operation and final stabilization ratios at 0.01 M calcium must thus include core micelles. The values of ~2 obtained in these experiments do, however, suggest an upper limit to a coat subunit weight ratio. Under conditions where core micelles would not be expected to contribute to stabilization ratios, but where equilibrium might not have been attained, stabilization ratios are as follows: 0.9 and 0.8 for solubilization of assay precipitates, 0.6 and 0.6 for solubilization of calcium α_s-caseinate precipitate, and 1 and 1.3 for stabilization on incremental addition of calcium during the descending limb of the dip. We suspect that these lower weight ratios cover a range which includes the coat subunit.

A source of coat material, which might be a reversibly dissociable coreless micelle or other coat subunit association product, would account for the apparent stability of micelle distributions to dilution (J). Such association products would serve as a buffer to maintain the necessary level of coat material in the environment. In addition, equilibrium levels of coat material might be sufficiently low so that dilution produces so slight a micelle coat dissociation that micelle characteristics are not affected. This is suggested by the extent and equivalence of resuspension of micelle pellets either in their supernatants or in standard KCl buffer containing appropriate calcium.

The degree of solvation of micelles and the variation in solvation with initial ratio (K) is expected from the characteristics of calcium α_s- and calcium κ-caseinates. This model readily accommodates these results; the high level of solvation associated with the coat would contribute to stability to close approach. The main difficulties with studies of solvation using pellet centrifugates are the extent to which supernatant is included in the pellet and the extent to which micelles are desolvated by the centrifugal field. Data, in addition to Table II, suggest that there are concomitant problems. More refined methods are required.

While mixtures of pure κ-casein and β-casein will not form micelles in any way comparable to those of mixtures of κ-casein and α_s-casein, considerable amounts of β-casein, at least up to 30% of the α_s-casein plus β-casein moiety, are readily incorporated. First Cycle casein behaves much like an α_s–κ-casein mixture of weight ratio 7. At 37° the solubility of calcium β-caseinate is comparable to that of calcium α_s-caseinate.[6] In this model the coating of a calcium α_s–β-caseinate core is readily accepted provided the β-casein fraction is not so high as to prevent formation of an effective coat layer.

There is a feature of this model which has additional meaning: κ-casein is placed at the surface where it can be attacked easily and quantitatively by rennin. Since carbohydrate is released into solution by rennin action,[27] the carbohydrate substituent of κ-casein would be placed near the micelle surface and might on account of its solvation characteristics be responsible for the stability of micelles to close approach. The placement of κ-casein at the surface also permits alteration of κ-casein without micelle dissociation, a circumstance which would allow rennin clotting of the extremely stable micelles formed by Cu, Ni, Co, Mn, Cd, and Zn.[17] As has been pointed out, κ-casein is the only casein component altered during the time required for rennin coagulation.[6] This model places α_s- and β-caseins so that they are not in contact with the environment. In addition, rennin action to produce para-α_s- and para-β-caseins is not only slower by a factor of ~10^3 but the products are nonprecipitable by calcium.[6]

(27) H. Nitschmann and R. Henzi, *Helv. Chim. Acta*, 42, 1985 (1957); H. Nitschmann and R. Beebe, *Chimia* (Aarau), 14, 318 (1960); P. Jolles, C. Alais, and J. Jolles, *Biochim. Biophys. Acta*, 51, 309 (1961); J. Garnier, *Ann. Biol. animale Biochim. Biophys.*, 3, 71 (1963).

Dismutation Reactions of Nucleoside Polyphosphates. I. General Features of the Reaction

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Adenosine 5′-triphosphate dissolved as its tributylamine salt in anhydrous pyridine at room temperature undergoes a rapid dismutation reaction. The main products of this reaction have been characterized as a homologous series of adenosine 5′-polyphosphates containing up to seven phosphate groups. A second minor series of products has been isolated and characterized as α,ω-di(adenosine-5′) polyphosphates. Similar types of products arise upon storage of anhydrous pyridine (or mixed solvents containing pyridine) solutions of adenosine 5′-di- or -tetraphosphate and of p-nitrobenzyl triphosphate. The presence of excess ortho- or pyrophosphate anions prevents the formation of products of increased polyphosphate chain length and leads to the accumulation of adenosine 5′-mono- and -diphosphates. A similar effect is produced by the addition of intermediate amounts of water. The stability of nucleoside polyphosphates in solvents such as dimethyl sulfoxide permits syntheses that would be extremely difficult in pyridine.

(1) (a) Syntex Postdoctoral Fellow, 1961–1962. (b) Syntex Postdoctoral Fellow, 1963–1964, supported in part by the "Stiftung für Stipendien auf dem Gebiete der Chemie" (Switzerland), whose aid is gratefully acknowledged.

Several years ago, during an attempted synthesis of adenosine 5′-triphosphate through the condensation of adenosine 5′-phosphoromorpholidate with tributylammonium pyrophosphate in pyridine, it was observed[2] that the initially formed triphosphate rapidly degraded to adenosine 5′-mono- and -diphosphates. While seeking an explanation for this unexpected degradation we noted that the 4-morpholine-N,N′-dicyclohexylcarboxamidine salt of adenosine 5′-triphosphate itself was equally unstable in anhydrous pyridine but decomposed giving a rather different spectrum of products. Thus, as well as adenosine 5′-mono- and -diphosphates, a major new product characterized as adenosine 5′-tetraphosphate was isolated. The mechanism by which a nucleoside polyphosphate "dismutates" into products containing both shorter and longer polyphosphate chains was obscure at the time and it is the purpose of these papers to clarify the issue. A preliminary account of our findings has already appeared.[3]

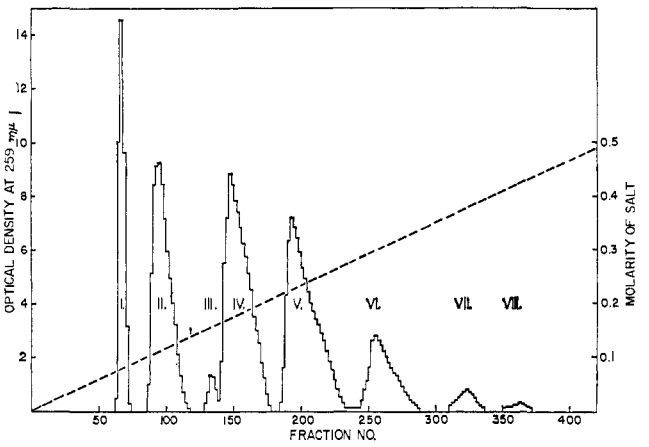


Figure 1. Ion-exchange chromatography of products from the dismutation of ATP in pyridine at room temperature for 3.5 days. See Table I for characterization.

Since our original observation[2] several other workers[4] have described the degradation of nucleoside 5′-triphosphates to mono- and diphosphates during syntheses in pyridine. No mention has been made, however, of the appearance of higher polyphosphates and, as will be seen later, this can be explained by the presence of excess inorganic phosphate ions present during the syntheses.

In this first paper of the series it is our intention simply to present a number of facts we have accumulated relating to the effects of a number of variables such as added anions, solvent, etc., upon the course of the dismutation reaction. In a later paper we will discuss the mechanism of this reaction and attempt to explain the present observations in a rational way.

It was felt that in our originally described experiment[2] the strongly basic 4-morpholine-N,N′-dicyclohexylcarboxamidine might have played a role in the observed dismutation reaction. Accordingly an anhydrous solution of the tributylamine salt of adenosine 5′-triphosphate in pyridine was allowed to stand at room temperature for 3.5 days and the products were then separated by ion-exchange chromatography on diethylaminoethyl (DEAE) cellulose in the bicarbonate form using a linear gradient of the volatile salt triethylammonium bicarbonate as eluent. Such a system facilitates the isolation of the products, particularly when dealing with minor components and is to be preferred to the use of Dowex-2 chloride resin. The results of this chromatography are shown in Figure 1. Clearly the reaction mixture is more complex than had previously been observed since eight well-resolved peaks were separated and isolated. Presumably peaks VI–VIII, which together represent only 10% of the total nucleotide material, were not previously detected due to the different chromatographic system and the much smaller scale reaction studied. The material in each peak was then further examined by paper chromatography which revealed the presence in peaks I, V, VI, VII, and VIII of a second series of readily separable adenosine nucleotides in minor amounts (5–20% of the peak). Peak III is a pure member of this second series. Also minor amounts of orthophosphate, pyrophosphate, tripolyphosphate, and perhaps other inorganic polyphosphates were detected in peaks I, II, IV, V, and VI but were not further investigated. Wherever necessary a portion of each peak (up to 200 optical density units) was freed of inorganic phosphates and separated into its major and minor nucleotide components by preparative paper chromatography. Each purified fraction was then analyzed for total phosphorus, acid-labile phosphorus, and ultraviolet absorption, the results being summarized in Table I,[5] together with an identification of the components as arrived at by these analyses, by comparison with authentic materials, and by enzymatic degradations.

Table I

Peak no.	Total O.D.^{260}	% of total	—Phosphorus:adenosine ratios— Total P	Labile P	Adenosine	Structure[5]
Ia	1847	12.3	1.06	0.07	1.00	AMP
Ib	118	0.8	1.04	0.06	1.00	AP_2A
II	3265	21.7	2.00	0.98	1.00	ADP
III	207	1.4	1.52	0.53	1.00	AP_3A
IV	4023	26.8	3.05	1.96	1.00	ATP
Va	3810	25.4	3.79	2.88	1.00	AP_4
Vb	256	1.7	2.04	0.97	1.00	AP_4A
VIa	1150	7.7	4.78	3.70	1.00	AP_5
VIb	79	0.5	2.45	1.51	1.00	AP_5A
VIIa	195	1.3	5.78	4.70	1.00	AP_6
VIIb	40	0.3	2.91	1.71	1.00	AP_6A
VIIIa	18	0.1	6.3	—	1.00	AP_7
VIIIb	7	0.05	2.90	—	1.00	AP_7A

The main series of products (peaks Ia, II, IV, Va, VIa, VIIa, and VIIIa) proved to be an homologous series of adenosine 5′-polyphosphates of structure I (n = 0–6) with from one to seven phosphate groups in a

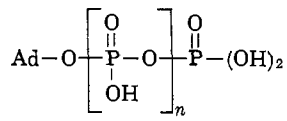
(2) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

(3) W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *ibid.*, **86**, 1254 (1964).

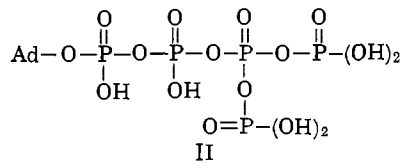
(4) *E.g.*, (a) J. Zemlicka, J. Smrt, and F. Sorm, *Collection Czech. Chem. Commun.*, **28**, 241 (1963); (b) M. Ikehara and E. Ohtsuka, *Chem. Pharm. Bull.* (Tokyo), 10, 536 (1962); (c) M. Ikehara and E. Ohtsuka, *ibid.*, 10, 997 (1962).

(5) The following abbreviations are used: AMP, ADP, ATP, AP_4, AP_5, AP_6, and AP_7 refer to the homologous series of adenosine 5′-mono-, -di-, -tri-, -tetra-, -penta-, -hexa-, and -heptaphosphates. AP_2A, AP_3A, AP_4A, AP_5A, AP_6A, and AP_7A refer to the homologous series of α,ω-di(adenosine-5′) polyphosphates; *e.g.*, AP_5A is P^1,P^5-di(adenosine-5′) pentaphosphate.

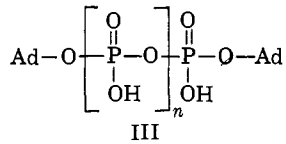
linear arrangement. The first four members of the series were chromatographically identical with authentic adenosine 5′-mono-, -di-, -tri-, and -tetraphosphates and the latter members appear to be of similar form. Thus, in addition to the observed total and acid-labile phosphorus content (see Table I), the purified compounds in peaks Ia, II, IV, Va, VIa, VIIa, and VIIIa were all found to be completely degraded in a stepwise fashion by *E. coli* alkaline phosphatase, ultimately giving only adenosine or, as frequently was observed in the case of the higher adenosine polyphosphates, a mixture of adenosine and lower adenosine polyphosphates. The stepwise degradation of ATP and other nucleoside polyphosphates by this enzyme has previously been noted by Heppel, *et al.*[6] In order to drive these degradations as nearly to completion as possible it is necessary to use a relatively high enzyme:substrate ratio since strong inhibition by orthophosphate has been demonstrated.[6] This complete degradation of the starting material does not exclude the possibility of a branched polyphosphate chain such as II but both stability considerations and other degradations make



I



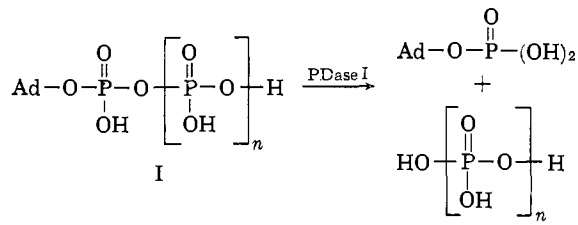
II



III

such structures unlikely. Complete degradation with *E. coli* alkaline phosphatase, however, provides the most exacting evidence for the absence in compounds of type I of contaminating dinucleoside polyphosphates of type III.

Degradation of nucleoside 5′-polyphosphates with purified phosphodiesterase-I from *Crotalus adamanteus* venom also provided useful information. In general we have qualitatively concluded that, whereas adenosine 5′-diphosphate (I, $n = 1$) is a poor substrate for this enzyme relative to *p*-nitrophenylthymidine 5′-phosphate, adenosine 5′-tri- and higher polyphosphates (I, $n = 2$–6) are efficiently cleaved to adenosine 5′-phosphate and the appropriate inorganic polyphosphate. This has been put on a quantitative basis by Razzell in these laboratories.[7] Such a degradation on the products I ($n = 1$–6) gave, in each case, adenosine 5′-phosphate and one of a series of inorganic polyphosphates (IV, $n = 1$–5) of decreasing mobility on paper chromatography in solvents A and D. The first four members of this series are chromatographically identical with available markers of ortho-, pyro-, tripoly-, and tetrapolyphosphoric acids. Thus no evidence of chain branching is to be found at least to the level of adenosine 5′-pentaphosphate, and it is considered unlikely to occur in the higher homologs. All products were periodate positive and were com-



I IV

pletely degraded to adenosine with crude *Crotalus adamanteus* venom thus confirming that no phosphate transfer to the 2′- or 3′-hydroxyl groups had occurred.

The series of minor products (peaks Ib, III, Vb, VIb, VIIb, and VIIIb) consistently gave a much lower ratio of total phosphorus to adenosine than did the nucleoside 5′-polyphosphate of type I found in the same peak. In contrast with products of structure I, these materials were completely resistant to the action of *E. coli* alkaline phosphatase and did not inhibit the degradation of adenosine 5′-triphosphate by that enzyme. Accordingly they do not possess a free, monosubstituted phosphate grouping. Conclusive evidence that these compounds form a series of α,ω-di(adenosine-5′) polyphosphates (III, $n = 1$–6) comes from their phosphorus analyses (Table I) and from degradations with purified phosphodiesterase-I. This enzyme rapidly degrades compounds of type III between the α- and β-phosphate at one end of the chain, giving adenosine 5′-phosphate and an adenosine polyphosphate of type I containing one phosphorus less than the starting material. This initial product is then more slowly degraded, as described above, to adenosine 5′-phosphate and an inorganic polyphosphate (IV). Both the initially produced nucleoside polyphosphate arising from a brief exposure to the enzyme and the ultimate inorganic polyphosphate can be identified chromatographically. Thus, for example, P^1,P^5-di-(adenosine-5′) pentaphosphate (AP_5A, III, $n = 4$) is initially degraded to adenosine 5′-tetraphosphate (I, $n = 3$) which then more slowly cleaves to tripolyphosphate. Schematically:

$$\text{A-P-P-P-P-P-A} \rightarrow \text{AP} + \text{A-P-P-P-P} \rightarrow \text{AP} + \text{P-P-P}$$

The major members of this series of compounds could also be directly compared chromatographically and electrophoretically with authentic dinucleoside polyphosphates synthesized by a different route.[8] The characterization of all the products listed in Table I thus seems assured.

Higher nucleoside 5′-polyphosphates (type I) have been known for some time as products of synthetic reactions[9] but have seldom been separated into pure components. The isolation of AP_4 and AP_5 has also been reported from natural sources[10] or as contam-

(6) L. A. Heppel, D. R. Harkness, and R. J. Hilmoe, *J. Biol. Chem.*, 237, 841 (1962).
(7) W. E. Razzell, *Methods Enzymol.*, 6, 236 (1963).
(8) J. R. Reiss and J. G. Moffatt, part III of this series, in preparation.
(9) See, *e.g.*, (a) M. Smith and H. G. Khorana, *J. Am. Chem. Soc.*, 80, 1141 (1958); (b) R. W. Chambers and H. G. Khorana, *ibid.*, 79, 3752 (1957); (c) A. M. Michelson, *Biochim. Biophys. Acta*, 91, 1 (1964); (d) W. Hasselbach, *Acta Biol. Med. Ger.*, 2, 13 (1959).
(10) I. Liebermann, *J. Am. Chem. Soc.*, 77, 3373 (1955).

inants in commercial ATP.[11] It has been shown by Hoh[12] that heating crystalline Na_2ATP at 80° results in the formation of 10% of a mixture of AP_4 and adenosine 2′(3′)-phosphate 5′-triphosphate by transphosphorylation. It seems unlikely that this phenomenon is in any way related to dismutation in pyridine. Of particular interest is the recent isolation of P^1,P^4-di(guanosine-5′) tetraphosphate from brine shrimp eggs.[13] The natural occurrence of this material increases our interest in compounds of type III.

With this understanding of the nature of the products from the simple dismutation of ATP in anhydrous pyridine we turned our attention to a study of the effects of a number of variables such as solvent, added anions, other polyphosphate esters, etc. Since a complete analysis of multiple aliquots from many reactions by ion-exchange chromatography would be extremely time consuming we have preferred to follow the course of these reactions by quantitative paper chromatography. In doing so, by spotting roughly 1 μmole of mixed nucleotides on paper and developing in solvent A one usually finds five, or perhaps six, well-separated spots which can be eluted and measured spectrophotometrically. These spots correspond mainly to the principal series of adenosine 5′-polyphosphates AMP to AP_5 (I, n = 0–4), but superimposed upon them are the spots of the minor series of diadenosine polyphosphates (see Table VI for R_f values). Since the latter series only constitutes a total of 5% of the mixture we have ignored the small error introduced and determined the extent of dismutation as if only simple nucleoside polyphosphates were formed. Quite good agreement in the product distribution of a single reaction was obtained by both ion-exchange and paper chromatography.

In order to provide a reason for the different pathways of breakdown of ATP during synthetic experiments (only degradation to shorter polyphosphate chains) and in isolated dismutation experiments (see above), we have examined the effect of an excess of inorganic phosphate ions. Anhydrous solutions of tributylammonium adenosine 5′-triphosphate were made up in pyridine containing 5 molar equiv. of tributylammonium orthophosphate and tributylammonium pyrophosphate, respectively. These were allowed to stand at room temperature for varying times and the distribution of products was compared to that in a similar reaction without added phosphate. The results are shown in Table II.

From the results it is clear that the addition of an excess of inorganic phosphate anions radically changes the reaction products. There is a much accelerated degradation of the ATP into ADP and then AMP and the formation of almost no products of increased polyphosphate chain length. This is certainly the reason that during syntheses of nucleoside polyphosphates[2] only the degradative effect is noted since such syntheses always utilize a considerable molar excess of the appropriate inorganic phosphate. In a later paper in this series[14] the effect of adding varying amounts of P^{32}-labeled orthophosphate to a dismutation reaction will be discussed.

Table II. Dismutation of ATP in the Presence of Excess Phosphates

	No added phosphate			Pyrophosphate, 5 equiv.			Orthophosphate, 5 equiv.		
	1 Day	3 Days	6 Days	1 Day	3 Days	6 Days	3 Days	6 Days	15 Days
AMP	17	26	34	27	57	78	45	64	81
ADP	24	25	30	53	29	16	51	34	18
ATP	31	24	20	18	11	6	4	2	1
AP_4	23	16	9	2	3	1	..	..	..
AP_5	5	6	3	..	..	..	..	..	..

The dismutation reaction is not unique to ATP but is apparently common to all monoesterified polyphosphates and, indeed, inorganic polyphosphates.[14] Confirmation of this comes from experiments on the stability of initially homogeneous adenosine 5′-diphosphate and adenosine 5′-tetraphosphate in anhydrous pyridine at room temperature. Once again it was necessary to use the tributylamine salts of the nucleotides in order to obtain homogeneous solutions. In both cases a reaction very similar to that occurring with ATP was observed with products containing both longer and shorter phosphate chains rapidly appearing. The results of these experiments are summarized in Table III.

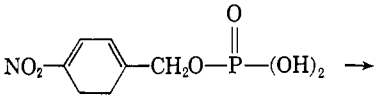
Table III. Dismutation of ADP and AP_4 in Anhydrous Pyridine

	ADP			AP_4		
	1 Day	3 Days	6 Days	1 Day	3 Days	6 Days
AMP	19	32	48	3	10	13
ADP	64	43	37	13	18	23
ATP	16	17	11	26	23	21
AP_4	1	6	3	40	32	30
AP_5	..	2	1	15	14	9
AP_6	..	..	..	3	3	3
AP_7	..	..	..	..	..	1

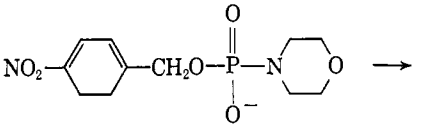
In order to prove that the purine (or pyrimidine) nucleoside structure is not in itself significant to the reaction we have synthesized and examined a simple monoester of tripolyphosphoric acid. The compound chosen was *p*-nitrobenzyl triphosphate (VII) since its strong ultraviolet absorption simplified quantitative measurements. The reaction of anhydrous triethylammonium orthophosphate with 3 molar equiv. of *p*-nitrobenzyl alcohol and trichloroacetonitrile at 50° for 40 min., essentially according to the method of Cramer,[15] gave a 72% yield of crystalline *p*-nitrobenzyl phosphate (V). In view of our more recent studies on similar reactions[16] we might expect an even better yield if the reaction were carried out at room temperature. Conversion of V to *p*-nitrobenzyl phosphoromorpholidate (VI), which was isolated in 70% yield, was accomplished by the usual method.[2] The condensation of VI with tributylammonium pyrophosphate in rigorously anhydrous dimethyl sulfoxide[17] gave *p*-

(11) (a) J. Sacks, *Biochim. Biophys. Acta*, **16**, 436 (1955); (b) C. Liebecq, K. Jaroszewicz, and A. Lallemand, *Bull. soc. chim. Biol.*, **43**, 571 (1961), and previous papers.
(12) F. Hoh, M.S. Thesis, University of Wisconsin, 1956. Cited by R. M. Bock, *Enzymes*, **2**, 1 (1960).
(13) F. J. Finamore and A. H. Warner, *J. Biol. Chem.*, **238**, 344 (1963).
(14) W. E. Wehrli and J. G. Moffatt, *J. Am. Chem. Soc.*, in press.
(15) F. Cramer and G. Weimann, *Chem. Ber.*, **94**, 996 (1961).
(16) K. E. Pfitzner and J. G. Moffatt, *Biochem. Biophys. Res. Commun.*, **17**, 146 (1964).
(17) J. G. Moffatt, *Can. J. Chem.*, **42**, 599 (1964).

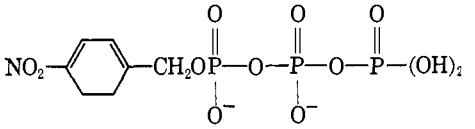
nitrobenzyl triphosphate (VII), which was isolated as the sodium salt by ion-exchange chromatography in 65% yield.



V



VI



VII

The tributylammonium salt of VII was then stored at room temperature in anhydrous pyridine for 3 days and the products were separated by ion-exchange chromatography on DEAE cellulose as shown in Figure 2. As can be seen from Figure 2 treatment in pyridine converted *p*-nitrobenzyl triphosphate into a series of compounds reminiscent of the products from ATP. The peaks I–VII were pooled, evaporated, and characterized by paper chromatography, spectra, phosphorus analysis, and enzyme degradation. The principal components were once again a homologous series of *p*-nitrobenzyl polyphosphates ranging from the mono- to the pentaphosphates. The first three members of this series (peaks I, III, and IV) were identified by direct comparison with the authentic mono-, di-, and triphosphates synthesized above, while the tetra- and pentaphosphates were characterized, following further purification by preparative paper chromatography, by phosphorus analysis and by their complete degradation to the lower members of the series with *E. coli* alkaline phosphatase. Peak II proved to be P^1,P^2-di(*p*-nitrobenzyl) pyrophosphate which was also synthesized by the general method of Khorana[18] from *p*-nitrobenzyl phosphate and dicyclohexylcarbodiimide in aqueous pyridine. As in the dismutation of ATP minor amounts of other α,ω-di(*p*-nitrobenzyl) polyphosphates were also detected admixed with the major components of each peak. These products had the typical ultraviolet spectrum of *p*-nitrobenzyl phosphate (λ_{max} 274 mμ; Σ_{max} 9350), moved as a series of spots of decreasing R_f in various paper chromatographic systems (Table VI), and were completely resistant to the action of *E. coli* alkaline phosphatase under conditions that degraded their monoesterified polyphosphate counterparts. These compounds were not studied further.

We have also examined the stability of ATP dissolved in anhydrous organic solvents other than pyridine. Such studies are handicapped by the limited solubility of nucleoside polyphosphates in nonaqueous media but it was possible to dissolve varying amounts of tributylammonium ATP in dimethyl sulfoxide, methanol, trimethyl phosphate, nitromethane, nitrobenzene, acetonitrile, and chloroform. After storage of these solutions at room temperature for up to ten days it was shown chromatographically that virtually no decomposition of the starting material had occurred. In dimethyl formamide a slight dismutation appeared to occur and after 6 days the mixture contained 2% AMP, 12% ADP, 76% ATP, and 10% AP_4. On the basis of later experiments, however, it now seems likely that this was due to a small amount of residual pyridine in the dimethylformamide since evaporation with pyridine was used to render the tributylammonium ATP anhydrous prior to addition of the other solvents.

A mixture of pyridine and dimethylformamide (1:1), however, led to rapid dismutation of tributylammonium ATP, giving a mixture of products very similar to that with pyridine alone except that AP_4 seemed to be present in consistantly larger amounts. Similar situations existed with ADP and AP_4 which were only slightly degraded by dimethylformamide alone but rapidly dismutated in pyridine–dimethylformamide (1:1). These results are gathered in Table IV.

Table IV. Dismutation of ADP, ATP, and AP_4 in Pyridine–Dimethylformamide (1:1)

	ADP			ATP			AP_4		
	1 Day	3 Days	6 Days	1 Day	3 Days	6 Days	1 Day	3 Days	6 Days
AMP	14	22	33	9	12	25	2	7	13
ADP	73	49	45	25	23	20	6	11	20
ATP	13	25	18	41	28	23	17	26	23
AP_4	..	4	4	25	31	24	56	34	28
AP_5	..	..	..	..	6	8	16	16	11
AP_6	..	..	..	..	..	..	3	6	4
AP_7	..	..	..	..	..	..	..	..	1

From a practical point of view the most significant feature of these studies on the stability of adenosine polyphosphates in various solvents lies in the realization that these compounds are very stable in certain solvents such as dimethyl sulfoxide. The combination of the excellent solvent power of anhydrous dimethyl sulfoxide for nucleotides, and the stability of nucleoside polyphosphates in it, has led to the development of a much improved selective synthesis of both ribo- and deoxyribonucleoside triphosphates through the condensation of nucleoside phosphoromorpholidates with tributylammonium pyrophosphate.[17] A particularly useful application of the knowledge that dismutation does not occur in dimethyl sulfoxide has been in the chemical synthesis of specifically β- or γ-P^{32}-labeled nucleoside 5′-triphosphates.[19] In pyridine, the solvent that has been most widely used in previous syntheses of nucleoside polyphosphates, extensive scrambling of the isotopic label would have resulted due to dismutation.[20] It is significant that one of the few other relatively efficient syntheses of adenosine 5′-triphosphate to have been described was also carried out in a pyridine-free medium of *o*-chlorophenol and tricresol.[21] Recently Michelson[9c] has described a synthesis of nucleoside 5′-triphosphates in pyridine but the method of con-

(18) H. G. Khorana, *J. Am. Chem. Soc.*, 76, 3517 (1954).

(19) D. L. M. Verheyden, W. E. Wehrli, and J. G. Moffatt, *J. Am. Chem. Soc.*, 86, 1253 (1964), and accompanying paper.

(20) The dismutation of γ-P^{32}-labeled ATP has been studied in these laboratories: ref. 3 and manuscript in preparation.

(21) K. Tanaka, M. Honjo, Y. Sanno, and H. Moriyama, *Chem. Pharm. Bull.* (Tokyo), 10, 220 (1962).

densation was such that the products were only in contact with the solvent for 40 min. and hence had but little opportunity to dismutate.

The effect of the presence of varying amounts of water in a pyridine solution of tributylammonium ATP was next investigated. This was done by adding 0.01%, 0.1%, 1.0%, 5%, 10%, and 50% water to otherwise anhydrous pyridine solutions of ATP. After 6 days at room temperature the distribution of products was determined by paper chromatography and the results are given in Table V.

Table V.[a] Effect of Water on the Dismutation of ATP in Pyridine

	Water added, %						
	0.01	0.1	1.0	5	10	50	100
AMP	26	26	34	36	24	..	..
ADP	35	30	34	52	44	13	7
ATP	20	23	18	7	27	85	90
AP_4	11	11	8	5	2	2	3
AP_5	6	10	4	..	..	..	..

[a] Data given for reactions after 6 days.

From Table V it can be seen that the result of adding up to 1% water to the pyridine does not dramatically change the product distribution. On reaching 5% water, however, there is a marked decrease in the proportion of products containing three or more phosphate groups and 88% of the mixture consists of AMP and ADP. As the proportion of water increases, however, the extent of degradation is once more reduced, and in 50% water ATP is essentially stable (85% remaining). Paper chromatographic examination of reactions that have undergone extensive degradation to ADP and AMP shows the accumulation of large amounts of orthophosphate. In a similar way, examination of dismutation reactions that have been driven toward the formation of ADP and AMP by the presence of excess orthophosphate anions shows an accumulation of inorganic pyrophosphate. It is thus apparent that in the presence of a large excess of added nucleophiles such as water or phosphate anions an activated phosphate originating from the ATP is competitively attacked to form ortho- or pyrophosphate, respectively, rather than reacting with other nucleoside polyphosphates to lengthen the polyphosphate chain. More convincing evidence for this type of mechanism will be presented in a later paper of this series dealing with isotopically labeled compounds.[14]

The over-all pattern of apparent transfer of the terminal phosphate of ATP to another phosphate species is somewhat reminiscent of the nonenzymatic transphorylations catalyzed by metal ions that have been described by Lowenstein.[22] The mechanism of these reactions are probably very different, however, and we are able to provide fairly convincing evidence for the direct participation of pyridine in the formation of the activated phosphorylating intermediate.[14]

In summary it appears that dismutation of monoesterified polyphosphates into products with both longer and shorter polyphosphate chains is a typical consequence of storing solutions of these compounds in anhydrous pyridine. The reaction appears to be relatively specific for pyridine or for mixed solvents containing pyridine. In the presence of excess ortho- or pyrophosphate or of intermediate amounts (~5–10%) of water the synthetic reaction leading to higher polyphosphate esters is inhibited and degradation to products with shorter polyphosphate chains predominates.

Experimental

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* tetrasodium ethylenediaminetetraacetic acid (100:60:1.6); solvent B, ethanol–0.5 *M* ammonium acetate buffer, pH 3.8 (5:2); solvent C, 1-propanol–29% ammonium hydroxide–water (6:3:1); solvent D, 2-propanol–water–trichloroacetic acid–29% ammonium hydroxide (75 ml.:25 ml.:5.0 g.:0.25 ml.). Paper electrophoresis was carried out on the same paper impregnated with 0.05 *M* ammonium bicarbonate (pH 7.6) or with 1 *M* acetic acid using 1000–2000 v. Phosphorus-containing compounds were visualized on paper chromatograms using the molybdate spray of Hanes and Isherwood[23] followed by ultraviolet irradiation.[24] Vicinal glycols were detected by the periodate–benzidine spray of Viscontini, *et al.*[25] Phosphorus analyses were obtained by the method of King[26] and acid-labile phosphorus was

Table VI. Paper Chromatography of Relevant Compounds[a]

Compound	R_f in solvent A	R_f in solvent B	R_f in solvent C
AMP	0.58	0.47	
AP_2A	0.58	0.25	
ADP	0.45	0.38	
AP_3A	0.48	0.20	
ATP	0.35	0.31	
AP_4	0.27	0.22	
AP_4A	0.41	0.16	
AP_5	0.22	0.13	
AP_5A	0.33	0.08	
AP_6	0.17	0.10	
AP_6A	0.28	0.07	
AP_7	0.15	0.07	
AP_7A	0.19	0.06	
p-Nitrobenzyl phosphate	0.78		0.45
p-Nitrobenzyl diphosphate	0.63		0.37
p-Nitrobenzyl triphosphate	0.51		0.32
p-Nitrobenzyl tetraphosphate	0.42		0.24
p-Nitrobenzyl pentaphosphate	0.33		0.17
P^1,P^2-Di(*p*-nitrobenzyl) pyrophosphate	0.82		0.74
P^1,P^3-Di(*p*-nitrobenzyl) triphosphate[b]	0.75		0.65
P^1,P^4-Di(*p*-nitrobenzyl) tetraphosphate[b]	0.71		0.56
P^1,P^5-Di(*p*-nitrobenzyl) pentaphosphate[b]	0.67		0.51

[a] All the adenosine phosphates were run simultaneously as were the *p*-nitrobenzyl phosphates; hence the relative mobility should be significant. [b] The characterization of these is tentative; see text.

(22) (a) J. M. Lowenstein, *Biochem. J.*, **75**, 269 (1960); (b) J. M. Lowenstein and M. N. Schutz, *J. Biol. Chem.*, **236**, 305 (1961).

(23) C. A. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).
(24) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(25) M. Viscontini, D. Hoch, and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).
(26) E. J. King, *Biochem. J.*, **26**, 292 (1932).

determined by the same method except that digestion with perchloric acid was replaced by treatment with 1 *N* HCl at 100° for 8 min. Ultraviolet measurements were made on Cary Model 15 and Zeiss PMQ-II spectrophotometers. Elemental analyses other than for phosphorus were obtained from the laboratory of A. Bernhardt, Mülheim, Germany. All evaporations were carried out at a pressure of roughly 1 mm. and a bath temperature of 30° using a Buchler flash evaporator, the condensing flask of which was cooled to −15° with circulating, refrigerated aqueous glycol.

Enzyme Degradations. 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 0.05 *M* Tris buffer, pH 9 [tris(hydroxymethyl)aminomethane].

B. Phosphodiesterase-I was a highly purified preparation from *Crotalus adamanteus* venom that was free of 5′-nucleotidase activity.[27] It was made up to contain 0.8 mg./ml. of protein in 0.05 *M* Tris buffer, pH 9, and had a specific activity of 9.3 μmole/min./ml. toward *p*-nitrophenyl thymidine 5′-phosphate or 8.3 μmole/hr./ml. toward ADP. The sample was kindly provided by Dr. W. E. Razzell.

Preparative Dismutation of Adenosine 5′-Triphosphate. Crystalline disodium adenosine 5′-triphosphate monohydrate[28] (559 mg., 1.0 mmole) was dissolved in water and converted to the pyridine salt by passage through a column containing 10 ml. of Dowex-50 (pyridinium) ion-exchange resin. The eluate and water wash were concentrated *in vacuo* with a bath temperature of 30° to a volume of roughly 10 ml. and then diluted with pyridine (30 ml.). To the homogeneous solution[29] was added distilled tri-*n*-butylamine (1.0 ml., 4 mmoles), and the mixture was swirled for a few minutes until it was once again homogeneous.[29] The solution was then evaporated to dryness and rendered anhydrous by three evaporations *in vacuo* with 10-ml. portions of pyridine. The final residue was dissolved in anhydrous pyridine[30] (10 ml.) and stored at room temperature for 3.5 days. The solvent was then evaporated to dryness *in vacuo* and residual pyridine was removed by evaporation with water. The final aqueous solution was adjusted to pH 8 with ammonium hydroxide and applied to a 3 × 38 cm. column of DEAE cellulose in the bicarbonate form. The column was washed with water until no further ultraviolet-absorbing material was released, and elution was commenced with a linear gradient of triethylammonium bicarbonate (8 l. ranging from 0.005 to 0.4 *M*). Fractions of 20 ml. were collected and the elution was followed by optical density at 259 mμ. The elution pattern is shown in Figure 1. The peaks were pooled, evaporated to dryness *in vacuo*, and freed from residual triethylammonium bicarbonate by three evaporations with 20-ml. portions of methanol. A portion of each peak (up to 200 optical density units at 259 mμ) was then purified by paper chromatography in solvent A. The resulting ultraviolet-absorbing bands (in Table I the slower and faster moving bands in each peak are referred to as "a" and "b," respectively) were eluted with water and analyzed for ultraviolet absorption and for total and acid-labile phosphorus content. From 2–5 optical density units of each compound were incubated at 37° with 10 μl. of 1 *M* Tris buffer, pH 9, 2 μl. of 0.1 *M* magnesium acetate, and either 2 μl. of phosphodiesterase-I or 20 μl. of *E. coli* alkaline phosphatase. The samples with alkaline phosphatase were incubated for 4 to 6 hr. and then examined by paper chromatography in solvent A. Under these conditions the dinucleoside polyphosphates were completely unchanged while the mononucleoside 5′-polyphosphates were converted into either adenosine or to mixtures of adenosine and lower adenosine polyphosphates. Samples with phosphodiesterase-I were chromatographically examined after 1 and 4 hr. in solvent A. After 1 hr. the diadenosine 5′-polyphosphates showed extensive degradation to adenosine 5′-phosphate and the appropriate adenosine 5′-polyphosphate, and after 4–6 hr. degradation to adenosine 5′-phosphate and an inorganic polyphosphate was almost complete. Analytical figures are given in Table I.

The Effect of Excess Phosphate Anions. A solution of 0.2 mmole of tributylammonium ATP in anhydrous pyridine was prepared as above and divided in two equal portions. Separately, distilled tributylamine (0.25 ml., 1.0 mmole) was added to a solution of orthophosphoric acid (0.5 mmole) in 80% pyridine (10 ml.) and the homogeneous solution was evaporated to dryness and rendered anhydrous by three evaporations with dry pyridine. The final residue was dissolved in pyridine (5 ml.) and added to one-half of the ATP solution. The mixture was evaporated once more, taken up in anhydrous pyridine (5 ml.), and stored at room temperature. The other half of the ATP solution was mixed with 0.5 mmole of tetrakis(tributylammonium) pyrophosphate which was prepared similarly to the orthophosphate above, and evaporated to dryness. The mixture was also dissolved in anhydrous pyridine (5 ml.) and stored at room temperature. After 1, 3, and 6 days 1-ml. aliquots of each reaction were removed and evaporated to dryness. The residue was dissolved in water and approximately 1 μmole of the mixture was separated by paper chromatography in solvent A.[31] The ultraviolet-absorbing spots were cut out, eluted with water, and quantitatively measured at 259 mμ. The results are given in Table II.

Dismutation of ADP and AP_4 in Pyridine. Chromatographically pure samples of the sodium salts of ADP and AP_4 (10 μmoles) were converted to the pyridine salts by passage through small columns (1 ml.) of Dowex-50 (pyridinium salt). Each aqueous solution was evaporated to about 0.5 ml. and 2 ml. of pyridine was added. Distilled tributylamine (9 μl., 30 μmoles for the ADP reaction and 12 μl., 50 μmoles for the AP_4 reaction) was added. The homogeneous solutions were evaporated to dryness, rendered anhydrous by three evaporations with 1-ml. portions of pyridine, and dissolved in 0.1 ml. of dry pyridine. After 1, 3, and 6 days aliquots of 10 μl. were removed and ex-

(27) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, 234, 2105 (1959).

(28) Obtained from Schwarz Bioresearch, Orangeburg, N. J., and chromatographically shown to contain only traces of ADP.

(29) It is important that the solution be homogeneous at this point.

(30) Distilled from, and stored over, calcium hydride.

(31) In the case of the reaction with orthophosphate the chromatographic separation was somewhat sharper if excess phosphate anions were first removed by bringing to pH 10 with lithium hydroxide, centrifugation, and readjustment to pH 6 with Dowex-50 (H^+) resin. Such measures were not necessary with any of the other reactions described.

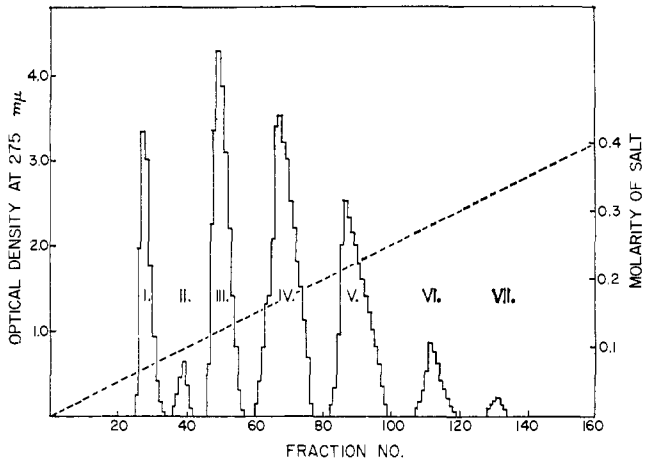


Figure 2. Ion-exchange chromatography of products from the dismutation of *p*-nitrobenzyl triphosphate in pyridine.

amined in solvent A. The distribution of products is given in Table III.

p-Nitrobenzyl Phosphate. Recrystallized *p*-nitrobenzyl alcohol (27.0 g., 0.18 mole) was dissolved in dry acetonitrile (45 ml.) and to the solution was added anhydrous, crystalline phosphoric acid[32] (5.8 g., 0.06 mole), distilled triethylamine (12.1 g., 0.12 mole), and trichloroacetonitrile[33] (28 ml., 0.28 mole). An exothermic reaction commenced rapidly and the mixture was stored at 50° for 40 min. The brown solution was evaporated *in vacuo* and the residue was dissolved in water and extracted several times with ether. The light-colored aqueous solution was passed through a column containing 300 ml. of Dowex-50 (H^+) resin and the acidic effluent was evaporated to dryness. Crystallization from acetone–chloroform gave *p*-nitrobenzyl phosphate (9.87 g., 72%) as chromatographically homogeneous white crystals, m.p. 156–159°, $\lambda_{max}^{H_2O}$ 274 mμ (Σ_{max} 9350).

Anal. Calcd. for $C_7H_8NO_6P$: C, 36.05; H, 3.46; N, 6.01; P, 13.29. Found: C, 35.97; H, 3.62; N, 5.90; P, 12.89.

p-Nitrobenzyl Phosphoromorpholidate. A solution of dicyclohexylcarbodiimide (4.13 g., 20 mmoles) in *t*-butyl alcohol (75 ml.) was added dropwise over 2 hr. to a refluxing solution of *p*-nitrobenzyl phosphate (1.165 g., 5 mmoles) and morpholine (1.70 ml., 20 mmoles) in 50% aqueous *t*-butyl alcohol (100 ml.). After a further 2 hr. under reflux, paper electrophoresis at pH 7.6 showed complete conversion of the starting material to the slower moving phosphoromorpholidate. The mixture was cooled to room temperature, dicyclohexylurea was removed by filtration, and the *t*-butyl alcohol was removed by evaporation. The aqueous solution was extracted twice with ether and evaporated to dryness. The residue was dissolved in methanol (5 ml.) and addition of anhydrous ether gave a gummy precipitate that was converted into a dry white powder on trituration with fresh ether. The yield[34] of the hygroscopic *p*-nitrobenzyl phosphoromorpholidate as its 4-morpholine N,N′-dicyclohexylcarboxamidine salt trihydrate was 2.28 g. (70%), $\lambda_{max}^{H_2O}$ 275 mμ. The product was chromatographically and electrophoretically homogeneous and was used as is.

p-Nitrobenzyl Triphosphate. *p*-Nitrobenzyl phosphoromorpholidate as the 4-morpholine N,N′-dicyclohexylcarboxamidine salt trihydrate (595 mg., 0.90 mmole) was dried by three evaporations with pyridine and then one with benzene to remove all residual pyridine. Separately, an anhydrous pyridine solution of tetrakis(tributylammonium) pyrophosphate (4 mmoles) was prepared as previously described[17] and then freed of pyridine by several evaporations with benzene. The pyrophosphate was dissolved in anhydrous dimethyl sulfoxide[35] (15 ml.) and added to the morpholidate. The homogeneous mixture was stored at room temperature for 6 days, diluted with water (120 ml.), and applied to a column (3 × 42 cm.) of DEAE cellulose (HCO_3^-). The column was well washed with water and eluted with a linear gradient of triethylammonium bicarbonate (6 l. from 0.005 to 0.4 *M*). Fractions of 25-ml. volume were collected and read at 274 mμ. The following products were isolated: *p*-nitrobenzyl phosphoromorpholidate (1%), *p*-nitrobenzyl phosphate (6%), *p*-nitrobenzyl diphosphate (12%) heavily contaminated with pyrophosphate, *p*-nitrobenzyl triphosphate (70%) containing a trace of tripolyphosphate, and *p*-nitrobenzyl tetraphosphate (1%). The triphosphate peak was evaporated to dryness *in vacuo* and residual triethylammonium bicarbonate was removed by four evaporations with methanol (20 ml.). The final residue was dissolved in methanol (5 ml.) and to it was added 1 *M* sodium iodide in acetone (10 ml.) followed by acetone (30 ml.). The resulting precipitate was washed three times with 20-ml. portions of acetone and dried *in vacuo* giving tetrasodium *p*-nitrobenzyl triphosphate (316 mg., 65%) which could be shown chromatographically to contain a trace of tripolyphosphate. For analysis a sample was purified by preparative paper chromatography in solvent C and then gave P:*p*-nitrobenzyl = 2.91 based upon Σ_{max} 9350.

Dismutation of p-Nitrobenzyl Triphosphate. The sodium salt of *p*-nitrobenzyl triphosphate (108 mg., 0.18 mmole) was converted to the tributylamine salt by the same procedure used for adenosine 5′-triphosphate. This was dried by three evaporations with pyridine, dissolved in anhydrous pyridine (1 ml.), and stored at room temperature for 3 days. The solvent was then evaporated and the residue was dissolved in water and applied to a 1.3 × 38 cm. column of DEAE cellulose (HCO_3^-). After a thorough water wash the column was eluted with a linear gradient of triethylammonium bicarbonate (3 l. from 0.005 to 0.40 *M*). Fractions (20 ml.) were collected and read at 275 mμ. The elution pattern is shown in Figure 2. Peak I (13%) is *p*-nitrobenzyl phosphate; peak II (2%) is P^1,P^2-di(*p*-nitrobenzyl) pyrophosphate[36]; peak III (24%) is *p*-nitrobenzyl diphosphate (P:*p*-nitrobenzyl = 1.99); peak IV (31%) is *p*-nitrobenzyl triphosphate containing a small amount of a diester presumably P^1,P^3-di(*p*-

(32) Fluka A. G., Buchs, Switzerland.
(33) Obtained from Aldrich Chemical Co.
(34) The low recovery in this reaction as compared with syntheses of nucleoside phosphoromorpholidates apparently lies in a partial solubility in ether. It could doubtless be improved by precipitation with petroleum ether rather than ether.
(35) Dried by distillation and storage over Linde Molecular Sieve, Type 4A.
(36) This compound was independently prepared by the reaction of *p*-nitrobenzyl phosphate in aqueous pyridine (20% water) with dicyclohexylcarbodiimide[18] and purified by paper chromatography in solvent C.

nitrobenzyl) triphosphate; peak V (23%) is *p*-nitrobenzyl tetraphosphate (P:*p*-nitrobenzyl = 3.65 after purification from a small amount of P^1,P^4-di[*p*-nitrobenzyl] tetraphosphate and tripolyphosphate in solvent C); and peak VI (6%) is *p*-nitrobenzyl pentaphosphate (P:*p*-nitrobenzyl = 4.58 after purification from a trace of P^1,P^5-di[*p*-nitrobenzyl] pentaphosphate in solvent C).

Aliquots of each peak (5 optical density units at 275 mμ) were treated in 10 μl. of water with 5 μl. of 1 *M* Tris buffer, pH 9, and 20 μl. of *E. coli* alkaline phosphatase at 37° for 1 and 24 hr., and examined by paper chromatography in solvent C. The various *p*-nitrobenzyl polyphosphates were all degraded to *p*-nitrobenzyl alcohol or (in the case of the higher polyphosphates) to mixtures of this alcohol and its lower polyphosphates. The minor impurities corresponding to di(*p*-nitrobenzyl) polyphosphates were, however, completely unchanged by this treatment.

Dismutation of ADP, ATP, and AP_4 in Pyridine–Dimethylformamide (1:1). Anhydrous solutions of the tributylamine salts of ADP, ATP, and AP_4 (10 μmoles) in pyridine were prepared by the general procedure described for the preparative experiment above and quickly evaporated to dryness. The residues were separately dissolved in 0.1 ml. of a mixture of pyridine–dimethylformamide and stored at room temperature. After 1, 3, and 6 days, 10-μl. aliquots were removed and separated in solvent A. The results are shown in Table IV.

In a similar way the anhydrous tributylamine salts were dissolved in dimethylformamide, dimethyl sulfoxide, trimethyl phosphate, nitromethane, methanol, nitrobenzene, acetonitrile, and chloroform (roughly 0.5 ml. for 10 μmoles), and any insoluble material was removed by centrifugation prior to removal of aliquots. In each case at least 93% of the nucleoside polyphosphate remained unchanged (see text for some reaction in dimethylformamide).

The Effect of Water on the Dismutation of ATP. Solutions of tributylammonium ATP (10 μmoles each) in anhydrous pyridine were prepared as above and evaporated to dryness. Each was then separately dissolved in 0.1 ml. of pyridine which had previously had 0.01, 0.1, 1.0, 5.0, 10.0, and 50.0% water added to it. The mixtures were stored at room temperature and 10-μl. aliquots were removed and examined in solvent A after 1, 3, and 6 days. The results after 6 days are given in Table V.

Dismutation Reactions of Nucleoside Polyphosphates. II. Specific Chemical Syntheses of α-, β-, and γ-P^{32}-Nucleoside 5′-Triphosphates[1]

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Specific chemical syntheses of α-, β-, γ-, and β,γ-P^{32}-labeled ATP have been developed in which no detectable isotope is present in other than the desired position. The reaction of adenosine 5′-diphosphate with morpholine and dicyclohexylcarbodiimide gave ADP-morpholidate (II) which was allowed to react with P^{32}-tributylammonium orthophosphate to give γ-P^{32}-ATP. The reaction of p-nitrobenzyl phosphoromorpholidate with P^{32}-orthophosphate gave β-P^{32}-p-nitrobenzyl diphosphate which was condensed with AMP-morpholidate giving γ-p-nitrobenzyl β-P^{32}-ATP which upon hydrogenolysis gave β-P^{32}-ATP. Phosphorylation of 2′,3′-O-isopropylideneadenosine with P^{32}-2-cyanoethyl phosphate gave P^{32}-AMP which was converted into the phosphoromorpholidate and condensed with pyrophosphate to give α-P^{32}-ATP. In a similar way, condensation of nucleoside phosphoromorpholidates with P^{32}-pyrophosphate gave β,γ-P^{32}-nucleoside 5′-triphosphates. The synthesis of ATP-morpholidate (IV) and its use in the synthesis of various adenosine 5′-tetraphosphates is also described, as well as direct syntheses of the terminal monomethyl esters of adenosine 5′-tri- and -tetraphosphates.

During the past ten years remarkable advances have been made in our understanding of the intimate mechanisms of biological reactions. Many of these reactions involve the participation of a nucleoside 5′-triphosphate, either as a direct substrate or as the progenitor of a phosphorylated intermediate or cofactor. It is clear that in various biological reactions nucleophilic attack can occur at any of the phosphorus atoms of a nucleoside 5′-triphosphate[3] thus leading to products containing the elements of the original nucleoside,[4] nucleotide,[5] ortho-,[6] or pyrophosphate.[7] Much information as to the actual course of many of these reactions has come from studies utilizing nucleoside 5′-triphosphates in which one or more of the

(1) For part I, see D. L. M. Verheyden, W. E. Wehrli, and J. G. Moffatt, *J. Am. Chem. Soc.*, **87**, 2257 (1965).

(2) (a) Syntex Postdoctoral Fellow, 1963–1964. Supported in part by the "Stiftung für Stipendien auf dem Gebiete der Chemie" (Switzerland), whose aid is gratefully acknowledged. (b) Syntex Postdoctoral Fellow, 1961–1962.

(3) See, *e.g.*, A. Kornberg, *Advan. Enzymol.*, **18**, 191 (1956).

(4) S. H. Mudd, *J. Biol. Chem.*, **238**, 2156 (1963).

(5) A. Kornberg, "Enzymatic Synthesis of DNA," John Wiley and Sons, Inc., New York, N. Y., 1961.

(6) R. Nordlie and H. Lardy, *Enzymes*, **6**, 3 (1962).

(7) H. G. Khorana, J. F. Fernandes, and A. Kornberg, *J. Biol. Chem.*, 230, 941 (1958).