[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, CANADA]

Nucleoside Polyphosphates. X.[1] The Synthesis and Some Reactions of Nucleoside-5′ Phosphoromorpholidates and Related Compounds. Improved Methods for the Preparation of Nucleoside-5′ Polyphosphates[2]

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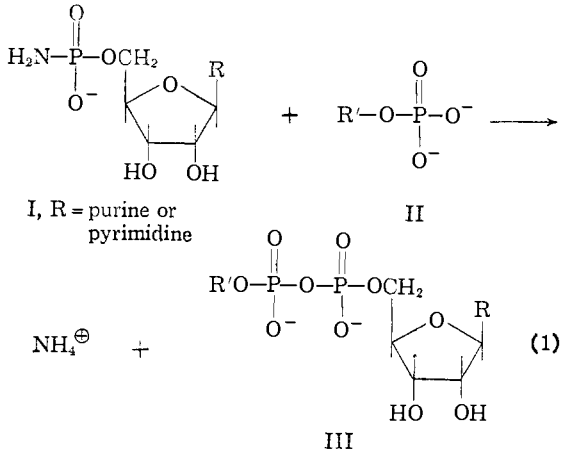
In an investigation of the reactivity of nucleoside-5′ phosphoramidates derived from different amines, adenosine-5′ phosphoro-*p*-anisidate (IV; R = *p*-methoxyphenyl), adenosine-5′ phosphoromorpholidate (V) and adenosine-5′ phosphoropiperidate (VI) have been prepared by the general method involving the reaction of adenosine-5′ phosphate with dicyclohexylcarbodiimide and the appropriate amine. A side reaction in these preparations is the direct addition of the amine to the carbodiimide to form the tri-substituted guanidines of the type VII. The guanidines, whose rate of formation is a function of the basicity of the amine, inhibited the formation of phosphoramidates. Studies on the reactivity of different phosphoramidates in pyrophosphate-forming reaction are reported, as well as their rates of acidic hydrolysis. The order of reactivity is IV < V < VI. The nucleoside-5′ phosphoromorpholidates (V) represented the best compromise between the ease of preparation and their effectiveness in pyrophosphate synthesis and, accordingly, a general method for their preparation in practically quantitative yields has been devised. Using the phosphoromorpholidates, improved procedures for the preparation of ribonucleoside-5′ diphosphates have resulted. The reaction of adenosine-5′ phosphoromorpholidate with trialkylammonium salt of inorganic pyrophosphate has been studied. Adenosine-5′ triphosphate, the major initial product, decreases in amount and adenosine-5′ diphosphate is ultimately the major product. After a 2 hr. reaction period, however, adenosine-5′ triphosphate has been isolated pure in 43% yield. Some other reactions of adenosine-5′ phosphoromorpholidate are reported, including the formation of IX and the preparation of methyl adenosine-5′ phosphate on reaction with methyl alcohol under acidic catalysis.

Introduction

The problems of the synthesis of nucleoside-5′ di- and tri-phosphates and the unsymmetrical diesters of pyrophosphoric acid, to which class the nucleotide coenzymes belong, have been the subject of extended studies in recent years.[3] A major advance in the field during the last few years has been the introduction of nucleoside-5′ phosphoramidates as the reactive intermediates in the specific synthesis of the pyrophosphate bond according to eq. 1.[3-5] The phosphoramidates themselves have been prepared in high yield by the direct condensation of the unprotected ribonucleoside-5′



I, R = purine or pyrimidine

II

III

(1)

phosphates with ammonia in the presence of dicyclohexylcarbodiimide.[6] The application of the nucleoside-5′ phosphoramidates in the synthesis of nucleotide coenzymes was demonstrated by the synthesis of uridine diphosphate glucose and flavin adenine dinucleotide in satisfactory yields.[8] Nevertheless a serious difficulty was encountered in the choice of a solvent for the pyrophosphate forming reaction.[7] Pyridine was found to be by far the preferred medium of reaction but of all the ribonucleoside-5′ phosphoramidates only the uridine derivative was soluble in anhydrous pyridine and then only as an amorphous lyophilized powder.[8]

Before undertaking further work, one of the major aims of which was an attack on the synthesis of coenzyme A, it was considered desirable to investigate nucleoside-5′ phosphoramidates derived from alkylamines of widely varying basicities. It was hoped, first, that more reactive and more conveniently soluble phosphoramidates might thus become available and, secondly, that a more thorough investigation of the properties and reactions of such "activated" mononucleotides might suggest other synthetic applications in the phosphate ester field. The present paper contains a detailed report of these investigations, which have shown the nucleoside-5′ phosphoromorpholidates to have the desired characteristics. Improved procedures for the synthesis of ribonucleoside-5′ pyrophosphates have consequently resulted and are also described herein. Subsequent work based on the use of the phosphoromorpholidates in the synthesis of a variety of other nucleotide coenzymes is described in the following two communications.[8,9] A preliminary announcement of the preparation of nucleoside-5′ phosphoromorpholidates has already been made.[10]

(1) Paper IX, H. G. Khorana and J. P. Vizsolyi, THIS JOURNAL, **81**, 4660 (1959).

(2) This work has been supported by grants from the Life Insurance Medical Research Fund, New York, and the National Research Council of Canada.

(3) For earlier references to the literature see J. G. Moffatt and H. G. Khorana, Paper VIII of this series, THIS JOURNAL, **80**, 3756 (1958).

(4) (a) R. W. Chambers and H. G. Khorana, *Chem. and Ind. (London)*, 1022 (1956); (b) R. W. Chambers and H. G. Khorana, THIS JOURNAL, **80**, 3749 (1958).

(5) Clark, Kirby and Todd [*J. Chem. Soc.*, 1497 (1957)] have reported on the use of monobenzylphosphoramidic acid in the synthesis of adenosine-5′ di- and tri-phosphates.

(6) (a) R. W. Chambers, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 4240 (1957); (b) R. W. Chambers and J. G. Moffatt, *ibid.*, **80**, 3752 (1958).

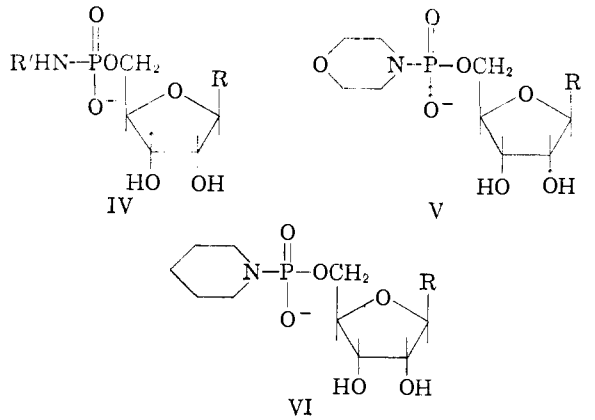
(7) See, for example, the last paragraph of discussion and footnote 19 in ref. 3.

(8) S. Roseman, J. J. Distler, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **82**, 659 (1960).

(9) J. G. Moffatt and H. G. Khorana, *ibid.*, **82**, 663 (1960).

(10) J. G. Moffatt and H. G. Khorana, *ibid.*, **81**, 1265 (1959).

The Preparation of Nucleoside-5′ Phosphoromorpholidates and Other Phosphoramidates.—The general procedure used in the initial work on the preparation of the different nucleoside-5′ phosphoramidates consisted of merely refluxing a solution of the nucleotide, for example, adenosine-5′ phosphate, with five equivalents of each of dicyclohexylcarbodiimide and the amine in 67% aqueous tertiary butyl alcohol. (Because of the greater solubility of the nucleotides as the alkylammonium salts than as the ammonium salts,[6b] the addition of formamide to the reaction mixture was unnecessary.) Using cyclohexylamine (pK, 10.64),[11] the reaction occurred rapidly to give 80–90% of the desired product (IV; R = adenine, R′ = C_6H_{11}) within 1 hr., but then it came to a halt and

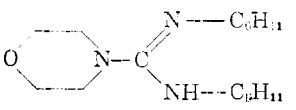


IV V VI

IV–VI, R = Purine or Pyrimidine

could not be driven to completion either by the addition of more of the reagents or by continued heating. Further experiments with different amines again showed that the formation of the phosphoramidates proceeded to different extents but, in general, did not go to completion. With piperidine, the strongest base (pK, 11.22)[11] studied, adenosine-5′ phosphoropiperidate (VI; R = adenine) was obtained in only 20% yield. On the other hand, the reaction went to completion with weak bases such as p-anisidine (pK, 5.29).[12] In all these experiments, the desired products could nevertheless be purified by chromatographic procedures for the purpose of comparative experiments on the pyrophosphate forming reaction. These experiments, which are discussed below, showed, in turn, that the amidates derived from strong bases, for example adenosine-5′ phosphoropiperidate (VI; R = adenine), were very reactive while the corresponding phosphoroanisidate (IV; R = adenine; R′ = p-methoxyphenyl) had very low reactivity. The best compromise between the ease of preparation and the reactivity was found in the use of morpholine (pK, 8.36).[11]

In further work aimed at designing preparative and general procedures for the nucleoside-5′ phosphoromorpholidates, the cause of the frequent failure of the condensation reaction to go to completion was found to be the side reaction already encountered in the previous work.[6] This is the addition of the amine itself to the carbodiimide and leads to the formation of the strongly basic trisubstituted guanidines.[13,14] Thus upon heating under reflux a mixture of morpholine and dicyclohexylcarbodiimide in aqueous t-butyl alcohol, the crystalline base 4-morpholine N,N′-dicyclohexylcarboxamidine (VII) formed in high yield.[15] A study of the reaction of the three bases p-



VII

anisidine, morpholine and piperidine showed (Fig. 1) that the rate of addition to dicyclohexylcarbodiimide was a function of the basicity of the amine. From these results it is then clear that the rate of accumulation of the strongly basic guanidines during the condensation reaction of the nucleotides with the amines will increase with increasing strength of the amine used. Furthermore, from the previous studies on the mechanism of the reactions of the phosphate esters with carbodiimides,[16] it seems reasonably certain that the highly basic guanidines inhibit the reaction of the nucleotide with the carbodiimide. Direct proof for this conclusion was obtained by setting up two identical reaction mixtures between one equivalent of adenosine-5′ phosphate and 5 equivalents each of morpholine and dicyclohexylcarbodiimide. To one reaction mixture was added five equivalents of 4-morpholine N,N′-dicyclohexylcarboxamidine (VII). The reaction without the added base gave about 95% of the desired phosphoromorpholidate within thirty minutes. In the presence of the base, however, the yield never exceeded 10%.

The foregoing results suggested the procedure which has uniformly given quantitative yields of nucleoside-5′ phosphoromorpholidates. This consists in the slow dropwise addition of a solution of dicyclohexylcarbodiimide in t-butyl alcohol to a refluxing solution of the nucleoside-5′ phosphate and morpholine in aqueous t-butyl alcohol.[17] Thus the phosphoromorpholidates of the four common ribonucleosides (V; R = adenine, guanine, uracil and cytosine) and of deoxycytidine and thymidine were formed quantitatively and isolated pure in over 90% yields as their guanidinium (VII) salts.[18] It remains unexplained, however,

(11) See H. K. Hall, Jr., THIS JOURNAL, **79**, 5441 (1957), for a compilation of the pK data on amines.

(12) E. Sawicki and F. E. Ray, *J. Org. Chem.*, **19**, 1686 (1954).

(13) The pK's of the guanidines must be over 13. See for example, S. J. Angyal and W. K. Warburton, *J. Chem. Soc.*, 2492 (1951).

(14) *Cf.*, H. G. Khorana, *Chem. Revs.*, **53**, 145 (1953).

(15) It may be noted that in the previous work[6b] the addition reaction between ammonia and dicyclohexylcarbodiimide could not be demonstrated without the addition of a phosphate ester. Thus there appears to be an alternative acid-catalyzed mechanism by which the guanidines can be formed during the phosphoramidate synthesis (*cf.* ref. 6b).

(16) These studies have given strong evidence in favor of the protonation of carbodiimides as an important step in their reactions with acids. [M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 6204 (1958)].

(17) The application of the dropwise addition technique increased the yield of adenosine-5′ phosphoropiperidate from 20 to 43% (see Experimental). It seems probable that in the same way nucleoside-5′ N-cyclohexyl phosphoramidates would be obtained in quantitative yields and these might then provide intermediates more reactive than the phosphoromorpholidates.

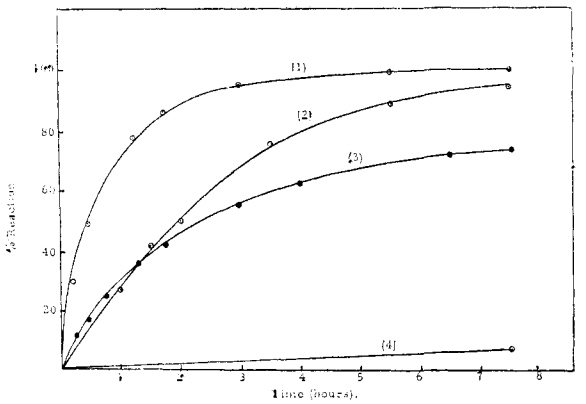


Fig. 1.—Rates of addition of different amines (0.4 *M*) to dicyclohexylcarbodiimide (0.1 *M*) in *t*-butyl alcohol at 100°; the rates were followed by the disappearance of dicyclohexylcarbodiimide. Curve 1, piperidine; curve 2, morpholine; curve 4, *p*-anisidine. Curve 3, using piperidine at 0.11 *M* concentration.

why even without the dropwise addition the morpholidates of adenosine-5′ and thymidine-5′ phosphates could often be formed quantitatively but those of the other nucleotides required the dropwise addition technique for similar results.

The Synthesis of Nucleoside-5′ Pyrophosphates.—With the assumption, which is supported by the results described below, that acidic lability of the nucleoside phosphoramidates may be a measure of their reactivity in pyrophosphate bond synthesis, the relative rates of acidic hydrolysis of adenosine-5′ phosphoramidate[6] and adenosine-5′ phosphoromorpholidate were first determined. The morpholidate (V; R = adenine) was much more labile than the parent amidate under all the conditions studied, (Fig. 2 and Experimental section), its half life in 0.1 *N* sulfuric acid at room temperature being about 5 minutes while the unsubstituted amidate was only 40% hydrolyzed in 6 hr. The difference in the rates of hydrolysis was less pronounced at *p*H 4 and 5 at 100°. It may be noted that in the latter experiments acetate buffers were employed and the possibility cannot be excluded that the acetate ions directly participated in the hydrolytic reaction by forming acetyl phosphate type of mixed anhydrides. At room temperature and under anhydrous conditions, the acetate anions have, however, been found to be ineffective in attacking the phosphoromorpholidate (see below).

The reactivities of the three phosphoramidates, adenosine-5′ phosphoropiperidate, phosphoromorpholidate and phosphoroanisidate in pyrophosphate formation were then determined under comparable conditions using three equivalents of monophenyl phosphate in anhydrous pyridine. (These experiments are analogous to those described earlier[3] with adenosine-5′ phosphoramidate itself.) The rates of the disappearance of the different phosphoramidates are shown in Fig. 3 and the composition of the reaction mixtures at the completion of the reactions in Table I. As can be seen the

(18) The nucleotide salts of the base (VII) have in general enhanced solubility in organic solvents such as pyridine and are being used in synthetic work in this Laboratory. See, for example, M. Smith, G. Drummond, and H. G. Khorana, THIS JOURNAL, **83**, 698 (1961).

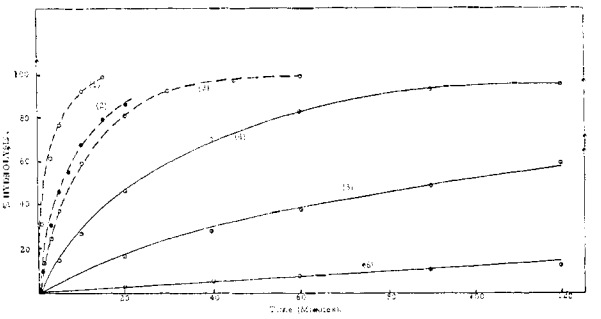


Fig. 2.—Rates of acidic hydrolysis of adenosine-5′ phosphoromorpholidate (broken lines) and adenosine-5′ phosphoramidate (solid lines). Curves 1 and 4, 1 *M* acetate buffer (*p*H 4) at 100°; curves 2 and 6, 0.1 *N* sulfuric acid at room temperature; curves 3 and 5, 1 *M* acetate buffer (*p*H 5) at 100°.

half-reaction times for the phosphoropiperidate, phosphoromorpholidate and the phosphoroanisidate were, respectively, 3 min., 6 min. and 30 hr. A further experiment was carried out using monophenyl phosphate as its bis-triethylammonium salt. The use of such aliphatic amines has frequently been found necessary to dissolve the phosphomonoester component in nucleotide coenzyme synthesis[3] (see also the following papers). Addition of the base increased the half reaction time in the case of the phosphoropiperidate (VI; R = adenine) from 3 to 25 min. but the product composition was essentially the same (Table I).

TABLE I

PRODUCTS OF REACTION OF DIFFERENT ADENOSINE-5-PHOSPHORAMIDATES WITH MONOPHENYL PHOSPHATE

Adenosine 5′-phosphoramidate (0.02 mmole) (as in column 1) as the guanidinium salt + monophenyl phosphate (0.06 mmole) in anhydrous pyridine (1 ml.) at room temperature. The products shown were obtained at the completion of the reaction and analyzed as in Experimental text.

	Products			
Phosphoramidate	P^1,P^2-diadenosine-5′ P^1-(substituted amino)-pyrophosphate[a] (%)	P^1,P^2-diadenosine-5′ pyrophosphate (%)	Adenosine-5′ phosphate (%)	P^1-adenosine-5′ P^2-phenyl pyrophosphate (%)
Piperidate[b]	8	4	5	83
Morpholidate[b]	10	7	5	78
p-Anisidate[c]	...[d]	2	8	90
Piperidate[b] + triethylamine	12	7	3	78
Electrophoretic mobility[e]	0.39	0.82	1.0	1.0

[a] These compounds are of the general structure IX (see text), the nature of the amino group depending upon the phosphoramidate used. [b] Used as the salt of 4-morpholine N,N′-dicyclohexylcarboxamidine (VII). [c] Used as the salt of N,N′-dicyclohexyl N″-(*p*-methoxyphenyl)-guanidine. [d] A trace was present in early aliquots but disappeared later. [e] At *p*H 7.5, relative to adenosine-5′ phosphate. The different phosphoramidates had a relative mobility of 0.54 and monophenyl phosphate, 1.50.

As can be seen from Table I the yields of the desired P′-adenosine-5′,P^2-phenyl pyrophosphate (III; R = adenine; R′=phenyl) in the above experiments varied between 78 and 90%. The highest yield was obtained using the phosphoroanisidate; however, the completion of the reaction took a very long time. The phosphoro-

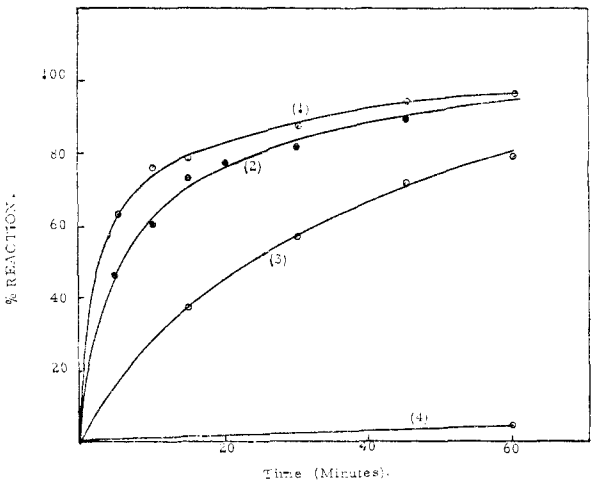


Fig. 3.—Rates of reaction of different adenosine-5′ phosphoramidates (0.04 *M*) with monophenyl phosphate (0.12 *M*) in pyridine at room temperature. Curve 1, adenosine-5′ phosphoropiperidate; curve 2, adenosine-5′ phosphoromorpholidate; curve 4, adenosine-5′ phosphoro-*p*-anisidate; curve 3, adenosine-5′ phosphoropiperidate in the presence of 0.24 *M* triethylamine.

piperidate is, in contrast, the most reactive but its use is severely limited by the difficulties in its preparation. The reactivity of the phosphoromorpholidates is intermediate and reasonably high and the conclusion from these experiments and those discussed in the preceding section is that these compounds offer the best compromise.

The above results, namely, the rates of acidic hydrolysis, the order of reactivity of the different phosphoramidates, the retarding effect of the strong tertiary bases and the much greater rates of nucleoside-5′ diphosphate synthesis under acidic conditions,[4,19] are all consistent with the following mechanism (eq. 2 and 3) for the formation of the pyrophosphate bond from the phosphoramidates. The first step is the reversible protonation of the nitrogen atom to form VIII and the latter is then attacked by a phosphomonoester anion at the phosphorus atom to form the pyrophosphate. Clark, *et al.*,[5] have also proposed a similar mechanism in their study of monobenzylphosphoramidic acid.

The first practical application of the nucleoside-5′ phosphoramidates has been in the specific synthesis of the nucleoside-5′ diphosphates. Previously[3,4,6,19] it was found necessary to use *o*-chlorophenol[20] as the solvent in order to obtain homogeneous reaction mixtures. In the present work, completely homogeneous solutions were obtained in anhydrous pyridine using the nucleoside-5′ phosphoromorpholidates and the mono-tri-*n*-butylammonium salt of orthophosphoric acid. Because of the use of the strong tertiary base salt, longer reaction periods (several days at room temperature) were necessary but the ultimate yields of the desired products were excellent. Thus by the procedures described in the Experimental section cytidine-5′ and uridine-5′ diphosphates were isolated pure in yields of 78% and 67%, respectively. The method, as it stands, is general for the preparation of the nucleoside 5′-diphosphates[21] and is particularly suited to the preparation of these compounds P^{32}-labelled in the terminal position. Further, in the synthesis of the acid-labile purine deoxyribonucleoside-5′ diphosphates, the use of the morpholidates in pyridine offers the advantage over the acidic conditions previously used with the parent amidates, that the glycosyl bonds would be stable.

It was of interest to extend the above work to the synthesis of nucleoside-5′ triphosphates. While a reasonably satisfactory and general method for the preparation of these compounds is available,[22] the reaction of the phosphoromorpholidates with inorganic pyrophosphate should provide a specific method, requiring simpler isolation procedures.[23] Homogeneous reaction mixtures were obtained in anhydrous pyridine using the bis-tri-*n*-butylammonium salt of inorganic pyrophosphate, a ten fold excess of the latter being used to avoid the possibility of both ends of this molecule reacting with the phosphoromorpholidate. The composition of the reaction mixture at different times, as determined by a combination of paper and ion exchange chromatography, is shown in Table II

TABLE II

PRODUCTS OF REACTION OF ADENOSINE-5′ PHOSPHOROMORPHOLIDATE WITH PYROPHOSPHORIC ACID

Adenosine-5′ phosphoromorpholidate (0.1 mmole), bis-(tri-*n*-butylammonium) pyrophosphate (1 mmole) in anhydrous pyridine (5 ml.) at room temperature

Time (hr.)	AMP[a] (%)	ADP[a] (%)	ATP[a] (%)
2	29[b]	14	57
24	9	76	15
48	18	73	9

AMP, adenosine-5′ phosphate; ADP, adenosine-5′ diphosphate; ATP, adenosine-5′ triphosphate. [b] Largely unreacted morpholidate.

(19) R. W. Chambers, THIS JOURNAL, **81**, 3032 (1959); R. W. Chambers, P. Shapiro and V. Kurkov, *ibid.*, **82**, 970 (1960).

(20) This is not a completely satisfactory solvent since, as shown below, phenols can also react with the phosphoramidates as nucleophiles.

(21) Guanosine-5′ phosphoromorpholidate has a limited solubility in dry pyridine, but a clear solution is usually obtained on the addition of the second component of the reaction; see, for example, the preparation of guanosine diphosphate mannose.[8]

(22) M. Smith and H. G. Khorana, (a) THIS JOURNAL, **80**, 1141 (1958); (b) in "Methods in Enzymology," Vol. V, Academic Press, Inc., New York, N. Y., 1960, in press.

(23) In the previously described procedures,[22a] a complex mixture of inorganic polyphosphates are concomitantly formed and this fact necessitates a charcoal adsorption step in the purification of the nucleoside-5′ triphosphates. Consequently, rather large losses in yields have been encountered. The present method should obviate the use of charcoal and the nucleoside-5′ triphosphates should be isolated by direct ion exchange chromatography of the reaction mixtures.

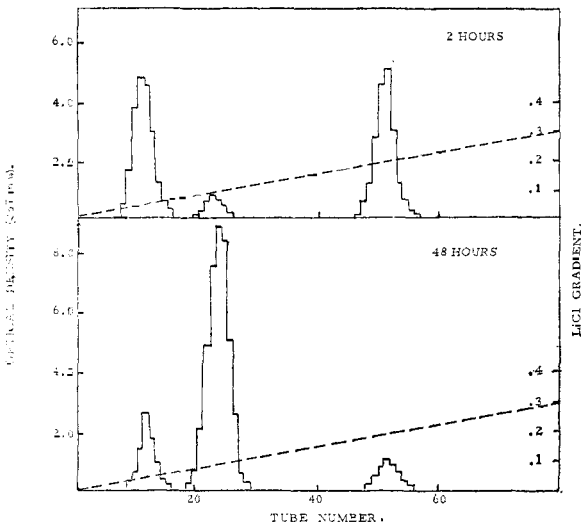


Fig. 4.—The reaction of adenosine-5′ phosphoromorpholidate (0.02 *M*) with bis-tri-*n*-butylammonium pyrophosphate (0.2 *M*) in pyridine at room temperature. Aliquots removed at 2 and 48 hr. and chromatographed on Dowex-1 (Cl^-) as in text. Peak 1, adenosine-5′-phosphate; peak 2, adenosine-5′-diphosphate; peak 3, adenosine-5′-triphosphate.

and Fig. 4. As can be seen, the morpholidate disappeared rapidly and the major product formed initially was the expected adenosine-5′ triphosphate. However, its concentration then fell and the corresponding diphosphate was ultimately the main product. Since the amount of the unreacted phosphoromorpholidate after 2 hr. is relatively small (about 25%), it is unlikely that the phenomenon is caused by the attack of the initially formed adenosine-5′ triphosphate on the morpholidate[24] and subsequent hydrolysis of the resulting P^1, P^4-diadenosine-5′ tetraphosphate to two molecules of adenosine-5′ diphosphate.[25a] Alternatively it seemed possible that adenosine-5′ triphosphate is inherently unstable in the medium employed and an experiment carried out by keeping adenosine-5′ triphosphate in anhydrous pyridine in the presence of tri-*n*-butylamine and the guanidine (VII) showed quite rapid disappearance of the starting material. The major new products after four days were found to be adenosine-5′ di- and tetraphosphates (Fig. 5). It seems very probable that the course of disproportionation of the triphosphate would be markedly influenced by the excess of the inorganic pyrophosphate, which was present in our reaction mixtures. While these dismutations of the nucleoside polyphosphates are highly interesting and merit further study, from the practical standpoint, the reaction of inorganic pyrophosphate with the nucleoside-5′ phosphoromorpholidates can be made the basis of practical procedures for the synthesis of the triphosphates provided that carefully controlled reaction times are employed. Thus in one experiment the products obtained after treatment of adenosine-5′ phosphoromorpholidate with pyrophosphate for 5 hr. were directly chromatographed and adenosine-5′ triphosphate was isolated pure in 43% yield.[25b]

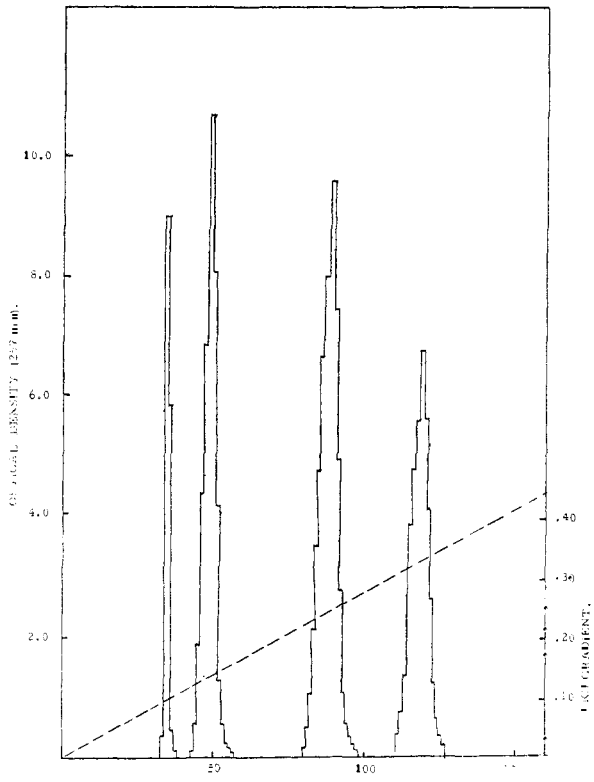
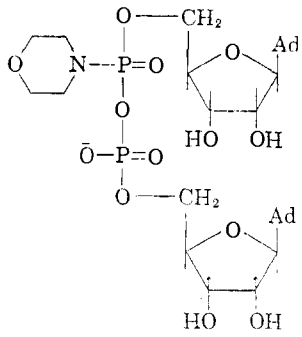


Fig. 5.—Ion exchange chromatography of products formed by treatment of adenosine-5′ triphosphate with a mixture of tri-*n*-butylamine, morpholine and the guanidine (VII) in pyridine for four days. For details see Experimental text. Peak I, adenosine-5′-phosphate; peak II, adenosine-5′-diphosphate; peak III, adenosine-5′-triphosphate; peak IV, adenosine-5′-tetraphosphate.

Other Reactions of Adenosine-5′ Phosphoromorpholidate.—Firstly, it was ascertained that an anhydrous solution of adenosine-5′ phosphoromorpholidate itself was fairly stable at room temperature. Even after five months, 73% of the starting material was intact and the only new product was P^1,P^2-diadenosine-5′ pyrophosphate. The formation of the latter product may be explained either by postulating a very slow hydrolysis (absorption of moisture) of the phosphoromorpholidate to adenosine-5′ phosphate followed by anionic attack of the latter on the unchanged starting material or by the self-condensation of the morpholidate to form IX followed by hydrolysis of the latter. The latter mechanism was proposed by Clark, *et al.*,[5]

(24) The reaction of adenosine-5′ triphosphate with adenosine-5′ phosphoromorpholidate has been briefly studied and found to give a highly complex mixture of products. A further study of this reaction will be reported upon later.

(25) (a) The reverse of this postulate, namely the dismutation of P^1, P^4-diadenosine-5′ tetraphosphate to form adenosine-5′ phosphate and adenosine-5′ triphosphate, has previously been invoked to explain the products of the reaction of adenosine-5′ diphosphate with dicyclohexylcarbodiimide. H. G. Khorana, unpublished experiments; J. M. Lowenstein, *Biochem. J.*, **65**, 197 (1955). (b) In a recent communication, D. Kessler, B. Moss and R. W. Chambers [*Biochim. Biophys. Acta*, **38**, 549 (1960)] have reported on the reaction of the parent ribonucleoside-5′ phosphoramidates with free pyrophosphoric acid. The formation of nucleoside-5′ diphosphates in amounts of 10–40% and of the triphosphates in amounts of 9–32% was observed in different cases. No isolation of the pure triphosphates was described, however.



IX, Ad = Adenine

for the formation of P^1,P^2-dibenzyl pyrophosphate from monobenzylphosphoramidic acid.[26]

The addition of stoichiometric amounts of strong acids (anhydrous hydrogen chloride, trifluoroacetic acid, tri-*n*-butylammonium hydrogen sulfate, di-*p*-nitrophenyl phosphate) to the anhydrous pyridine solution of the phosphoromorpholidate (V; R = adenine) caused rapid disappearance of the latter and the major new product then was indeed the one postulated above (IX). For its maximal formation, stoichiometric amounts of the strong acids seemed necessary, since in one experiment when one-half equivalent of trifluoroacetic acid was added, roughly one-half of the morpholidate disappeared within 30 minutes and no further reaction occurred until a further amount of the acid was added. In the presence of an excess of the strong acids, IX tended to slowly decompose to form P^1,P^2-diadenosine-5′ pyrophosphate. The isolation of IX in good yield was accomplished by ion exchange chromatography and its structure was proved by a combination of chemical and enzymic degradation. Thus, it was rapidly degraded by crude rattle snake venom to give adenosine and adenosine-5′ phosphoromorpholidate in equal amounts. (Adenosine arose, as expected, by the dephosphorylation of the initially formed adenosine-5′ phosphate.) The substance which was remarkably stable to alkali was degraded by 1 *N* sodium hydroxide at room temperature (42% in 8 hr.) to give equal amounts of adenosine-5′ phosphate and adenosine-5′ phosphoromorpholidate. Treatment with 1 *N* hydrochloric acid at room temperature gave P^1,P^2-diadenosine-5′ pyrophosphate. It is interesting that while 4 hr. were required for the completion of this reaction (39% in 39 min.), adenosine-5′ phosphoromorpholidate itself was completely converted to adenosine-5′ phosphate in five minutes.

From the standpoint of the use of the phosphoromorpholidates in pyrophosphate syntheses (preceding section), it should be mentioned that small amounts of IX were observed during reactions with monophenyl phosphate (Table I) but that in the case of the more nucleophilic phosphate esters, such as those used in coenzyme syntheses the compound was not detected.

(26) In their experiment, Clark, *et al.*,[3] used the free benzylphosphoramidic acid. Since the latter was present as a hemihydrate, the alternative mechauism, namely that the phosphoramidic acid underwent acidic hydrolysis to monobenzylphosphoric acid and the latter then attacked the remainder of the phosphoramidic acid, would explain their results equally well.

A number of other reagents have been studied for their ability to react with adenosine-5′ phosphoromorpholidate. It was hoped, for example, that the reaction of carboxylate anions with the phosphoromorpholidate would lead to the specific formation of the mixed anhydrides, acyl adenylates,[27] compounds which have profound biological significance.[28] Surprisingly, however, no reaction occurred between an excess of either acetate or benzoate anions with the morpholidate in anhydrous pyridine. With benzoic acid even after three months, 43% of the starting material was present and the only new ultraviolet absorbing product was P^1,P^2-diadenosine-5′ pyrophosphate. Although more than one explanation can be advanced for the formation of the pyrophosphate,[29] it, nevertheless, is clear that the carboxylate anions are not effective nucleophiles in this reaction. Furthermore the stability of benzoyl adenylate[30] is such that if formed its presence would have been demonstrated chromatographically.

The anions of diesters of phosphoric acid are known to be very poor nucleophiles relative to the anions derived from the monoesters of phosphoric acid (see *e.g.*, ref. 16). In an experiment in which adenosine-5′ phosphoromorpholidate was treated with an excess of dibenzylphosphoric acid in anhydrous pyridine, no reaction was detected for several days. This experimental demonstration of the lack of reactivity of diesters of phosphoric acid in the pyrophosphate synthesis is of significance in the work described on the synthesis of coenzyme A.[9,10]

In the earlier experiments,[3] during the reaction of adenosine-5′ phosphoramidate with monophenyl phosphate in a mixture of *o*-chlorophenol and pyridine, an appreciable amount of *o*-chlorophenyl adenosine-5′ phosphate was formed. Three reactive phenols, (*p*-nitrophenol, 2,4-dinitrophenol and 2,4-dinitrothiophenol) have now been examined for their reaction with adenosine-5′ phosphoromorpholidate. *p*-Nitrophenol (pK 7.2) was essentially inert except for a minor initial formation of IX. With 2,4-dinitrophenol (pK 4.0) reaction occurred rapidly, and 2,4-dinitrophenyl adenosine-5′ phosphate was formed as a major product, other products present being IX and P^1,P^2-diadenosine-5′ pyrophosphate. 2,4-Dinitrothiophenol,[31] which must be a much stronger acid than the corresponding phenol,[32] proved to be a powerful catalyst for

(27) These compounds have previously been prepared either by the direct reaction of acid anhydrides with phosphate esters, a method introduced by A. W. D. Avison [*J. Chem. Soc.*, 732 (1955)], or by the reaction of the acids and the nucleotides with dicyclohexylcarbodiimide [*e.g.*, P. T. Talbert and F. M. Huennekens, THIS JOURNAL, **78**, 4671 (1956)].

(28) See for example, the recent reivew by A. Kornberg, *Advances in Enzymology*, **18**, 191 (1957).

(29) During the early stages, a small amount of what appeared to be IX was detected on paper-chromatography and electrophoresis but this then disappeared. It is possible that the carboxylic acids slowly catalyzed the formation of IX, as has been discussed above with the strong acids, and the diadenosine pyrophosphate arose from IX. Alternatively, it is possible but not likely that the acetate anions did slowly react to form acetyladenylate which subsequently disproportionated to give the symmetrical dinucleoside pyrophosphate, as has previously been demonstrated by Khorana and Vizsolyi.[1]

(30) G. M. Kellerman, *J. Biol. Chem.*, **231**, 427 (1958).

(31) C. Willgerodt, *Ber.*, **17**, 354 (1884).

(32) G. Schwarzenbach and E. Rudin, *Helv. Chim. Acta*, **22**, 360 (1939).

the formation of IX, which then slowly hydrolyzed to P^1,P^2-diadenosine-5′ pyrophosphate. The action is thus similar to that of the other strong acids described above.

Much interest attaches to methods for the formation of phosphodiester bonds in connection with polynucleotide synthesis,[33] and the possibility of using the phosphoromorpholidates as the activated monoesters for the phosphorylation of alcohols has been investigated. Under the catalytic action of a strong acid adenosine-5′ phosphoromorpholidate was converted quantitatively to adenosine-5′ methyl phosphate in anhydrous pyridine in the presence of an excess (50 equivalents) of methyl alcohol. The diester was isolated as its calcium salt in 95% yield and was identical with a sample of the substance prepared previously.[16] It has, however, not been ascertained how small a molar excess of an alcohol can be used without concomitant formation of compounds of the type IX. The probable mechanism for the above esterification is the protonation of the amidate nitrogen followed by nucleophilic attack on the phosphorus atom by the alcohol. This is formally analogous to the mechanism for the esterification of nucleoside-5′ phosphates on reaction with carbodiimides in the presence of a large excess of alcohols.[16]

Experimental[34]

Chromatography and Electrophoresis.—Descending paper chromatography on Whatman No. 1 paper was carried out in the following solvent systems: Solvent I, isopropyl alcohol–concd. ammonium hydroxide (sp. gr. 0.9)–water (7:1:2); Solvent II, ethyl alcohol–1 *M* ammonium acetate (*p*H 7.5) (5:2); Solvent III, isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* ethylenediamine tetraacetic acid (100:60:1.6); Solvent IV, ethyl alcohol–0.5 *M* ammonium acetate, *p*H 3.8 (5:2); Solvent V, *n*-butyl alcohol–acetic acid–water (5:2:3). The compounds were located by observation under an ultraviolet lamp or by the phosphate spray of Hanes and Isherwood,[35] using ultraviolet development of the color.[36]

Paper electrophoresis was carried out in an apparatus similar to that described by Markham and Smith[37] using 0.05 *M* triethylammonium bicarbonate (*p*H 7.5)[38] or 0.1 *M* ammonium acetate (*p*H 3.8) buffer.

The R_f values in solvents I, II and III of all compounds described are given in Table III. The relative values should be reproducible since all compounds were run simultaneously. Some hydrolysis of the substituted phosphoramidates was apparent in Solvent III and caused minor streaking.

Nucleoside-5′ Phosphoromorpholidates (V).—General Method.—A solution of dicyclohexylcarbodiimide (824 mg., 4 mmole) in *t*-butyl alcohol (15 ml.) is added dropwise to a refluxing solution of the nucleoside-5′ phosphate (1 mmole of free acid) in a mixture of water (10 ml.), *t*-butyl alcohol (10 ml.) and purified morpholine (0.34 ml., 4 mmole). The addition is completed in 3–4 hr., and the mixture is further refluxed for several hours until electrophoresis at *p*H 7.5 shows only a single spot with a mobility roughly one-half that of the starting nucleotide. If after 3 hr. refluxing any starting material remains, further amounts (2 mmole of each) of dicyclohexylcarbodiimide and morpholine are added and refluxing is continued until a single spot is seen on electrophoresis. The mixture is then cooled to room temperature and any crystalline material present is removed by filtration and washed with *t*-butyl alcohol. The filtrate is evaporated *in vacuo* until the *t*-butyl alcohol is largely removed and the remaining aqueous phase is extracted three times with ether, with filtration if necessary after the first extraction. The clear aqueous solution is then evaporated to dryness *in vacuo* and last traces of water are removed on an oil pump. The glassy residue is transferred as a solution in the minimum volume of methyl alcohol to a 50-ml. centrifuge tube and the volume of alcohol carefully reduced *in vacuo* to roughly 3 ml. The addition of dry ether (35 ml.) precipitates a gummy solid which on trituration with fresh ether changes to a dry white powder. After a further wash with dry ether the product is dried *in vacuo* at room temperature.

The nucleoside-5′ phosphoromorpholidates are obtained as salts of 4-morpholine N,N′-dicyclohexylcarboxamidine and are hydrated as directly obtained[39] but can sometimes be rendered anhydrous by drying *in vacuo* at 100°. Crystallization has not yet been achieved, but the products are chromatographically and electrophoretically homogeneous (see Table III).

(a) Adenosine-5′ phosphoromorpholidate was obtained in 94% yield as a tetrahydrate.

Anal. Calcd. for $C_{31}H_{52}N_9O_8P$: C, 52.47; H, 7.39; N, 17.77. Found (after drying at 100°): C, 52.72; H, 7.19; N, 18.20.

(b) Uridine-5′ phosphoromorpholidate was obtained in 92% yield as an octahydrate.

Anal. Calcd. for $C_{30}H_{51}N_6O_{16}P$: C, 52.45; H, 7.49; N, 12.24. Found (after drying at 100°): C, 52.50; H, 7.68; N, 11.64.

(c) Cytidine-5′ phosphoromorpholidate was obtained in 95% yield as a trihydrate.

Anal. Calcd. for $C_{30}H_{52}N_7O_9P\cdot 3H_2O$: C, 48.70; H, 7.90; N, 13.20. Found (after drying at 100°): C, 48.97; H, 7.80; N, 12.85.

(d) Guanosine-5′ phosphoromorpholidate was obtained in 92% yield as a tetrahydrate.

TABLE III

R_f VALUES OF THE REPORTED COMPOUNDS

Compound	R_f values in solvents I	II	III
Adenosine-5′ phosphate	0.08	0.16	0.57
Uridine-5′ phosphate	.07	.22	.31
Cytidine-5′ phosphate	.06	.17	.51
Guanosine-5′ phosphate	.03	.10	.23
Deoxycytidine-5′ phosphate	.11	.25	.60
Thymidine-5′ phosphate	.15	.36	.52
Adenosine-5′ phosphoromorpholidate	.41	.53	.80
Uridine-5′ phosphoromorpholidate	.32	.58	.56
Cytidine-5′ phosphoromorpholidate	.35	.52	.70
Guanosine-5′ phosphoromorpholidate	.20	.45	.51
Deoxycytidine-5′ phosphoromorpholidate	.44	.59	.51
Thymidine-5′ phosphoromorpholidate	.50	.67	.77
Adenosine-5′ phosphoropiperidate	.49	.61	.81
Adenosine-5′ phosphoro-*p*-anisidate	.40	.50	.84
Monophenyl phosphate	.27	.45	.72
P^1,P^2-diadenosine-5′ pyrophosphate	.09	.17	.53
P^1-adenosine-5′,P^2-phenyl pyrophosphate	.32	.47	.67
P^1,P^2-diadenosine-5′,P^1-(4-morpholine) pyrophosphate (IX)	.23	.35	.77
Adenosine-5′ methyl phosphate	.37	.48	.67
Adenosine-5′ dinitrophenyl phosphate	.53	.58	.75
Cytidine-5′ diphosphate	.03	.09	.34
Uridine-5′ diphosphate	.04	.12	.21
Adenosine-5′ diphosphate	.03	.10	.46
Adenosine-5′ triphosphate	.02	.05	.33
Adenosine-5′ tetraphosphate	.01	.02	.24

(33) H. G. Khorana, in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Vol. III, in press.

(34) Elemental analyses by W. Manser, Herrliberg, Switzerland.

(35) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(36) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(37) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(38) J. Porath, *Nature*, **175**, 478 (1955).

(39) While these compounds do not appear to be crystalline, the degree of hydration encountered in different preparations of the same substance was found to be remarkably constant.

Anal. Calcd. for $C_{31}H_{52}N_9O_9P\cdot 4H_2O$: C, 47.80; H, 7.50; N, 16.19. Found (after drying at 100°): C, 47.25; H, 7.36; N, 16.61.

(e) Thymidine-5′ phosphoromorpholidate was obtained in 93% yield as a monohydrate.

Anal. Calcd. for $C_{21}H_{33}N_6O_9P\cdot H_2O$: C, 53.00; H, 7.89; N, 11.95. Found (after drying at 100°): C, 53.36; H, 8.14; N, 11.22.

(f) Deoxycytidine-5′ phosphoromorpholidate was obtained in 94% yield as a tetrahydrate.

Anal. Calcd. for $C_{30}H_{52}N_7O_8P\cdot 4H_2O$: C, 48.60; H, 8.16; N, 13.22. Found (after drying at 100°): C, 48.97; H, 7.64; N, 13.31.

Adenosine-5′ Phosphoropiperidate (VI, R = adenine).—Using a procedure similar to that for the morpholidates and using distilled piperidine in place of morpholine, a single product could not be obtained and a large amount (57%) of adenosine-5′ phosphate remained unreacted. On working up exactly as above, a mixture of adenosine-5′ phosphoropiperidate and adenosine-5′ phosphate, presumably as salts of 1-piperidine N,N′-dicyclohexylcarboxamidine, was obtained. This mixture was dissolved in water and applied to a column (2 × 8 cm.) of Dowex 2 (8% cross-linked) resin in the carbonate form.[40] After washing the column well with water, elution was commenced, using a linear gradient of triethylammonium bicarbonate (*p*H, 7.5). The mixing vessel initially contained 2 liters of 0.005 *N* triethylammonium bicarbonate (*p*H 7.5) and the reservoir 2 liters of 0.15 *N* triethylammonium bicarbonate. Two well resolved ultraviolet absorbing peaks were obtained, the first corresponding to the desired compound. This peak was concentrated to dryness under reduced pressure and last traces of triethylammonium bicarbonate were removed by several further evaporations after additions of small amounts of water and, finally, suction on an oil pump. The residue was dissolved in methyl alcohol (10 ml.), and 4-morpholine N,N′-dicyclohexylcarboxamidine (176 mg., 0.6 mmole) was added. The solution was evaporated to dryness and the evaporation repeated from a further amount of methyl alcohol. The residue was then dissolved in the minimum volume of methyl alcohol, transferred to a centrifuge tube and precipitated with ether as in the case of the morpholidates, giving 290 mg. (40%) of adenosine-5′ phosphoropiperidate as its 4-morpholine N,N′-dicyclohexylcarboxamidine salt.

Anal. Calcd. for $C_{32}H_{54}N_9O_7P\cdot 5H_2O$: C, 48.19; H, 8.03; N, 15.80. Found: C, 48.14; H, 7.93; N, 15.53.

A similar ion exchange method was used for the purification of some of the nucleoside phosphoromorpholidates in the early work whenever the reactions failed to go to completion.

Adenosine-5′ Phospho-*p*-anisidate.—A mixture of adenosine-5′ phosphoric acid (1 mmole), water (2 ml.), *t*-butyl alcohol (10 ml.), *p*-anisidine (816 mg., 7 mmole) and dicyclohexylcarbodiimide (1.02 g., 5 mmole) was heated under gentle reflux on a water-bath. After fifteen minutes a clear solution resulted and refluxing was continued for 3 hr. The solvent was then largely removed *in vacuo* and the residue was suspended in water (15 ml.) and extracted with ether three times, some insoluble material (mostly dicyclohexylurea) being removed after the first extraction. The aqueous solution was then evaporated *in vacuo* and finally dried on an oil pump. The residual glass was then dissolved in methyl alcohol (3 ml.) and precipitated by the addition of ether (30 ml.). Adenosine-5′ phospho-*p*-anisidate was obtained as its N,N′-dicyclohexyl N″-*p*-methoxyphenylguanidinium salt (721 mg., monohydrate) in 90% yield.

Anal. Calcd. for $C_{37}H_{52}N_9O_8P\cdot H_2O$: C, 55.60; H, 6.80; N, 15.77. Found: C, 56.03; H, 6.76; N, 15.77.

The guanidine base, which is visible under the ultraviolet light, separates from the nucleotide derivative on paper chromatograms developed in Solvents I (R_f, 0.90) and II (R_f, 0.90) and by paper electrophoresis.

4-Morpholine N,N′-dicyclohexylcarboxamidine (VIII). (a) **Synthesis.**—A solution of dicyclohexylcarbodiimide (4.1 g., 20 mmole) and distilled morpholine (4.0 ml., 40 mmole) in *t*-butyl alcohol (10 ml.) was refluxed for 4 hr. and the solution then allowed to cool overnight. The resulting white crystals were removed and washed first with a little *t*-butyl alcohol and then with petroleum ether. Concentration of the mother liquors gave a second crop. The combined yield was 5.2 g. (89%) of chunky white crystals of m.p. 105–105.5°, unchanged after recrystallization from aqueous methyl alcohol.

Anal. Calcd. for $C_{17}H_{31}N_3O$: C, 69.60; H, 10.65; N, 14.32. Found: C, 69.36; H, 10.67; N, 14.19.

(b) **From Adenosine-5′ Phosphoromorpholidate.**—4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate (342 mg., 0.44 mmole) was dissolved in water (1 ml.), and lithium hydroxide (0.5 ml. of 4 *N*) was added. After storage at 0° the white precipitate (100 mg., 78%) was removed and dried. After recrystallization from aqueous methyl alcohol, the compound melted at 105–105.5° and was identical with the product described above.

The Rates of Addition of Amines to Dicyclohexylcarbodiimide.—Distilled piperidine (0.19 ml., 1.2 mmole) was added to a 0.1 *M* solution (3 ml.) of dicyclohexylcarbodiimide in anhydrous *t*-butyl alcohol. A number of roughly 0.05 ml. aliquots of this mixture were sealed in fine Pyrex tubes and immersed in a boiling water-bath. At intervals tubes were removed and the spectra of the contents directly examined in the region 2000–2200 cm.$^{-1}$ with a Perkin–Elmer Model 21 Infrared Spectrophotometer fitted with sodium chloride optics. The intensity of the characteristic N=C=N stretching frequently[14,41] of the carbodiimide at 2120 cm.$^{-1}$ provides a direct measure of the extent of reaction.

Parallel experiments under the same conditions were run using morpholine and *p*-anisidine in place of piperidine. The results are shown in Fig. 1, the half times for the reaction with piperidine being 0.50 hr. and with morpholine 1.85 hr. After 32 hr. only 20% reaction had occurred in the case of *p*-anisidine. As would be expected when only 1.1 molar equivalents of piperidine were used in place of four, the half time of the reaction was increased to 2.40 hr.

Rates of Acidic Hydrolysis of Adenosine-5′ Phosphoromorpholidate and Adenosine-5′ Phosphoramidate.—Solutions of 4-morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate and of N,N′-dicyclohexylguanidinium adenosine-5′ phosphoramidate (5 μmole/ml.) were made up in (a) 1 *M* ammonium acetate buffer *p*H 4.0, (b) 1 *M* ammonium acetate buffer *p*H 5.0, (c) 0.1 *N* sulfuric acid. Approximately 20 μl. aliquots of the solutions a and b were sealed in Pyrex capillaries and heated in a boiling water-bath while the solution c was stored at room temperature. At varying intervals samples were removed, spotted directly on sheets of Whatman No. 1 paper[42] and developed in Solvent I. Spots corresponding to the unreacted phosphoramidates and to adenosine-5′ phosphate were widely separated and were eluted with 0.01 *N* hydrochloric acid. The relative intensities were measured by determination of the optical densities at 257 mμ. In the *p*H 5.0 hydrolysis of the morpholidate a small amount (maximum 4.9%) of a transitory product (R_f 0.23), which is probably IX, appeared during the hydrolysis, while in the case of the *p*H 5.0 hydrolysis of the amidate some cleavage to adenosine began to occur after the hydrolysis was 80% complete. The results are shown in Fig. 2.

Rates of Reaction of Substituted Phosphoramidates with Monophenyl Phosphate.—(a) Three parallel experiments were set up using 4-morpholine N,N′-dicyclohexylcarboxamidinium salts of adenosine-5′ phosphoromorpholidate and −5′ phosphoropiperidate and N,N′-dicyclohexyl N″-*p*-methoxyphenylguanidinium adenosine-5′ phospho-*p*-anisidate. In each case the substituted phosphoramidate (20 μmole) was dried by three evaporations with 1 ml. portions of anhydrous pyridine (dried over calcium hydride). Crystalline monophenylphosphoric acid (10.4 mg., 60 μmole) was then added and the mixture was rapidly evaporated twice with anhydrous pyridine (1 ml.). The final residue was dissolved in anhydrous pyridine (0.5 ml.) and sealed.[43] At various times up to one hour aliquots (0.05 ml.) were

(40) Anion exchange columns in the carbonate form and their elution with volatile triethylammonium bicarbonate[38] are commonly used in this Laboratory (*cf.* ref. 22b). The greater volatility of triethylammonium bicarbonate than of ammonium bicarbonate makes it more attractive in this work. Ammonium bicarbonate has recently been used in oligonucleotide work [M. Staehelin, H. A. Sober and E. A. Peterson, *Arch. Biochem. Biophys.*, **85**, 289 (1959)].

(41) G. D. Meakins and R. J. Moss, *J. Chem. Soc.*, 993 (1957).

(42) Aliquots from the hydrolyses in 0.1 *N* sulfuric acid were immediately quenched by overspotting with concentrated ammonium hydroxide prior to chromatography.

(43) The total operation up to this point after the addition of the monophenylphosphoric acid was completed in approximately 1.5 min.

quickly removed and evaporated to dryness, traces of pyridine then being removed by twice evaporating with water (0.1 ml.). The final residues were dissolved in water (0.1 ml.) and examined by paper chromatography and paper electrophoresis at *p*H 7.5. The various ultraviolet absorbing spots were identified by comparison with known markers and their relative intensities determined by elution with 0.1 *N* hydrochloric acid (2 ml.) and measuring the optical densities at 257 mμ. In the cases of the morpholidate and anisidate all the reaction components with the exception of P^1-adenosine-5′ P^2-phenyl pyrophosphate and adenosine-5′ phosphate were separable by electrophoresis at *p*H 7.5; the relative amounts of the latter compounds were determined by paper chromatography in Solvent I. With the piperidate paper chromatography in Solvent I alone cleanly separated the substituted phosphoramidate from all other nucleotides. Monophenyl phosphate, however, did not completely separate from the piperidate and the latter was measured at 280 mμ. Electrophoresis at *p*H 7.5 was used to obtain the product distribution after the reaction was complete. The reaction rates and final reaction compositions are shown in Fig. 3 and Table I, respectively.

(b) **Effect of Tertiary Bases on the Reaction Rate.**—A reaction mixture was set up exactly as above using adenosine-5′ phosphoropiperidate, except that purified triethylamine[44] (0.017 ml., 120 μmole) was added. The reaction was followed by chromatography and electrophoresis as above and the rate and products are shown in Fig. 1 and Table I.

Stability of Adenosine-5′ Phosphoromorpholidate in Anhydrous Pyridine.—4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate (77 mg., 0.1 mmole) was dried by three consecutive evaporations of its solutions in pyridine (3 ml.). The final residue was dissolved in anhydrous pyridine (1 ml.) and stored. After seven days an aliquot was removed and examined by chromatography in Solvents I and II and by electrophoresis at *p*H 7.5. The material was virtually unchanged and after five months a similar examination showed the mixture to contain 73% adenosine-5′ phosphoromorpholidate and 27% P^1,P^2-diadenosine pyrophosphate as the only detectable products.

P^1,P^2-Diadenosine-5′ P^1-4-Morpholine Pyrophosphate (IX).—4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate (155 mg., 0.2 mmole) was dried by three evaporations with pyridine (5 ml.), and anhydrous hydrogen chloride in dioxane (0.06 ml. of 3.65 *N*, 0.22 mmole) was added. After thirty minutes the reaction was not complete but there was no change during the next hour. A further 0.02 ml. of hydrogen chloride in dioxane was added and after thirty minutes the mixture was evaporated to dryness. The residue was dissolved in water (10 ml.), and after adding concentrated ammonium hydroxide (0.1 ml.), the solution was applied to a 2 × 20 cm. column of DEAE cellulose in the carbonate form. After washing with water the column was eluted using a linear gradient. The mixing vessel contained 2 liters of 0.005 *M* triethylammonium bicarbonate and the reservoir 2 liters of 0.05 *M* triethylammonium bicarbonate. Fifteen milliliter fractions were collected at a flow rate of 3 ml. per minute. A large ultraviolet absorbing peak was eluted at roughly 0.009 *N* bicarbonate concentration, the first few fractions of which were found to contain a little adenosine-5′ phosphoromorpholidate by electrophoresis at *p*H 7.5. The main portion of the peak was pooled and contained 2010 optical density units at 257 mμ (67%). The solution was evaporated to dryness *in vacuo* and all remaining triethylammonium bicarbonate removed by a further evaporation from a little water. The final residue was dissolved in ethyl alcohol (10 ml.) containing a little methyl alcohol and a 1 *M* solution of calcium chloride in ethyl alcohol (0.15 ml.) was added. Precipitation was completed by adding acetone (30 ml.) and the resulting precipitate was collected by centrifugation and washed twice with ethyl alcohol (10 ml.) and acetone (30 ml.) and then dried *in vacuo*, giving 50 mg. of the calcium salt of P^1,P^2-diadenosine-5′ P^1-4-morpholine pyrophosphate (IX).

Anal. Calcd. for $C_{24}H_{32}N_{11}O_{14}P_2Ca \cdot 0.5H_2O$: N, 19.70; P, 7.91. Found (after drying at 100°): N, 19.70; P, 8.10.

Cytidine-5′ Diphosphate.—4-Morpholine N,N′-dicyclohexylcarboxamidinium cytidine-5′ phosphoromorpholidate (716 mg., 1 mmole) was dried by two evaporations of its solution in pyridine (10 ml.). Separately 85% orthophosphoric acid (0.205 ml., 3 mmole) was dissolved in pyridine (10 ml.) containing purified tri-*n*-butylamine (0.715 ml., 3 mmole) and the compound rendered anhydrous by three evaporations of pyridine (10 ml.) solutions. The two pyridine solutions were mixed and evaporated twice more *in vacuo*, the flask being opened each time to dry air. The final residue was dissolved in dry pyridine (10 ml.) and shaken for 1 hr. in order to dissolve a little precipitate which separated. The clear solution was stored at room temperature for 50 hr., after which time the morpholidate had completely disappeared. The solution was evaporated to dryness and residual pyridine removed by addition and evaporation of water (5 ml.). The residue was dissolved in water (10 ml.) containing lithium acetate (408 mg., 4 mmole) and extracted with ether. The *p*H of the aqueous phase was then brought to 12 with lithium hydroxide and the mixture was stored at 0° for thirty minutes. The precipitated tri-lithium phosphate was removed and washed free of nucleotides with 0.01 *N* lithium hydroxide. The combined filtrates were adjusted to *p*H 8.0 by the gradual addition of Dowex 50 (H^+) resin, filtered from the latter and applied to a 2 × 10 cm. column of Dowex 2 (Cl^-) resin. After washing with water the products were eluted with a linear gradient of lithium chloride in hydrochloric acid. The mixing chamber contained 0.003 *N* hydrochloric acid and the reservoir 2 liters of 0.05 *M* lithium chloride in 0.003 *N* hydrochloric acid. Fractions of 18 ml. volume were collected at a flow rate of 2.5 ml. per minute. Two ultraviolet absorbing peaks were eluted, the first (1600 optical density units at 280 mμ, 12%) being cytidine-5′ phosphate, and the second (10,900 density units at 280 mμ, 83%) being cytidine-5′ diphosphate. The pooled second peak was adjusted to *p*H 4.5 with lithium hydroxide and evaporated to dryness *in vacuo*. The residue was dried to a white solid on an oil pump, then well stirred with methyl alcohol (10 ml.), and acetone (70 ml.) was added. The white precipitate was collected by centrifugation and washed three times with smaller portions of methyl alcohol and acetone, after which the supernatant was free of chloride ions. The precipitate was washed with ether and dried *in vacuo* at room temperature, giving the lithium salt of cytidine-5′ diphosphate (375 mg., 78% based upon the morpholidate) as the trihydrate.

Anal. Calcd. for $C_9H_{13}N_3O_{11}P_2Li_3 \cdot 3H_2O$: C, 22.76; H, 3.82; N, 8.85. Found (after drying at 100°): C, 23.06; H, 3.93; N, 8.53.

The product was chromatographically and electrophoretically pure under a variety of conditions and contained no inorganic phosphate.

Uridine-5′ Diphosphate.—A reaction mixture was set up essentially as in the case of cytidine-5′ diphosphate, except that the final anhydrous pyridine solution (50 ml.) contained 4-morpholine N,N′-dicyclohexylcarboxamidinium uridine-5′ phosphoromorpholidate (6 mmole) and bis-(tri-*n*-butylammonium) orthophosphate (24 mmole).[45] The phosphoromorpholidate had completely disappeared after four days and the pyridine was then evaporated *in vacuo*. Subsequently, after partial evaporation of an aqueous solution to remove residual pyridine, the *p*H was brought to 12 with lithium hydroxide[46] and the resulting mixture was stored at 0° for 1 hr. The precipitated trilithium phosphate was centrifuged and washed free of ultraviolet absorbing compounds with 0.01 *N* lithium hydroxide. The combined filtrates were adjusted to *p*H 8.0 with Dowex 50 (H^+) resin, filtered and applied to a 3 × 12 cm. column of Dowex 2 (Cl^-) resin. After washing the column with water it was eluted with 0.04 *N* lithium chloride in 0.003 *N* hydrochloric acid, which gave uridine-5′ phosphate and two smaller ultraviolet absorbing peaks (total of 10,400 optical density units at 262 mμ). Elution with 0.1 *N* lithium chloride in 0.003 *N* hydrochloric acid then gave a large peak of uridine-5′ diphosphate (45,700 optical density units at 262 mμ, 76% based on the morpholidate). The pooled peak was worked up in a similar manner to that used for cytidine-5′ diphosphate and gave the chromatographically and electrophoretically pure lithium salt of uridine-5′ diphosphate (1.75 g., 67%) as the monohydrate.

(44) Distilled from *p*-toluenesulfonyl chloride.

(45) The reaction would have been faster had *mono* tri-*n*-butylammonium orthophosphate been used as in the preparation of cytidine-5′ diphosphate.

(46) On the addition of alkali, the amine separates and several phases are formed. Subsequent work-up is much easier if tri-*n*-butylamine is first extracted at neutral *p*H, as in the cytidine-5′ diphosphate experiment.

Anal. Calcd. for $C_9H_{12}N_2O_{12}P_2Li_2 \cdot H_2O$: C, 24.91; H, 3.23; N, 6.46. Found: C, 24.99; H, 3.06; N, 6.46.

The Reaction of Adenosine-5′ Phosphoromorpholidate with Pyrophosphoric Acid.—4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate (78 mg., 0.1 mmole) was dried by three evaporations of its solution in anhydrous pyridine. Separately a solution of bis-(tri-*n*-butylammonium) pyrophosphate[47] (1 mmole) was dried by repeated evaporation of a solution in pyridine. The two solutions were mixed and evaporated once more and and the residue kept in anhydrous pyridine (5 ml.) at room temperature. Some precipitate (non-ultraviolet absorbing) began to separate and the mixture was shaken mechanically. After 2, 24 and 48 hr., 1.5 ml. aliquots were removed, evaporated to dryness and examined by paper and ion exchange chromatography. The bulk of each aliquot was adjusted to *p*H 8 with ammonia and applied to a 1 × 13 cm. column of Dowex 2 (Cl^-) resin. After washing with water the column was eluted with a linear salt gradient, the mixing chamber containing 500 ml. of 0.003 *N* hydrochloric acid and the reservoir 500 ml. of 0.40 *N* lithium chloride in 0.003 *N* hydrochloric acid. Ten milliliter fractions were collected at a flow rate of 1 ml. per minute. The composition of the aliquots thus determined are shown in Table II and Fig. 4.

Adenosine-5′ Triphosphate.—In another experiment as above using adenosine-5′ phosphoromorpholidate (0.2 mmole) and bis-tributylammonium pyrophosphate (1.0 mmole) in pyridine (6 ml.), one half of the reaction mixture was evaporated to dryness after 5 hr. and directly applied to a 1 × 20 cm. column of Dowex 2 (Cl^-) resin. Gradient elution using 0.45 *N* lithium chloride in 0.003 *N* hydrochloric acid (700 ml.) in the reservoir and 0.003 *N* hydrochloric acid (700 ml.) in the mixing vessel cleanly separated AMP (8%), ADP (36%), ATP (54%) and A-tetraP (2%). The triphosphate peak was adjusted to *p*H 7.5 with lithium hydroxide and evaporated to dryness. The dry residue was thoroughly mixed with methyl alcohol (5 ml.) and acetone (45 ml.) was added. The white precipitate was freed of lithium chloride by repeated treatment with methyl alcohol and an excess of acetone and the final product was dried *in vacuo* at room temperature, giving the lithium salt of ATP as the octahydrate (28 mg., 43%).

Anal. Found: Total P: labile P: adenosine = 2.93: 1.94: 1.00. Theor.: 3:2:1.

The remainder of the reaction mixture was worked up in the same way after 48 hr., giving AMP (25%), ADP (57%), ATP (15%) and A-tetraP (3%). The diphosphate peak was isolated as above, giving the lithium salt of ADP as the pentahydrate (24 mg., 45%).

Anal. Found: Total P:labile P:adenosine = 1.93: 1.03:1.00. Theor: 2:1:1.

The Stability of Adenosine-5′ Triphosphate.—Chromatographically pure adenosine-5′ triphosphate (0.1 mmole) was converted to the pyridinium salt and dissolved in 80% pyridine. Tri-*n*-butylamine (1 mmole) was added, the solution evaporated to dryness and then rendered anhydrous by three further evaporations with dry pyridine. The final residue was dissolved in pyridine (5 ml.), and 4-morpholine N,N′-dicyclohexylcarboxamidine (0.1 mmole) and morpholine (0.1 mmole) were added. Some precipitation slowly occurred and the mixture was shaken. At intervals aliquots were removed and examined chromatographically in several solvent systems, particularly Solvent III. Within 2 hr. spots corresponding to adenosine-5′ di- and tetra-phosphates were observed and after four days the entire mixture was evaporated to dryness, brought to *p*H 8 with ammonium hydroxide and applied to a 1 × 15 cm. column of Dowex 2 (Cl^-) resin. After washing with water the column was eluted using a linear salt gradient. The mixing vessel contained 0.003 *N* hydrochloric acid (600 ml.) and the reservoir 0.40 *N* lithium chloride in 0.003 *N* hydrochloric acid (600 ml.). Four well separated peaks resulted (Fig. 5) and no ultraviolet absorbing material was eluted subsequently with 1.0 *N* lithium chloride in 0.003 *N* hydrochloric acid. Each peak was adjusted to *p*H 7.5 with lithium hydroxide, evaporated to dryness and freed from salts by precipitation with methyl alcohol and acetone as usual. Peak I (9.4%) was adenosine-5′ phosphate. Peak II (27.5%) was adenosine-5′ diphosphate (Ratio of adenosine:total P:labile P = 1.0:1.90: 0.95). Peak III (35.5%) was unchanged adenosine-5′ triphosphate (adenosine: total P:labile P = 1.0:2.85:1.80). Peak IV (27.5%) was adenosine-5′ tetraphosphate (adenosine: total P:labile P = 1.00:4.00:2.85). All peaks were chromatographically pure and I, II and III were identical with known markers. Peak IV had the expected mobility of the tetraphosphate.

Adenosine-5′ Methyl Phosphate.—4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate (155 mg., 0.2 mmole) was dried by three evaporations of pyridine solutions (5 ml. each time). Anhydrous methyl alcohol (0.4 ml., 10 mmole) was then added followed by anhydrous hydrogen chloride in dioxane (0.05 ml. of 4 *N*). The clear solution was left overnight and then evaporated to dryness, last traces of pyridine being removed by evaporation of an aqueous solution. After drying the residue on an oil pump, it was dissolved in ethyl alcohol (3 ml.), and calcium chloride (0.2 ml. of 1 *M* solution in ethyl alcohol) was added. Precipitation was completed by the addition of acetone (6 ml.), and the white precipitate was collected and re-treated several times with ethyl alcohol and acetone until the supernatant was free of chloride ions. The precipitate was washed with ether and dried *in vacuo*, giving the calcium salt of adenosine-5′ methyl phosphate (86 mg., 95%) as the tetrahydrate. The product was chromatographically and electrophoretically homogeneous[48] and identical with a previously described sample.[16]

2,4-Dinitrophenyl Adenosine-5′ Phosphate.—4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate (78 mg., 0.1 mmole) was dried by three evaporations of its solutions in anhydrous pyridine (5 ml.). 2,4-Dinitrophenol (55 mg., 0.3 mmole) was then added and the mixture evaporated once more with pyridine. The final residue was stored in dry pyridine (2 ml.) at room temperature for 4 hr. and then evaporated to dryness. The residue was dissolved in water (5 ml.) and extracted three times with chloroform. The combined chloroform extracts were chromatographically shown to contain only 2,4-dinitrophenol. The aqueous solution was concentrated *in vacuo*, applied to three 8″-wide strips of Whatman 3 MM paper and the chromatograms developed in Solvent V. The strong band of R_f 0.50 was eluted with water and lyophilized, giving 14 mg. of chromatographically pure 2,4-dinitrophenyl adenosine-5′ phosphate as a somewhat hygroscopic yellowish solid.[49] Good elemental analyses could not be obtained, but the compound was rapidly hydrolyzed both by purified venom phosphodiesterase and by alkali to adenosine-5′ phosphate and 2,4-dinitrophenol.

The Attempted Reaction of Adenosine-5′ Phosphoromorpholidate with Carboxylic Acids.—(a) 4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate (77 mg., 0.1 mmole) was dried by three evaporations of solutions in pyridine and then dried benzoic acid (37 mg., 0.3 mmole) was added. A pyridine solution of the mixture was evaporated once more and the final residue dissolved in dry pyridine (3 ml.). After two weeks an aliquot was examined chromatographically and the relative intensities of the spots determined by elution and ultraviolet absorption measurement. By use of a combination of Solvents II and IV the mixture was shown to contain unreacted adenosine-5′ phosphoromorpholidate (72%), P^1,P^2-diadenosine-5′ pyrophosphate (19%), adenosine-5′ phosphate (1%) and a compound with R_f 0.38 in Solvent II (8%) which was probably IX. After three months the mixture contained only adenosine-5′ phosphoromorpholidate (43%), P^1,P^2-diadenosine-5′ pyrophosphate (54%) and adenosine-5′ phosphate (3%).

(b) A comparable experiment using acetic acid in place of benzoic acid showed a somewhat faster degradation of the morpholidate. After five months the mixture contained roughly equal amounts of P^1,P^2-diadenosine-5′-pyrophosphate and adenosine-5′ phosphate.

(47) Prepared by passing a solution of crystalline tetrasodium pyrophosphate through a column of pyridinium Dowex 50 and adding two equivalents of tri-*n*-butylamine to the product in 80% pyridine.

(48) In some reactions the product was contaminated with 2–3% adenosine-5′ phosphate.

(49) This compound has a marked solubility in both aqueous and non-aqueous solvents and accordingly its isolation and purification by precipitation techniques led to extensive losses.