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).

phosphorus atoms are isotopically labeled with P^{32}. Many different enzymatic reactions have been used, with varying success, to prepare these P^{32}-labeled nucleoside polyphosphates. By far the most frequently prepared compounds have been the various P^{32}-labeled adenosine 5′-triphosphates (α-, β-, and γ-P^{32}-ATP) since such a wealth of adenosine nucleotide-specific enzymes are available. Syntheses of γ-P^{32}-ATP have been accomplished primarily by phosphorylation of ADP by a P^{32}-labeled phosphate donor such as acetyl phosphate,[8] carbamyl phosphate,[9] phosphoenolpyruvate,[9] or polyphosphoric acid[10] in the presence of an appropriate kinase. All these methods depend upon the availability of kinases that are free of nucleoside monophosphate kinase or other contaminating enzymes in order to produce ATP labeled exclusively in the γ-phosphorus, and are virtually restricted to the adenosine series. Syntheses of β-P^{32}-ATP have generally been accomplished by first preparing β,γ-P^{32}-ATP with a nucleotide kinase, removing the γ-phosphorus by transfer to glucose with hexokinase, and finally replacing the terminal unlabeled phosphorus by one of the methods above.[7,9] The preparation of both β- and γ-P^{32}-nucleoside triphosphates has recently been accomplished with reasonable selectivity using photophosphorylation in the presence of chloroplasts.[11]

In general, the specific synthesis of β- or γ-P^{32}-labeled nucleoside triphosphates by purely chemical methods has not been successful although Tanaka[9] has enzymatically converted chemically synthesized β-P^{32}-ADP into β-P^{32}-ATP containing 96% of its isotope in the β-position. Lowenstein[12] has attempted to prepare γ-P^{32}-ATP by condensation of P^{32}-orthophosphate with ADP in the presence of dicyclohexylcarbodiimide, but in the presence of such a powerful activating agent it is not surprising that one-third of the isotope was found to be in the β-position of the product. Only in the case of α-P^{32}-nucleoside triphosphates has chemical synthesis been of general value. Methods are available for the chemical synthesis of P^{32}-labeled nucleotides which can be enzymatically or chemically converted into the corresponding triphosphates.[11,13c,14]

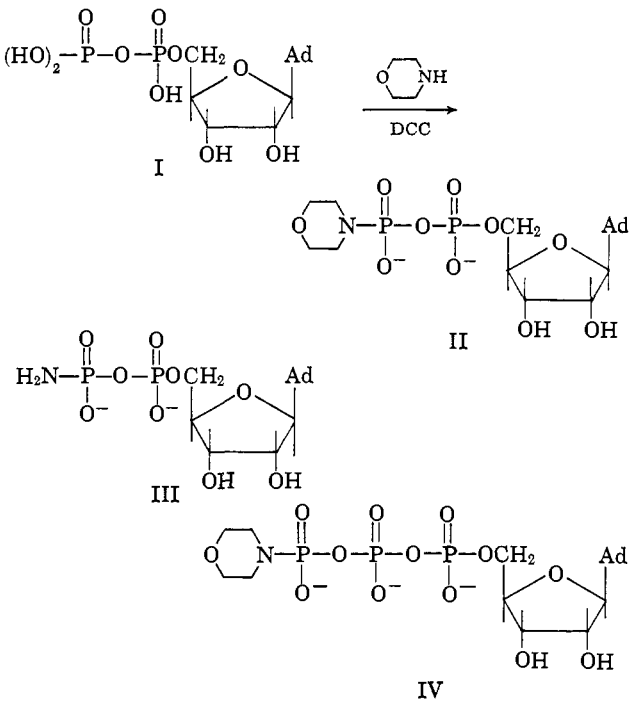
It is the purpose of this paper to describe completely chemical syntheses of α-, β-, and γ-P^{32}-labeled adenosine 5′-triphosphates with greater than 99% of the isotope in each case being in the position specified. The methods of synthesis are such that it is reasonable to expect that they can be applied to any nucleoside triphosphate, thus obviating any restrictions due to enzyme specificities.

In connection with our studies on the mechanism of dismutation reactions of adenosine 5′-triphosphate in anhydrous pyridine[1,15] we required reasonable quantities of γ-P^{32}-ATP, and this was our first goal. A widely used method for the chemical synthesis of pyrophosphate bonds consists of the reaction of a compound containing an activated P–N bond with orthophosphate or with monosubstituted orthophosphates (including pyrophosphate) under anhydrous conditions.[16] It has previously been shown[17] that monoesters of phosphoric acid are readily converted to disubstituted derivatives by reaction with either alcohols or amines in the presence of dicyclohexylcarbodiimide. Phosphoric acid diesters are, however, inert toward conversion into trialkyl phosphates or dialkyl phosphoramidates by this method. From this it could be predicted that adenosine 5′-diphosphate (I) would react with an amine and dicyclohexylcarbodiimide exclusively at its terminal monosubstituted phosphate to form a phosphoramidate, the internal α-phosphate being disubstituted and, hence, unreactive. This proved to be correct. The reaction of I with morpholine and dicyclohexylcarbodiimide under the conditions usually used for the synthesis of nucleoside phosphoromorpholidates[17b] gives a 75% yield of P^1-(adenosine-5′)-P^2-(4-morpholine) pyrophosphate (II, ADP-morpholidate) which was isolated by ion-exchange chromatography. The only other product of this reaction was adenosine 5′-phosphoromorpholidate (17%) which must arise through some decomposition of I to adenosine 5′-phosphate under the reaction conditions. The compound II was readily isolated as either its analytically pure calcium salt or, more conveniently, as its 4-morpholine N,N′-dicyclohexylcarboxamidine salt which can be directly used in subsequent reactions. As expected, II was completely inert toward the action of *E. coli* alkaline phosphatase but was readily cleaved by purified phosphodiesterase-I from rattlesnake venom to AMP and phosphoromorpholidic acid[18] which subsequently decomposed to orthophosphate during the incubation and chromatography.

By a similar reaction between adenosine 5′-diphosphate and ammonium hydroxide in the presence of dicyclohexylcarbodiimide the potentially interesting analog P^1-(adenosine-5′)-P^2-amino pyrophosphate (III, ADP-NH_2) was obtained in 42% yield. In this case the isolation of the product proved to be more difficult since III and adenosine 5′-phosphate emerged together from the DEAE cellulose (HCO_3^-) column and this peak had then to be rechromatographed at pH 5. Once again this product (III) proved to be completely resistant to *E. coli* alkaline phosphatase, thus confirming the absence of a monosubstituted phosphate group.

This general reaction can also be extended to the activation of the terminal phosphate group in nucleoside triphosphates. Thus, adenosine 5′-triphosphate

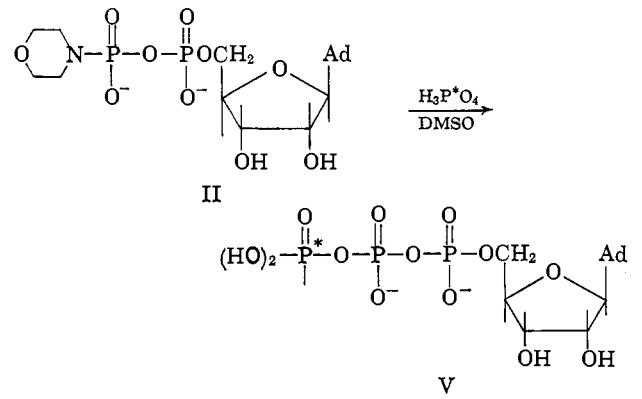
(8) A. Kornberg, S. R. Kornberg, and E. S. Simms, *Biochim. Biophys. Acta*, 20, 215 (1956).
(9) R. Tanaka, *J. Biol. Chem.* (Tokyo), 47, 207 (1960).
(10) S. R. Kornberg, *Biochim. Biophys. Acta*, 26, 294 (1957).
(11) (a) U. Z. Littauer, Y. Kimki, and A. Avron, *Anal. Biochem.*, 9, 85 (1964); (b) M. Avron, *ibid.*, 2, 535 (1961).
(12) J. M. Lowenstein, *Biochem. J.*, 65, 197 (1957).
(13) (a) G. M. Tener, *Biochem. Prepn.*, 9, 7 (1964); (b) K. E. Pfitzner and J. G. Moffatt, *Biochem. Biophys. Res. Commun.*, 17, 146 (1964); (c) D. B. Strauss and E. Goldwasser, *J. Biol. Chem.*, 236, 849 (1961); (d) R. W. Chambers, *J. Am. Chem. Soc.*, 81, 3032 (1959).
(14) See, *e.g.*, E. D. Gray, S. M. Weissman, J. Richards, D. Bell, H. M. Keir, R. M. S. Smellie, and J. N. Davidson, *Biochim. Biophys. Acta*, 45, 111 (1960).
(15) W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *J. Am. Chem. Soc.*, 86, 1254 (1964), and paper in preparation.
(16) For a review, see H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 4.
(17) (a) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, 80, 6204 (1958); (b) J. G. Moffatt and H. G. Khorana, *ibid.*, 83, 649 (1961); (c) J. G. Moffatt and H. G. Khorana, *ibid.*, 83, 663 (1961).
(18) Compounds of this type have been prepared in this laboratory by hydrogenolysis of N-substituted dibenzylphosphoramidates (K. E. Pfitzner and J. G. Moffatt, unpublished observations). They are quite unstable and tend to hydrolyse, alkylate, or polymerize depending upon the solvent.



was allowed to react with morpholine and dicyclohexylcarbodiimide and the products were separated by ion-exchange chromatography on Dowex-2 (HCO_3^-). The main product, isolated in 75% yield, proved to be P^1-(adenosine-5′)-P^3-(4-morpholine) triphosphate (IV, ATP-morpholidate). Once again some decomposition of the starting material occurred during the reaction since ADP-morpholidate (II) was also isolated in 11% yield. As expected, V was completely resistant to the action of *E. coli* alkaline phosphatase but was rapidly cleaved by venom phosphodiesterase-I to adenosine 5′-phosphate and, presumably, the monomorpholidate of pyrophosphoric acid. The latter compound appears to be considerably more stable than the analogous orthophosphate derivative and only slowly decomposed to pyrophosphate during prolonged incubation with the enzyme.

Both II and IV can, therefore, be considered as readily available, and useful, synthetic intermediates.

The condensation of ADP-morpholidate (II) with orthophosphate to form adenosine 5′-triphosphate (ATP, V) directly was then studied under several condi-



tions prior to undertaking isotopic experiments. It was initially shown that II and tributylammonium orthophosphate react in anhydrous pyridine to give ATP but, as expected from our studies on the stability of ATP in pyridine,[1,15] the initially formed product quite rapidly degraded to ADP, AMP, and lesser amounts of adenosine tetraphosphate. After relatively short reaction times (4–6 hr.) ATP was present in not unreasonable yields but it was felt that under these conditions there was a serious risk of nonspecific introduction of P^{32} when using isotopic orthophosphate. Fortunately we had already shown that anhydrous dimethyl sulfoxide is a highly satisfactory solvent for pyrophosphate syntheses[19] and does not promote dismutation reactions.[1] The reaction of II with tributylammonium orthophosphate in rigorously anhydrous dimethyl sulfoxide was rather slow at 20°, roughly 80% of the morpholidate being unchanged after 22 hr. In view of the short half-life of P^{32} it was desirable to complete the reaction as quickly as possible and, accordingly, comparable reactions were studied at 37 and at 50°. Reaction at 50° was rapid, roughly 75% of the morpholidate having disappeared after 4 hr. Tributylammonium ATP itself, however, proved to be somewhat unstable in dimethyl sulfoxide at this temperature,[20] appreciable amounts of AMP and ADP arising in 15 hr. At 37° the reaction proceeded at a reasonable rate, roughly one-third of the morpholidate remaining after 1 day. At this temperature ATP underwent but little decomposition in dimethyl sulfoxide during several days and it was felt that these conditions promised the best compromise between rapidity of the reaction and stability of the product. For the synthesis of γ-P^{32}-ATP the 4-morpholine N,N′-dicyclohexylcarboxamidine salt of ADP-morpholidate (II) was condensed with 3 equiv. of tributylammonium orthophosphate (specific activity of 6.6 mc./mmole) in rigorously anhydrous dimethyl sulfoxide at 35° for 45 hr. The mixture was then separated by ion-exchange chromatography on a column of DEAE cellulose (HCO_3^-) using a linear gradient of triethylammonium bicarbonate. The elution diagram is shown in Figure 1 and a summary of the contents of the various peaks is given in Table I.

From Table I it can be seen that chromatographically pure ATP was obtained in over 65% yield. Autoradiography of the isolated product showed that all the radioactivity coincided with the ultraviolet absorption. The only other nucleotides containing isotope were adenosine tetraphosphate and AP_5A. The specific activity of the adenosine tetraphosphate was roughly twice that of the ATP, which indicated that two of its phosphorus atoms were labeled. Labeling of both the γ- and δ-phosphorus atoms could arise through some minor dismutation of γ-P^{32}-ATP[21] or through reaction of ADP-morpholidate (II) with P^{32}-pyrophosphate. Also, the specific activity of the AP_5A (roughly 60% that of the ATP) indicates that it was formed by reaction of 1 mole of II (unlabeled) with a mole of γ-P^{32}-ATP, thus introducing only one P^{32} atom for two adenosine moieties. The presence of trace amounts of unlabeled AP_3A and AP_4A is also consistent with their formation from II and AMP or ADP, respectively.

The determination of the distribution of P^{32} in the various phosphorus atoms of labeled ATP has most frequently been determined by transfer of the γ-phos-

(19) J. G. Moffatt, *Can. J. Chem.*, **42**, 599 (1964).
(20) It has not been determined whether this is actual thermal instability or degradation due to traces of residual pyridine which had been used to render the tributylammonium ATP anhydrous.
(21) W. E. Wehrli and J. G. Moffatt, in preparation.

Table I. Products from the Synthesis of γ-P^{32}-ATP

Tube no.	O.D.[a]	% yield[b]	Total c.p.m.	% of P^{32}	c.p.m. per adenosine	Identification[c,e]
32–34	3	...	6.51×10^5	0.05	...	Not adenosine spectrum
42–49	...	...	9.95×10^8	71.0	...	Orthophosphate
50–52	152	19	1.05×10^7		...[d]	PO_4^{2-} + ADP-morph.
53–57	160		...	...	...	ADP–morph.
58–62	25	1.5	...	...	...	AMP
71–73	2	...	7.35×10^5	0.05	...	Unknown
85–92	153	9	3.29×10^7	2.6	...[d]	ADP + pyrophosphate
93–94	2	0.1	4.25×10^6		...[d]	Pyrophosphate + unknown
95–99	6.5	0.4	1.75×10^6	0.12	...[d]	AP_3A + polyphosphate
103–120	1080	65.3	3.37×10^8	23.8	4.68×10^6	ATP
124–127	6.6	0.4	7.57×10^4	0.005	...[d]	AP_4A + polyphosphate
132–140	46	2.8	3.17×10^7	2.18	1.03×10^7	AP_4
149–154	12	0.7	2.36×10^6	0.17	2.95×10^6	AP_5A

[a] Total optical density units at 259 mμ. [b] Based on optical density. [c] By paper chromatography, autoradiography, and enzyme degradation. [d] The nucleotide component contained no detectable isotope by autoradiography. All the P^{32} was located in a nonultraviolet-absorbing compound. [e] AP_3A, AP_4A, and AP_5A refer to α,ω-diadenosine tri-, tetra-, and pentaphosphates, respectively.

phate to glucose with hexokinase followed by acid hydrolysis of the remaining ADP.[8,11] Since for our other studies we required a method of analysis that was also applicable to nucleotides other than ATP we have developed a new and general technique for this purpose. Partial degradation of our labeled ATP with *E. coli* alkaline phosphatase gave a mixture of adenosine, AMP, ADP, and unreacted ATP. The distribution of label in each of the original phosphorus atoms can then be directly obtained by determination of the specific activity (c.p.m. per optical density unit or μmole of adenosine) of each of the degradation products. Thus, the specific activity of the AMP determines the isotope in the α-position of the ATP, and the specific activity of the ADP minus that of the AMP determines the isotope in the β-position, etc. We have separated the phosphatase products by paper chromatography but it is difficult to separate completely the labeled orthophosphate that is released from the various nucleotides with any single solvent system. This difficulty has been overcome by the use of micro ion-exchange columns of DEAE cellulose (HCO_3^-).

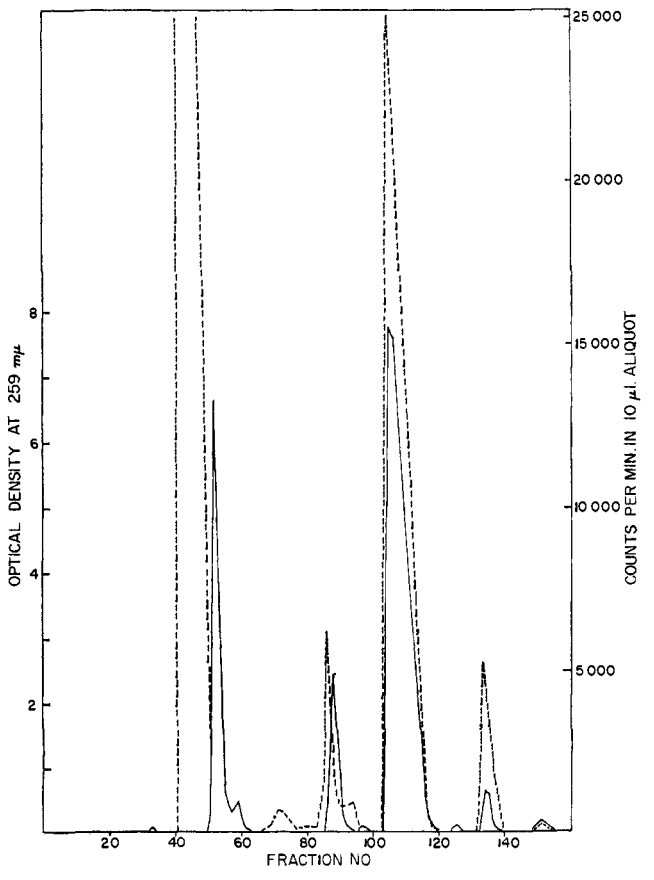


Figure 1. Ion-exchange chromatography of the products resulting from the synthesis of γ-P^{32}-ATP. The solid line represents optical density at 259 mμ and the broken line counts per minute in a 10-μl. aliquot. See Table I for the nature of the various peaks.

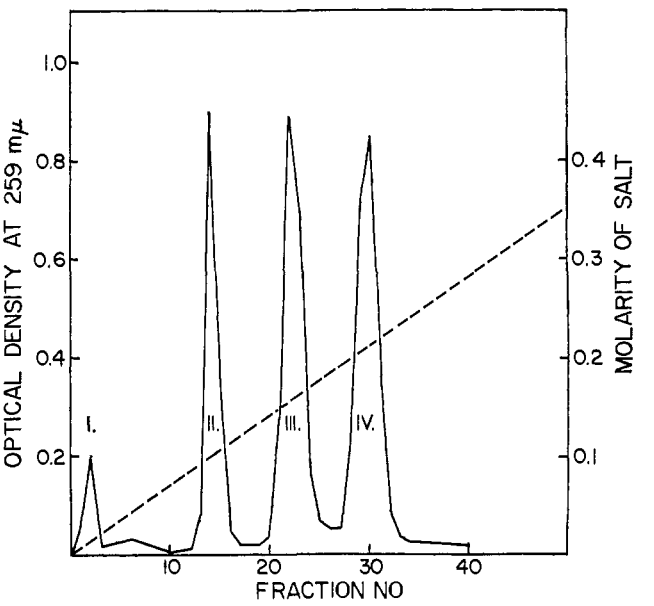


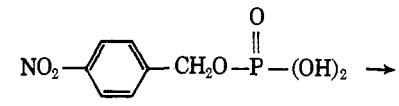
Figure 2. Enzymatic degradation of ATP. Separation of the products resulting from partial hydrolysis of 0.5 μmole of ATP with *E. coli* alkaline phosphatase as described in Experimental. Peaks 1, 2, 3, and 4 represent adenosine and adenosine 5′-mono-, -di-, and -triphosphates, respectively.

Degradation of roughly 0.5–1.0 μmole of the labeled nucleoside polyphosphate followed by chromatography on a 0.4 × 18 cm. column of the ion exchanger using a linear gradient gives a complete separation of the products in 2–3 hr. Most important is that orthophosphate emerges just before, and clearly separated from, the AMP peak. The results of the degradation of 0.5 μmole of ATP is shown in Figure 2. Applied to our synthetic product, which had an initial specific activity of 74,600 c.p.m./O.D.U., the AMP peak contained no isotope, the entire ADP peak contained 740 c.p.m./O.D. unit, and the undegraded ATP peak showed 75,400 c.p.m./O.D.U. Thus, at least 99.1% of the P^{32} was located in the γ-position. Pooling of the ADP peak followed by paper chromatography and autoradiography showed that the isotope was not in ADP itself but rather in an unidentified, nonultraviolet-absorbing spot moving near ATP in solvent A. This indicates that no isotope could be detected in the β- or α-positions of the ATP but the exact nature of the 0.9% impurity remains obscure. It is not impossible that this represents phosphorylated enzyme.[22]

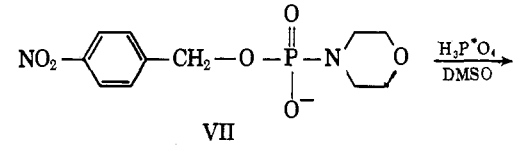
The advantage of this method of analysis lies in the fact that it is applicable to any nucleoside polyphosphate regardless of the base or the number of phosphorus atoms in the polyphosphate chain. Since *E. coli* alkaline phosphatase is inhibited by orthophosphate the enzyme reaction tends to slow down following partial hydrolysis, thus making it easy to stop the reaction at a suitable point. Further applications of this technique will be described in another paper of this series.[21] As a practical point we have observed that during hydrolysis of P^{32}-labeled compounds with the enzyme in Tris buffer a nonultraviolet-absorbing, P^{32}-containing compound (R_f 0.36 in solvent A) accumulates and is detected by autoradiography. The amount of this compound increases with higher concentrations of the buffer and it appears likely to be the phosphate ester of tris(hydroxymethyl)aminomethane arising by transphosphorylation. No such by-product occurs if dialyzed enzyme is used without buffer (at low substrate concentration) or in the presence of bicarbonate buffer rather than Tris. Recently Dayan and Wilson[23] have provided kinetic evidence of transphosphorylation by this enzyme in Tris buffer.

The specific chemical synthesis of β-P^{32}-labeled ATP was somewhat more complex. It appears quite possible to synthesize this material by converting β-P^{32}-ADP (from AMP-morpholidate and P^{32}-orthophosphate) into β-P^{32}-ADP-morpholidate (II) and then condensing this with cold orthophosphate as above. We have, however, decided to use a different approach as follows (VI–X).

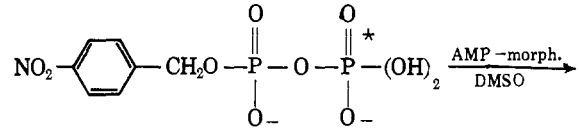
The synthesis of *p*-nitrobenzyl phosphate (VI), through condensation of *p*-nitrobenzyl alcohol and orthophosphate in the presence of trichloroacetonitrile, was accomplished in 72% yield as described earlier.[1] This was converted into *p*-nitrobenzyl phosphoromorpholidate (VII)[1] which in turn was allowed to react with P^{32}-labeled tributylammonium orthophosphate (0.25 mc./mmole) in dimethyl sulfoxide to give



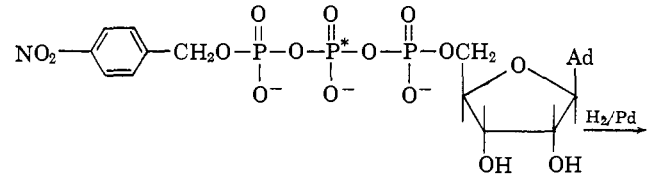
VI



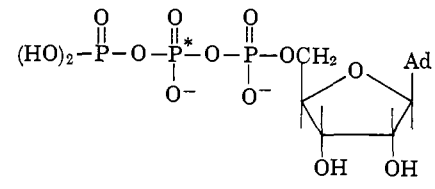
VII



VIII



IX



X

β-P^{32}-*p*-nitrobenzyl diphosphate (VIII) which was isolated in 83% yield by ion-exchange chromatography. This material was chromatographically homogeneous and identical with unlabeled VIII obtained previously as a by-product during the synthesis of *p*-nitrobenzyl triphosphate.[1] It contained 1.99 phosphorus atoms per *p*-nitrobenzyl group (from ultraviolet spectra) and was cleaved by *E. coli* alkaline phosphatase to P^{32}-orthophosphate, unlabeled VI, and *p*-nitrobenzyl alcohol. Pure VIII was directly obtained from the ion-exchange purification as its triethylamine salt and was then condensed with 0.7 molar equiv. of AMP-morpholidate[17b] in anhydrous dimethyl sulfoxide at 37° for 4 days. Here advantage was taken of the fact that disubstituted phosphates are not sufficiently nucleophilic to react with phosphoromorpholidates[17b,c] and hence VIII will be expected to react exclusively at its unlabeled terminal phosphate giving the γ-(*p*-nitrobenzyl) ester of β-P^{32}-ATP (IX). The products of this reaction were separated by ion-exchange chromatography on DEAE cellulose and the various fractions were assayed for both ultraviolet absorption and radioactivity as shown in Figure 3. The nonultraviolet absorbing radioactive peak between peaks I and II was shown to be orthophosphate and the various ultraviolet-absorbing peaks were characterized as follows: peak I (λ_{max} 259 mμ, total 162 O.D.U.) was excess AMP-morpholidate; peak II (λ_{max} 263 mμ, total 1690 O.D.U.) was an almost equal mixture of AMP and *p*-nitrobenzyl phosphate; peak III (λ_{max} 259 mμ, total 2280 O.D.U.) was mainly AMP; peak IV (λ_{max} 276 mμ, total 1710 O.D.U.) was *p*-nitrobenzyl diphosphate with a specific activity

(22) J. H. Schwartz and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.*, 47, 1996 (1961).

(23) (a) J. Dayan and I. B. Wilson, *Biochim. Biophys. Acta*, 81, 620 (1964); (b) I. B. Wilson, J. Dayan, and K. Cyr, *J. Biol. Chem.*, 239, 4182 (1964).

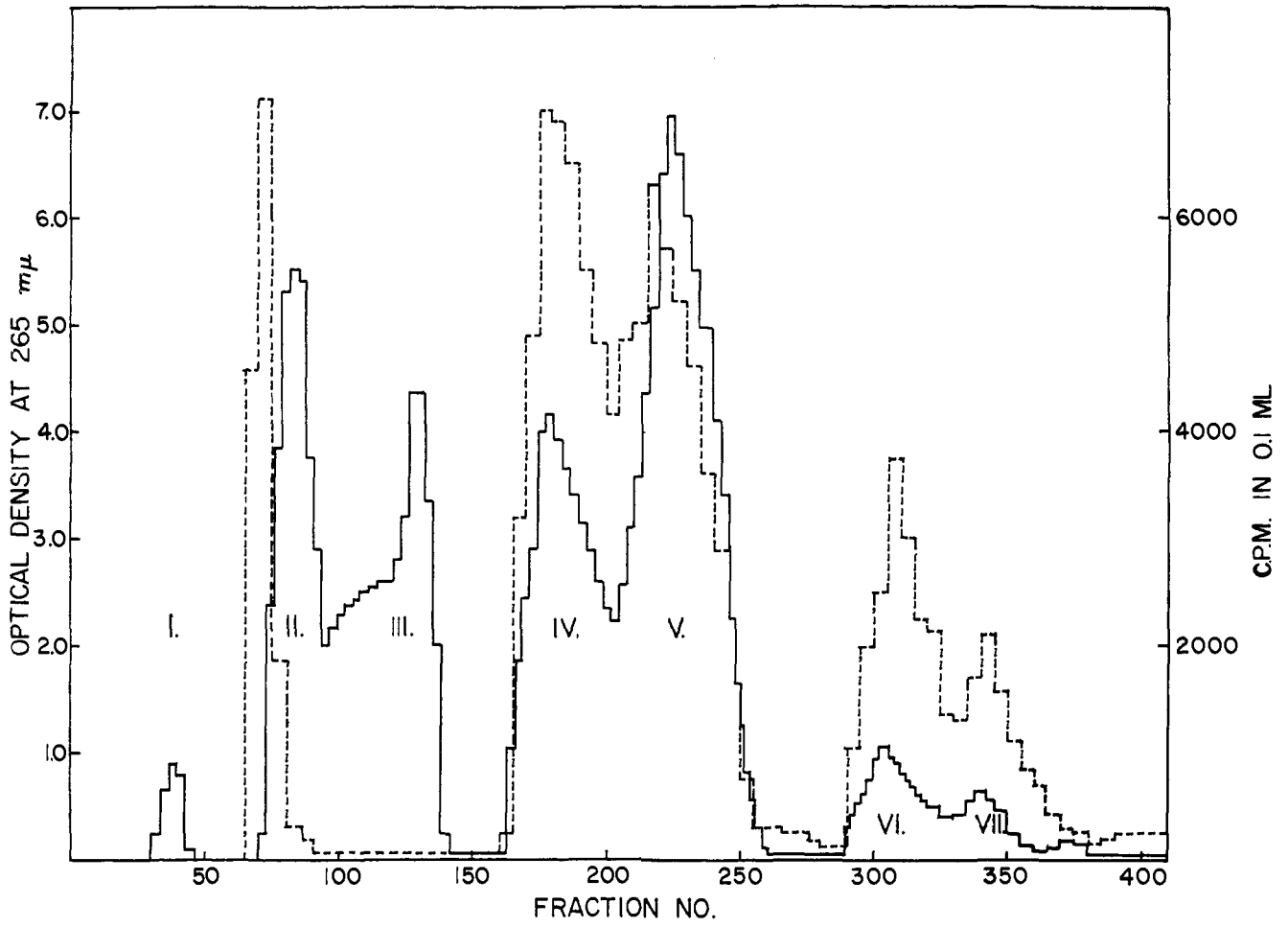


Figure 3. Ion-exchange chromatography of the products from the condensation of β-P^{32}-*p*-nitrobenzyl diphosphate and AMP-morpholidate. The solid line represents ultraviolet absorption at 265 mμ and the broken line counts per minute in a 0.1-ml. aliquot. The desired product, γ-*p*-nitrobenzyl β-P^{32}-ATP, is in peak V, and the nature of the other peaks is described in the text.

of 1.23×10^5 c.p.m. per μmole; peak V (λ_{max} 263 mμ, total 4700 O.D.U. and 4.6×10^7 c.p.m.) was mainly γ-(*p*-nitrobenzyl) β-P^{32}-ATP (IX) contaminated with some P^{32}-*p*-nitrobenzyl diphosphate from peak IV and a trace of P^{32}-ADP; peak VI (λ_{max} 270 mμ, total 502 O.D.U. and 1.54×10^7 c.p.m.) was largely β,γ-P^{32}-*p*-nitrobenzyl triphosphate contaminated with a little β,γ-P^{32}-ATP; peak VII (λ_{max} 259 mμ, total 217 O.D.U. and 6.86×10^6 c.p.m.) was predominantly β,γ-P^{32}-ATP.

A portion of peak V was purified by paper chromatography in solvent C, and the major product was characterized as having the desired structure IX as follows: Based upon an assumed Σ_{max} of 22,640 at 263 mμ, determined by summation of the spectra of AMP and *p*-nitrobenzyl phosphate at pH 7, IX was found to contain 3.03 phosphate groups per molecule. The material was completely resistant toward the action of *E. coli* alkaline phosphatase but was quantitatively cleaved to equimolar amounts of AMP and *p*-nitrobenzyl diphosphate by purified snake venom phosphodiesterase-I.

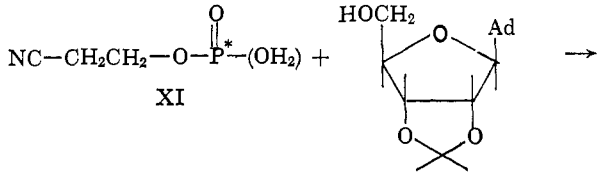
Without further purification the remainder of the crude *p*-nitrobenzyl ester (IX) was hydrogenated at room temperature in the presence of a palladium on barium sulfate catalyst.[24] The products were then separated on a column of DEAE cellulose (HCO_3^-) and the results are shown in Figure 4. This column clearly separated β-P^{32}-labeled ATP (X, fractions 80–105) from a minor amount of ADP and an appreciable amount of P^{32}-pyrophosphate arising from the P^{32}-*p*-nitrobenzyl diphosphate known to be present in the crude IX. The main peak was isolated as its chromatographically homogeneous sodium salt in an over-all yield of 33.5% from AMP-morpholidate. Isolated X analyzed for 2.98 phosphorus atoms per molecule of adenosine and had a specific activity of 9.68×10^4 c.p.m./μmole.

The location of the isotope in X was checked by two methods. First, the material was partially degraded by *E. coli* alkaline phosphatase and the AMP, ADP, and unreacted ATP were separated on a micro DEAE cellulose (HCO_3^-) column as previously described. Each ultraviolet-absorbing fraction in each peak was then analyzed for P^{32}, thus providing four or five independent checks on the specific activity of each compound. The average deviation between tubes was roughly 1%. By this method the AMP peak was shown to contain no isotope whatsoever, while the ADP had a specific activity of 9.29×10^4 c.p.m./μmole, and the ATP a specific activity of 9.41×10^4 c.p.m./μmole. Clearly there is no significant contribution to the specific activity from the γ-phosphorus, and at least 98.8% of the P^{32} could be assigned to the β-position. A sample of this material was also assayed

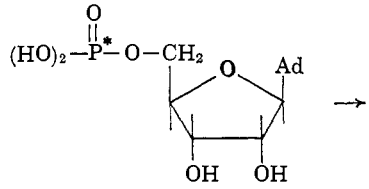
(24) R. Kuhn and H. J. Hass, *Angew. Chem.*, 67, 785 (1955).

by cleavage of the terminal phosphorus with rabbit muscle ATPase.[25] In this assay 1.0 μmole of ATP with a specific activity of 14,000 c.p.m./μmole was hydrolyzed with the purified enzyme until 0.46 μmole of orthophosphate had been released. This phosphate was selectively precipitated by the method of Sugino and Miyoshi[26] and found to have no counts above background. Assuming this determination to be sensitive at at least 25 c.p.m. above background, this would indicate a maximum of 0.3% isotope in the γ-position. The two methods together show the P^{32} to be predominantly, if not exclusively, in the β-position.

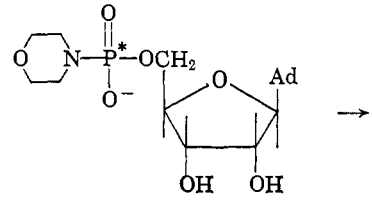
Synthesis of α-P^{32}-ATP is chemically less demanding than that of the β- or γ-isomers since the isotopic phosphorus is in a stable ester linkage and would not be expected to equilibrate with other positions under normal conditions. We have recently described a very convenient and efficient synthesis of P^{32}-labeled 2-cyanoethyl phosphate (XI)[13b] which can be used for the preparation of a wide variety of P^{32}-phosphate esters according to the elegant method of Tener.[27]



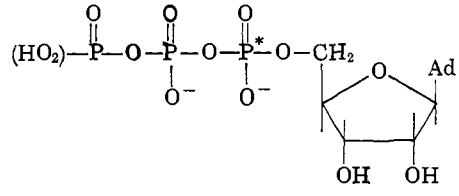
XI XII



XIII



XIV



XV

Phosphorylation of 2′,3′-O-isopropylideneadenosine (XII) with this reagent and dicyclohexylcarbodiimide gave, after mild alkaline and acid hydrolysis, a 75% yield of P^{32}-AMP (XIII) which was isolated by ion-exchange chromatography. This material was chromatographically pure and had a specific activity of 10.3 μc./μmole. This compound was converted into P^{32}-AMP-morpholidate (XIV) of specific activity 9.7 μc./μmole by the usual method.[17b] During this reaction 10.5% of the starting material (XIII) stubbornly remained unreacted but it was not considered necessary to purify the product prior to the next step. Accordingly XIV was allowed to react with 4 molar equiv. of tributylammonium pyrophosphate in rigorously anhydrous dimethyl sulfoxide according to our general method.[19] The products were separated by ion-exchange chromatography on DEAE cellulose giving α-P^{32}-ATP (XV) which was isolated as its sodium salt in 71% yield with a specific activity of 7.45 μc./μmole. This compound was contaminated with only a faint trace (less than 1%) of ADP and the isotope was shown to reside exclusively in the α-position. This was done by degradation of 1 μmole of the compound (7.5×10^6 c.p.m.) with purified snake venom phosphodiesterase-I which quantitatively gave AMP and pyrophosphoric acid. No trace of radioactivity was found in the pyrophosphate by autoradiography while the AMP contained all the counts.

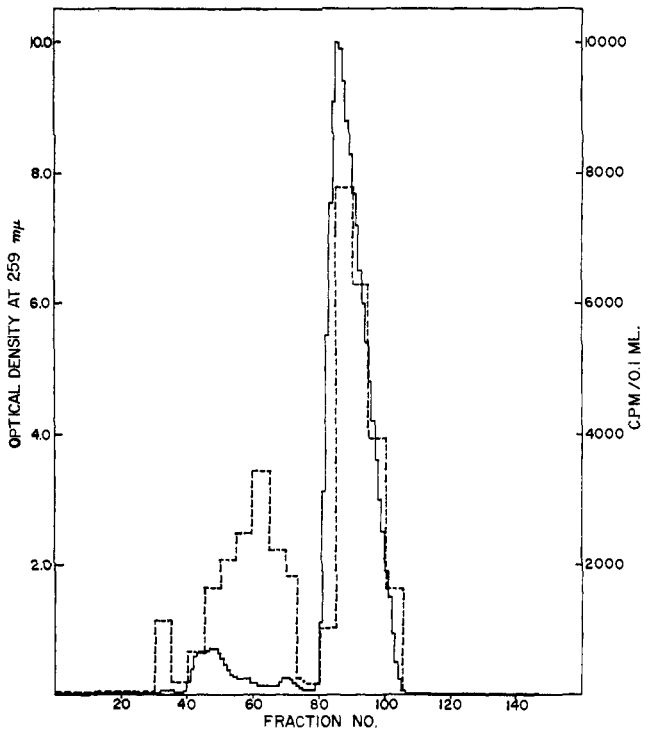
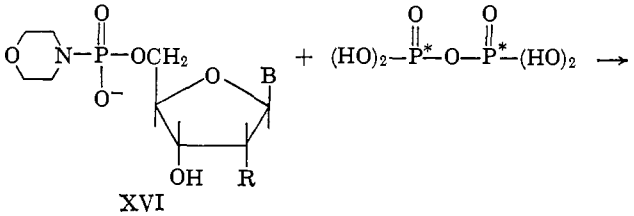


Figure 4. Ion-exchange chromatography of the products from hydrogenation of crude γ-nitrobenzyl β-P^{32}-ATP. The solid line represents ultraviolet absorption at 259 mμ and the broken line gives counts per minute in a 0.1-ml. aliquot.

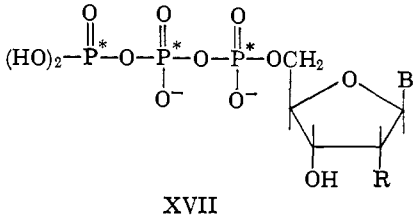
We have also had occasion, as part of another study,[28] to prepare β,γ-P^{32}-labeled ATP and thymidine triphosphate of fairly high specific activity. These were prepared through condensation of tributylammonium P^{32}-pyrophosphate[29] with the nucleoside phosphoro-

(25) W. W. Kielley, *Methods Enzymol.*, 2, 588 (1955). We are very grateful to Dr. R. A. Smith of U.C.L.A. for performing this assay for us.
(26) Y. Sugino and Y. Miyoshi, *J. Biol. Chem.*, 239, 2360 (1964).
(27) G. M. Tener, *J. Am. Chem. Soc.*, 83, 159 (1961).
(28) W. E. Wehrli and A. Kornberg, unpublished experiments.
(29) This was prepared essentially according to the method of P. Berg [*J. Biol. Chem.*, 233, 601 (1958)], by Mr. L. L. Bertsch of the Dept. of Biochemistry, Stanford University. We are very grateful to Mr. Bertsch for his help. As obtained the material contained 90% of its P^{32} as pyrophosphate, 9% as orthophosphate, and 1% as tripolyphosphate, as determined by paper chromatography and determination of radioactivity in eluted spots.

morpholidates (XVI) in anhydrous dimethyl sulfoxide.[19] Thus the reaction of thymidine 5′-phosphoromorpholi-



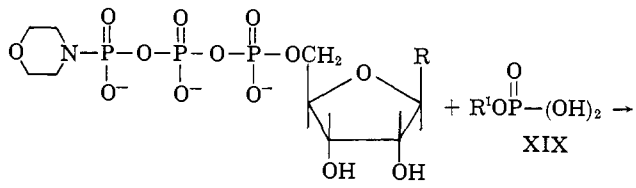
XVI



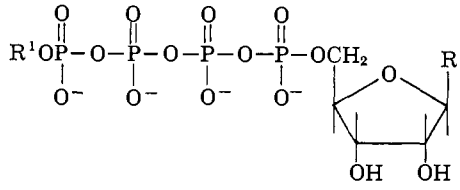
XVII

date (XVI, B = thymine, R = H)[17b] with 4 equiv. of pyrophosphate with a specific activity of roughly 2 mc./μmole in anhydrous dimethyl sulfoxide followed by ion-exchange chromatography on DEAE cellulose (HCO_3^-) gave a 72% yield of β,γ-P^{32}-thymidine 5′-triphosphate (XVII, B = thymine, R = H) with a specific activity of 1.47×10^9 c.p.m./μmole (gas flow counter). This material was chromatographically homogeneous with respect to P^{32} content and was incorporated into deoxy-AT-copolymer by DNA polymerase with the same efficiency as unlabeled thymidine 5′-triphosphate.[28] This experiment also conclusively demonstrated the complete absence of label in the α-position. By a similar reaction between P^{32}-pyrophosphate and adenosine 5′-phosphoromorpholidate (XVI, B = adenine, R = OH),[17b] β,γ-P^{32}-ATP (XVII, B = adenine, R = OH) was obtained in 62% yield. In this case the product was contaminated by a trace (less than 1%) of tripolyphosphate which, unlike the case of the thymidine analog, did not completely separate on the column. This impurity could be removed completely by charcoal adsorption but only at the cost of a considerable loss in yield. For most purposes the directly obtained material is quite satisfactory.

Finally, while we have not done any isotopic experiments, we have demonstrated the utility of the γ-morpholidates of nucleoside 5′-triphosphates in the synthesis of nucleoside tetraphosphates. This, of course, should be directly applicable to the synthesis of, for example, δ-P^{32}-adenosine 5′-tetraphosphate and related compounds. Thus condensation of ATP-morpholidate (XVIII, R = adenine) with 4 molar equiv. of tributylammonium orthophosphate (XIX, R = H) in anhydrous dimethyl sulfoxide gave a 72% yield of adenosine 5′-tetraphosphate (XX, R = adenine) which was isolated by ion-exchange chromatography. This synthesis of adenosine 5′-tetraphosphate proved to be somewhat superior to that *via* condensation of AMP-morpholidate with tripolyphosphate in dimethyl sulfoxide, since in the latter case it is difficult to separate completely excess tripolyphosphate from the product by ion-exchange chromatography. In a similar reaction ATP-morpholidate (XVIII, R = adenine) was condensed with adenosine 5′-phosphate (XIX, R = adenosine) in anhydrous dimethyl sulfoxide giving P^1,P^4-di(adenosine-5′) tetraphosphate (XX, R =



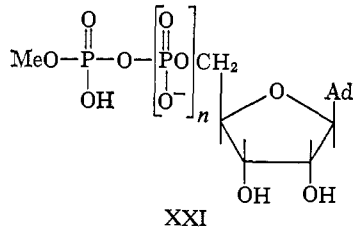
XVIII



XX

adenine, R^1 = adenosine-5′) in 31% yield. Similar reactions are now being investigated for the synthesis of the naturally occurring P^1,P^4-di(guanosine-5′) tetraphosphate[30] (R = guanine, R^1 = guanosine) and related compounds.

We have also prepared simple terminal monoalkyl esters of nucleoside polyphosphates by direct esterification mediated by a carbodiimide. Thus the reaction of the tributylamine salt of ATP in anhydrous methanol with dicyclohexylcarbodiimide gave the γ-monomethyl ester of ATP (XXI, n = 2) in 67% yield. As in the preparation of the terminal morpholidate (IV) some decomposition of the starting material occurred during the reaction since the β-monomethyl ester of ADP (XXI, n = 1) and the monomethyl ester of AMP (XXI, n = 0) were formed as by-products.



XXI

In a similar reaction starting with adenosine 5′-tetraphosphate the terminal monomethyl ester (XXI, n = 3) together with its lower homologs (XXI, n = 0, 1, 2) was obtained. In each case the compound was not affected by *E. coli* alkaline phosphatase but was rapidly degraded to AMP and a stable monomethyl polyphosphate by phosphodiesterase-I from snake venom. As in the synthesis of the terminal phosphoromorpholidates, the exclusive formation of a monoester at the terminal position of the polyphosphate chain is to be predicted in such a carbodiimide reaction.[17]

In summary, completely chemical methods have been developed for the synthesis of α-, β-, γ-, and β,γ-P^{32}-labeled ATP. The over-all yields in each case are quite good and we have been unable to detect any isotope in other than the desired position. In view of the generality of reactions utilizing nucleoside phosphoromorpholidates it seems safe to predict that all these syntheses would be equally applicable to the synthesis of specifically labeled triphosphates derived from any ribo- or deoxyribonucleoside. They may also

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

Table II. Paper Chromatography of Relevant Compounds[a]

Compound	R_f in solvent A	B	C
AMP	0.51	0.17	0.23
ADP	0.35	0.08	0.18
ATP	0.25	0.04	0.13
AP_4	0.18	0.02	0.09
AMP-morpholidate	0.72	0.54	0.53
ADP-morpholidate	0.52	0.34	0.38
ATP-morpholidate	0.25	0.22	0.29
ADP-amide	0.40	0.17	0.25
p-Nitrobenzyl phosphate	0.75	0.54	0.54
p-Nitrobenzyl phosphoro-morpholidate	0.82	0.83	0.83
p-Nitrobenzyl diphosphate	0.60	0.34	0.47
p-Nitrobenzyl triphosphate	0.48	0.26	0.41
γ-*p*-Nitrobenzyl ATP	0.53	0.29	0.43
2′,3′-O-Isopropylidene-adenosine	0.93	0.79	0.81
Cyanoethyl 2′,3′-O-isopropylidene AMP	...	0.72	0.71
2′,3′-O-Isopropylidene AMP	...	0.42	0.42
P^1,P^4-Di(adenosine-5′) tetraphosphate	0.33	0.04	0.11
γ-O-Methyl ATP	0.30	0.20	0.25
δ-O-Methyl AP_4	0.22	0.12	0.18

[a] The various compounds were run simultaneously and the relative R_f values should be significant.

be extended to the synthesis of labeled tetraphosphates through the use of the γ-morpholidates of nucleoside 5′-triphosphates.

Experimental

General Methods. Chromatographic and analytical techniques were similar to those described previously.[1] The paper chromatographic systems used were: solvent A, isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* tetrasodium ethylenediaminetetraacetic acid (100:60:1.6); solvent B, ethanol–1 *M* ammonium acetate, pH 7.5 (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.). Enzyme degradations were conducted using the enzyme preparations previously described.[15] In addition, chromatographically purified *E. coli* alkaline phosphatase[31] was freed from salts by dialysis against 0.05 *M* ammonium bicarbonate at 0° for 15 hr. and then against three changes of distilled water. It was then made up to a concentration of 100 μg. of protein/ml. and showed the same specific activity as an equivalent concentration of the undialyzed enzyme. Elemental analyses were obtained in the laboratory of A. Bernhardt, Mulheim, Germany, and P^{32} was measured on aluminum planchets using a Nuclear Chicago gas flow counter.

P^1-(Adenosine-5′)-P^2-(4-morpholine) Pyrophosphate. (ADP-morpholidate, II). Dicyclohexylcarbodiimide (1.0 g., 4.8 mmoles) in *t*-butyl alcohol (35 ml.) was added dropwise over 3 hr. to a refluxing solution of free acid adenosine 5′-diphosphate (0.8 mmole) and morpholine (0.50 ml., 5 mmoles) in 50% aqueous *t*-butyl alcohol (16 ml.). The mixture was then further refluxed for 3 hr., cooled, and evaporated to roughly half its volume. The remaining aqueous mixture was shaken with ether (50 ml.) and filtered to remove dicyclohexylurea. The aqueous layer was applied to a 2 × 25 cm. column of Dowex-2 (HCO_3^-) resin and thoroughly washed with water. Elution was effected with a linear gradient of 6 l. of triethylammonium bicarbonate from 0.005 to 0.5 *M*. Two ultraviolet-absorbing peaks were obtained. The first (2050 optical density units at 259 mμ, 17%) was eluted at roughly 0.13 *M* salt and was shown to be AMP-morpholidate while the second (9000 O.D.U., 75%) was eluted at roughly 0.3 *M* and was ADP-morpholidate. The pooled second peak was evaporated to dryness *in vacuo* at 30° and residual triethylammonium bicarbonate was removed by four evaporations with 50-ml. portions of methanol. The final residue (which could be precipitated from methanol with ether as a rather hygroscopic solid) was dissolved in ethanol (3 ml.) and quantitatively precipitated as the calcium salt by addition of 1 *M* ethanolic calcium chloride (1 ml.) followed by excess ethanol. The precipitate was washed free of chloride ions with ethanol and dried *in vacuo* at room temperature giving 300 mg. of the calcium salt of ADP-morpholidate dihydrate.

Anal. Calcd. for $C_{14}H_{20}N_6O_{10}P_2Ca \cdot 2H_2O$: C, 29.48; H, 4.24; N, 14.73; total P, 10.86; acid-labile P, 5.43. Found: C, 30.36; H, 4.62; N, 14.50; total P, 10.68; acid labile P, 5.14.

In subsequent preparations the evaporated, salt-free peak from the column was dissolved in methanol and to it was added 3 molar equiv. of 4-morpholine N,N′-dicyclohexylcarboxamidine.[17b] The solution was evaporated to dryness and the residue was dissolved in methanol (3 ml.) and precipitated by addition of ether (30 ml.). Trituration of the resulting sirup with fresh ether gave the bis(4-morpholine N,N′-dicyclohexylcarboxamidine) salt of II as a chromatographically pure white solid which could be directly used in later experiments. The ammonium salt of II (1 μmole) was completely degraded to AMP by 10 μl. of phosphodiesterase-I within 30 min. at 37° at pH 9, but remained totally unchanged after incubation with 20 μl. of *E. coli* alkaline phosphatase and 10 μl. of Tris buffer, pH 9, for 4 hr.

P^1-(Adenosine-5′)-P^2-amino Pyrophosphate (ADP-amide, III). A solution of dicyclohexylcarbodiimide (309 mg., 1.5 mmoles) in *t*-butyl alcohol (5 ml.) was added dropwise over 1.5 hr. to a refluxing solution of adenosine 5′-diphosphate (0.20 mmole of ammonium salt) and concentrated ammonium hydroxide (0.065 ml., 1 mmole) in 50% aqueous *t*-butyl alcohol (10 ml.). The mixture was then refluxed for a further 8 hr., cooled, and evaporated to half its volume. The mixture was shaken with ether (25 ml.) and filtered, and the aqueous layer was extracted once more with ether. It was then adjusted to pH 8 and applied to a 1.5 × 30 cm. column of DEAE cellulose (HCO_3^-) (total of 3240 optical density units at 259 mμ). Elution was effected with a linear gradient of 2 l. of triethylammonium bicarbonate (0.005 to 0.4 *M*). Three ultraviolet-absorbing peaks were obtained. Peak I (607 O.D.U. at 259 mμ, 20%) was not degraded by *E. coli* alkaline phosphatase and was electrophoretically and chromatographically identified as adenosine 5′-phosphoramidate.[32] Peak II (2000 O.D.U.) was a mixture of AMP and the desired ADP-amide (III),

(31) Worthington Biochemical Co., Freehold, N. J.

(32) R. W. Chambers and J. G. Moffatt, *J. Am. Chem. Soc.*, 80, 3752 (1958).

which could be readily distinguished by electrophoresis at pH 5. Peak III (580 O.D.U., 19%) was unreacted ADP. Most of peak II (1800 O.D.U.) was evaporated to dryness, freed from salt by several evaporations with methanol, and applied at pH 8 to a 1.2 × 26 cm. column of DEAE cellulose (acetate). Elution was effected with a linear gradient of 1 l. of ammonium acetate buffer, pH 5.0 (0.005 to 0.2 *M*). Two well-resolved peaks were obtained, the first (621 O.D.U.) being AMP and the second (1158 O.D.U., 42% from ADP) being ADP-amide. The second peak was diluted roughly sevenfold with water, adjusted to pH 7.8 with 4 *M* lithium hydroxide, and adsorbed on a 2 × 8 cm. column of DEAE cellulose (HCO_3^-). After a thorough water wash III was quantitatively recovered by elution with 50 ml. of 0.7 *M* triethylammonium bicarbonate. The pooled eluate was evaporated to dryness, freed of salt by several evaporations with methanol, and dissolved in ethanol (10 ml.). Addition of 1 *M* calcium chloride in ethanol (0.2 ml.) and acetone (20 ml.) precipitated the calcium salt of ADP-amide (43 mg.) which was washed free of chloride ions with ethanol–acetone and dried *in vacuo*. The material was chromatographically and electrophoretically homogeneous and showed a phosphorus–adenosine ratio of 2.08. Treatment of 1 μmole of the ammonium salt of III with 20 μl. of *E. coli* alkaline phosphatase and 5 μl. of 1 *M* Tris buffer, pH 9, for up to 5 hr. at 37° resulted in no degradation, while comparable treatment of ADP led to complete hydrolysis.

P^1-(Adenosine-5′)-P^3-(4-morpholine) Triphosphate (ATP-morpholidate, IV). Dicyclohexylcarbodiimide (1.24 g., 6 mmoles) in *t*-butyl alcohol (25 ml.) was added dropwise over 2 hr. to a refluxing solution of the morpholine salt of adenosine 5′-triphosphate (1 mmole), and morpholine (0.32 ml., 4 mmoles) in 50% aqueous *t*-butyl alcohol (50 ml.). The mixture was then refluxed for a further 4 hr., cooled, and evaporated to roughly half its volume. The mixture was shaken with ether (50 ml.) and filtered, and the aqueous solution was applied to a 2 × 25 cm. column of Dowex-2 (HCO_3^-) resin. Elution was effected with a linear gradient of 5 l. of triethylammonium bicarbonate (0.2 to 1.0 *M*) and gave ADP-morpholidate (11%), followed by a large peak of ATP-morpholidate (11,510 optical density units, 75%) and a small peak of unreacted ATP. The major peak was evaporated to dryness, evaporated five times with 25-ml. portions of methanol, and then dissolved in methanol in the presence of 4-morpholine N,N′-dicyclohexylcarboxamidine. This mixture was evaporated to dryness, dissolved in methanol (2 ml.), and precipitated with ether (30 ml.). The precipitate was washed three times with ether and dried *in vacuo* giving the chromatographically pure 4-morpholine N,N′-dicyclohexylcarboxamidine salt of ATP-morpholidate (997 mg.), which was ideally suited for direct use in other reactions. In a separate experiment the ATP-morpholidate was isolated as its calcium salt by addition of an ethanolic solution of calcium chloride to the evaporated product from the column.

Anal. Calcd. for $C_{14}H_{20}N_6O_{13}P_3Ca_{1.5}\cdot 4H_2O$: C, 23.84; H, 4.00; N, 11.94; total P:labileP:adenosine, 3.00:2.00:1.00. Found: C, 24.14; H, 3.97; N, 11.06; total P:labile P:adenosine, 2.96:1.94:1.00.

Incubation of 1 μmole of the ammonium salt of IV with 10 μl. of Tris buffer, pH 9, and 10 μl. of phosphodiesterase-I for 30 min. at 37° resulted in complete cleavage to AMP and a nonultraviolet-absorbing phosphorus-containing spot of R_f 0.30 in solvent C which slowly was replaced by pyrophosphate (R_f 0.11) on prolonged incubation. Compound IV was unchanged after incubation for 4 hr. at 37° with 20 μl. of *E. coli* alkaline phosphatase and 10 μl. of Tris buffer, pH 9.

γ-P^{32}-Adenosine 5′-Triphosphate. The 4-morpholine N,N′-dicyclohexylcarboxamidine salt of ADP-morpholidate (0.1 mmole) was dissolved in dry pyridine (3 ml.), evaporated to dryness, and rendered anhydrous by three further evaporations with 2-ml. portions of pyridine.[33] Residual pyridine was then removed by two evaporations with 2-ml. portions of anhydrous benzene.[33] Separately a solution of 1 *M* phosphoric acid (0.3 ml., 0.3 mmole) was mixed with 2 mc. of free acid P^{32}-phosphoric acid[34] and diluted with 1.2 ml. of pyridine. Distilled tri-*n*-butylamine (0.072 ml., 0.3 mmole) was added to the clear solution which was then evaporated to dryness and made anhydrous by three evaporations with 2-ml. portions of pyridine. After two further evaporations with 2-ml. portions of benzene the residue was dissolved in rigorously anhydrous dimethyl sulfoxide[35] (two 1-ml. portions) and added to the morpholidate. The clear solution was sealed and stored at 35° for 45 hr. It was then diluted with water (40 ml.), applied to a 2 × 33 cm. column of DEAE cellulose (HCO_3^-), and thoroughly washed with water. Elution was effected with a linear gradient of 3 l. of triethylammonium bicarbonate (0.005 to 0.35 *M*) and fractions of 20 ml. were collected as shown in Figure 1. The fractions were pooled as shown in Table I and fractions 103–120 (0.24 *M* salt) were carefully evaporated to dryness *in vacuo*. Residual bicarbonate was removed by three evaporations with 20-ml. portions of methanol and the final residue was dissolved in 2 ml. of methanol. Addition of 1 *M* sodium iodide in acetone (0.5 ml.) followed by excess acetone (10 ml.) quantitatively precipitated the sodium salt of γ-P^{32}-ATP which was washed three times with 10-ml. portions of acetone. The final yield of sodium salt was 42 mg. and the specific activity was 4.68×10^6 c.p.m./μmole. It was chromatographically homogeneous both by ultraviolet absorption and P^{32} content and was indistinguishable from authentic ATP in several solvent systems.

Determination of the Labeling Pattern in ATP. ATP (0.5 μmole) was incubated with 20 μl. of dialyzed *E. coli* alkaline phosphatase and 5 μl. of ammonium carbonate buffer (pH 8) in 40 μl. of water for 40 min. at 35°. Water (1 ml.) and chloroform (1 ml.) were then added and the mixture was vigorously agitated for several minutes on a Vortex Jr. mixer.[36] The mixture was filtered through glass wool and the aqueous layer was applied to a 0.4 × 18 cm. column of DEAE

(33) Distilled and stored over calcium hydride.

(34) From Abbott Laboratories and containing only a trace of pyrophosphate.

(35) This material, a generous gift from the Crown Zellerbach Corp., Camas, Wash., was distilled *in vacuo* and stored over Linde Molecular Sieve, Type 4A.

(36) Scientific Industries, Inc., Queens Village, N. Y. If the enzyme is not denatured prior to the chromatography step, some hydrolysis occurs on the column and gives unsharp peaks.

cellulose (HCO_3^-). Elution was effected with a linear gradient of triethylammonium bicarbonate (100 ml. from 0.005 to 0.35 *M*). Fractions of 2.5 ml. were collected and assayed by ultraviolet absorption at 259 mμ. In some experiments the specific activity of each ultraviolet-absorbing fraction was determined (*e.g.*, with the β-P^{32}-ATP), and in others the peaks were pooled and the specific activity was determined. The results for the various labeled ATP's are given in the text and a representative elution pattern is shown in Figure 2.

β-P^{32}-p-Nitrobenzyl Diphosphate (VIII). The 4-morpholine N,N′-dicyclohexylcarboxamidine salt of *p*-nitrobenzyl phosphoromorpholidate trihydrate[1] (595 mg., 0.9 mmole) was dried by three evaporations to dryness *in vacuo* with 5-ml. portions of anhydrous pyridine. Residual pyridine was then removed by two evaporations with anhydrous benzene. Separately bis(tri-*n*-butylammonium) orthophosphate (0.4 mmole) containing 1 mc. of P^{32} was dried with pyridine and benzene in the same way and was then added to the morpholidate in 10 ml. of rigorously anhydrous dimethyl sulfoxide. The clear solution was stored at 37° for 3 days, diluted with water (25 ml.), and applied to a 2.3 × 40 cm. column of DEAE cellulose (HCO_3^-). After a thorough water wash the column was eluted with a linear gradient of triethylammonium bicarbonate (6 l. from 0.005 to 0.25 *M*). Two small peaks of unreacted morpholidate and *p*-nitrobenzyl phosphate were eluted at 0.025 and 0.07 *M* salt, respectively, and were followed by a large peak (7000 optical density units at λ_{max} 276 mμ, 83%) of β-P^{32}-*p*-nitrobenzyl diphosphate. This peak was evaporated to dryness and freed from triethylammonium bicarbonate by four evaporations with 25-ml. portions of methanol. The product was homogeneous both by ultraviolet absorption and P^{32} content and contained 1.99 phosphate groups per molecule based upon its ultraviolet spectrum (λ_{max} 276 mμ, Σ_{max} 9350). Incubation of 1 μmole of VIII with 20 μl. of dialyzed *E. coli* alkaline phosphatase for 30 min. at 37° gave *p*-nitrobenzyl alcohol, isotope-free *p*-nitrobenzyl phosphate, and P^{32}-orthophosphate as shown by autoradiography using solvent C.

γ-(p-Nitrobenzyl)-β-P^{32}-adenosine 5′-Triphosphate (IX). The 4-morpholine N,N′-dicyclohexylcarboxamidine salt of adenosine 5′-phosphoromorpholidate[17b] (0.5 mmole) was dried by three evaporations with pyridine (5 ml.) followed by two evaporations with benzene. Separately triethylammonium β-P^{32}-*p*-nitrobenzyl diphosphate (6400 O.D.U., 0.7 mmole), as directly obtained above, was similarly dried by evaporation with pyridine and then benzene, and the two compounds were mixed in anhydrous dimethyl sulfoxide (3 ml.). After reaction at 37° for 4 days the mixture was diluted with water (25 ml.) and applied to a 2 × 40 cm. column of DEAE cellulose (HCO_3^-). After a thorough water wash the column was eluted with a linear gradient of triethylammonium bicarbonate (8 l. from 0.005 to 0.2 *M*). The elution was followed by ultraviolet absorption at 265 mμ and is shown in Figure 3. Radioactivity was also determined on 0.1-ml. aliquots. The various peaks were pooled and quantitatively assayed for ultraviolet absorption and P^{32} content as described in the text. Peak V was pooled (fractions 190–260) so as to include some of peak IV but all of the desired product IX as shown by paper chromatography in solvent A. Peak V was evaporated to dryness and freed from salt by four evaporations with 25-ml. portions of methanol. It contained a total of 4700 optical density units at λ_{max} 263 mμ and 4.6 × 10^7 c.p.m. and was mainly IX contaminated with some P^{32}-*p*-nitrobenzyl diphosphate and a trace of P^{32}-ADP. A portion was purified by chromatography in solvent C and then showed the presence of 3.03 phosphate groups per molecule based upon an assumed Σ_{max} of 22,640 at 263 mμ. The material (0.5 μmole) remained completely unchanged upon incubation for 3 hr. with 20 μl. of *E. coli* alkaline phosphatase but was completely hydrolyzed to AMP and P^{32}-*p*-nitrobenzyl diphosphate upon incubation for the same time with 10 μl. of purified phosphodiesterase-I.

β-P^{32}-Adenosine 5′-Triphosphate (X). Roughly 4600 optical density units of the mixed peak V described above (predominantly IX) was dissolved in water (15 ml.) and vigorously stirred with Kuhn's catalyst[24] (150 mg.) in an atmosphere of hydrogen. Hydrogen uptake was complete within 1 hr. and paper electrophoresis at pH 7 showed the complete disappearance of IX. The catalyst was removed by centrifugation and the aqueous solution was adjusted to pH 8 and applied to a 2 × 30 cm. column of DEAE (HCO_3^-). After a thorough water wash, elution was effected with a linear gradient of triethlammonium bicarbonate (3 l. from 0.005 to 0.33 *M*). The fractions were examined for ultraviolet absorption and P^{32} content as shown in Figure 4. The mixed peak from fractions 40–75 contained 269 optical density units (259 mμ) of slightly radioactive ADP and 1.54 × 10^7 c.p.m. of pyrophosphate. The main peak (fractions 80–105) contained 2540 optical density units of ATP and 2.05 × 10^7 c.p.m. It was evaporated to dryness and the residue was evaporated with methanol (25-ml. portions) four times. The final residue was dissolved in methanol (4 ml.) and to it was added 1 *M* sodium iodide in acetone (1 ml.) and acetone (25 ml.). The resulting precipitate was washed free of iodide ions with acetone and dried *in vacuo* giving 100 mg. (33.5% from AMP-morpholidate) of tetrasodium β-P^{32}-ATP·$5H_2O$. This material was chromatographically homogeneous both by ultraviolet absorption and P^{32} content and was identical with authentic ATP. It showed a P:adenosine ratio of 2.98 and had a specific activity of 9.68 × 10^4 c.p.m./μmole.

Degradation of 1 μmole of this compound with 20 μl. of dialyzed *E. coli* alkaline phosphatase and 5 μl. of 1 *M* ammonium carbonate, pH 9.4, for 40 min. at 37° was followed by ion-exchange chromatography as described for γ-P^{32}-ATP. As described in the text at least 98.8% of the P^{32} was shown to be in the β-position.

P^{32}-Adenosine 5′-Phosphate (XIII). The barium salt of P^{32}-2-cyanoethyl phosphate[13b] (0.23 mmole with a specific activity of 10.2 μc./μmole) was converted into its pyridine salt with Dowex-50 (pyridinium) resin and then evaporated to dryness. It was then rendered anhydrous by three evaporations with 5-ml. portions of pyridine. Dried 2′,3′-O-isopropylideneadenosine (230 mg., 0.75 mmole) and dicyclohexylcarbodiimide (206 mg., 1 mmole) were then added and the mixture was left overnight at room temperature in

5 ml. of pyridine. Water (1 ml.) was added and after 1 hr. the solvent was evaporated *in vacuo*. The residue was partitioned between water and ether and filtered through glass wool, and the water layer was extracted once more with ether. The aqueous layer was evaporated to dryness, taken up in concentrated ammonium hydroxide (10 ml.), and kept at 37° for 4 hr. At this point virtually all the radioactivity was present as 2′,3′-O-isopropylideneadenosine 5′-phosphate (R_f 0.42 in solvent C). After evaporation to dryness and several evaporations with ethanol the residue was dissolved in 15% acetic acid (10 ml.) and heated at 100° for 1.5 hr. The solvent was then evaporated to dryness and the residue applied at pH 8 to a 2 × 30 cm. column of DEAE cellulose (HCO_3^-). Adenosine was eluted with water and the nucleotide with a linear gradient of triethylammonium bicarbonate (1500 ml. from 0.005 to 0.1 *M*). This separated very small amounts of a faster moving material (perhaps cyanoethyl AMP) and P^{32}-orthophosphate from the main product. Since the tail of the orthophosphate peak overlapped the first two tubes of the AMP, only product with a constant specific activity (c.p.m. per optical density unit) was pooled. This gave 2720 optical density units (75% from cyanoethyl phosphate) of chromatographically pure P^{32}-AMP of specific activity 10.3 μc./μmole, which was evaporated to dryness and freed from salt by several evaporations with methanol leaving 79 mg. of the monotriethylammonium salt.

P^{32}-Adenosine 5′-Phosphoromorpholidate (XIV). Triethylammonium P^{32}-AMP (0.17 mmole) as directly obtained above was dissolved in 10 ml. of 50% aqueous *t*-butyl alcohol containing morpholine (0.06 ml., 0.7 mmole). A solution of dicyclohexylcarbodiimide (144 mg., 0.7 mmole) in *t*-butyl alcohol (5 ml.) was added dropwise over 30 min.[37] and the mixture was refluxed for a further hour. Morpholine (0.06 ml.) and dicyclohexylcarbodiimide (140 mg.) were added and the mixture was refluxed for 1 more hr. The mixture was evaporated to half its volume, extracted three times with ether, filtered, and evaporated to dryness. The residue contained 2600 optical density units at 259 mμ (96% recovery) and consisted of 89.5% P^{32}-AMP-morpholidate (specific activity 9.7 μc./μmole) and 10.5% AMP (of identical specific activity) as determined by both chromatography in solvent C and electrophoresis at pH 7.5.

α-P^{32}-Adenosine 5′-Triphosphate (XV). The crude morpholidate (2500 optical density units containing 0.14 mmole of XIV) was dried by three evaporations with pyridine (5 ml.) and two with benzene. Separately tributylammonium pyrophosphate (0.5 mmole) was prepared and dried by similar evaporations with pyridine and benzene[19] and added to the morpholidate in anhydrous dimethyl sulfoxide (2.5 ml.). After 2.5 days at room temperature the mixture was diluted with water (20 ml.) and chromatographed on a 1.5 × 41 cm. column of DEAE cellulose (HCO_3^-) using a linear gradient of triethylammonium bicarbonate (2 l. from 0.005 to 0.35 *M*). Four peaks were obtained as follows: AMP-morpholidate (6%), AMP (14%), ADP (6%), and ATP (71%). The specific activity of each peak was the same. The main peak was evaporated to dryness, freed from bicarbonate by four evaporations with methanol, and isolated as its sodium salt with sodium iodide in the usual way. The final product (68 mg., 71%) contained less than 1% ADP, analyzed for 2.97 phosphate groups per molecule, and had a specific activity of 7.45 μc./μmole.

Incubation of 1 μmole of this compound with 15 μl. of purified phosphodiesterase-I for 5 hr. at 37° resulted in complete conversion to P^{32}-AMP and completely nonisotopic pyrophosphate.

β,γ-P^{32}-Adenosine 5′-Triphosphate and β,γ-P^{32}-Thymidine 5′-Triphosphate (XVII). P^{32}-Phosphoric acid (12 mc.) was evaporated to dryness five times with 1-ml. portions of water to remove traces of hydrochloric acid, mixed with 10 μmoles of Na_2HPO_4 in 0.5 ml. of water, and evaporated to dryness. The residue was heated in a muffle furnace at 400° for 1 hr., dissolved in water, and passed through a column containing Dowex-50 (pyridinium resin, 1 ml.). The resulting solution, which contained 90% pyrophosphate, 9% orthophosphate, and 1% tripolyphosphate, was dissolved in 0.2 ml. of 75% pyridine, and tributylamine (10 μl., 42 μmoles) was added. The resulting solution was dried by four evaporations with pyridine (0.2 ml.) and two with benzene. This was then mixed in 0.1 ml. of rigorously dry dimethyl sulfoxide with the 4-morpholine N,N′-dicyclohexylcarboxamidine salt of thymidine 5′-phosphoromorpholidate (1.0 mg., 1.2 μmoles) which had been dried with pyridine and benzene in the usual way. The mixture was stored at room temperature for 5 days, diluted with water, and chromatographed on a 0.5 × 40 cm. column of DEAE cellulose (HCO_3^-) using a linear gradient of 300 ml. (0.005 to 0.35 *M*) of triethylammonium bicarbonate. Fractions of 2 ml. were collected and followed by radioactivity. The main peak (8.3 optical density units at 267 mμ, 72%) was eluted without contamination by either pyrophosphate or tripolyphosphate which occurred on either side of it, and was evaporated to dryness. After removing bicarbonate by several evaporations with methanol the sodium salt of β,γ-P^{32}-thymidine 5′-triphosphate was precipitated with sodium iodide in acetone in the usual way with minor losses. The final product was chromatographically homogeneous and had a specific activity of 1.47 × 10^9 c.p.m./μmole.

By exactly the same procedure β,γ-P^{32}-adenosine 5′-triphosphate was prepared and isolated in 62% yield from AMP-morpholidate and P^{32}-pyrophosphate. In this case a trace amount (less than 1%) of P^{32}-tripolyphosphate emerged from the column with the product. This could be quantitatively removed by adsorption of the nucleotide on charcoal and elution with 2% ammonia in 50% aqueous ethanol but only with loss of 50% of the product.

Adenosine 5′-Tetraphosphate. The 4-morpholine N,N′-dicyclohexylcarboxamidine salt of ATP-morpholidate (IV, 134 mg., 0.1 mmole) was dried by three evaporations with pyridine (2 ml.) and two with benzene. Separately tributylammonium orthophosphate (0.4 mmole) was dried in the same way and mixed with the morpholidate in anhydrous dimethyl sulfoxide (2 ml.). After 3 days at room temperature, water (10 ml.) was added and the mixture was applied to a

(37) A more quantitative reaction would probably have resulted if this addition had been extended over a longer period.

2 × 25 cm. column of DEAE cellulose (HCO_3^-). Following a water wash the nucleotides were eluted with a linear gradient of 2 l. of triethylammonium bicarbonate (0.005 to 0.35 *M*). Three peaks were obtained containing ATP-morpholidate (3%), ATP (18%), and adenosine 5′-tetraphosphate (72%). The main peak was evaporated to dryness, evaporated with methanol four times, and precipitated as the sodium salt with sodium iodide in acetone giving 54 mg. of the chromatographically pure sodium salt of adenosine 5′-tetraphosphate as the pentahydrate.

Anal. Calcd. for $AP_4Na_5 \cdot 5H_2O$: P, 15.74; total P:acid labile P:adenosine = 4:3:1. Found: P, 15.46; total P:acid labile P:adenosine = 3.95:3.09:1.00.

P^1,P^4-Di(adenosine 5′) Tetraphosphate (*XX*, *R* = *Adenine*, *R^1* = *Adenosine*). Adenosine 5′-phosphate (139 mg., 0.4 mmole) was dissolved in a mixture of dimethyl formamide (1 ml.), pyridine (2 ml.), and tri-*n*-butylamine (0.2 ml., 0.8 mmole) and evaporated to dryness. The residue was then dried by three evaporations with pyridine (2 ml.) and two with benzene and mixed in anhydrous dimethyl sulfoxide (2 ml.) with the 4-morpholine N,N′-dicyclohexylcarboxamidine salt of ATP-morpholidate (134 mg., 0.1 mmole) that had been dried as above. After 3 days at room temperature the mixture was diluted with water and chromatographed on a 2 × 20 cm. column of DEAE cellulose (HCO_3^-) using a linear gradient of triethylammonium bicarbonate (2 l. from 0.005 to 0.35 *M*). Five ultraviolet-absorbing peaks were obtained as follows: peak I (4470 O.D.U.) was excess AMP; peaks II and III (108 O.D.U. and 88 O.D.U., respectively) were unreacted ATP-morpholidate and ADP; peak IV (780 O.D.U., 51%) was ATP; and peak V (950 O.D.U., 31%) was the desired AP_4A. The product from peak V was isolated as its chromatographically homogeneous sodium salt (35 mg.) using sodium iodide in acetone in the usual way.

Anal. AP_4ANa_4 requires: total P:acid labile P:adenosine = 2:1:1. Found: total P:acid labile P:adenosine = 2.05:1.06:1.00.

The material (1 μmole) was unaffected by incubation for three hr. with 50 μl. of *E. coli* alkaline phosphatase but was rapidly cleaved to AMP and ATP (and ultimately completely to AMP) by 20 μl. of phosphodiesterase-I.

γ-Methyladenosine 5′-Triphosphate (*XXI*, *n* = *2*). The anhydrous tributylamine salt of ATP (0.5 mmole) was prepared as usual and dissolved in methanol (50 ml.) together with dicyclohexylcarbodiimide (510 mg., 2.5 mmoles). After 2 days at room temperature the solvent was evaporated and the residue partitioned between ether and water. The filtered water layer was chromatographed on a 1.5 × 20 cm. column of DEAE cellulose (HCO_3^-) using a linear gradient of 3 l. of triethylammonium bicarbonate (0.005 to 0.3 *M*). Three peaks were obtained and shown to contain AMP-methyl ester[17b] (1%), β-methyl ADP (32%), and γ-methyl ATP (67%). The latter compound was isolated as its calcium salt by addition of ethanolic calcium chloride to a solution of the salt-free triethylamine salt in ethanol and washed free of chloride ion with ethanol.

Anal. Calcd. for $C_{11}H_{15}N_5O_{13}P_3Ca_{1.5} \cdot 6H_2O$: C, 19.25; H, 3.97; N, 10.20; total P, 13.53; labile P, 4.51; phosphorus:adenosine, 3.0. Found: C, 19.34; H, 4.07; N, 10.29; total P, 13.53; labile P, 5.13; phosphorus:adenosine, 3.02.

Incubation of the ammonium salt of the compound (0.7 μmole) with 50 μl. of phosphodiesterase-I for 20 min. resulted in complete conversion to AMP and a single nonultraviolet-absorbing phosphate compound (presumably monomethyl pyrophosphate) moving faster than AMP in solvent I.

δ-Methyladenosine 5′-Tetraphosphate (*XXI*, *n* = *3*). A reaction similar to that above was carried out using the ammonium salt of adenosine 5′-tetraphosphate (37 μmoles) and dicyclohexylcarbodiimide (0.7 mmole) in methanol (20 ml.). Chromatography on DEAE cellulose (HCO_3^-) with a linear gradient of 800 ml. of triethylammonium bicarbonate (0.005 to 0.4 *M*) gave a 45% yield of pure δ-methyladenosine 5′-tetraphosphate as well as lesser amounts of the methyl esters of AMP, ADP, and ATP. The desired product was isolated as its chromatographically pure calcium salt which showed ratios of adenosine:total P:labile P of 1.0:3.95:2.10. It was resistant toward *E. coli* alkaline phosphatase but quantitatively converted into AMP and presumably monomethyl tripolyphosphate (R_f 0.18 in solvent A) by incubation with 50 μl. of phosphodiesterase-I per μmole of nucleotide for 30 min.