[CONTRIBUTION NO. 12 FROM THE SYNTEX INSTITUTE FOR MOLECULAR BIOLOGY, PALO ALTO, CALIFORNIA]

Possible Intermediates in the Biosynthesis of Deoxyribonucleotides. I. The Synthesis of Cytidine 2′-Phosphate 5′-Diphosphate

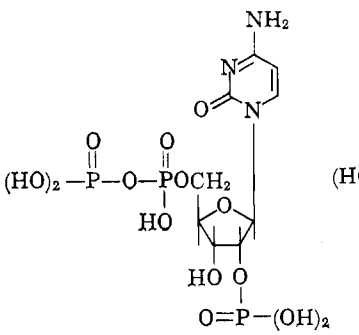
BY J. P. H. VERHEYDEN AND J. G. MOFFATT

RECEIVED OCTOBER 18, 1963

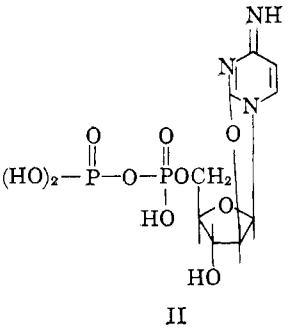
The synthesis of cytidine 2′-phosphate 5′-diphosphate (I), a possible intermediate in the biosynthesis of deoxycytidine, is described. Phosphorylation of cytidine with polyphosphoric acid gave cytidine 2′(3′),5′-diphosphate (IX) which was treated with either morpholine or *p*-anisidine in the presence of dicyclohexylcarbodiimide to give cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (Va) or 5′-phosphoroanisidate (Vb), respectively. These compounds were treated with orthophosphoric acid in pyridine–dimethylformamide to give cytidine 2′,3′-cyclic phosphate 5′-diphosphate (VI) which was converted selectively into cytidine 2′-phosphate 5′-diphosphate (I) by treatment with beef brain ribonucleoside 2′,3′-cyclic phosphodiesterase. A preparation of I labeled with C^{14} in the pyrimidine ring is also described.

While the biosynthetic pathways leading to both the purine and pyrimidine ribonucleosides and ribonucleotides have been clearly defined,[1] the origin of the corresponding deoxynucleotides has remained more obscure until recently. It was, however, possible to show that in the rat isotopically labeled uridine and cytidine were incorporated into DNA as the corresponding deoxynucleotides without fission of the glycosidic bond.[2] More recent studies by Reichard, *et al.*,[3] have greatly clarified this picture and it has been possible to demonstrate the direct deoxygenation of both purine and pyrimidine ribonucleotides to 2-deoxynucleotides by cell free preparations from *E. coli* and from chick embryo. Similar systems have been demonstrated in mammalian cells.[4]

The striking observation was made by Reichard, *et al.*,[3] that the conversion of cytidine to 2-deoxycytidine occurred at the level of the 5′-diphosphate and still required adenosine 5′-triphosphate (ATP), magnesium ion, and reduced lipoic acid. Subsequently, it was shown[3d] that the requirement for reduced lipoic acid may be replaced by a reduced pyridine nucleotide and a purified enzyme fraction. The cofactor requirements for ATP and magnesium ion are reminiscent of those necessary during many enzymatic phosphorylations,[5] and it was attractive to speculate upon the possible activation of the 2′-position of the ribose by phosphorylation to give cytidine 2′-phosphate 5′-diphosphate (I) prior to reduction to the deoxynucleoside. The reduction step could then be looked upon as proceeding through either direct displacement of phosphate by the biological equivalent of hydride ion, or by elimination of phosphate accompanied by introduction of a 1′,2′ or 2′,3′ double bond[6] which is subsequently reduced. The latter mechanism appears less likely in view of the results of Thomson, *et al.*,[4c] who demonstrated no loss of stable tritium from the ribose moiety during conversion of cytidine to deoxycytidine.[7] A further possibility involves elimination of phosphate by participation of the cytosine ring oxygen leading to an $O^2,2'$-cyclonucleoside 5′-diphosphate (II) which is subsequently reduced.



I



II

The possibility of cyclonucleoside intermediates in deoxynucleoside biosynthesis has been considered previously[8] but largely ignored in view of the metabolic inertness of $O^2,2'$-cyclouridine itself. As pointed out by Pizer and Cohen,[8b] however, this may only indicate a requirement for phosphorylation prior to cyclonucleoside formation. A subsequent paper from this laboratory will describe the synthesis of cyclouridine nucleotides suitable for testing this hypothesis.[9]

The present paper describes the synthesis of cytidine 2′-phosphate 5′-diphosphate (I) which has been tested by Dr. Peter Reichard[3c] at Uppsala University, Uppsala, Sweden, and was shown not to be an intermediate in the pathway from cytidine to deoxycytidine. The possibility still remains that the activation of the 2′-hydroxyl group is through formation of a pyrophosphate,[3c] or an adenosine phosphate derivative of cytidine 5′-diphosphate rather than of a simple orthophosphate ester. Such structures provide a more formidable synthetic challenge.

The synthesis of cytidine 2′-phosphate 5′-diphosphate (I) was accomplished by a route formally similar in design to that used for Coenzyme A[10] and outlined in Chart I.

Treatment of cytidine with a large excess of polyphosphoric acid (P_2O_5–H_3PO_4) at 60° for 21 hr., essentially according to the procedure of Hall and Khorana,[11] gave, after acidic hydrolysis of polyphosphates and ion exchange chromatography, a 65% yield of the

(1) For comprehensive reviews see: (a) J. M. Buchanan and C. S. Hartman, *Advan. Enzymol.*, **21**, 199 (1959); (b) P. Reichard, *ibid.*, **21**, 263 (1959).

(2) (a) E. Hammarsten, P. Reichard, and E. Saluste, *J. Biol. Chem.*, **183**, 105 (1950); (b) I. A. Rose and B. S. Schweigart, *ibid.*, **202**, 635 (1953).

(3) (a) P. Reichard, A. Baldesten, and L. Rutberg, *ibid.*, **236**, 1150 (1961); (b) P. Reichard, *ibid.*, **236**, 2511 (1961); (c) P. Reichard, *ibid.*, **237**, 3513 (1962); (d) E. C. Moore and P. Reichard, *ibid.*, **238**, PC2244 (1963); (e) A. Larsson, *Acta Chem. Scand.*, **17**, 891 (1963).

(4) (a) E. C. Moore and R. B. Hurlbert, *Biochim. Biophys. Acta*, **55**, 651 (1962); (b) R. Abrams, L. Libenson, and M. Edmonds, *Biochem. Biophys. Res. Commun.*, **3**, 272 (1960); (c) R. Y. Thomson, G. T. Scotto, and G. B. Brown, *J. Biol. Chem.*, **237**, 3510 (1962).

(5) R. Nordlie and H. Lardy, "The Enzymes," Vol. 6, Academic Press, New York, N. Y., 1962, p. 3.

(6) Quite similar dehydration mechanisms obtain during the conversion of mevalonic acid 5-pyrophosphate to isopentenylpyrophosphate [M. Lindberg, C. Yuan, A. deWaard, and K. Block, *Biochemistry*, **1**, 182 (1962)] and in the conversion of homoserine to valine [M. Flavin and C. Slaughter, *J. Biol. Chem.*, **235**, 1112 (1960)].

(7) The conclusiveness of this experiment is diminished, however, by the recent work of Simon, *et al.* [H. Simon, H. D. Dorrer, and K. H. Ebert, *Z. Naturforsch.*, **18b**, 360 (1963)], who have shown that the conditions used by Thomson, *et al.*,[4c] for the tritiation of ribose lead to the introduction of 93% of the stabled tritium at C-4 and only 0.7% at C-3.

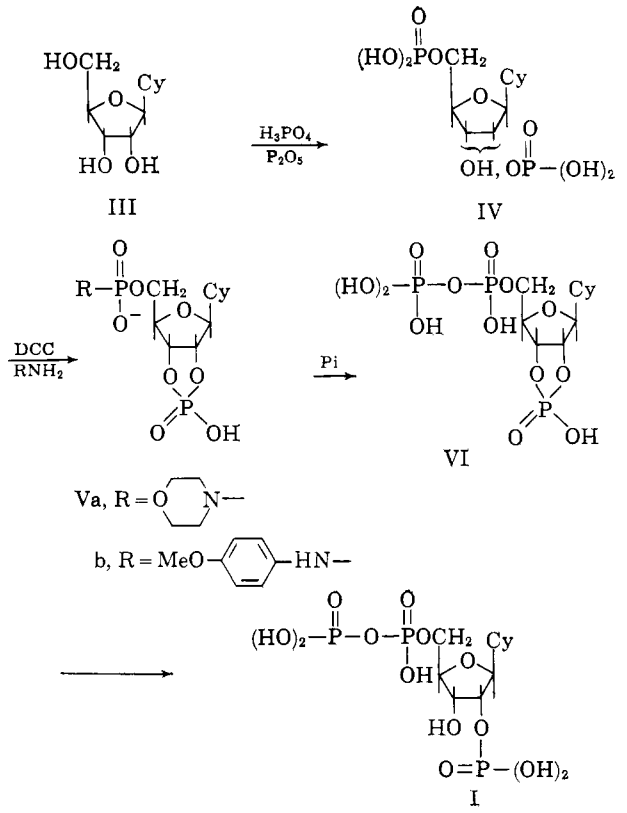
(8) (a) P. Reichard, *J. Biol. Chem.*, **234**, 2719 (1959); (b) L. I. Pizer and S. S. Cohen, *ibid.*, **235**, 2387 (1960); (c) S. S. Cohen, H. D. Barner, and J. Lichtenstein, *ibid.*, **236**, 1448 (1961).

(9) J. P. H. Verheyden and J. G. Moffatt, unpublished results.

(10) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961).

(11) R. H. Hall and H. G. Khorana, *ibid.*, **77**, 1871 (1955).

CHART I



mixed cytidine 2′(3′),5′-diphosphates (IV) which were isolated as their lithium salts. In view of the very rapid formation of monophosphates with this reagent,[11,12] it is surprising that such a long reaction period should be necessary, but examination of aliquots removed after shorter times revealed a rather slow conversion of the monophosphates to diphosphates, the yield being considerably lower after 6 or 10 hr. The product contained almost equal amounts of the 2′,5′- and 3′,5′-diphosphate isomers as shown by incubation with a purified preparation of rye grass 3′-nucleotidase[13,14] which degraded 55% of the mixture to cytidine 5′-phosphate, the remainder being unaffected. In view of the observations of Walwick, *et al.*,[15] on the formation of phosphate esters of O²,2′-cyclonucleosides (and of the arabinosyl nucleosides arising from their hydrolysis) during vigorous phosphorylation of nucleosides and nucleotides with polyphosphoric acid, we have carefully examined our product for such contamination. Following complete dephosphorylation of IV with *E. coli* alkaline phosphatase,[16] the resulting nucleoside was shown by paper chromatography in the system 2-propanol–ammonium hydroxide–0.1 *M* boric acid (7 : 1 : 2) to consist of almost exclusively cytidine, with only the faintest trace (less than 1%) of cytosine arabinoside[17] being detected. It, therefore, appears that under the present conditions cyclonucleoside formation is not a significant side reaction.

(12) R. W. Chambers, P. Shapiro, and V. Kurkov, *J. Am. Chem. Soc.*, **82**, 970 (1960).

(13) L. Shuster and N. O. Kaplan, "Methods in Enzymology," Vol. II, Academic Press, New York, N. Y., 1955, p. 551.

(14) This preparation was kindly provided by Dr. R. J. Hilmoe of the National Institutes of Health, Bethesda, Md. It showed less than 0.1% activity toward adenosine 2′- and 5′-monophosphate relative to that toward adenosine 3′-phosphate.

(15) (a) E. R. Walwick, W. K. Roberts, and C. A. Dekker, *Proc. Chem. Soc.*, 84 (1959); (b) W. K. Roberts, Ph.D. Dissertation, University of California, 1960. We are very grateful to Dr. C. A. Dekker for allowing us to read this dissertation prior to publication.

(16) Obtained from Worthington Biochemical Corp., Freehold, N. J.

(17) We are grateful to Dr. C. A. Dekker for a sample of cytosine arabinoside.

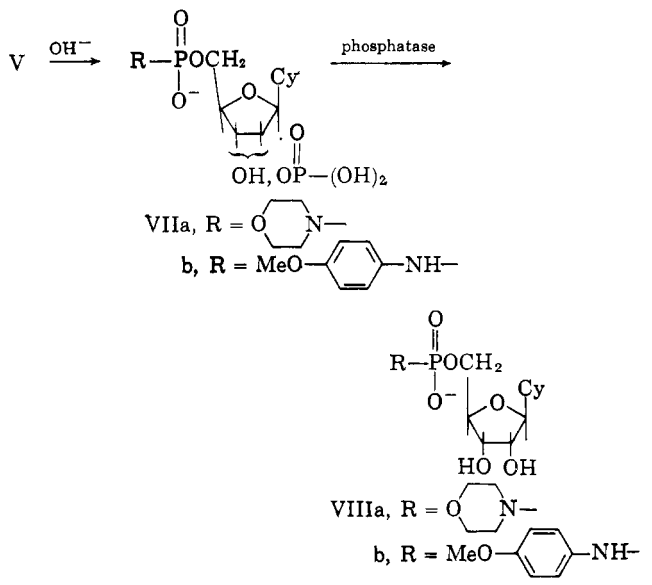
While the isomeric cytidine 2′(3′),5′-diphosphates can be separated by ion exchange chromatography on Dowex-2 formate resin,[15b] such a step was unnecessary and the mixed isomers were directly treated with dicyclohexylcarbodiimide (DCC) and an amine in aqueous *t*-butyl alcohol.[18,10] Both morpholine and *p*-anisidine were used as the amines in this reaction and each presented somewhat different problems. The reaction with morpholine proved to be rather variable and under seemingly identical conditions gave cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (Va) in yields varying from 50–100%. The product could be isolated in pure form by ion exchange chromatography on Dowex-2 (HCO_3^-) resin, or, in the case of reactions that went nearly to completion, could be used directly without serious side effects. Unlike the corresponding adenosine compound,[10] Va was quite insoluble in pyridine both as its tributylammonium and 4-morpholine-N,N′-dicyclohexylcarboxamidinium salts. It was, however, soluble in dimethylformamide and in a 1:1 mixture of pyridine and dimethylformamide. On the other hand, reaction of IV with *p*-anisidine and DCC readily gave cytidine 2′,3′-cyclic phosphate 5′-phosphoroanisidate (Vb) uncontaminated by other products. This derivative was readily soluble in pyridine or in mixtures of pyridine–dimethylformamide, but was quite inert on attempted reaction with tributylammonium orthophosphate. Examination of the simpler model compound, cytidine 5′-phosphoroanisidate,[18] showed that while this substance reacted slowly but completely with the pyridinium salts of orthophosphoric acid or its monoesters in pyridine[18] or in dimethyl sulfoxide, it was completely inert toward reaction with the corresponding tributylammonium salts even over reaction periods of a month or more. The nucleoside 5′-phosphoromorpholidates, however, react readily with both the pyridinium and tributylammonium salts of orthophosphoric acid and its monoesters.[19] In view of the more reliable synthesis of the phosphoroanisidate (Vb) and its greater solubility in suitable anhydrous solvents, we have used this derivative in most of the syntheses reported in this paper. The structures of Va and Vb were confirmed by elemental analysis and by chemical and enzymatic degradations. Thus Vb was completely resistant to the action of *E. coli* alkaline phosphatase under conditions which resulted in the complete dephosphorylation of cytidine 2′(3′)-phosphate. It was, however, hydrolyzed at room temperature by 0.2 *N* sodium hydroxide to cytidine 2′(3′)-phosphate 5′-phosphoroanisidate (VIIb) which was then susceptible to dephosphorylation by the phosphomonoesterase. The sole product of this last degradation was chromatographically and electrophoretically identical with an independently prepared sample of cytidine 5′-phosphoroanisidate (VIIIb). A similar pattern was obtained starting with Va → VIIa → VIIIa. V was also a good substrate for the ribonucleoside 2′,3′-cyclic phosphodiesterase of Drummond, *et al.*,[20] giving the 2′-phosphate isomer of VII. Mild acidic hydrolysis of Va or Vb regenerated cytidine 2′(3′),5′-diphosphates (IV) quantitatively.

Since, as mentioned above, Vb failed to react with trialkylammonium orthophosphate, and since pyridinium phosphate is insoluble in pyridine, it was necessary to conduct the synthesis of cytidine 2′,3′-cyclic

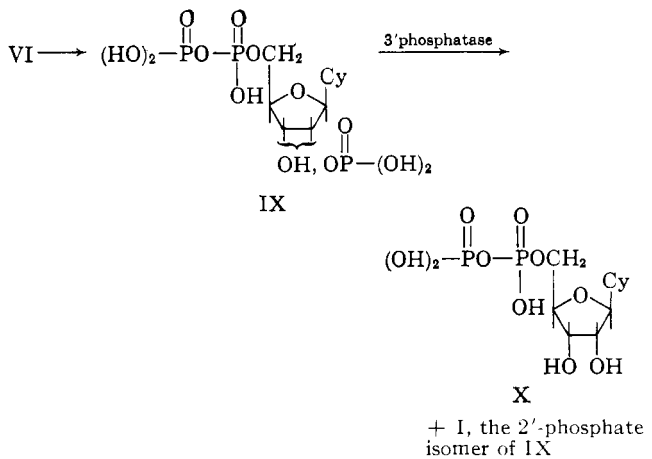
(18) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

(19) As pointed out previously,[18] the reaction with the triethylamine salt of monophenyl phosphate is markedly slower than that with the pyridine salt.

(20) G. I. Drummond, N. T. Iyer, and J. Keith, *J. Biol. Chem.*, **237**, 3535 (1962). We are very grateful to Dr. Drummond for a generous gift of the purified enzyme.



phosphate 5′-diphosphate (VI) in a mixture of pyridine and dimethylformamide. An initially homogeneous solution of the anhydrous components in pyridine–dimethylformamide (3 : 2) slowly deposited an insoluble gum during several days reaction at room temperature. The products of the reaction were then separated by ion exchange chromatography on either Dowex 2 (HCO_3^-) or DEAE-cellulose (HCO_3^-) using a linear gradient of triethylammonium bicarbonate as eluent. The main product, which usually comprised about 50% of the total optical density, was isolated as its chromatographically homogeneous calcium salt and shown to be cytidine 2′,3′-cyclic phosphate 5′-diphosphate (VI) as follows. The compound had a typical cytidine spectrum (λ_{max} 280 mμ at pH 2) and contained 3.00 phosphorus atoms per cytidine of which 1.1 was labile to *N* HCl at 100° for 8 min. Mild acidic or alkaline hydrolysis resulted in opening of the cyclic phosphate structure to give a more negatively charged species (IX) which was approximately 50% degraded to cytidine 5′-diphosphate (X) by rye grass 3′-nucleotidase. Reisolation of the portion undegraded by the



+ I, the 2′-phosphate isomer of IX

3′-nucleotidase gave a compound shown to be resistant to the enzyme on retreatment and chromatographically identical with either IX or the pure isomer (I) obtained in the next step.

While it was thus possible to obtain the desired compound I by chemical hydrolysis of the cyclic phosphate group in VI followed by degradation of the undesired 3′-phosphate isomer of IX with the specific 3′-nucleotidase, it was more efficient to utilize the highly specific ribonucleoside 2′,3′-cyclic phosphodiesterase obtained by Drummond, *et al.*,[20] from beef brain. This enzyme has been shown by Drummond, and in the present work, to open ribonucleoside 2′,3′-cyclic phosphates selectively to the 2′-phosphates in compounds bearing a wide variety of phosphorylated substituents at the 5′-position. Incubation of VI with the enzyme resulted in a rapid hydrolysis, as shown by paper chromatography of aliquots, and the formation of one major and two minor products. These were clearly separated on a column of DEAE-cellulose in the bicarbonate form from which cytidine 2′-phosphate 5′-diphosphate (I) was isolated as its calcium or sodium salt in yields of 70–90% in several runs. The product was chromatographically and electrophoretically pure under a variety of conditions and showed ratios of cytidine : total phosphorus : labile phosphorus of 1.00 : 3.00 : 0.98 (expected 1 : 3 : 1). The phosphomonoester group was shown to reside exclusively in the 2′-position by enzymatic tests. Thus I was completely unaffected on incubation with purified rye grass 3′-nucleotidase[14] under conditions which resulted in the expected conversion of one-half of a roughly equimolar mixture of cytidine 2′(3′)-phosphate 5′-diphosphate (IX) to cytidine 5′-diphosphate (X). Also it was possible to selectively cleave the pyrophosphate bond in I by incubation with a large excess of purified snake venom phosphodiesterase[21] giving cytidine 2′,5′-diphosphate. The latter compound, after purification by paper chromatography, was also shown to be completely resistant to the action of rye grass 3′-nucleotidase under conditions known to degrade cytidine 3′,5′-diphosphate to cytidine 5′-phosphate. The pyrophosphate bond in I was cleaved only very slowly by venom phosphodiesterase as would be expected from both the slow cleavage of adenosine 5′-diphosphate compared to that of adenosine 5′-triphosphate, and the inhibitory action of extra phosphate groups on the 2′- or 3′-position.[22] Hence a lengthy incubation of I with a large amount of enzyme was necessary for complete reaction and under these conditions a small amount of a monophosphate, identified as exclusively cytidine 5′-phosphate by paper chromatography in the presence of borate, arose. The homogeneity of this by-product is rather unusual in view of the largely random action of the nonspecific phosphatase present in venom.[23] A similar release of a little cytidine 5′-phosphate also occurred on incubating cytidine 2′(3′),5′-diphosphate under the same conditions. The above degradations clearly prove the structure of I as indicated.

In order to provide a greater sensitivity in testing of this compound in the cytidine diphosphate reductase system, we have also prepared I bearing a C^{14} label in C-2 of the cytosine ring. Since a high specific activity was not necessary for this purpose, the synthesis was done so as to provide material of only 0.05 μc. per μmole and the route was similar to that described above using the unlabeled phosphoroanisidate. Some minor variations were used to accommodate the small scale synthesis and are outlined in the Experimental section.

Finally, we might comment upon the structures of two compounds isolated from unsuccessful reactions during this work. During one attempt to prepare cytidine 2′,3′-cyclic phosphate 5′-phosphomorpholidate (Va), we obtained, after a prolonged reaction period, a single ultraviolet-absorbing product which was more negatively charged than the desired Va and

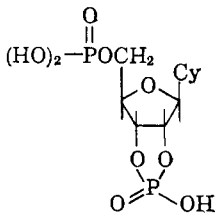
(21) This extremely active preparation from *Crotalus adamanteus* venom was kindly provided by Dr. W. E. Razzell of this Institute.

(22) Unpublished experiments by Dr. W. E. Razzell.

(23) E. Sulkowski, W. Bjork, and M. Laskowski, *J. Biol. Chem.*, **238**, 2477 (1963).

was chromatographically and electrophoretically identical with the product (VIIa) of the alkaline hydrolysis of Va. Its identity as cytidine 2′(3′)-phosphate 5′-phosphoromorpholidate (VIIa) was confirmed as before by its inertness to ribonucleoside 2′,3′-cyclic phosphodiesterase, and by its conversion by *E. coli* alkaline phosphatase to cytidine 5′-phosphoromorpholidate (VIIIa). This product apparently arose through hydrolysis of the cyclic phosphate structure in the desired Va, brought about through accumulation of the strongly basic by-product 4-morpholine-N,N′-dicyclohexylcarboxamidine. The latter compound always arises as a by-product during the synthesis of phosphoromorpholidates by this route,[18] and in shorter term reactions its base-catalyzed hydrolytic effect is counteracted by rapid recyclization in the presence of excess carbodiimide. Presumably in this more lengthy reaction, the excess carbodiimide was all converted to amidine and simple hydrolysis occurred. The hydrolysis of cytidine 2′,3′-cyclic phosphate in hot aqueous *t*-butyl alcohol containing an excess of 4-morpholine-N,N′-dicyclohexylcarboxamidine was readily demonstrated in a separate experiment.

In another experiment, the condensation of C^{14}-labeled cytidine 2′,3′-cyclic phosphate 5′-phosphoroanisidate (Vb) with pyridinium orthophosphate was attempted in dimethyl sulfoxide.[24] Ion exchange chromatography of the reaction mixture after 5 days at room temperature revealed, in addition to 24% unreacted phosphoroanisidate, a 59% yield of a chromatographically homogeneous and more negatively charged product. This compound was isolated as its sodium salt and was shown to still contain only two phosphorus atoms (cytidine:total P = 1:1.95). It was rapidly converted into cytidine 2′(3′),5′-diphosphate by beef brain ribonucleoside 2′,3′-cyclic phosphodiesterase and into cytidine 2′,3′-cyclic phosphate by *E. coli* phosphatase. Its structure was therefore shown to be cytidine 2′,3′-cyclic phosphate 5′-phosphate (XI).



XI

Phosphoramidate condensations in dimethyl sulfoxide are extremely sensitive to traces of moisture,[24] and it appears as if this reaction had not been rendered sufficiently anhydrous. A relatively successful condensation of Va with tributylammonium phosphate in dimethyl sulfoxide is described in the Experimental section. Final proof for the structure of XI came through its quantitative reconversion into the phosphoroanisidate (Vb) on treatment with dicyclohexylcarbodiimide and *p*-anisidine.

Experimental

General Methods.—Paper chromatography was conducted by the descending technique on sheets of Schleicher and Schuell No. 589 Orange Ribbon paper using the following systems: Solvent I, ethanol–*M* ammonium acetate (pH 7.5, 5:2); Solvent II, *n*-propyl alcohol–ammonium hydroxide–water (6:3:1); Solvent III, isopropyl alcohol–ammonium hydroxide–water (7:1:2); Solvent IV, saturated ammonium sulfate–isopropyl alcohol–*M* sodium acetate (80:2:18); Solvent V, isobutyric acid–*M* ammonium hydroxide–0.1 *M* tetrasodium ethylenediamine tetraacetate (100:60:1.6). The R_f values of most pertinent compounds in Solvents I–III are given in Table I. Paper electrophoresis was carried out at 1000–1500 v. on the same paper impregnated with 0.02 *M* sodium acetate buffer, pH 4.0, or with 0.02 *M* phosphate buffer, pH 7.4. Phosphorus-containing compounds were visualized by use of the Hanes and Isherwood spray[25] followed by ultraviolet irradiation.[26] Vicinal glycols were detected by the periodate–benzidine spray of Viscontini, *et al.*[27]

TABLE I

R_f VALUES OF COMPOUNDS

Compound	R_f in solvent I	II	III
Cytidine	0.73	0.52	0.72
Cytidine 2′(3′)-phosphate	.37	.25	.30
Cytidine 5′-phosphate	.30	.17	.20
Cytidine 2′(3′),5′-diphosphate	.13	.07	.05
Cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate	.57	.39	.56
Cytidine 2′,3′-cyclic phosphate 5′-phosphoroanisidate	.51	.44	.56
Cytidine 2′,3′-cyclic phosphate 5′-diphosphate	.18	.11	.10
Cytidine 2′-phosphate 5′-diphosphate	.11	.05	.03
Cytidine 2′(3′)-phosphate 5′-phosphoromorpholidate	.35	.21	.24
Cytidine 2′,3′-cyclic phosphate 5′-phosphate	.22	.18	.18
Cytidine 5′-phosphoroanisidate	.69	.59	.72

Phosphorus analyses were obtained by the method of King[28] and other elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind. Ultraviolet spectra were recorded on a Cary Model 15 spectrophotometer, and analytical optical densities on a Zeiss PMQ-II spectrophotometer. Measurements of C^{14} were made using a Nuclear Chicago gas flow counter with an efficiency of 21.4%.

Enzyme Degradations. A.—*E. coli* alkaline phosphatase[16] (specific activity 207 μmoles of thymidine 5′-phosphate cleaved/hr./mg.) was made up to a concentration of 100 μg./ml. in 0.05 *M* TRIS buffer, pH 9 [tris(hydroxymethyl)aminomethane].

B.—Beef brain ribonucleoside 2′,3′-cyclic phosphodiesterase[20] was made up to a concentration of 5 mg./ml. in 0.005 *M* TRIS hydrochloride buffer, pH 7.5. Two μl. of this preparation will hydrolyze 1.5 μmoles of cytidine 2′,3′-cyclic phosphate within 20 min. at 30° and pH 7. A small amount (200 μg.) of egg albumin was added to the incubation as a preservative in some experiments.

C.—Rye grass 3′-nucleotidase[14] was a solution capable of hydrolyzing 1.4 μmoles of adenosine 3′-phosphate/hr./μl. in 0.1 *M* TRIS buffer, pH 7.6. The activity towards adenosine 2′- and 3′-phosphates was less than 0.1% of this.

D.—Venom phosphodiesterase[21] was a highly purified preparation free of 5′-nucleotidase and containing 8.1 mg. of protein/ml. The specific activity was 211 μmoles/min./ml. of *p*-nitrophenyl thymidine 5′-phosphate or 83 μmoles of adenosine 5′-diphosphate/hr./ml. in pH 9 TRIS buffer.

Cytidine 2′(3′),5′-Diphosphate (IV).—Cytidine (2.43 g., 10 mmoles) was added to a mixture of phosphorus pentoxide (6.5 g.) and 85% orthophosphoric acid (8.5 g.) and stored at 60° for 21 hr. under anhydrous conditions with occasional stirring. Water (100 ml.) then was added, and the mixture was heated to 100° for 20 min. After cooling in ice the pH was brought to 11 with 4 *N* lithium hydroxide, and the resulting precipitate of Li_3PO_4 was washed with several portions of 0.01 *N* lithium hydroxide. The combined filtrates were adjusted to pH 7.5 with Dowex 50 (H^+) resin and filtered. The filtrate was applied to a 3 × 13-cm. column of Dowex 2 (Cl^-) resin, and a little unreacted cytidine (3.4%) was washed through with water. Elution with 0.015 *N* lithium chloride in 0.003 *N* HCl gave 5.9% mixed cytidine monophosphates. Cytidine 2′(3′),5′-diphosphate (65%) then was eluted with 0.03 *N* lithium chloride in 0.003 *N* HCl, and finally a little cytidine 2′,3′,5′-triphosphate (3.2%) was removed with 0.2 *N* lithium chloride in 0.003 *N* HCl. The pooled diphosphate peak was adjusted to pH 7.5 with lithium hydroxide and evaporated to dryness. The white residue was freed from lithium chloride by repeated stirrings with methanol and addition of five volumes of acetone. The final precipitate was washed with ether and dried *in vacuo* giving the chromatographically homogeneous tetralithium salt of cytidine 2′(3′),5′-diphosphate (2.70 g., 57%) as the trihydrate.

(24) The use of this solvent in extremely efficient syntheses of nucleoside 5′-triphosphates through condensation of nucleoside 5′-phosphoromorpholidates with tributylammonium pyrophosphate has been thoroughly studied in this laboratory (J. G. Moffatt, *Can. J. Chem.*, to be published).

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

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

(27) M. Viscontini, D. Hoch, and P. Kaner, *Helv. Chim. Acta*, **38**, 642 (1955).

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

Anal. Calcd. for $C_9H_{11}Li_4N_3O_{11}P_2 \cdot 3H_2O$: P, 6.44; P:cytidine, 2.00. Found: P, 6.57; P:cytidine, 2.04.

Incubation of 1 μmole of this compound with 20 μl. of rye grass 3′-nucleotidase and 5 μmoles of TRIS buffer, pH 7.8, in a total volume of 0.125 ml. for 1 hr. gave 45% cytidine 2′,5′-diphosphate and 55% cytidine 5′-phosphate.

2-C^{14}-Cytidine 2′(3′),5′-Diphosphate (IV).—2-C^{14}-Cytidine (23 mg., 95 μmoles, containing 9.3 μc. of C^{14}) was phosphorylated as above in 0.2 ml. of polyphosphoric acid mixture. After acidic hydrolysis, the nucleotides were adsorbed on 2.0 g. of acid-washed Takeda charcoal.[29] After thorough washing with water, the nucleotides were eluted with 50% ethanol containing 2% ammonium hydroxide (98% recovery of C^{14}). After evaporation of the solvent, the products were separated by preparative paper chromatography on four 20-cm. wide strips of Whatman 3MM paper. Development with *n*-butyl alcohol–acetic acid–water (5:2:3) cleanly separated some residual orthophosphate from the nucleotides which were then rerun in Solvent V giving pure 2-C^{14}-cytidine 2′(3′),5′-diphosphate (62 μmoles, 66%, 2.9×10^6 c.p.m.) which was well separated from 2-C^{14}-cytidine 5′-phosphate (5%) and 2-C^{14}-cytidine 2′,3′,5′-triphosphate (21%).

Cytidine 2′,3′-Cyclic Phosphate 5′-Phosphoromorpholidate (Va).—Tetralithium cytidine 2′(3′),5′-diphosphate (48 mg., 0.1 mmole) was converted into its triethylammonium salt with Dowex 50 (Et_3N) resin and evaporated to a volume of 2 ml. To this was added *t*-butyl alcohol (2 ml.) and morpholine (0.09 ml., 1 mmole). This solution was refluxed gently while a solution of dicyclohexylcarbodiimide (206 mg., 1 mmole) in *t*-butyl alcohol (3 ml.) was added slowly over 1.5 hr. Gentle reflux was maintained for a further hour and then another 0.04 ml. of morpholine and 105 mg. of dicyclohexylcarbodiimide were added. After 1 hr. under reflux the mixture was cooled, evaporated, and partitioned between water (10 ml.) and ether (10 ml.) with filtration of the insoluble dicyclohexylurea. The water layer was extracted twice more with ether and evaporated to dryness. The glassy residue was dissolved in methanol (1 ml.) and precipitated with ether (10 ml.). After trituration with fresh ether, the hydrated 4-morpholine N,N′-dicyclohexylcarboxamidine salt of cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate was collected and dried *in vacuo* (100 mg., 81%). Quantitative paper chromatographic examination in Solvent I showed the product to be 93% pure and contaminated by 1% cytidine 2′(3′),5′-diphosphate, 4% cytidine 2′(3′)-phosphate 5′-phosphoromorpholidate, and 2% cytidine 2′,3′-cyclic phosphate 5′-phosphate. Such a preparation could be directly used for subsequent reactions.

Ion Exchange Purification of Cytidine 2′,3′-Cyclic Phosphate 5′-Phosphoromorpholidate.—A preparation similar to that above from 0.2 mmole of cytidine 2′(3′),5′-diphosphate was purified by ion exchange chromatography on a 1.5 × 30-cm. column of DEAE-cellulose (HCO_3^-). Elution was effected using a linear gradient of triethylammonium bicarbonate (2 l.) with the salt varying from 0.005 *M* to 0.12 *M*. One large peak appeared followed by two extremely small ones. The main peak was evaporated to dryness and freed of residual salt by four evaporations with 20 ml. portions of methanol. 4-Morpholine N,N′-dicyclohexylcarboxamidine (140 mg.) was added, and after three further evaporations with methanol the product was precipitated with ether. It was then washed twice with ether and dried *in vacuo* giving chromatographically pure cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate as the trihydrate of the 4-morpholine N,N′-dicyclohexylcarboxamidinium salt.

Anal. Calcd. for $C_{47}H_{82}N_{10}O_{12}P_2 \cdot 3H_2O$: C, 51.58; H, 8.10; N, 12.80. Found: C, 51.50; H, 8.40; N, 12.99.

The ammonium salt of Va (1 μmole) was completely resistant to the action of *E. coli* alkaline phosphatase (50 μl.) at pH 9 for 5 hr. After hydrolysis with 0.2 *N* sodium hydroxide at 37° for 4 hr., however, it was rapidly and quantitatively degraded under the same enzymatic conditions to cytidine 5′-phosphoromorpholidate.

Cytidine 2′,3′-Cyclic Phosphate 5′-Phosphoroanisidate (Vb).—This compound was prepared from triethylammonium cytidine 2′(3′),5′-diphosphate[30] exactly as above for the phosphoromorpholidate (Vb), except that morpholine was replaced by recrystallized *p*-anisidine and the reaction was carried out under nitrogen. The chromatographically homogeneous N,N′-dicyclohexyl-N″-*p*-methoxyphenylguanidinium salt[31] of Vb (175 mg., 75%) was precipitated with peroxide-free ether and dried *in vacuo*.

Anal. Calcd. for $C_{56}H_{82}N_{10}O_{12}P_2 \cdot 3H_2O$: C, 55.89; H, 7.37; N, 11.64. Found: C, 55.66; H, 7.36; N, 11.89.

The ammonium salt of Vb was completely resistant towards *E. coli* alkaline phosphatase (as with Va above) but after alkaline hydrolysis was degraded completely to cytidine 5′-phosphoroanisidate.

In a separate experiment the product was purified from some minor impurities by ion exchange chromatography on a column of Dowex 2 (HCO_3^-) resin (1 × 10 cm. for 0.4 mmole). Elution was effected with a linear gradient of triethylammonium bicarbonate (2 l.) with the salt varying from 0.004 to 0.4 *M*. The main ultraviolet absorbing peak (eluted with 0.2 *M* salt) was evaporated to dryness and freed from residual triethylammonium bicarbonate by several evaporations with methanol. The final residue was dissolved in methanol (2 ml.) and addition of ether (20 ml.) gave, after careful drying *in vacuo*, anhydrous bis(triethylammonium)cytidine 2′,3′-cyclic phosphate 5′-phosphoroanisidate in chromatographically pure form.

Anal. Calcd. for $C_{16}H_{18}N_4O_{10}P_2 \cdot 2Et_3N$: N, 12.13; P, 8.94. Found: N, 12.12; P, 8.89.

2-C^{14}-Cytidine 2′,3′-Cyclic Phosphate 5′-Phosphoroanisidate (Vb).—In a reaction similar to that above, 2-C^{14}-cytidine 2′(3′),5′-diphosphate (59 μmoles, 3.4×10^6 c.p.m.) as the triethylamine salt was treated with *p*-anisidine (97 mg.) and dicyclohexylcarbodiimide (170 mg.) in aqueous *t*-butyl alcohol. After evaporation and three extractions of a filtered, aqueous solution of the residue with ether, the aqueous phase contained chromatographically homogeneous Vb (3.3×10^6 c.p.m., 97%) as its N,N′-dicyclohexyl-N″-*p*-methoxyphenylguanidinium salt.[31] The product was lyophilized and used directly in the next step.

2-C^{14}-Cytidine 2′,3′-Cyclic Phosphate 5′-Phosphate (XI).—2-C^{14}-Cytidine 2′,3′-cyclic phosphate 5′-phosphoroanisidate (58 μmoles, 3.3×10^6 c.p.m.) as its N,N′-dicyclohexyl-N″-*p*-methoxyphenylguanidinium salt was rendered anhydrous by three evaporations *in vacuo* with small amounts of dry pyridine and dissolved in anhydrous dimethyl sulfoxide.[32] Pyridine (0.17 ml., 2.3 mmoles) and anhydrous phosphoric acid[33] (70 mg., 0.7 mmole) were added, and the clear solution was stored at room temperature for 5 days. Water (50 ml.) and triethylamine (0.32 ml., 2.3 mmoles) were added, and the entire mixture was applied to the top of a column containing 50 ml. of DEAE-cellulose (HCO_3^-). After a thorough washing with water (500 ml.), the column was eluted with a linear gradient of triethylammonium bicarbonate (0.03 to 0.2 *M* in a total volume of 4 l.). Three sharp peaks were eluted as follows: Peak I, at 0.05 *M* bicarbonate, contained 14 μmoles of starting material, Vb; peak II, at 0.08 *M* bicarbonate, contained 34 μmoles (59%) of 2-C^{14}-cytidine 2′,3′-cyclic phosphate 5′-phosphate (XI); peak III, at 0.13 *M* bicarbonate, contained 10 μmoles of 2-C^{14}-cytidine-2′(3′),5′-diphosphate. Peak II was pooled, evaporated to dryness, and freed from residual triethylammonium bicarbonate by four evaporations with 10 ml. portions of methanol. The residue was dissolved in methanol (1 ml.) and a *M* solution of sodium iodide in acetone (0.25 ml.) was added followed by pure acetone (20 ml.). The resulting white precipitate was washed several times with fresh acetone and dried *in vacuo* giving the sodium salt of 2-C^{14}-cytidine 2′,3′-cyclic phosphate 5′-phosphate.

Anal. Calcd.: Total phosphorus per cytidine, 2.00. Found: 1.95; labile phosphorus, 0%.

Incubation of this compound (0.32 μmole) with *E. coli* alkaline phosphatase (10 μl.) and 10 μl. of *M* TRIS buffer, pH 8, for 2 hr. resulted in quantitative conversion to cytidine 2′,3′-cyclic phosphate as shown by chromatography in Solvent I. Treatment of XI (0.3 μmole) with ribonucleoside 2′,3′-cyclic phosphodiesterase (5 μl.) and 10 μl. of *M* TRIS buffer, pH 6.9, for 2 hr. at 34° resulted in complete conversion to cytidine 2′,5′-diphosphate (Solvent I).

2-C^{14}-Cytidine 2′,3′-Cyclic Phosphate 5′-Phosphoranisidate (Vb) from XI.—Triethylammonium 2-C^{14}-cytidine 2′,3′-cyclic phosphate 5′-phosphate (XI, 46 μmoles, 1.2×10^6 c.p.m.) was dissolved in a gently refluxing mixture of *t*-butyl alcohol (1 ml.), water (1 ml.), and *p*-anisidine (65 mg.), and to it was added a solution of dicyclohexylcarbodiimide (115 mg.) in *t*-butyl alcohol (2 ml.) over 1 hr. The mixture then was refluxed for another hour, and after evaporation of the solvent the residue was partitioned between water and ether. The aqueous layer contained only pure Vb as the N,N′-dicyclohexyl-N″-*p*-methoxyphenylguanidinium salt as judged by both ultraviolet examination[31] and autoradiography. The recovery was 1.1×10^6 c.p.m. (92%). This material was lyophilized and used directly in subsequent condensations.

Cytidine 2′,3′-Cyclic Phosphate 5′-Diphosphate (VI). A. From the Phosphoromorpholidate.—Bis(triethylammonium)-

(29) This particular brand of carbon, obtained through the kindness of Dr. K. Tanaka of Takeda Chemical Industries, Osaka, Japan, has been found to be particularly valuable since virtually quantitative recoveries of nucleotides may be obtained upon subsequent elution.

(30) It was necessary to use a trialkylamine salt of the starting material in order to avoid the formation of N-phosphorylureas which arose if weakly basic *p*-anisidine (p*K* 5.29) was the only amine present [M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958)].

(31) On chromatography or electrophoresis, the ultraviolet absorbing guanidine base separates from the nucleotide and has R_f 0.90 in Solvent III and 0.90 in Solvent I.

(32) Distilled and dried over Molecular Sieve Type 4A from Linde Co.

(33) Obtained from Fluka A. G., Buchs, Switzerland.

cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (0.25 mmole) was rendered anhydrous by three evaporations with a mixture of pyridine (3 ml.) and dimethylformamide (2 ml.).[32] Separately, orthophosphoric acid (0.75 mmole) was dried in the same way and the two materials then were mixed and evaporated one further time. The final residue was dissolved in a mixture of dimethylformamide (3 ml.) and pyridine (4 ml.) and shaken for 18 hr. at room temperature.[34] The solvent was evaporated *in vacuo*, and an aqueous solution of the residue (adjusted to pH 7.5) was applied to the top of a 2.5 × 25-cm. column of Dowex 2 (HCO_3^-) resin. After a thorough water wash, the products were eluted with a linear gradient of triethylammonium bicarbonate (4 l. total ranging from 0.05 to 0.35 *M* bicarbonate). Four ultraviolet absorbing peaks resulted as follows: Peak I (23%) was unreacted starting material; peak II (52%) was pure cytidine 2′,3′-cyclic phosphate 5′-diphosphate (VI); peak III (9%) was cytidine 2′(3′)-phosphate 5′-diphosphate (IX); peak IV (4%) was unidentified. Peaks I, II, and III were identified by paper chromatography and by mild acidic hydrolysis or enzyme degradation. Peak II was pooled and evaporated to dryness. After several evaporations with methanol (25 ml. each), the residue was dissolved in ethanol (5 ml.) and a *M* solution of calcium chloride in ethanol (0.5 ml.) was added. The resulting calcium salt was collected by centrifugation and freed of chloride ion by washing with ethanol. It was then dried *in vacuo* giving 57 mg. of the chromatographically pure calcium salt of VI.

Anal. Calcd.: Cytidine:total P:labile P, 1.0:3.0:1.0. Found: 1.00:3.00:1.13.

On treatment with 0.1 *N* hydrochloric acid at room temperature for 1 hr. or with 0.2 *N* sodium hydroxide at 37° for 2 hr., this compound was completely converted into cytidine 2′(3′)-phosphate 5′-diphosphate which was chromatographically identical with the product of the action of the 2′,3′-cyclic phosphodiesterase (see below).

Incubation of the acid treated compound (1 μmole) with rye grass 3′-nucleotidase (20 μl.), pH 7.8 TRIS buffer (10 μmoles), and water (50 μl.) for 1 hr. at 37° gave an equimolar mixture of cytidine 2′-phosphate 5′-diphosphate (I) and cytidine 5′-diphosphate (X).

Overnight treatment of 0.3 μmole of VI with 10 μl. of *E. coli* alkaline phosphatase resulted in complete conversion to cytidine 2′,3′-cyclic phosphate.

B. From the Phosphoroanisidate.—N,N′-Dicyclohexyl-N″-*p*-methoxyphenylguanidinium cytidine 2′,3′-cyclic phosphate 5′-phosphoroanisidate (183 mg., 0.25 mmole) was treated for 10 days with phosphoric acid (0.2 mmole) in pyridine–dimethylformamide exactly as above in A. Chromatography of the reaction mixture gave 30% unreacted phosphoroanisidate and 40% cytidine 2′,3′-cyclic phosphate 5′-diphosphate which was isolated as above. The resulting material was chromatographically pure and identical with that obtained from the phosphoromorpholidate.

C. From the Phosphoromorpholidate in Dimethyl Sulfoxide.—4-Morpholine-N,N′-dicyclohexylcarboxamidinium cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (70 μmoles) was dried by three evaporations with a mixture of pyridine–dimethylformamide (1:1) and then freed of residual pyridine by one evaporation with benzene. Separately bis(tributylammonium) orthophosphate (0.3 mmole) was dried by evaporation with pyridine and then benzene, and the two components rapidly were dissolved and rigorously mixed in anhydrous dimethyl sulfoxide (2 ml.).[32] After 3 days at room temperature, water (30 ml). was added and the mixture was applied to a 2 × 20-cm. column of DEAE-cellulose (HCO_3^-). Elution was effected by a linear gradient (2 l. total) of triethylammonium bicarbonate (0.005 to 0.33 *M*). Three well separated peaks resulted. Peak I (34 μmoles, 49%) was unreacted phosphoromorpholidate, peak II (10.5 μmoles, 15%) was cytidine 2′,3′-cyclic phosphate 5′-phosphate, and peak III (21 μmoles, 30%) was the desired product(VI). Peak III was pooled and isolated as the sodium salt (10 mg.) in the usual way. The product was chromatographically, chemically, and enzymatically identical with that obtained by methods A, B, or D.

D. 2-C^{14}-Cytidine 2′,3′-Cyclic Phosphate 5′-Diphosphate.—2-C^{14} labeled Vb (42 μmoles, 1.1 × 10^6 c.p.m., directly obtained as above) was dried and reacted with phosphoric acid (0.5 mmole) in pyridine–dimethylformamide as in B for 10 days. After evaporation of the solvent, the mixture was separated by chromatography on a 2 × 15-cm. column of DEAE-cellulose (HCO_3^-). Elution with 2 l. of a linear gradient (0.005 to 0.3 *M*) of triethylammonium bicarbonate gave one main peak (53%, eluted with 0.18 *M* salt) and three well separated minor peaks. The pooled main peak was evaporated to dryness, freed of residue bicarbonate by four evaporations with methanol, and precipitated as the sodium salt with sodium iodide in acetone as described above for XI. The resulting white sodium salt (17 mg.) was chromatographically pure both by ultraviolet absorption and C^{14} content.

Cytidine 2′-Phosphate 5′-Diphosphate (I).—Ammonium cytidine 2′,3′-cyclic phosphate 5′-diphosphate (VI, 82 μmoles) was obtained by passing the corresponding calcium salt through a small column containing 2 ml. of Dowex 50 (NH_4^+) resin. This solution was concentrated to 3 ml., and to it was added 0.5 *M* TRIS acetate buffer (pH 7, 2.5 ml.), 1% egg albumin (1 ml.), and the stock solution of ribonucleoside 2′,3′-cyclic phosphodiesterase (0.5 ml.). After incubation for 2.5 hr. at 31° the solution was diluted to a volume of 50 ml. with water and passed through a 2 × 21-cm. column of DEAE-cellulose (HCO_3^-). After a thorough water wash (200 ml.), the products were eluted with a linear gradient (4 l.) of triethylammonium bicarbonate (0.10 to 0.40 *M*). One major ultraviolet absorbing peak (58 μmoles, 71% at 0.20 *M* salt) and two minor peaks (4 and 6%) were clearly separated. The major peak was pooled, evaporated, and freed of bicarbonate in the usual way. The residue was then dissolved in ethanol (5 ml.) and precipitated as the calcium salt by addition of *M* ethanolic calcium chloride (0.5 ml.). After repeated washings with ethanol, the calcium salt was dried *in vacuo*.

Anal. Calcd. for $C_9H_{11}Ca_{2.5}O_{14}P_3N_3 \cdot 9H_2O$: P, 12.55; cytidine: total P:labile P, 1.0:3.0:1.0. Found: P, 12.35; cytidine: total P:labile P, 1.00:3.00:0.98.

After conversion to the ammonium salt, the following enzymatic tests were done. Incubation of I (0.5 μmole) with rye grass 3′-nucleotidase (10 μl.) and 0.2 *M* TRIS buffer, pH 7.8 (10 μl.), for one hour at 37° resulted in no observable reaction[35] (*cf.* similar treatment of acid treated VI.) Incubation of I (1 μmole) with venom phosphodiesterase (50 μl.) and *M* TRIS buffer, pH 9 (50 μl.), in water (0.2 ml.) at 45° for 50 hr. in the presence of thymol gave roughly 90% cytidine 2′,5′-diphosphate and 10% cytidine 5′-phosphate. The isolated cytidine 2′,5′-diphosphate was completely unchanged by subsequent incubation with rye grass 3′-nucleotidase as above.

2-C^{14}-Cytidine 2′-Phosphate 5′-Diphosphate.—The sodium salt of 2-C^{14}-cytidine 2′,3′-cyclic phosphate 5′-diphosphate (15.5 μmoles, 4.06 × 10^5 c.p.m.) was dissolved in water (0.6 ml.) containing *M* TRIS buffer, pH 6.9 (0.3 ml.), and ribonucleoside 2′,3′-cyclic phosphodiesterase (0.15 ml.). After incubation at 34° for 1.5 hr. the mixture was diluted with water (15 ml.) and chromatographed on a 1.5 × 30-cm. column of DEAE-cellulose (HCO_3^-) as before. The main peak, containing 13.9 μmoles (90%) of the total optical density and C^{14}-counts was evaporated and isolated as its chromatographically homogeneous sodium salt (8 mg.).

Anal. Calcd. for pentasodium salt·$8H_2O$: P, 12.52; cytidine: total P:labile P, 1.00:3.00:1.00. Found: P, 1285; cytidine:total P:labile P, 1.00:3.04:0.99. Specific activity was 2.63 × 10^4 c.p.m. per μmole (0.055 μc. per μmole).

(34) A small precipitate initially separated and then redissolved within the first 3 hr.

(35) Under these conditions, cytidine 3′,5′-diphosphate is completely degraded. Complete hydrolysis of adenosine 3′-phosphate results with 1 μl. of enzyme under these conditions but considerably more enzyme is required for the complete digestion of the polyphosphorylated compounds.