent A. The release of orthophosphate was separately measured by the Lowry and Lopez method.

P^1,P^3-Di(adenosine-5′)triphosphate (AP_3A).—Orthophosphoric acid (0.1 mmole) was dissolved in 50% aqueous pyridine (2 ml.) containing 25 μl. (0.1 mmole) of distilled tributylamine and evaporated to dryness. The residue was evaporated three times with 5-ml. portions of anhydrous pyridine and then mixed with a similarly dried solution of the 4-morpholine N,N′-dicyclohexylcarboxamidine salt of adenosine 5′-phosphoromorpholidate[2] in anhydrous pyridine (10 ml.). After 3 days at room temperature the solvent was evaporated, and the residue was applied to a 2 × 15 cm. column of DEAE cellulose (HCO_3^-). After a water wash elution was effected with a linear gradient of 1600 ml. of triethylammonium bicarbonate (0.005 to 0.4 M). Seven ultraviolet-absorbing peaks were obtained and characterized as follows: peak I (23%) was unreacted AMP-morpholidate; peak II (11%) was AMP; peak III (9%) was diadenosine pyrophosphate; peak IV (11%) was ADP; peak V (37%) was the desired AP_3A; peaks VI and VII (5% and 4%) were not identified. Peak V was evaporated to dryness and freed from bicarbonate by four evaporations with 25-ml. portions of methanol. The final residue was dissolved in ethanol and precipitated as the calcium salt (18 mg.) by addition of a 1 M solution of calcium chloride in ethanol. The product was chromatographically homogeneous and identical with the AP_3A isolated from the pyrophosphate reaction above. AP_3A requires that total P–labile P–adenosine = 1.50:0.50:1.00. Found: total P–labile P–adenosine = 1.40:0.52:1.00. The compound was identical with the AP_3A previously described in its enzymatic degradations.

The Stability of AP_3A and AP_4A in Anhydrous Pyridine.—The hydrated calcium salts of AP_3A and AP_4A (7 mg. and roughly 8 μmoles each) were separately converted to their pyridinium salts with Dowex-50 resin and evaporated to dryness. The residues were dissolved in 80% pyridine (1 ml.) containing 6 and 8 μl. of distilled tributylamine, respectively, and evaporated to dryness. The materials were then dried by three evaporations with 2-ml. portions of pyridine and stored at room temperature in anhydrous pyridine (1 ml.). After 2, 5, and 10 days the mixtures were examined by chromatography in solvents A and C. After 2 days only single spots of the starting materials were visible, and after 5 and 10 days only trace amounts of decomposition products (principally AMP) were visible.

The Reaction of Thymidine 5′-Phosphoromorpholidate and Pyrophosphate (2:1).—The reaction between tributylammonium pyrophosphate (1 mmole) and 4-morpholine N,N′-dicyclohexylcarboxamidinium thymidine 5′-phosphoromorpholidate (2

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(28) Elemental analyses were performed by Midwest Microlabs, Indianapolis, Ind.

mmoles) in 50 ml. of anhydrous pyridine was set up exactly as described for the analogous adenosine reaction. After 2 days at room temperature the solvent was evaporated, and the residue was applied in water to a 4 × 25 cm. column of DEAE cellulose (HCO_3^-). After a water wash the products were eluted with a linear gradient of 8 l. of triethylammonium bicarbonate (0.005 to 0.25 *M*). Five peaks were obtained as follows: peak I (21%) was unreacted TMP-morpholidate; peak II (10%) was mainly dithymidine pyrophosphate plus a little TMP; peak III (26%) was mainly TP_3T plus a little TDP; peak IV (26%) was mainly TP_4T plus some TTP; peak V (15%) was TP_5T and TP_4.

(a) **P^1,P^3-Di(thymidine-5')triphosphate (TP_3T).**—Peak III (4900 optical density units at 267 mμ) was evaporated to dryness and freed from excess bicarbonate by evaporation with methanol. The material was dissolved in water, adjusted to pH 7 with ammonia, and applied to a 2 × 25 cm. column of DEAE cellulose (acetate). Elution with a linear gradient of 2 l. of ammonium acetate (pH 5.0, 0.005 to 0.30 *M*) cleanly separated TTP (13%) from TP_3T (87%). The latter peak was diluted fourfold with water and adjusted to pH 7.7 with ammonium hydroxide. It was then passed through a column containing 50 ml. of DEAE cellulose (HCO_3^-) and washed thoroughly with water. The product was then quantitatively eluted with 0.5 *M* triethylammonium bicarbonate, evaporated to dryness, and isolated in the usual way as the hydrated calcium salt (131 mg.) which was chromatographically homogeneous in several solvents: R_f in solvent A, 0.20; in solvent B, 0.20.

TP_3T requires that total P-labile P-thymidine = 1.50:0.50:1.00. Found: total P-labile P-thymidine = 1.58:0.53:1.00. The ammonium salt of TP_3T (0.5 μmole) remained completely unchanged upon incubation with 50 μl. of *E. coli* alkaline phosphatase and released only 1.6% of its total phosphorus as orthophosphate by this treatment. The action of venom phosphodiesterase-I (50 μl.) and Tris buffer, pH 9 (5 μl.), on 0.5 μmole of TP_3T resulted in complete conversion to TMP and TDP within 15 min., the latter compound being only slowly hydrolyzed to TMP and orthophosphate.

(b) **P^1,P^4-Di(thymidine-5')tetraphosphate (TP_4T).**—The majority of peak IV (4100 optical density units) was purified on a 3 × 20 cm. column of DEAE cellulose (acetate) using a linear gradient of 2 l. of ammonium acetate, pH 5 (0.005 to 0.5 *M*) giving 38% TTP and 62% TP_4T. The latter compound was desalted and isolated as its calcium salt (130 mg.) by the same methods used for TP_3T.

TP_4T requires that total P-labile P-thymidine = 2.00:1.00:1.00. Found: total P-labile P-thymidine = 2.01:0.86:1.00. The product was chromatographically homogeneous and had R_f 0.15 in solvent A and 0.14 in solvent B. Incubation with 50 μl. of *E. coli* alkaline phosphatase produced no new ultraviolet-absorbing products but did release 4.8% of the total phosphorus as orthophosphate. Venom phosphodiesterase (10 μl.) converted TP_4T into equimolar amounts of TMP and TTP within 30 min. and gave only TMP and pyrophosphate after overnight incubation.

Conformational and Configurational Studies on Some Acetylated Aldopyranosyl Halides[1,2]

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A series of poly-*O*-acetylated aldopyranosyl halide derivatives (I–X), each in the thermodynamically more stable anomeric form, has been studied by n.m.r. spectroscopy. The halides examined included derivatives of all four pentoses, three hexoses (glucose, galactose, and mannose), and three derivatives of 2-amino-2-deoxyglucose. All compounds studied were of the D series. Comparison spectra of α-D-lyxose tetraacetate and β-D-mannose pentaacetate were obtained. In deuteriochloroform solution, the signal of the anomeric (C-1) proton is observed at lower field than the signals of the other ring protons. Analysis of the spectra of the halides reveals that the stable anomer in every case has the halogen atom axial when the molecule concerned is in its favored conformation. All of the halides studied exhibited a high degree of conformational purity in solution. The n.m.r. data confirm anomeric configurational assignments previously based solely upon optical rotatory data. The stable form of tri-*O*-acetyl-D-lyxopyranosyl bromide (known only as a sirup) is shown to be the α-D anomer in the *C1* conformation.

The poly-*O*-acetylaldopyranosyl halides theoretically can exist in either of two anomeric forms, differing in configuration at C-1, and both forms have been prepared in several instances.[3] The configurations assigned to these derivatives are based on optical rotatory data through application of Hudson rules of rotation,[4] and are supported by mechanistic data[5] on the reactions they undergo. In the case of the poly-*O*-acetylaldopyranosyl halides of those simple sugars where both anomeric forms are known, there can be little reasonable doubt as to the correctness of these assignments, although direct proof by methods such as X-ray crystallographic analysis is lacking. When only one anomeric form is known, configurational assignment on the basis of optical rotation may be more difficult. When one of the acetoxy groups is replaced by a different substituent, as in the *O*-acetylated glycosyl halides of amino sugars,[6] further problems in configurational assignment may be introduced, since it has been noted[7,8] that certain substituent groups at C-2 may cause a complete reversal of normal rotatory relationships between anomers.

Poly-*O*-acetylaldopyranosyl halides readily undergo anomeric interconversion[5] in the acetic acid-hydrogen halide and similar mixtures commonly used to introduce the halogen atom at C-1. The major product of the equilibrated reaction is consequently the thermodynamically more stable anomer.

Rules have been formulated,[3] based on configurational relationships of the ring substituents, for predicting

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