(b) **N^6,3'-O-Diacetyldeoxycytidine-5' Phosphate.**—The lyophilized material prepared as described previously was kept at room temperature in 50% acetic acid. Most of the N-acetyl group had been removed in 3–4 days. After a total of 7 days, the total products were streaked on a strip of paper which was developed in solvent G. Although there were three other trace bands present, most of the nucleotide material was contained in two bands. Of these, the faster travelling band (band 1) was very much stronger than the slower band (band 2). Bands 1 and 2 were eluted and treated with concentrated ammonia, to remove *O*-acetyl group, and the resulting products were dephosphorylated by incubation with alkaline phosphomonoesterase. The nucleosides obtained were chromatographed in solvent H. Band 1 was found to give mostly deoxycytidine but also some deoxyuridine (the amount of the latter was estimated to be 12%). Band 2 also gave deoxycytidine and a barely visible trace of deoxyuridine. From these results, it is concluded that band 1 originally contained mostly 3'-*O*-acetyldeoxycytidine-5' phosphate and some deoxyuridine-5' phosphate, while band 2 contained mostly deoxycytidine-5' phosphate, some deacetylation of the 3'-*O*-acetyl group having occurred. The extent of deamination to uracil nucleotide was 10%.

Paper Chromatography and Paper Electrophoresis.—Paper chromatography was performed using double-acid washed papers (Whatman No. 40 or 44) and the descending technique. The solvent systems used were: solvent A, isopropyl alcohol–1 *M* ammonium acetate (*p*H 6) (5–2, v./v.); solvent B, isopropyl alcohol–concd. ammonia–water (7–1–2); solvent C, *n*-propyl alcohol–concd. ammonia–water (5.5–1–3.5); solvent D, ethyl alcohol–0.5 *M* ammonium acetate (*p*H 3.8), (5–2); solvent E, ethyl alcohol–1 *M* ammonium acetate (*p*H 7.5) (5–2); solvent F, isobutyric acid (100 ml.)–1 *N* ammonia (60 ml.)–0.1 ethylenediaminetetraacetate-disodium (1.6 ml.); solvent G, *n*-butyl alcohol–acetic acid–water (5–2–3); solvent H, *n*-butyl alcohol–water (86–14). The R_f's of different compounds in the different solvent systems are listed in Table III.

Paper electrophoresis was carried out in an apparatus similar to that described by Markham and Smith,[61] using 0.05 *M* triethylammonium bicarbonate (*p*H 7.5) buffer and 0.05 *M* ammonium acetate (*p*H 3.5) buffer or simply 1 *M* acetic acid for acidic *p*H.

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

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL AND THE DEPARTMENT OF PHARMACOLOGY OF THE UNIVERSITY OF BRITISH COLUMBIA, VANCOUVER 8, B. C., CANADA]

Cyclic Phosphates. IV.[1] Ribonucleoside-3',5' Cyclic Phosphates. A General Method of Synthesis and Some Properties

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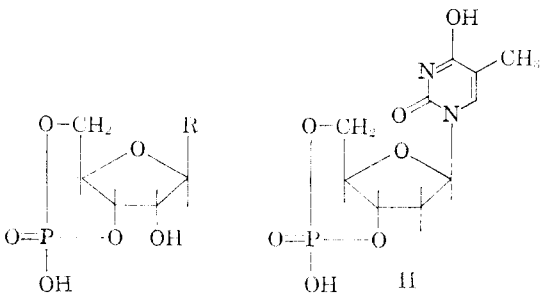
A general method for the synthesis of ribonucleoside-3',5' cyclic phosphates is described. It involves the reaction of a 4-morpholine-N,N'-dicyclohexylcarboxamidinium (III) salt of a ribonucleoside-5' phosphate with dicyclohexylcarbodiimide under dilute conditions. Using this method, adenosine-, guanosine-, cytidine- and uridine-3',5' cyclic phosphates have been prepared in yields of 60–85%. The preparation of N^6-benzoyl cytidine-5' and N-benzoylguanosine-5' phosphates, the starting materials in two of the above syntheses, is described. Acidic, alkaline and enzymic degradations of the cyclic phosphates have been studied. The results show that the six-membered phosphate ring exerts a profound and characteristic influence on the stabilities of the glycosyl bonds in the nucleotides. Purine glycosyl bonds are rendered remarkably stable to acid while the glycosyl bond in uridine-3',5' cyclic phosphate is strikingly labilized. Barium ions catalyze the alkaline hydrolysis of the six-membered phosphate ring, the products being the nucleoside-3' and nucleoside-5' phosphates in the ratio of 5:1. Some observations on the action of different phosphodiesterases on the cyclic phosphates are recorded.

An adenine nucleotide was recognized by Sutherland and co-workers[5,6] as a factor stimulating the conversion of inactive glycogen phosphorylase to the active form in liver preparations. The substance,[6] which proved to be identical with a product arising from the barium hydroxide-catalyzed degradation of adenosine-5' triphosphate,[7a] was shown by Lipkin and co-workers[7b] to be adenosine-3',5' cyclic phosphate (I, R = adenine).

A minor product formed during the polymerization of thymidine-5' phosphate was independently identified as thymidine-3',5' cyclic phosphate[8] (II)



I, R = purine or pyridine

and at the same time several methods for its synthesis were described.[8] In further work, uridine-3',5' cyclic phosphate (I, R = uracil) was synthesized in good yield from uridine-5' phosphate and was used as the key intermediate in the specific synthesis of the naturally occurring C_3'–C_5' interribonucleotidic linkage.[9]

The recognition of the biological importance of adenosine-3',5' cyclic phosphate and of the potentialities of this class of compounds as intermediates in the synthesis of ribopolynucleotides made it

(1) Paper III, H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, THIS JOURNAL, **79**, 430 (1957).

(2) British Columbia Research Council. The work has been supported by grants from The National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and the National Research Council of Canada, Ottawa.

(3) The Institute for Enzyme Research, University of Wisconsin, Madison 5, Wis.

(4) Department of Pharmacology, University of British Columbia. The work in this department has been supported by a grant from The Life Insurance Medical Research Fund, New York.

(5) T. W. Rall, E. W. Sutherland and J. Berthet, *J. Biol. Chem.*, **224**, 463 (1957); T. W. Rall and E. W. Sutherland, *ibid.*, **232**, 1065 (1958).

(6) E. W. Sutherland and T. W. Rall, THIS JOURNAL, **79**, 3608 (1957); *J. Biol. Chem.*, **232**, 1077 (1958).

(7) (a) W. H. Cook, D. Lipkin and R. Markham, THIS JOURNAL, **79**, 3607 (1957); (b) D. Lipkin, W. H. Cook and R. Markham, *ibid.*, **81**, 6198 (1959).

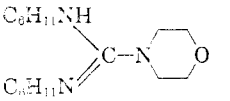
(8) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *ibid.*, **80**, 6223 (1958).

(9) M. Smith and H. G. Khorana, *ibid.*, **81**, 2911 (1959).

highly desirable that a more thorough investigation of the methods for their synthesis and of their chemical and enzymic properties be undertaken. The present communication describes a simple and generally satisfactory method for the preparation of ribonucleoside-3′,5′ cyclic phosphates, by which adenosine-, guanosine-, uridine- and cytidine-3′,5′ cyclic phosphates have been prepared in good yield. The acidic and alkaline degradations of this class of compound have been studied and show highly unusual features. A phosphodiesterase purified from rabbit brain hydrolyzes all of the ribonucleoside-3′,5′ cyclic phosphates, except the cytidine analog, to the corresponding ribonucleoside-5′ phosphates.[10] A forthcoming paper contains a detailed report of the use of uridine-3′,5′ cyclic phosphate in the synthesis of the C_3'–C_5', linked di-ribonucleoside phosphates.[11]

Preparation.—The formation and properties of different types of cyclic phosphate esters have been studied extensively in recent years.[1] While several methods for the preparation of the cyclic esters exist,[1] a generally useful method has been the activation of a phosphomonoester by reaction with dicyclohexylcarbodiimide (DCC) followed by an intramolecular cyclization reaction with a suitably placed hydroxyl function. The ease of cyclic phosphate formation is very much a function of the stereochemistry of the phosphomonoester and the hydroxyl groups. In the extensive earlier studies of reactions involving nucleotides and carbodiimides,[12] it was found that, while ribonucleoside-2′- (or 3′) phosphates gave quantitatively the ribonucleoside-2′,3′ cyclic phosphates,[13] ribonucleoside-5′ phosphates underwent *bimolecular* pyrophosphate-forming reactions.[14] The absence of the intramolecular cyclization reaction under the usual conditions of the reaction of ribonucleoside-5′ phosphates with carbodiimides[12] must be due to the unfavorable spacial relationship of the phosphoryl and the 3′-hydroxyl groups, which requires that the six-membered cyclic phosphate ring be fused *trans* to a five-membered ring.[15] Cyclization to form the nucleoside-3′,5′ cyclic phosphate (II) was nevertheless the major result when thymidine-5′ phosphate reacted with DCC under high dilution conditions.[8] This discovery formed the starting point of the present work.

The reaction of uridine-5′ phosphate with DCC in anhydrous pyridine at 25° was studied extensively and conditions (see Experimental) were devised which gave uridine-3′,5′ cyclic phosphate (I, R = uracil) as the major product, the yield after isolation being about 60%.[9] The technique as developed for working in pyridine alone was not, however, directly applicable to the synthesis of other ribonucleoside-3′,5′ cyclic phosphates because of the insolubility in this medium of adenosine-, guanosine- and cytidine-5′ phosphates. Therefore, it was decided to investigate the use of aliphatic tertiary amines[17] to solubilize the nucleotides and to carry out the reaction at an elevated temperature.[18] The use of tri-*n*-butyl- and tri-*n*-octylamines was unsatisfactory in the case of adenosine-5′ phosphate, which was first studied.[19] The base which proved to be an effective solubilizing agent was 4 - morpholine - N,N′ - dicyclohexyl - carboxamidine (III).[20] A study of the reaction with equimolar amounts of the base III and adenosine-5′



III

phosphate with DCC at different temperatures showed that at lower temperatures (25–60°) P^1, P^2-diadenosine-5′ pyrophosphate was produced exclusively. On increasing the temperature (60–100°) increasing amounts of adenosine-3′,5′ cyclic phosphate resulted. Eventually, the best conditions for the preparation of the cyclic phosphate proved to be the dropwise addition[21] of a solution of the guanidinium (III) salt of the nucleotide in pyridine to a boiling solution of DCC in pyridine. The crystalline cyclic phosphate was isolated in 80–86% yields.[22] Application of this procedure to uridine-5′ phosphate followed by ion-exchange chromatography gave uridine-3′,5′ cyclic phosphate in 74% yield.

Cytidine-5′ and guanosine-5′ phosphates were insoluble in pyridine as salts of the base III and rather than modify the reaction medium further, the nucleotides were converted to the more soluble N-benzoyl derivatives IV and V.[23] A simple

(10) For details see G. I. Drummond and S. Perrott-Yee, *J. Biol. Chem.*, in press.

(11) M. Smith, D. H. Rammler, I. Goldberg and H. G. Khorana, THIS JOURNAL, **83**, forthcoming publication (1961).

(12) For references see (a) M. Smith, J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 6204 (1958); and (b) H. G. Khorana, *Federation Proc.*, in press (1960).

(13) C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

(14) H. G. Khorana, *ibid.*, **76**, 3517 (1954).

(15) It is interesting, however, that the alkaline hydrolysis of thymidine-3, *p*-nitrophenyl phosphate[16] seems to proceed in good part *via* thymidine-3′,5′ cyclic phosphate, while the hydrolysis of uridine-5′ *p*-nitrophenyl phosphate is free from the corresponding participation (footnote 14 in ref. 16) from the 3′-hydroxyl group. The effective participation in the former case could be due either to the greater acidity of the 5′-hydroxyl group or to its more favorable conformation.

(16) A. F. Turner and H. G. Khorana, THIS JOURNAL, **81**, 4651 (1959). These authors noted that thymidine-5′ phosphate was present in the amount of 11% in the mixture of thymidine phosphates obtained on alkaline hydrolysis of thymidine-3′ *p*-nitrophenyl phosphate. Since the alkaline hydrolysis of thymidine-3′,5′ cyclic phosphate gives approximately 20% of thymidine-5′ phosphate,[8] more than 50% of the hydrolysis must have occurred *via* the cyclic phosphate.

(17) M. Smith and H. G. Khorana, *ibid.*, **80**, 1141 (1958).

(18) Lipkin, *et al.*,[7b] have reported on the preparation of adenosine 3′,5′ cyclic phosphate (29% yield) by reaction of tri-*n*-butylammonium adensoine-5, phosphate with DCC at 100°. As shown previously, at room temperature, the tri-*n*-alkylammonium salts of nucleotides give[12a,17] the corresponding P^1,P^2-dinucleoside-5′ pyrophosphate as the stable ultimate products.

(19) Although the nucleotide dissolved at moderate temperatures, it separated as a solid immediately at 100°. The insolubility is presumably the cause of the low yield in the earlier work.[18]

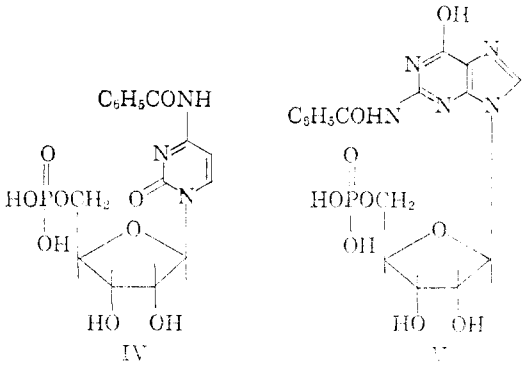
(20) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **83**, 663 (1961).

(21) The dropwise addition technique had been used earlier in the above-mentioned satisfactory preparations of uridine-3′,5′ and thymidine-3′,5′ cyclic phosphates in pyridine alone at room temperature.

(22) With reaction periods longer than 4 hr. the yield of the cyclic phosphate decreases and a by-product is produced. The side product, however, can be removed either by ion exchange chromatography or by crystallization. The by-product has a mobility on paper chromatograms like the 3′,5′-cyclic phosphate but is much more stable to treatment with barium hydroxide. Further structural work on the substance will be reported subsequently.

(23) The location shown of the benzoyl group in V is arbitrary. In cytidine derivatives IV the monobenzoyl group is probably on the N^6

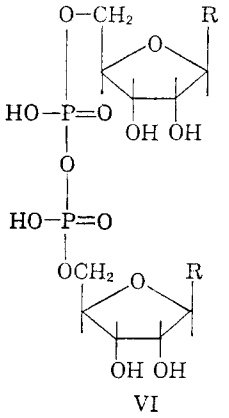
method for the preparation of this class of compounds has been developed recently.[24]



IV V

Reaction of IV and V with DCC under the conditions described above, followed by removal of the benzoyl groups with concentrated ammonia[24] and ion exchange chromatography, gave cytidine-3′,5′ and guanosine-3′,5′ cyclic phosphates.

Two reaction paths are possible for the conversion of nucleoside-5′ phosphates to the 3′,5′ cyclic phosphates on reaction with DCC. In the first one, analogous to the formation of ribonucleoside-3′,5′ cyclic phosphates from ribonucleoside-2′ (or 3′) phosphates,[12a] direct cyclization would occur after the formation of the isourea adduct with carbodiimide. In the alternative route, the first step would be the formation of P^1,P^2-dinucleoside-5′ pyrophosphate (VI). Certainly, the diadenosine-5′ pyrophosphate (VI, R = adenine)



VI

was efficiently converted to the 3′,5′-cyclic phosphate in the presence of DCC at reflux temperature (see Experimental). This conversion could proceed as a base-catalyzed intramolecular phosphorylation reaction to give equimolar amounts of the cyclic phosphate and the original nucleoside-5′ phosphate, as is known to occur with several nucleotide coenzymes and related pyrophosphates.[1] However, the necessity for further reaction with DCC during the above conversion of VI to the 3′,5′-cyclic phosphate was evidenced by the fact that without the carbodiimide the rate of reaction was slow.[25]

Whichever of the pathways discussed is the true mechanism of the formation of the cyclic phosphate under the present experimental conditions, the essential feature is an intramolecular phosphodiester bond synthesis and as such it is to be distinguished from the more complex mechanism of phosphodiester bond synthesis involving bimolecular phosphorylations of hydroxyl groups.[21,26]

Properties.—These studies are concerned mainly with the behavior of the cyclic phosphates to acidic and alkaline hydrolysis. While the alkaline hydrolysis, especially as catalyzed by barium ions, also serves as structural proof for all the members, the single overwhelming conclusion of these studies is that the formation of the six-membered ring fused *trans* to the ribofuranose ring has a very profound and characteristic influence on each one of the members so as to invalidate the familiar pattern of stabilities associated with the parent ribonucleosides and ribonucleotides.

Acid-catalyzed Hydrolysis.—As noted previously,[6,7] the final product of the acidic hydrolysis of adenosine-3′,5′ cyclic phosphate is adenine. However, the purine is released much more slowly from the cyclic nucleotide ($t_{1/2}$ 30 min. at 100° in 1 *N* hydrochloric acid) than it is from adenosine-5′ or adenosine-2′ (or -3′) phosphates ($t_{1/2}$ 2–4 min. under the above conditions). Similarly, guanine is released only very slowly on heating guanosine-3′,5′ cyclic phosphate in 1 *N* hydrochloric acid at 100° ($t_{1/2}$ for guanosine-3′,5′ cyclic phosphate 28 min.). Cytidine-3′,5′ cyclic phosphate under identical conditions has a half-life of 26 min. and the major products formed are cytidine-5′ phosphate and, presumably, a mixture of cytidine-2′ and cytidine-3′ phosphates[27] in the ratio 1:5.[28] Since with this cyclic phosphate the major reaction observed on acidic treatment is the opening of the phosphodiester ring, the rate observed may be regarded as an estimate of the stability of this type of six-membered cyclic phosphate.[29] Furthermore, since this rate of the ring opening is similar to the rates of release of purine bases from the purine ribonucleoside cyclic phosphates, it seems very probable that the rate-controlling step in the release of the purines is the hydrolysis of the cyclic phosphate ring in these compounds. The transient appearance of acyclic adenosine and guanosine phosphates during the hydrolysis of the corresponding cyclic phosphates (see Experimental) provides support for this mechanism. While the formation of the 3′,5′-cyclic phosphate ring brings about stabilization of the purine glycosyl bonds, the glycosyl bond in uridine-3′,5′ cyclic phosphate is, in contrast, rendered strikingly labile. The half-life in 1 *N* hydrochloric acid at 100° is only 8 min. and the

group; see D. M. Brown, A. R. Todd and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956).

(24) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, THIS JOURNAL, **83**, 686 (1960).

(25) For further discussion of the carbodiimide reactions see H. G. Khorana "Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1960, in press.

(26) H. G. Khorana, in "The Nucleic Acids," Vol. III, eds. E. Chargaff and J. N. Davidson, Academic Press, Inc., New York, N.Y., 1960, in press.

(27) Equilibration of the initially formed cytidine-3′ phosphate to a mixture of the 2′- and 3′-phosphates would be expected to occur under these conditions.

(28) A trace of cytosine is also present. In a comparable experiment, cytosine-5′ phosphate also gave a trace of the pyrimidine.

(29) For a comparison of the stability of different types of cyclic phosphates, see ref. 1.

major ultraviolet absorbing product is uracil (67%). (Uridine-5′ phosphate (6%) and uridine-2′(3′)[27,30] phosphates (27%) account for the remainder of the material. Comparable labilization of the pyrimidine glycosyl bond was previously noted for thymidine-3′,5′ cyclic phosphate.[8] This cyclic phosphate was completely hydrolyzed in 0.1 *N* hydrochloric acid at 100° in less than 5 min., thymidine-5′ phosphate being essentially unaffected under these conditions after 3 hr.

The fate of the sugar and phosphate components of adenosine-3′,5′ cyclic phosphate has been investigated by Sutherland and Rall.[6] Using the acidic catalysis of a cation-exchange resin, they were able to demonstrate the formation of ribose-2, ribose-3 and ribose-5 phosphates by anion-exchange chromatography. Under the acidic conditions used in the present work, which are rather more drastic, adenosine-3′,5′ and uridine-3′,5′ cyclic phosphates gave ribose and inorganic phosphate as the major non-ultraviolet-absorbing products, ribose phosphates being present only in trace amounts.

Alkaline Hydrolysis.—On treatment with 1 *M* sodium hydroxide at 100°, adenosine-3′,5′ cyclic phosphate yields adenine as the final product.[7b] Hydrolysis of uridine-3′,5′ cyclic phosphate has been studied under similar conditions. The ultraviolet-absorbing products are uridine-5′ and uridine-3′ phosphates, the latter predominating, and a small amount of uracil. During the hydrolysis of both adenosine and uridine cyclic phosphates, there is considerable destruction of the chromophores (*cf.* ref. 7b). The release of the purine and pyrimidine bases, respectively, in the above two cases is again a result peculiar to this class of ribonucleotide derivatives and is probably a consequence of distortion in the ribofuranose ring caused by the *trans*-fused six-membered phosphate ring. It is interesting that, in contrast, a corresponding derivative in the 2′-deoxyribofuranose series, namely, thymidine-3′,5′ cyclic phosphate, did not release any thymine on alkaline treatment and the only products were thymidine-3′ and 5′-phosphates in the approximate ratio 4:1.[8,31]

Polyvalent ions are known to catalyze the hydrolysis of diesters of phosphoric acid.[34–36] The effect has also been, noted in the hydrolysis of five-[35] and six-membered[7,36] cyclic phosphates. In the present work, heating in 0.2 *M* barium hydroxide at 100° for 30 min.[37] was found to hydrolyze completely all of the cyclic phosphates. The products were the corresponding nucleoside-5′ and 3′ phosphates in the ratio 1:5, respectively, as found by Lipkin, *et al.*,[7b] for adenosine-3′,5′ cyclic phosphate. The identification of the products in all cases was accomplished by ion exchange chromatography on columns previously standardized with synthetic mixtures of the three isomers of each ribonucleotide. The positions of elution of the nucleoside-5′ and nucleoside-3′ phosphates obtained from alkaline degradation corresponded exactly to those of markers. No nucleoside-2′ phosphate was detected in the hydrolysis of any of the cyclic phosphates, showing that cyclization occurred exclusively on to the 3′-hydroxyl group of the ribonucleosides.[38a,b] In the hydrolysis of cytidine-3′,5′ cyclic phosphate there was extensive deamination to uracil nucleotides so that the 3′- and 5′-phosphates of both uridine and cytidine were produced. In addition, both the pyrimidine cyclic phosphates yielded on hydrolysis small amounts (less than 5%) of new nucleotides which emerged from the ion exchange columns in between the 5′- and 3′-phosphates. The structures of these products remain to be investigated.[39]

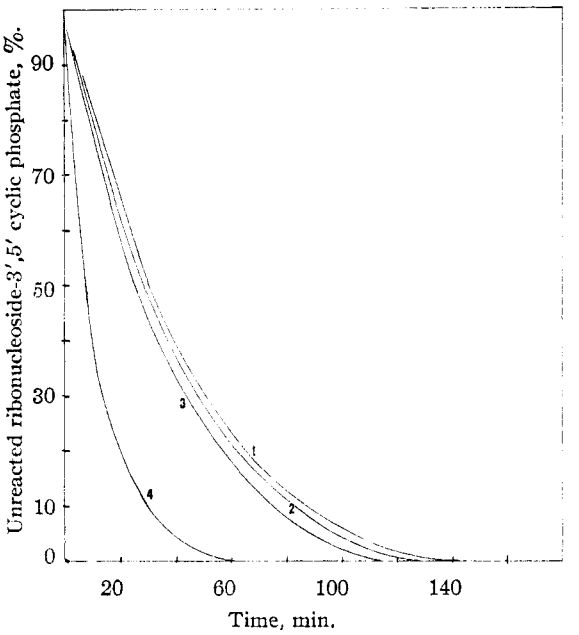


Fig. 1.—Hydrolysis of ribonucleoside-3′,5′ cyclic phosphates with *N* hydrochloric acid at 100°: 1, adenosine-3′,5′ cyclic phosphate; 2, guanosine 3′,5′ cyclic phosphate; 3, cytidine-3′,5′ cyclic phosphate; 4, uridine-3′,5′ cyclic phosphate.

(30) Uridine-5′, phosphate is completely stable in 1 *N* hydrochloric acid at 100° for 2 hr.

(31) It is a reasonable assumption that the half-life (about 2 hr.) of thymidine-3′,5′ cyclic phosphate (II) in 1 *N* sodium hydroxide at 100° is a fairly good measure of the stability of a six-membered cyclic phosphate *trans*-fused to a five-membered ring. The six-membered ring is more strained than the same sized phosphate ring *cis*-fused to a furanose ring ($t_{1/2}$ for 1,2-*O*-isopropylidene xylofuranose-3′,5′ cyclic phosphate under the same conditions, 4 hr.).[1,32] The latter system in turn is less stable than the simple propane-1,3-cyclic phosphate[1] ($t_{1/2}$ 72 hr.).[33]

(32) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 1194 (1957).

(33) Extrapolated from data in ref. 1.

(34) P. Fleury, J. Lecocq and L. Le Dizet, *Compt. rend.*, **242**, 420 (1956); K. Dimroth, H. Witzell, W. Hülsen and H. Mirbach, *Ann.*, **620**, 94, 109, 126 (1959).

(35) F. H. Westheimer, *Chem. Soc., Special Publications*, **8**, Chemical Society, London, 1957, p. 55.

(36) (a) J. Baddiley and E. M. Thein, *J. Chem. Soc.*, 3421 (1951); (b) J. Baddiley, J. G. Buchanan and L. Szabo, *ibid.*, 3826 (1954); (c) P. Szabo and L. Szabo, *Compt. rend.*, **247**, 1748 (1958).

(37) Lipkin, *et al.*,[7b] used 0.4 *M* barium hydroxide. The lower concentration was used in the present work in an effort to minimize the deamination of cytidine derivatives.

(38) (a) Further evidence in favor of the exclusive involvement of the C-3′ hydroxyl group is provided by the syntheses of uridylyl-(3′ → 5′)-uridine and uridyl-(3′ → 5′)-adenosine[11] using uridine-3′,5′ cyclic phosphate as an intermediate. The synthetic dinucleoside phosphates are uncontaminated with "C_3'-C_5' linked isomers." (b) The absence of participation by the 2′-hydroxyl group in the base-catalyzed hydrolysis of the ribonucleoside-3′,5′ cyclic phosphates has been noted by Lipkin, Markham and Cook.[7b]

(39) The absorption spectra of the new nucleotides are similar, in both series, to those of the corresponding major products (5′- and 3′-

The marked catalysis by barium ions which affects the cyclic phosphate ring opening to the exclusion of complications noted with other reagents is clearly of great practical value in the use of cyclic phosphates in ribopolynucleotide synthesis. Alternative means for the selective phosphate ring opening of cytidine-3′,5′ cyclic phosphate, where deamination was observed, are being sought.[40]

The proportion of nucleoside-3′ to nucleoside-5′ phosphate formed during the barium hydroxide-catalyzed hydrolysis of the four ribonucleoside-3′,5′ cyclic phosphates was consistently 5:1.[41] A similar ring opening to form a prepondering amount of the secondary alkyl phosphate has been noted for the base-catalyzed hydrolysis of methyl α-D-glucose-4,6[36b] and methyl α-D-galactoside-4,6 cyclic phosphates.[36c] The greater stability of the secondary phosphate ester linkage relative to the primary alkyl ester linkage[42] is analogous to the greater stability of secondary alkyl esters of carboxylic acids relative to primary alkyl esters.[43]

Enzymic Hydrolysis.—Sutherland and Rall have reported on the hydrolysis of adenosine-3′,5′ cyclic phosphate by an enzyme preparation from heart[5] and the product was shown to be adenosine-5′ phosphate. A phosphodiesterase[10] purified from rabbit brain has also been found to hydrolyze the adenosine cyclic phosphate to the 5′-phosphate.[44] The preparation also hydrolyzed guanosine-3′,5′ and uridine-3′,5′ cyclic phosphates, but not the cytidine analog, to the corresponding nucleoside-5′ phosphates. The rates with guanosine-3′,5′ and uridine-3′,5′ cyclic phosphates were one-third and one-ninth, respectively, of that observed with adenosine-3′,5′ cyclic phosphate.

Snake venom phosphodiesterase[45] attacks adenosine-[7b] and uridine-3′,5′ cyclic phosphates very slowly to give a mixture of the corresponding nucleoside-3′ and nucleoside-5′ phosphates. In agreement with the observation of previous workers,[7b] the proportions of the isomeric products varied considerably under apparently similar conditions.

A purified spleen phosphodiesterase preparation[45,46] was completely inert toward uridine-3′,5′ cyclic phosphate.

Experimental

General Methods.—Reagent grade pyridine was used and it was dried over calcium hydride or potassium hydroxide for several days. All evaporations were carried out using a rotary evaporation at about 10 mm. pressure, the bath temperature being kept below 40°. Phosphorus analysis was carried out by the method of King.[47]

Paper chromatography was performed by the descending technique using Whatman No. 40 (double acid-washed) paper. Nucleotides and related compounds were detected by viewing under an ultraviolet lamp, phosphorus-containing compounds by the molybdate–perchloric acid spray[48] and reducing sugars by the aniline hydrogen phthalate spray.[49]

The solvent systems used for paper chromatography were: isopropyl alcohol–concd. ammonia–water (7:1:2) (solvent A); isopropyl alcohol–concd. ammonia–0.1 *M* boric acid (7:1:2) (solvent B); *n*-butyl alcohol–acetic acid–water (5:2:3) (solvent C); isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* disodium ethylenediaminetetraacetic acid (100:60:1.6) (solvent D); ethyl alcohol–0.5 *M* ammonium acetate, *p*H 3.8 (5:2) (solvent E); ethyl alcohol–1 *M* ammonium acetate, *p*H 7.5 (5:2) (solvent F); saturated aqueous ammonium sulfate–1 *M* sodium acetate–isopropyl alcohol (80:18:2) (solvent G); *n*-butyl alcohol–ethyl alcohol–5 *N* hydrochloric acid (3:2:2) (solvent H). The R_f's of different compounds are given in Table I.

Uridine-3′,5′ Cyclic Phosphate.—(a) Disodium uridine-5′ phosphate (2.175 mmoles) was converted to the free acid by passing its aqueous solution through a column of Amberlite IR-120 resin (H^+ form). The eluate was concentrated to dryness and residual traces of water removed by co-evaporation with pyridine. The resulting gummy pyridinium salt was dissolved in pyridine (250 ml.) and the solution added dropwise over a period of 8 days to a vigorously stirred solution of DCC (5.15 g., 25 mmoles) in pyridine (500 ml.) at 25°. Dicyclohexylurea separated during the last 2 days. After the addition of the nucleotide solution was complete, the mixture was kept at 25° for 24 hours. Subsequently, water (250 ml.) was added and after 2 hr. at room temperature the mixture was concentrated to dryness. The resulting white semi-solid product was suspended in a mixture of water (250 ml.) and ether (250 ml.). The insoluble dicyclohexylurea was removed by filtration and the aqueous layer after treatment with concentrated ammonia (2 ml.) was evaporated to dryness. During this evaporation, ethyl alcohol was added to the solution to prevent foaming. The residual gum was dissolved in 20 ml. of the solvent mixture, isopropyl alcohol–concd. ammonia–water (7:1:2) and to the solution were added a few drops of brom thymol blue solution. The solution was applied to a column (65 cm. × 6.5 cm. dia.) of Whatman standard grade cellulose powder packed in the above solvent mixture. Elution was carried out with the same solvent at a flow rate of 1 ml./min. After the blue indicator had reached the bottom of the column, fractions (20 ml.) were collected and the nucleotide content estimated spectrophotometrically at 261 mμ. The fractions (20–80) contained the major peak and were examined by

phosphates). The formation of a new nucleotide with unchanged spectrum was also noted in the hydrolysis of deoxycytidine-3′,5′ cyclic phosphate.[34] The position of elution of this product was similar to that of the above products in the ribonucleotide series. The formation of the new nucleotides does not, therefore, appear to depend on the 2′-hydroxyl group in the ribonucleotides. It seems reasonable to postulate that the new nucleotides arise by participation from the 2-keto group in the pyrimidines during the alkaline hydrolysis and the intermediate formation of cyclonucleoside-5′ phosphates. They would therefore be xylofuranosyl-pyrimidine-5′ phosphates. [See *e.g.*, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 816 (1955), for cyclonucleoside formation from 3′-*O*-substituted thymidine and related compounds.]

(40) Cadmium hydroxide (*p*H 7.5) at 100° was ineffective in hydrolyzing the 3,5, cyclic phosphate ring in uridine-3′,5′ cyclic phosphate.

(41) It is of interest that the acid-catalyzed hydrolysis of the pyrimidine-3′,5′ cyclic phosphates also gave the secondary and primary phosphates in the same proportion.

(42) Examples of cyclic phosphates where the primary ester linkage is more stable than the secondary ester are known. Thus, 1,2-*O*-isopropylidene-D-xylofuranose-3,5 cyclic phosphate on treatment with base gave the 5- and the 3-phosphate in the ratio 2.5:1.[32] There the 3-phosphate is probably labilized by interaction with the eclipsing 5-methylene group. Pantothenic acid 2,4-cyclic phosphate yields exclusively the primary (4-) phosphate on reaction with barium hydroxide,[35b] probably because of the presence of the adjacent carboxyamide group. The latter could exert a polar effect or a hydrogen bond effect involving the proton on the amide nitrogen or could participate in chelation with barium ion.[35]

(43) M. S. Newman, "Steric Effects in Organic Chemistry," J. Wiley and Sons, Inc., New York, N. Y., 1956, p. 219.

(44) With adenosine-3′,5′ cyclic phosphate as substrate, the phosphodiesterase can be coupled with purified adenosine-5′ phosphate deaminase. The rate of hydrolysis by the diesterase can then be followed spectrophotometrically by measuring the decrease in adsorption at 265 mμ. This provides a convenient assay system for determining the distribution of the enzyme in various tissues.[10]

(45) H. G. Khorana in "The Enzymes," eds. P. D. Boyer, H. A. Lardy and K. Myrback, Academic Press, Inc., New York, N. Y., 1960, in press.

(46) L. A. Heppel and R. J. Hilmoe in "Methods in Enzymology," Vol. II, eds. S. P. Colowick and N. O. Kaplan, Academic Press, Inc., New York, N. Y., 1955, p. 565.

(47) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(48) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949), R. S. Bandursky and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(49) S. M. Partridge, *Nature*, **164**, 443 (1949).

TABLE I

R_f'S OF DIFFERENT COMPOUNDS ON PAPER CHROMATOGRAMS

Solvent	A	B	C	D	E	F	G	H
Adenosine-2′ phosphate	0.17	0.11		0.56			0.31	
Adenosine-3′ phosphate	.17	.11		.56			.20	
Adenosine-5′ phosphate	.12	.07	0.10	.44	0.43	0.17	.34	
Adenosine-3′,5′ phosphate	.47	.37	.17	.56	.51	.43		
Diadenosine pyrophosphate	.13				.20	.17		
Adenosine	.59							
Adenine	.53							
Cytidine-2′(3′) phosphate	.15	.09						
Cytidine-5′ phosphate	.10	.05	.09	.32	.42	.17		
Cytidine-3′,5′ phosphate	.39	.33	.11	.33	.49	.44		
N-Benzoylcytidine-5′ phosphate[a]	.25[b]		.32	.60	.62	.35		
Cytosine	.55							
Guanosine-2′ phosphate	.07	.05		.21			.56	0.29
Guanosine-3′ phosphate	.07	.05		.21			.45	.29
Guanosine-5′ phosphate	.03	.01	.07	.14	.35	.11	.56	
Guanosine-3′,5′ phosphate	.26	.18	.11	.16	.43	.37		.29
N-Benzoylguanosine-5′ phosphate[c]	.13		.25	.43	.54	.27		
Guanine								.16
Uridine-2′(3′) phosphate	.18	.12						
Uridine-5′ phosphate	.07	.04	.11	.15	.48	.24		
Uridine-3′,5′ phosphate	.35	.26	.25	.20	.64	.52		
Uridine	.48							
Uracil	.56							
Ribose	.59							
Orthophosphoric acid	.10							

[a] Blue fluorescence under ultraviolet light. [b] Tailing due to N-debenzoylation. [c] White fluorescence under ultraviolet light.

paper chromatography in the same solvent (solvent A). The earlier fractions contained uridine-3′,5′ cyclic phosphate as the major component, and a faster-moving material (R_f 0.45). Fractions 34–59 contained only uridine-3′,5′ cyclic phosphate and the later fractions contained the cyclic phosphate contaminated with some slower-traveling material. Fractions 34–59 were combined, evaporated to dryness and the residual gum dissolved in water. Lyophilization of this solution yielded the ammonium salt of uridine-3′,5′ cyclic phosphate (436 mg., 56%). The molecular weight estimated spectrophotometrically (see below) was 358; $C_9H_{14}N_3O_3P\cdot 2H_2O$ has mol. wt. 359.2.

In earlier experiments employing shorter reaction times, the amounts of the faster-traveling material (uridine?) and slower-traveling materials (oligonucleotides?) formed were appreciably higher.

(b) Uridine-5′ phosphate (0.88 mmole, free acid), pyridine (25 ml.) and tri-*n*-butylamine (0.5 ml., 2.0 mmoles) were treated with DCC (103 g., 5.0 mmoles) under reflux for 1 hour with exclusion of moisture. After working up, as described above, uridine-3′,5′ cyclic phosphate as its ammonium salt in 32% yield was obtained.

(c) Uridine-5′ phosphate (1.0 mmole, free acid) was converted to its 4-morpholine N,N′-dicyclohexylcarboxamidinium salt by treatment with the carboxamidine[20] (293 mg., 1.0 mmole) in pyridine (25 ml.). After removal of traces of water by co-evaporation with pyridine, the nucleotide was dissolved in pyridine (100 ml.) and the resulting solution added dropwise (2 hr.), *via* the reflux condenser, to a boiling solution of DCC (412 mg., 2.0 mmoles) in pyridine (100 ml.), the system being protected from moisture. Heating was continued for a further 2 hr. and then the solution was concentrated to dryness. After removal of DCC and dicyclohexylurea, as described above, the residual aqueous solution of the product was applied to a column (30 cm. × 3.5 cm. dia.) of diethylaminoethyl cellulose in the carbonate form. Elution was carried out using a linear salt gradient with 0.002 *M* triethylammonium bicarbonate, *p*H 7.5, (4 l.) in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (4 l.) in the reservoir. Fractions (20 ml.) were collected at 5-min. intervals, the elution of nucleotides being followed spectrophotometrically at 261 mμ. Uridine-3′,5′ cyclic phosphate appeared in fractions 79–105. The combined fractions were concentrated to dryness and the solid residue was taken up in water (50 ml.) and the solution evaporated to dryness to remove residual triethylammonium carbonate. The resultant gummy product was dissolved in ethyl alcohol (2 ml.). Crystallization of the nucleotide began immediately and was completed by addition of ether (5 ml.). The yield of the anhydrous triethylammonium uridine-3′,5′ cyclic phosphate was 300 mg. (74 %); ultraviolet adsorption: λ_{max} 261 mμ (ε 9,940) at *p*H 7.0, λ_{max} 260 mμ (ε 7,740) at *p*H 12.0.

Anal. Calcd. for $C_9H_{11}N_2O_3P\cdot C_6H_{15}N$: C, 44.60; H, 6.40; N, 10.3; P, 7.60. Found: C, 44.08; H, 6.09; N, 10.61; P, 7.59.

Adenosine-3′,5′ Cyclic Phosphate.—Adenosine-5′ phosphate (1.0 mmole of the free acid) and 4-morpholine-N,N′-dicyclohexylcarboxamidine (293 mg., 1.0 mmole) were dissolved in pyridine (25 ml.) containing water (5 ml.) and the solution concentrated to dryness. After removal of water by co-evaporation with pyridine, the nucleotide salt was dissolved in pyridine (100 ml.). The solution was added dropwise to a boiling solution of DCC (412 mg., 2.0 mmoles) in pyridine (100 ml.) as in preparation (c) of uridine-3′,5′ cyclic phosphate described above. Occasionally, the adenosine-5′ phosphate salt partially crystallized in the dropping funnel. In this event, the crystals which remained in the dropping funnel, after the solution of nucleotide had run out, were rinsed into the reaction flask with small amounts of pyridine. After completion of addition of the adenosine-5′ phosphate solution, the reaction mixture was heated under reflux for 1 hour. The product was then worked up and chromatographed on the diethylaminoethyl cellulose column as in the preparation of uridine-3′,5′ cyclic phosphate, the elution of nucleotides being followed spectrophotometrically at 260 mμ (Fig. 2). The major product of the reaction (peak C) was adenosine-3′,5′ cyclic phosphate. The fractions containing this peak were combined and concentrated to dryness. The residue was dissolved in water (2 ml.) containing ethyl alcohol (2 ml.); *M* hydrochloric acid was added to bring the solution to *p*H 2.0. Adenosine-3′,5′ cyclic phosphate crystallized immediately. After cooling to −15° the solid was collected, washed with ethyl alcohol and dried *in vacuo* to yield the cyclic phosphate as the monohydrate (280 mg., 80%); ultraviolet adsorption: λ_{max} 256 mμ (ε 14,500) at *p*H 2.0, λ_{max} 258 mμ (ε 14,650) at *p*H 7.0.

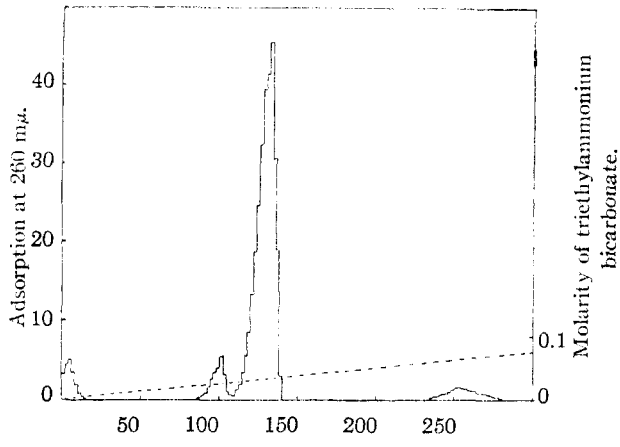


Fig. 2.—Isolation of adenosine-3′,5′ cyclic phosphate by chromatography on a diethylaminoethyl cellulose (carbonate form) column; details as in text. The major peak is that of the cyclic phosphate.

Anal. Calcd. for $C_{10}H_{12}N_5O_6P \cdot H_2O$: C, 34.58; H, 4.06; N, 20.17; P, 8.92. Found: C, 35.55; H, 4.05; N, 19.94; P, 8.75.

In earlier experiments on the preparation of adenosine-3′,5′ cyclic phosphate by the above method, the reaction medium was heated under reflux for prolonged periods (up to 10 hours). In these experiments more of the material in peak B (Fig. 2) was produced. This substance had the same mobility on paper chromatograms developed in solvent A as adenosine-3′,5′ cyclic phosphate. It could be removed from the latter by crystallization from ethyl alcohol because of its higher solubility in the solvent. Thus, in one experiment, performed as described above, the crude adenosine-3′,5′ cyclic phosphate obtained prior to anion-exchange chromatogaphy was dissolved in water (4 ml.) containing ethyl alcohol (4 ml.) and the solution adjusted to *p*H 2.0 with 4 *M* hydrochloric acid (0.3 ml.). Pure adenosine-3′,5′ cyclic phosphate crystallized immediately. The solution was cooled to −15° and the nucleotide collected, washed with ethyl alcohol and dried *in vacuo* to yield adenosine-3′,5′ cyclic phosphate monohydrate (300 mg., 86%).

Reaction of Adenosine-5′ Phosphate with DCC in the Presence of 4-Morpholine-N,N′-dicyclohexylcarboxamidine at Different Temperatures. (a) **At 60°.**—Adenosine-5′ phosphate (0.1 mmole) and 4-morpholine-N,N′-dicyclohexylcarboxamidine (0.1 mmole) in pyridine (30 ml.) were warmed under anhydrous conditions until a clear solution resulted; DCC (0.24 mmole) was added and the solution kept at 60°. The progress of reaction was followed by paper chromatography in solvent E, which clearly separates adenosine-5′ phosphate, adenosine-3′,5′ cyclic phosphate and P^1,P^2-diadenosine-5′ pyrophosphate. P^1,P^2-Diadenosine-5′ pyrophosphate was the product, no adenosine-3′,5′ phosphate being produced under these conditions. There was an indication that the pyrophosphate was slowly converted to another substance (see also below) with a slightly higher mobility in the above-mentioned solvent.

(b) **At 100°.**—An identical experiment to the above carried out at 100° showed that adenosine-5′ phosphate reacted completely in less than 30 min., the major product being P^1,P^2-diadenosine-5′ pyrophosphate. After 2 hr. the amount of adenosine-3′,5′ cyclic phosphate had increased to about 50% (visual estimation) of the total ultraviolet adsorbing products and an appreciable amount of the material moving just ahead of P^1,P^2-diadenosine-5′ pyrophosphate in the solvent E had formed. This substance, which remains unidentified, moves midway between adenosine-3′,5′ cyclic phosphate and adenosine-5′ phosphate on paper chromatograms in solvent D as well as on paper electrophoresis at *p*H 7.5.

(c) **Under Reflux.**—The experiment was carried out as above but at reflux temperature. Adenosine-5′ phosphate had reacted completely in 10 minutes to give P^1,P^2-diadenosine-5′ pyrophosphate as the major product. There were also present small amounts of adenosine-3′,5′ cyclic phosphate and the unidentified substance described above. After 1 hour, the major product was adenosine-3′,5′ cyclic phosphate, but appreciable amounts of the products mentioned above were also present. Subsequent work-up and chromatography on a diethylaminoethyl cellulose column gave the 3′,5′-cyclic phosphate in 56% yield.

Adenosine-3′,5′ Cyclic Phosphate from P^1,P^2-Diadenosine Pyrophosphate.—Adenosine-5′ phosphate (1.0 mmole) and 4-morpholine-N,N′-dicyclohexylcarboxamidine (293 mg., 1.0 mmole) were dissolved in pyridine (200 ml.) under anhydrous conditions at 100°; DCC (500 mg., 2.4 mmoles) was added and the solution kept at 100° for 18 min. The solution was cooled to 0°, whereupon it set to a gel. Chromatographic examination of the product in solvent E showed P^1,P^2-diadenosine-5′ pyrophosphate to be the only ultraviolet adsorbing product.

The gel was liquefied by warming and the resultant viscous solution was run dropwise into a solution of DCC (500 mg., 2.4 mmole) in pyridine (100 ml.) under reflux over a period of 2 hr.

Paper chromatography showed adenosine-3′,5′ cyclic phosphate to be the only product. Continued heating under reflux for an additional 1 hr. gave at race amount of the material traveling just ahead of P^1,P^2-diadenosine-5′ pyrophosphate. After removal of pyridine, DCC and dicyclohexylurea in the usual way, adenosine-3′,5′ cyclic phosphate was crystallized from aqueous ethyl alcohol at *p*H 2.0 (yield 332 mg., 95%). Chromatographic examination in solvent E showed that the product contained a trace amount of the slower-moving impurity.

In another experiment, P^1,P^2-diadenosine-5′ pyrophosphate (0.5 mmole) prepared as above, but freed from dicyclohexylurea and unreacted carbodiimide, was dissolved in pyridine (20 ml.) and the solution heated under reflux. Paper chromatography in solvent E showed that the pyrophosphate had completely disappeared in 8 hr. and the products were adenosine-3′,5′ cyclic phosphate and adenosine-5′ phosphate.

N-Benzoylguanosine-5′ Phosphate.—Guanosine-5′ phosphate (1.0 mmole) as its pyridinium salt was dissolved in water (100 ml.) and the solution lyophilized. The resulting finely divided white powder was suspended in a mixture of benzoyl chloride (2.5 ml.) and pyridine (15 ml.). After being shaken for a few minutes, a homogeneous solution resulted which was kept in the dark at 25° for 1 hr. Water (50 ml.) was added and, after 5 minutes at 25°, the solution was extracted with chloroform (3 × 50 ml.). The combined chloroform extracts were washed with water (2 × 10 ml.) and then concentrated to dryness. The residual oil was dissolved in a mixture of water (10 ml.) and pyridine (20 ml.). To this clear solution was added 2 *M* sodium hydroxide (30 ml.), whereupon the solution became cloudy and then clear bright orange. After 4 min. at 25°, freshly prepared pyridinium Amberlite IR-120 (50 ml. of wet resin) was added. The solution turned almost colorless and the *p*H dropped to 7.0. The resin was removed by filtration and washed thoroughly with water. The combined filtrate and washings were concentrated to a small volume (25 ml.) and benzoic acid, which separated during this concentration, was removed by extraction with ether (2 × 25 ml.). The aqueous solution was passed through a column (15 cm. × 2.5 cm. dia.) of Amberlite IR-120 resin in the pyridinium form, the column being subsequently washed with water (100 ml.). The combined eluate and washings were concentrated to a small volume (25 ml.) and washed with ether (2 × 25 ml.). Pyridine (50 ml.) was added to the aqueous solution and the mixture concentrated to dryness. The residual pale yellow gum was dissolved in water (10 ml.) and treated with *M* calcium chloride in 95% ethyl alcohol (2 ml.). The resulting solution was concentrated to dryness and then dissolved in 95% ethyl alcohol (10 ml.). Acetone (30 ml.) was added to the solution and the resulting precipitate of the calcium salt of N-benzoyl guanosine-5′ phosphate was collected by centrifugation, washed with ethyl alcohol-acetone, acetone and ether, and dried *in vacuo* at 25°. The yield of calcium N-benzoylguanosine-5′ phosphate monohydrate was 350 mg. (67%). The loss occurred during the precipitation of the calcium salt, since benzoylation was quantitative and furthermore, after the alkaline treatment, the only detectable product was the desired one; ultra-violet adsorption: λ_{max} 259 mμ (ε 17,350) and 290 mμ (ε, 16,200) with inflection at 242 mμ (ε 14,700) at *p*H 7.0.

Anal. Calcd. for $C_{17}H_{16}N_5O_9P$ Ca.H_2O: C, 39.06; H, 3.45; N, 13.40; P, 5.92. Found: C, 39.78; H, 3.89; N, 13.03; P, 5.72.

Guanosine-3',5' Cyclic Phosphate.—Calcium N-benzoylguanosine-5' phosphate monohydrate[50] (273 mg., 0.5 mmole) was converted to the pyridinium salt by treatment with Amberlite IR-120 resin (pyridinium form) in the usual manner. The resulting aqueous solution of the nucleotide was rendered anhydrous by co-evaporation with pyridine. The resultant gum was dissolved in dry pyridine (50 ml.) containing 4-morpholine-N,N'-dicyclohexylcarboxamidine (147 mg., 0.5 mmole) and the solution run dropwise over a period of 2 hr. into a boiling solution of DCC (412 mg., 2.0 mmoles) in pyridine (50 ml.). After a further hour at reflux temperature, the solution was concentrated to dryness and the residue extracted thoroughly with water and the extract was concentrated to dryness. The resulting gum was dissolved in 95% ethyl alcohol (5 ml.) and concentrated ammonia (10 ml.) and the solution heated in a sealed tube at 100° for 2 hr. After cooling, the solvent was removed and the residue taken up in water (100 ml.). After an ether wash, the solution was applied to a column (25 cm. × 3.5 cm. dia.) of diethylaminoethyl cellulose in the carbonate form. The column was eluted as in the preparation of adenosine-3',5' cyclic phosphate, the elution of nucleotides being followed spectrophotometrically at 252 mμ. The fractions containing the major peak were combined and concentrated to dryness. Attempts to crystallize the nucleotide as the free acid were unsuccessful and therefore guanosine-3',5' cyclic phosphate was isolated as its calcium salt, 150 mg. (66%); ultraviolet adsorption: λ_{max} 256.5 mμ (ε 11,350) at *p*H 1.0; λ_{max} 254 mμ (ε 12,950) at *p*H 7.0; λ_{max} 262 mμ (ε 12,400) at *p*H 12.0.

Anal. Calcd. for $C_{10}H_{11}N_5O_7P$ $Ca_{0.5}\cdot 5H_2O$: P, 6.82. Found: P, 6.92.

N-Benzoylcytidine-5' phosphate was prepared by the same procedure as described for N-benzoylguanosine-5' phosphate and isolated as the calcium salt; 325 mg. (65%), ultraviolet adsorption: λ_{max} 304 mμ (ε 9,800) and 256 mμ (ε 18,800) at *p*H 7.0

Anal. Calcd. for $C_{16}H_{16}N_3O_9P$ Ca·$2H_2O$: C, 38.35; H, 4.02; N, 8.39; P, 6.18. Found: C, 37.20; H, 3.99; N, 7.42; P, 6.38.

Cytidine-3',5' Cyclic Phosphate.—The procedure used in the preparation of guanosine-3',5' cyclic phosphate was used except that the N-benzoyl group was removed by treatment with concentrated ammonia at 25° for 15 hr. Cytidine-3',5' cyclic phosphate isolated after ion-exchange chromatography was crystallized from aqueous ethyl alcohol at *p*H 2.0 and dried over phosphorus pentoxide to give the anhydrous nucleotide; ultraviolet adsorption: λ_{max} 279 mμ (ε 12,430) at *p*H 2.0; λ_{max} 272 mμ (ε 9,340) at *p*H 7.0.

Anal. Calcd. for $C_9H_{12}N_3O_7P$: C, 35.42; H, 3.96; N, 13.77; P, 10.14. Found: C, 35.21; H, 4.25; N, 14.61; P, 9.58.

Acid Hydrolysis of Ribonucleoside-3',5' Cyclic Phosphates.—Aliquots (about 20 μl.) of a solution of the nucleoside-3',5' cyclic phosphate (0.01 mmole) in *M* hydrochloric acid (0.5 ml.) were sealed in capillary tubes. The sealed tubes were heated in a boiling water-bath. At suitable intervals the capillary tubes were removed and the contents examined chromatographically.

(a) **Cytidine-3',5' Cyclic Phosphate.**—The products of hydrolysis were separated in solvent A and the ultraviolet adsorbing spots were eluted with 0.1 *M* hydrochloric acid (2 ml.) and estimated spectrophotometrically at 280 mμ; $t_{1/2}$ for the disappearance of the cyclic phosphate was 26 min., the reaction being complete in 2 hr. The products of the reaction were cytosine (identified chromatographically, 9%) and cytidylic acids, 81%. Examination of the nucleotide fraction in solvent B showed that it was a mixture of cytidine-5' phosphate (14%) and cytidine-2'(3') phosphate (86%).

Under identical conditions, cytosine (9%) was released from cytidine-2'(3') phosphate.

(b) **Uridine-3',5' Cyclic phosphate.**—The reaction was carried out as above, the products being estimated at 262 mμ. The $t_{1/2}$ for the disappearance of the cyclic phosphate was 8 min., the reaction being complete in 1 hour. Ultraviolet-adsorbing products were uracil (67%, identified chromatographically and spectrophotometrically), uridine-5' phosphate (6%) and uridine-2'(3') phosphate (27%). The only other detectable products were ribose and orthophosphoric acid. Uridine-5' phosphate was completely unaffected in 1 *M* hydrochloric acid at 100° for 2 hr.

(c) **Adenosine-3',5' cyclic phosphate** was hydrolyzed as above, except that the ultraviolet-absorbing spots were eluted with 1 *M* hydrochloric acid (2 ml.) and estimated at 257 mμ; the $t_{1/2}$ for the disappearance of the cyclic phosphate was 30 min., the reaction being complete in 3 hr. The sole product was adenine (identified chromatographically and spectrophotometrically), although an adenosine phosphate was produced transiently (5% after 30 min.) during the early stages of the reaction. Other products detected were orthophosphoric acid, ribose and a trace of ribose phosphate(s) with R_f 0.13 in solvent A.

Under the same conditions, $t_{1/2}$ for the release of adenine from adenosine-5' and adenosine-2'(3') phosphate was 2–4 min.

(d) **Guanosine-3',5' Cyclic Phosphate.**—The reaction was carried out as above. The initial separation of the guanine from the nucleotide fraction was effected in solvent H because of the insolubility of guanine in neutral or basic systems. The chromatograms were dried when the guanosine phosphate was well separated from that of guanine. The chromatograms were then cut in two just below the slower traveling guanine spots and development of the lower section of the paper was continued in solvent A to resolve guanosine-3',5' cyclic phosphate from the guanosine-2' or 3' or -5' phosphates. Ultraviolet-adsorbing spots were eluted with 1 *M* hydrochloric acid and estimated at 256 mμ; $t_{1/2}$ for the disappearance of guanosine-3',5' cyclic phosphate was 28 min., the reaction being complete in 3 hr. The ultimate ultraviolet-absorbing product of the reaction was guanine (identified chromatographically and spectrophotometrically), although guanosine phosphate(s) were produced transiently (13% after 30 min.).

Under the same conditions, $t_{1/2}$ for the release of guanine from guanosine-2'(3') phosphate was 1.5 min.

Barium Hydroxide-catalyzed Hydrolysis of Ribonucleoside-3',5' Cyclic Phosphates. (a) Uridine-3',5' Cyclic Phosphate.—The nucleotide (0.01 mmole) and barium hydroxide octahydrate (0.1 mmole) in water (0.5 ml.) were heated at 100° in a stoppered polyethylene tube. The clear solution which resulted on warming turned cloudy as the reaction proceeded. Aliquots were removed at 5, 15 and 30 min., treated with Amberlite IR-120 (H^+) resin, and examined chromatographically in solvent A. Hydrolysis of the cyclic phosphate was complete in 30 min.

In another experiment, uridine-3',5' cyclic phosphate (0.05 mmole) and barium hydroxide octahydrate (0.5 mmole) in water (2.5 ml.) were kept at 100° for 30 min. Barium ions were removed by treatment with Amberlite-IR-120 (H^+) resin, the latter being washed thoroughly with 1 *M* ammonium hydroxide to ensure complete recovery of the nucleotides. The nucleotide solution was concentrated to a small volume (10 ml.) and applied to a column (10 cm. × 1.0 cm. dia.) of Dowex-1 resin (8% cross-linked, 200–400 mesh) in the formate form.[51] After a water wash (20 ml.) and a wash with 0.1 *M* ammonium formate (about 100 ml.) to reduce the *p*H of the effluent to 7, the column was eluted with a solution containing 0.00125 *M* formic acid and 0.05 *M* ammonium formate,[51] the flow rate being 0.8 ml./min. Fractions were collected at 10-minute intervals and examined at 261 mμ. A total of 2.5 l. of eluent was required to remove all the nucleotidic material. The same ion exchange column was then calibrated by separation of a synthetic mixture of uridine-5', uridine-2' and uridine-3' phosphates under identical conditions. From this, the products resulting from the hydrolysis of uridine-3',5' cyclic phosphate were shown to be uridine-5' phosphate (14%) and uridine-3' phosphate (81%). No uridine-2' phosphate was detected. A small amount of unidentified material (3%) with a uridine spectrum was eluted from the column just after uridine-5' phosphate. The proportions of uridine-3' and uridine-5' phosphates produced (5:1) were also estimated after chromatographic separation of the products in solvent B.

(50) Because of the loss in yield during the precipitation of the calcium salt, it is advisable to use the pyridinium N-benzoylguanosine-5, phosphate obtained above directly. The product prior to precipitation is pure.

(51) W. E. Cohn and J. X. Khym in "Biochemical Preparations," ed. D. Shemin, John Wiley and Sons, Inc., New York, N. Y., 1957, Vol. 5, p. 40.

(b) **Cytidine-3′,5′ Cyclic Phosphate.**—The nucleotide (0.05 mmole) and barium hydroxide (0.5 mmole) in water (2.5 ml.) were heated at 100° and the product adsorbed onto a column (10 cm. × 1.0 cm. dia.) of Dowex 1 resin (8% cross-linked, 200–400 mesh) in the formate form, as in the hydrolysis of uridine-3′,5′ cyclic phosphate. Cytidine phosphates were eluted with 0.01 *M* formic acid[51] (1.1 l.), the nucleotides being estimated at 280 mμ. Subsequently, the uridine phosphates were eluted as in the preceding experiment. The column was then calibrated with a mixture of cytidine-5′, cytidine-2′, cytidine-3′ and the corresponding uridine nucleotides. The products thus were shown to be cytidine-5′ phosphate (8%), cytidine-3′ phosphate (41%), uridine-5′ phosphate (7%) and uridine-3′ phosphate (37%). In addition, an unidentified product with a cytidine spectrum (3%) was eluted immediately after cytidine-5′ phosphate. No cytidine-2′ or uridine-2′ phosphate was detected.

(c) **Adenosine-3′,5′ Cyclic Phosphate.**—The nucleotide (0.05 mmole) was hydrolyzed with barium hydroxide (0.5 mmole) in water (2.5 ml.) at 100° as above and the product analyzed on a column (10 cm. × 1.0 cm. dia.) of Dowex 1 resin (8% cross-linked, 200–400 mesh) in the chloride form. Elution was carried out with 0.002 *M* hydrochloric acid,[51] the eluate being examined at 257 mμ. The products were adenosine-3′ phosphate (84%) and adenosine-5′ phosphate (16%). No adenosine-2′ phosphate was detected. Continued washing with a solution 0.02 *N* in sodium chloride and 0.01 *N* in lithium acetate, *p*H 5.5,[52] showed the absence of any inosine nucleotides. The hydrolysis products were also characterized paper chromatographically in solvents B and G.

(d) **Guanosine-3′,5′ Cyclic Phosphate.**—Hydrolysis was as for uridine-3′,5′ cyclic phosphate and chromatography on the standard ion exchange column in the chloride form. Elution was carried out using 0.005 *M* hydrochloric acid. The products were guanosine-3′ phosphate (80%) and guanosine-5′ phosphate (20%). No xanthosine phosphates were detected on continued elution with 0.025 *M* hydrochloric acid and 0.05 *M* sodium chloride. The products were also characterized paper chromatographically in the same way as described for the products of hydrolysis of adenosine-3′,5′ cyclic phosphate.

Hydrolysis of Uridine-3′,5′ Cyclic Phosphate by Sodium Hydroxide.—The nucleotide (0.1 mmole) in *M* sodium hydroxide (1.0 ml.) was heated under nitrogen at 100° in a polyethylene tube. The solution turned brown during the course of reaction and some gummy material separated. Aliquots were removed at suitable intervals, treated with Amberlite IR-120 resin (ammonium form) and examined chromatographically in solvent A. Ultraviolet adsorbing spots were eluted with 0.1 *M* hydrochloric acid (2 ml.) and estimated spectrophotometrically at 261 mμ; $t_{1/2}$ for the conversion of uridine-3′,5′ cyclic phosphate into other ultraviolet adsorbing substances was 1.5 hr. There was concomitant destruction of the chromophore. After 2 hr. the products were uridine-5′ phosphate (trace), uridine-3′ phosphate (major product), uracil (about 1/4 the amount of uridine-3′ phosphate), and an unidentified substance, R_f 0.77 (trace), some unreacted uridine-3′,5′ cyclic phosphate being also present.

Under the same conditions (2 hr. at 100°) uridine-5′ phosphate was completely unaffected by 1 *M* sodium hydroxide. Adenosine-5′ phosphate was partially (about 10%) degraded to adenine (identified chromatographically and spectrophotometrically) and two minor unidentified substances (R_f's 0.05 and 0.55 in solvent A).

When uridine-3′5′ cyclic phosphate (0.01 mmole) in water (0.1 ml.) containing freshly precipitated cadmium hydroxide (10 mg.) at *p*H 7.5 was heated at 100°, only a faint trace of hydrolysis could be detected after 24 hr.

Enzymic Hydrolysis of Uridine-3′,5′ Cyclic Phosphate. (a) **With Crude Snake Venom.** Crude *Crotalus adamanteus* venom (10 mg.) was added to a solution of the nucleotide (0.02 mmole) in water (1.8 ml.) containing *M* tris-(hydroxymethyl)-aminomethane buffer *p*H 8.9 (0.2 ml.). A drop of toluene was added and the mixture incubated at 37° for 24 hr. At this stage, 51% of the uridine-3′,5′ cyclic phosphate was unreacted, the products being uridine (41%) and uridine-3′ phosphate (8%). After 108 hr., 11% of the uridine-3′,5′ cyclic phosphate remained, the products being uridine (53%), uracil (15%) and uridine-3′ phosphate (11%). In another experiment, a mixture of uridine-3′,5′ cyclic phosphate (3.0 μmoles of ammonium salt) and the crude venom (3.3 mg.) in 0.05 *M* tris-(hydroxymethyl)-aminomethane, *p*H 8.2 (0.4 ml.), was kept at 35° for 13 hr. The products were uridine-3′,5′ cyclic phosphate (40%), uridine (15%) and uridine-3′ phosphate (45%).

(b) **With Purified Spleen Diesterase.**[46] The nucleotide (2.5 μmoles of ammonium salt) in *M* ammonium acetate, *p*H 6.1 (0.1 ml.), and the spleen diesterase preparation (36 μg. protein) in 0.01 *M* pyrophosphate buffer, *p*H 6.1 (0.1 ml.), were incubated together at 37° for 24 hr. No hydrolysis of the uridine-3′,5′ cyclic phosphate was detectable. The activity of the spleen phosphodiesterase used was such that 0.02 ml. of the preparation hydrolyzed completely 1 μmole of thymidylyl-(3′→5′)-thymidine in 1 hr. under the above conditions.

Hydrolysis of Adenosine-3′,5′ Cyclic Phosphate with Crude Snake Venom.[7]—The nucleotide (20 μmoles) and the crude venom (10 mg.) in water (1.8 ml.) containing *M* tris-(hydroxymethyl)-aminomethane, *p*H 8.9 (0.2 ml.), were incubated at 37° for 24 hr. in the presence of a drop of toluene. Chromatography in solvent A showed that 27% of adenosine-3′,5′ cyclic phosphate remained, the products being adenosine (36%) and adenosine-3′ phosphate (37%). After 108 hr., reaction was complete, the products being adenosine (51%) and adenosine-3′ phosphate (49%).

(52) W. E. Cohn in "The Nucleic Acids," eds. E. Chargaff and J. N. Davidson, Academic Press, Inc., New York, N. Y., 1955, Vol. I, p. 228.

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Organic Phosphorus Compounds. VI.[1] Effects of Structural Variations in Systox and Isosystox Analogs on their Reactions with Cholinesterase

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A series of Systox and Isosystox analogs of the general formulas $RP(S)(OR')OCH_2CH_2SC_2H_5$ (I) and $RP(O)(OR')SCH_2CH_2SC_2H_5$ (II), respectively, was prepared; R represents CH_3 and C_2H_5 and R′ is an alkyl group with 2–5 carbon atoms. The effect of the variation of R′ on the rate of the reaction of these compounds with eel cholinesterase was studied.

Introduction.—The higher rate of inactivation of eel cholinesterase by isopropyl methylphosphonofluoridate (Sarin), as compared to diisopropyl phosphorofluoridate (DFP),[3] and the increase in anticholinesterase activity realized by the replacement of one ethoxy group of S-(2-ethylthioethyl) phosphorothioate (Isosystox) by an ethyl[4,5] or propyl[5] radical demonstrate a pronounced effect of

(1) Paper V of this series, *J. Am. Chem. Soc.*, **81**, 148 (1959).
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(4) H. S. Aaron, H. O. Michel, B. Witten and J. I. Miller, *J. Am. Chem. Soc.*, **80**, 456 (1958).
(5) T. R. Fukuto and R. L. Metcalf, *ibid.*, **81**, 372 (1959).