Nucleotide Synthesis. III. Preparation of Nucleoside-3′,5′ Cyclic Phosphates in Strong Base[1,2]

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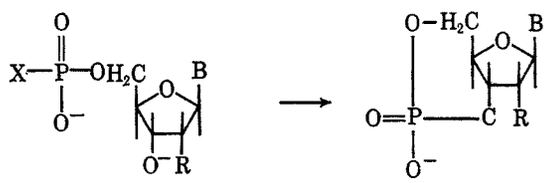
The *p*-nitrophenyl esters of thymidine-3′ and thymidine-5′ phosphates react rapidly with potassium *t*-butoxide in dimethyl sulfoxide at 20° to yield thymidine-3′,5′ cyclic phosphate and *p*-nitrophenol, the 3′ ester reacting most rapidly. The *p*-nitrophenyl esters of deoxyadenosine-5′, deoxycytidine-5′, and deoxyguanosine-5′ phosphates also react to give the corresponding deoxyribonucleoside-3′,5′ cyclic phosphates. Corresponding *p*-nitrophenyl esters of ribonucleoside-5′ phosphates react even more readily than *p*-nitrophenyl thymidine-3′ phosphate to yield the ribonucleoside-3′,5′ cyclic phosphates. Nucleoside-5′ phosphorofluoridates, nucleoside-5′ 2,4-dinitrophenyl phosphates and P[1]-nucleoside-5′ P[2]-diphenyl pyrophosphates also give nucleoside-3′,5′ cyclic phosphates on reaction with potassium *t*-butoxide in dimethyl sulfoxide.

Interest in the synthesis of phospho diesters receives its major stimulus from their occurrence in deoxyribo- and ribonucleic acids.[4] While a variety of techniques is available for the synthesis of simple phospho diesters[5,6] only one basic approach has been extensively applied to the synthesis of polynucleotides with the natural (3′→5′)-internucleotide linkage. In this method the phosphoryl group in a nucleoside monophosphate is activated by reaction with a carbodiimide or an anhydride of a strong acid to produce a phosphorylating agent believed to be derived from the nucleoside metaphosphate.[5,7] This type of phosphorylating agent, which is generally used in anhydrous pyridine or dimethylformamide,[8,9] reacts with nucleotide sugar hydroxyl groups and also with the heterocyclic bases and phosphate groups.[10,11] Therefore methods have been developed to protect the heterocyclic bases with acyl groups and to break down the pyrophosphates produced during polynucleotide synthesis.[12–14] The aim of the present study was to develop a phosphorylation procedure where the sugar hydroxyl group was the dominant nucleophile. In this way it was hoped that the need for protection of nucleotide heterocyclic bases and that problems associated with polyphosphate formation could be eliminated.[15] The most obvious approach is to carry out reactions in media where the sugar hydroxyl groups are ionized. The simplest application in the nucleotide field is the synthesis of nucleoside-3′,5′ cyclic phosphates (1 → 2) with which this communication is concerned.[16,17]



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Considerations of the chemistry of nucleotides indicate that aqueous alkali would not be an appropriate medium for this type of reaction, because of the danger of hydrolysis of the phosphate derivatives and the resultant cyclic phosphates and of deamination or other breakdown of the heterocyclic bases.[6,13,18–22] The introduction of alkali metal alkoxides dissolved in solvents such as dimethyl sulfoxide and dimethylformamide as reagents in organic chemistry[23,24] provided a convenient system for the present work since both dimethyl sulfoxide and dimethylformamide are powerful solvents for nucleotides.[25,26] Other studies suggested

(1) Part II: R. K. Borden and M. Smith, *J. Org. Chem.*, **31**, 3241 (1966).

(2) This work was supported in part by grants from the Division of General Medical Sciences, National Institutes of Health, U. S. Public Health Service (GM 09241) and from the Medical Research Council of Canada (MA 1706).

(3) Part of this investigation was carried out by R. K. Borden in fulfillment of the requirements for a B.S. degree in Biochemistry from Cornell University, Ithaca, N. Y.

(4) D. M. Brown and A. R. Todd in "The Nucleic Acids," Vol. 1, E. Chargaff and J. N. Davidson, Ed., Academic Press Inc., New York, N. Y., 1955, p 409.

(5) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961.

(6) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic Press Inc., New York, N. Y., 1963.

(7) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964).

(8) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).

(9) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).

(10) G. M. Tener, *ibid.*, **83**, 159 (1961); P. T. Gilham and H. G. Khorana, *ibid.*, **81**, 4647 (1959).

(11) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962); T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965).

(12) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *ibid.*, **87**, 2954 (1965), and papers cited therein.

(13) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

(14) H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, *ibid.*, **84**, 414 (1962).

(15) A method free of these problems could also be useful in linking oligonucleotide fragments.[11]

(16) A brief report on part of this work has appeared: M. Smith, *ibid.*, **86**, 3586 (1964).

(17) Preliminary experiments, in which thymidylyl-(5′→3′)-thymidine and thymidylyl-(5′→3′)-deoxycytidine were obtained in low yield, indicate that the reaction can be extended to internucleotide diester synthesis: M. Smith, unpublished results.

(18) H. S. Loring, ref 4, p 191.

(19) R. W. Chambers, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 3747 (1957).

(20) M. Smith, G. I. Drummond, and H. G. Khorana, *ibid.*, **83**, 698 (1961).

(21) D. Lipkin, W. H. Cook, and R. Markham, *ibid.*, **81**, 6198 (1959).

(22) The production of thymidine-3′,5′ and adenosine-3′,5′ cyclic phosphates as intermediates during the breakdown in aqueous alkali of thymidine-3′ *p*-nitrophenyl phosphate[13] and of adenosine-5′ triphosphate,[21] respectively, encouraged the present study.

(23) D. J. Cram, B. Rickborn, C. A. Kingsbury, and P. Haberfield, *J. Am. Chem. Soc.*, **83**, 3678 (1961).

(24) A. J. Parker, *Quart. Rev.* (London), **16**, 163 (1962).

(25) J. G. Moffatt, *Can. J. Chem.*, **42**, 599 (1964); R. W. Chambers and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 3752 (1957).

(26) The conversion of sodium ribonucleate to ribonucleoside-2′,3′ cyclic phosphates catalyzed by potassium *t*-butoxide in formamide [D. Lipkin and P. T. Talbert, *Chem. Ind.* (London), 143 (1955)] and the alkoxide-catalyzed transesterification of ribonucleoside-2′,3′ cyclic phosphates [C. A. Decker and H. G. Khorana, *J. Am. Chem. Soc.*, **76**, 3522 (1954); G. R. Barker, M. D. Montague, R. J. Moss, and M. A. Parsons, *J. Chem. Soc.*, 3786 (1957)] are interesting precedents.

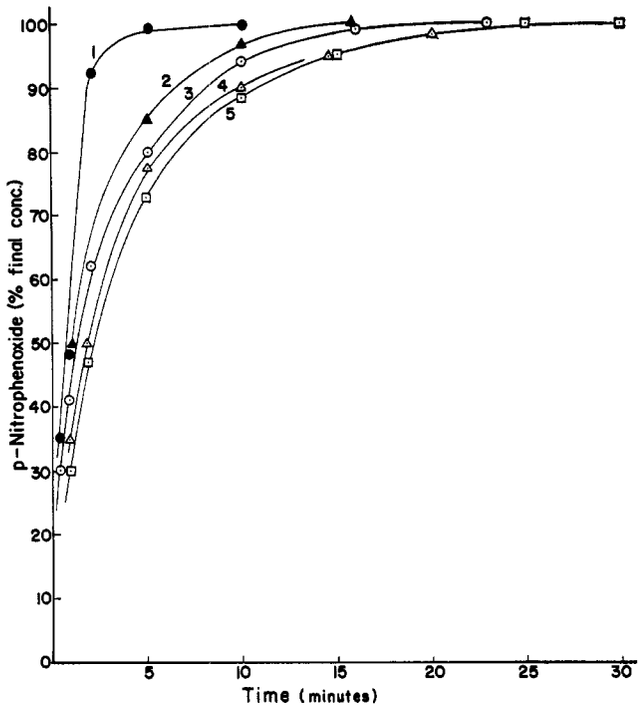


Figure 1.—Rates of reaction of deoxyribonucleoside *p*-nitrophenyl phosphates (10 μmoles) with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) in dimethyl sulfoxide (1.9 ml) at 20°: curve 1, thymidine-3′ *p*-nitrophenyl phosphate; curve 2, deoxycytidine-5′ *p*-nitrophenyl phosphate; curve 3, thymidine-5′ *p*-nitrophenyl phosphate; curve 4, deoxyguanosine-5′ *p*-nitrophenyl phosphate; curve 5, deoxyadenosine-5′ *p*-nitrophenyl phosphate.

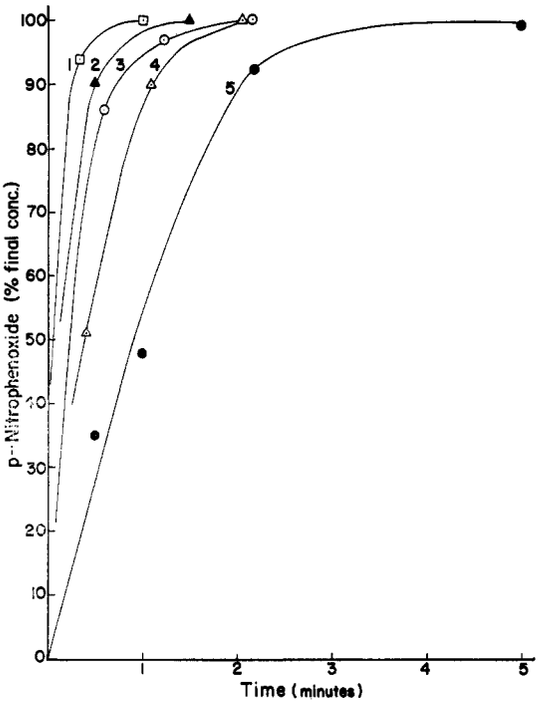
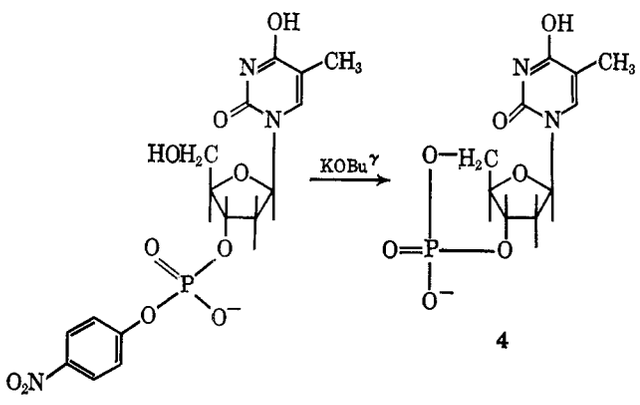


Figure 2.—Rates of reaction of ribonucleoside-5′ *p*-nitrophenyl phosphates (10 μmoles) with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) in dimethyl sulfoxide (1.9 ml) at 20°: curve 1, adenosine-5′ *p*-nitrophenyl phosphate; curve 2, cytidine-5′ *p*-nitrophenyl phosphate; curve 3, uridine-5′ *p*-nitrophenyl phosphate; curve 4, guanosine-5′ *p*-nitrophenyl phosphate; curve 5, thymidine-3′ *p*-nitrophenyl phosphate.

that *p*-nitrophenoxide would be an appropriate leaving group (X) in **1**.[13,19,27,28]

Reaction of Thymidine-3′ and Thymidine-5′ *p*-Nitrophenyl Phosphates with Potassium *t*-Butoxide.—The ammonium salt of thymidine-3′ *p*-nitrophenyl phosphate (**3**)[1,13] was readily soluble in anhydrous dimethyl sulfoxide. On treatment with potassium *t*-butoxide in *t*-butyl alcohol at 20° there was a rapid release of *p*-nitrophenoxide (Figure 1). A preparative scale reaction in which the products were isolated after chroma-



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tography gave thymidine-3′,5′ cyclic phosphate in over 90% yield.[29] The nucleotide was characterized by its ready hydrolysis to thymine in *M* hydrochloric acid at 50°, as well as by its chromatographic and electrophoretic identity with authentic thymidine-3′,5′ cyclic phosphate.[30,31] Thus an initial aim of this investigation had been accomplished, *i.e.*, the synthesis of a nucleoside-3′,5′ cyclic phosphate by intramolecular phosphorylation in strong base under anhydrous conditions. However, for the reaction to have widest utility, especially for the synthesis of the biologically important ribonucleoside-3′,5′ cyclic phosphates,[20,32] it should also be applicable to nucleoside-5′ *p*-nitrophenyl phosphates.[1,33] When the sodium salt of thymidine-5′ *p*-nitrophenyl phosphate[1,34] in dimethyl sulfoxide was treated with excess potassium *t*-butoxide in *t*-butyl alcohol at 20° the release of *p*-nitrophenoxide was slower than in the reaction of the thymidine-3′ derivative (Figure 1). However, the product was again thymidine-3′,5′ cyclic phosphate, together with a small amount of thymidine-5′ phosphate.

(27) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 1194 (1957).

(28) In our experience *p*-nitrophenyl esters of nucleotides are stable at 20° in pyridine or dimethylformamide.[1] However, in a study of the reactions of nucleotides with tri-*p*-nitrophenyl phosphite in the presence of *p*-nitrophenol in similar media, such esters have been proposed as active phosphorylating agents: K. L. Agarwal and M. M. Dhar, *Indian J. Chem.*, **2**, 493 (1962).

(29) Apart from *p*-nitrophenol, the only other product was a thymidine phosphate, presumably produced by direct hydrolysis of the *p*-nitrophenyl ester. The extent of this hydrolysis was reduced by using freshly prepared potassium *t*-butoxide in *t*-butyl alcohol.

(30) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Am. Chem. Soc.*, **79**, 430 (1957).

(31) G. I. Drummond, M. W. Gilgan, E. J. Reiner, and M. Smith, *ibid.*, **86**, 1626 (1964).

(32) E. W. Sutherland and T. W. Rall, *Pharmacol. Rev.*, **12**, 265 (1960); D. F. Ashman, R. Lipton, M. M. Melicow, and T. D. Price, *Biochem. Biophys. Res. Commun.*, **11**, 330 (1963).

(33) Available data, both on the reaction of *p*-nitrophenyl esters with aqueous alkali,[13] and on the reaction of nucleotides with dicyclohexyl carbodiimides [M. Smith and H. G. Khorana, unpublished results; see D. H. Rammler, Y. Lapidot, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1989 (1963), footnote 21; D. Soll and H. G. Khorana, *ibid.*, **87**, 350 (1965)], indicate that 3′ derivatives are more easily converted to nucleoside-3′,5′ cyclic phosphates.

(34) J. G. Moffatt, reported in W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

Reaction of Deoxyadenosine-5′, Deoxycytidine-5′, and Deoxyguanosine-5′ *p*-Nitrophenyl Phosphates with Potassium *t*-Butoxide.—The reactions of the thymidine nucleotides described above established the practicability of intramolecular phosphorylation in strong base and also that there were no competing reactions involving either the ionized pyrimidine ring[6,35] or the phosphate group. The extension of the reaction to the *p*-nitrophenyl esters of deoxyadenosine-5′, deoxycytidine-5′, and deoxyguanosine-5′ phosphates was next investigated.[36] All of the esters as their ammonium salts were readily soluble in dimethyl sulfoxide and reacted with potassium *t*-butoxide in *t*-butyl alcohol at about the same rate as did the thymidine-5′ ester (Figure 1). However, there was some precipitation of the *p*-nitrophenyl esters of deoxyadenosine-5′ and deoxyguanosine-5′ phosphate under conditions which were satisfactory for thymidine nucleotides. This problem was eliminated by use of more dilute conditions in dimethyl sulfoxide. The conversion of each of the *p*-nitrophenyl esters to the deoxyribonucleoside-3′,5′ cyclic phosphate again was the major reaction, the only side product being a small amount of nucleoside phosphate presumably resulting from direct hydrolysis of the *p*-nitrophenyl esters.[29]

Reaction of Adenosine-5′, Cytidine-5′, Guanosine-5′, and Uridine-5′ *p*-Nitrophenyl Phosphates with Potassium *t*-Butoxide in *t*-Butyl alcohol.—The success of the base-catalyzed transesterification as a general synthetic route to deoxyribonucleoside-3′,5′ cyclic phosphates encouraged the extension of the reaction to the synthesis of ribonucleoside-3′,5′ cyclic phosphates both because of the differing chemistry and also because of the biochemical importance of this class of compounds.[38] All of the ribonucleoside-5′ *p*-nitrophenyl phosphates[1,19,39] reacted more readily with potassium *t*-butoxide in *t*-butyl alcohol than did thymidine-3′ *p*-nitrophenyl phosphate (Figure 2).[40] A preparative-scale reaction of adenosine-5′ *p*-nitrophenyl phosphate[1] produced adenosine-3′,5′ cyclic phosphate in excellent yield (90%) the only other nucleotide product being adenosine-5′ phosphate.[41] Adenosine-3′,5′ cyclic phosphate was characterized by its electrophoretic, chromatographic, and spectral properties, by its stability to acid, and by the ease of its hydrolysis by brain adenosine-3′,5′ cyclic phosphate diesterase.[20,42] There was no evidence for the presence of the 2′,5′ cyclic phosphate, because barium hydroxide hydrolysis yield only adenosine-5′ and adenosine-3′ phosphates.[20,43,44] The optical rotation of the cyclic phosphate was virtually identical with that of authentic adenosine-3′,5′ cyclic phosphate[45] as was its pmr spectrum.[46,47]

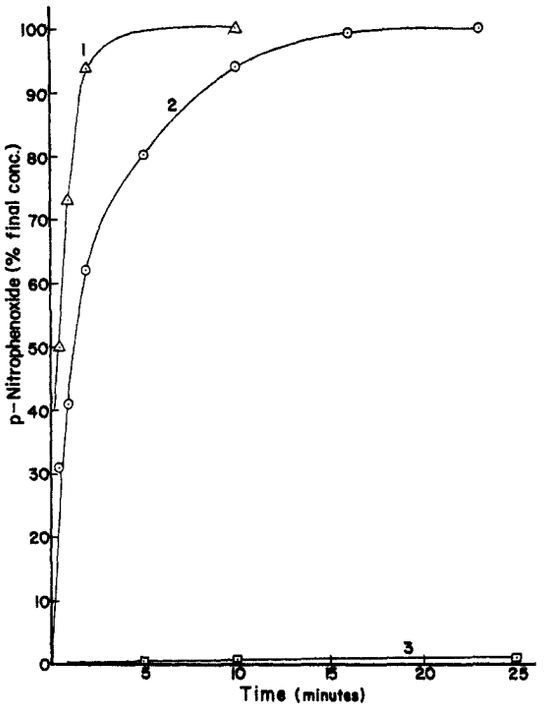

Figure 3.—Effect of solvent (1.9 ml) on rate of reaction of thymidine-5′ *p*-nitrophenyl phosphate (10 μmoles) with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml): curve 1, dimethylformamide; curve 2, dimethyl sulfoxide; curve 3, formamide.

The synthesis of cytidine-3′,5′, guanosine-3′,5′, and uridine-3′,5′ cyclic phosphates from the appropriate nucleoside-5′ *p*-nitrophenyl phosphates was achieved with equivalent ease.[48] Thus, the new procedure provides a general and efficient route to both deoxyribonucleoside-3′,5′ and ribonucleoside-3′,5′ cyclic phosphates.

Effect of Solvent and Base on Transesterification of Nucleoside-5′ *p*-Nitrophenyl Phosphates.—While dimethyl sulfoxide has been a favored solvent for potassium *t*-butoxide catalyzed reactions,[28] it was of interest to determine the utility of acetonitrile, formamide, and dimethylformamide[49] as solvents for the transesterification reaction. The sodium salt of thymidine-5′ *p*-nitrophenyl phosphate was insoluble in acetonitrile but was soluble in formamide and dimethylformamide. However, addition of potassium *t*-butoxide in *t*-butyl alcohol to the formamide solution did not result in the release of *p*-nitrophenoxide (Figure 3).[50] In dimethyl-

(35) R. Letters and A. M. Michelson, *J. Chem. Soc.*, 1410 (1961).

(36) The corresponding deoxyribonucleoside-3′ esters[37] were not investigated. By analogy with thymidine-3′ *p*-nitrophenyl phosphate, a rapid reaction would be anticipated.[38]

(37) W. Fiers and H. G. Khorana, *J. Biol. Chem.*, **238**, 2780 (1963). An alternate route to deoxyribonucleoside-3′ *p*-nitrophenyl phosphates is provided by the use of *p*-nitrophenyl phosphate and dicyclohexylcarbodiimide in dimethyl formamide as phosphorylating agent.[1]

(38) E. W. Sutherland, I. Øye, and R. W. Butcher, *Recent Progr. Hormone Res.*, **21**, 623 (1965); J. G. Hardman and E. W. Sutherland, *J. Biol. Chem.*, **240**, 3704 (1965).

(39) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 3741 (1957).

(40) This parallels the case of hydrolysis of nucleoside acyl esters.[6] Presumably intramolecular hydrogen bonding increases the ease of ionization of the 3′-hydroxyl group.

(41) The adenosine-5′ phosphate was shown, chromatographically, to be free of adenosine-3′ phosphate. This excludes the possibility of the nucleoside phosphate being produced from the nucleoside-3′,5′ cyclic phosphate.[20]

(42) G. I. Drummond and S. Perrott-Yee, *J. Biol. Chem.*, **236**, 1126 (1961).

(43) E. W. Sutherland and T. W. Rall, *ibid.*, **232**, 1077 (1958); D. Lipkin, W. H. Cook, and R. Markham, *J. Am. Chem. Soc.*, **81**, 6198 (1959).

(44) The ribonucleoside-2′ hydroxyl group is thought to be more acidic than the 3′-hydroxyl group.[6] Therefore, if the ease of cyclic phosphate formation is related to the acidity of the hydroxyl group, then the course of this reaction must be determined by the stereochemistry.

(45) D. Lipkin, R. Markham, and W. H. Cook, *J. Am. Chem. Soc.*, **81**, 6075 (1959).

(46) C. D. Jardetsky, *ibid.*, **84**, 62 (1962).

(47) Both these observations indicate that there was no appreciable anomerization at the glycosidic linkage.

(48) Guanosine-5′ *p*-nitrophenyl phosphate was least soluble in dimethyl sulfoxide containing potassium *t*-butoxide.

(49) These solvents have previously been useful in nucleotide chemistry.[25,28]

(50) Presumably this is a more acidic solution because of the amide protons in formamide. It is interesting to note that this system is completely adequate for the more facile transesterification of ribonucleate to ribonucleoside-2′,3′ cyclic phosphates.[26]

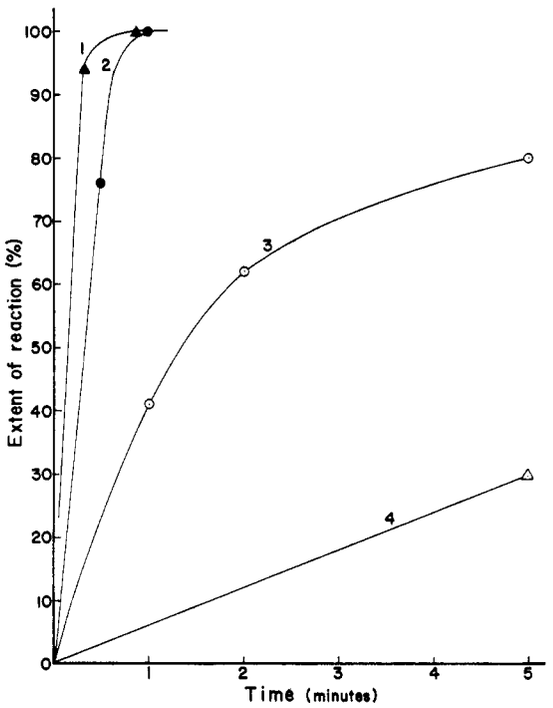

Figure 4.—Rates of reaction of derivatives of nucleoside-5′ phosphates (10 μmoles) and 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) with dimethyl sulfoxide (1.9 ml) as solvent: curve 1, adenosine-5′ *p*-nitrophenyl phosphate; curve 2, thymidine-5′ 2,4-dinitrophenyl phosphate; curve 3, thymidine-5′ *p*-nitrophenyl phosphate; curve 4, adenosine-5′ phosphorofluoridate.

formamide, reaction with potassium *t*-butoxide was more rapid than in dimethyl sulfoxide. However, at the concentrations employed, there was some precipitation of the *p*-nitrophenyl ester and reaction was incomplete.[51] Thus dimethyl sulfoxide is the preferred solvent, with dimethylformamide being next most useful.

The need for an ionizing base as catalyst for the transesterification of nucleoside-5′ *p*-nitrophenyl phosphates was next investigated. When the adenosine-5′ ester was treated with triethylamine in dimethyl sulfoxide at 100°, there was no reaction after 2 hr. This contrasts with the rapid conversion of ribonucleate to ribonucleoside-2′,3′ cyclic phosphates when treated with anhydrous ammonia in formamide,[52,53] and also with the studies of Agarwal and Dhar.[28]

Leaving Groups Other than *p*-Nitrophenoxide.—The scope of the transesterification reaction with regard to the leaving ester group was next investigated. The 2,4-dinitrophenyl group is known to hydrolyze readily[54] and therefore a nucleoside-5′ 2,4-dinitrophenyl phosphate[1,54,55] should be readily converted to the nucleoside-3′,5′ cyclic phosphate on reaction with potassium *t*-butoxide in anhydrous solvent. Thymidine-5′ 2,4-dinitrophenyl phosphate reacted more readily than did the corresponding *p*-nitrophenyl ester (Figure 4) and the major product was the nucleoside-3′,5′ cyclic phosphate.[56] Turning to alkyl esters of nucleotides, the stability of thymidylyl-(5′→3′)-thymidine[57] to potassium *t*-butoxide in dimethyl sulfoxide was next investigated. There was no detectable decomposition under conditions used in the synthesis of nucleoside-3′,5′ cyclic phosphates, nor under more extreme conditions. This, of course, encourages the hope that intermolecular phosphorylation in anhydrous alkali will be applicable to polynucleotide synthesis.[17]

Thus far, studies have been concentrated on intramolecular transesterification as the route to nucleoside-3′,5′ cyclic phosphates. However, other leaving groups, principally phosphate, have been effective in base-catalyzed cyclic phosphate formation.[5,58] Particularly relevant in the present study are the formation of six-membered cyclic phosphates; pantothenic-2′,4′ cyclic phosphate from coenzyme A,[59] adenosine-3′,5′ cyclic phosphate from adenosine-5′ triphosphate on reaction with barium hydroxide,[43,60] and adenosine-3′,5′ cyclic phosphate from P^1,P^2-diadenosine-5′ pyrophosphate in hot pyridine.[20]

When thymidine-5′ pyrophosphate or P^1,P^2-dithymidine-5′ pyrophosphate was treated with potassium *t*-butoxide in dimethyl sulfoxide at 20°, no reaction was detected. However, P^1-diphenyl-P^2-nucleoside-5′ pyrophosphate[55] was rapidly converted to the nucleoside-3′,5′ cyclic phosphate. Similarly, adenosine-5′ phosphorofluoridate,[61] which is partially converted to the nucleoside-3′,5′ cyclic phosphate in aqueous alkali, was quantitatively converted to the cyclic phosphate in potassium *t*-butoxide in dimethyl sulfoxide. Thus the scope of the reaction is not restricted to transesterification. The ease with which the differing leaving groups are displaced decreases in the order diphenyl phosphate, 2,4-dinitrophenoxide, *p*-nitrophenoxide, and fluoride.[62] The successful application of phosphorylation in strong anhydrous base to a general synthesis of nucleoside-3′,5′ cyclic phosphates encourages the hope that this type of reaction will find wider utility in nucleotide chemistry.

Experimental Section

General Methods.—Molar potassium *t*-butoxide was prepared by dissolving the appropriate amount of potassium in *t*-butyl alcohol which had been recently distilled from calcium hydride. Reagent grade dimethyl sulfoxide, dimethylformamide, formamide, and acetonitrile were dried over Linde 4-A molecular sieves. Chromatography was carried out by descending technique in solvent 1, isopropyl alcohol–concentrated ammonia–water (7:1:2); solvent 2, *n*-butyl alcohol–acetic acid–water (5:2:3); solvent 3, isobutyrate acid–concentrated ammonia–water(66:1:33); solvent 4, saturated ammonium sulfate–1 *M* sodium acetate–isopropyl alcohol (80:18:2); and solvent 5, 0.5 *M* ammonium acetate–ethyl alcohol (2:5). Nucleotides were detected under ultraviolet light. Chromatographic mo-

(51) When the reaction was followed chromatographically rather than spectrophotometrically, the precipitated ester made the reaction appear to be slower than it was.[16]

(52) K. Tanaka, *J. Biochem.* (Tokyo), **47**, 398 (1960).

(53) This again emphasises the relative ease with which five-membered cyclic phosphates are produced by transeterification.[5]

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

(55) A. M. Michelson, *Biochem. Biophys. Acta*, **91**, 1 (1964).

(56) The instability of nucleoside-5′ 2,4-dinitrophenyl phosphates[1,54,55] makes them less convenient precursors than the *p*-nitrophenyl esters.

(57) H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3828 (1963).

(58) H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *ibid.*, **79**, 430 (1957).

(59) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 3783 (1952).

(60) Some conversion of adenosine-5′ triphosphate to adenosine-3′,5′ cyclic phosphate has been detected in less basic aqueous solution: J. B. Posner, K. E. Hammermeister, G. E. Bratvold, and E. G. Krebs, *Biochemistry*, **3**, 1040 (1964).

(61) R. Witmann, *Chem. Ber.*, **96**, 771 (1963).

(62) With the techniques available, it was not possible to differentiate the rates of displacement of diphenyl phosphate and 2,4-dinitrophenoxide. However, the work of Michelson[55] indicates that the diphenyl phosphate should be most easily displaced.

bilities are recorded in Table I. Electrophoresis was carried out on Whatman 3MM paper using a field strength of 20 v/cm in 0.05 *M* acetic acid, pH 3.0, and in 0.05 *M* triethylammonium carbonate, pH 9.0. Hydrolyses of nucleoside-3′,5′ cyclic phosphates in 1 *M* hydrochloric acid were carried out as previously described as were enzymic hydrolyses using adenosine-3′,5′ cyclic phosphate diesterase from brain.[20,31,42] Ultraviolet absorption spectra were recorded on Beckman DK or DB spectrophotometers and compared with those obtained from authentic samples of nucleotides at pH 7.0 and 12.0 for thymine and uracil nucleotides, at pH 2.0 and 7.0 for adenine and cytosine nucleotides, and at pH 2.0, 7.0, and 12.0 for guanine nucleotides.

TABLE I

R_f VALUES OF NUCLEOTIDES

Compound	Solvent 1	Solvent 2	Solvent 3	Solvent 4	Solvent 5
Adenosine-5′ phosphate	0.08	0.15	0.30	0.32	0.20
Adenosine-2′ phosphate	0.15			0.27	
Adenosine-3′ phosphate	0.15			0.18	
Adenosine-3′,5′ cyclic phosphate	0.39	0.31	0.50		0.51
Adenosine-5′ *p*-nitrophenyl phosphate	0.53	0.49			
Adenosine-5′ 2,4-dinitrophenyl phosphate	0.55	0.55			
Adenosine-5′ phosphorofluoridate	0.26	0.44			
P¹,P²-Diadenosine-5′ pyrophosphate	0.08	0.05	0.15		0.13
Adenine	0.49				
Cytidine-5′ phosphate	0.07		0.25		
Cytidine-3′,5′ cyclic phosphate	0.34	0.25	0.36		
Cytidine-5′ *p*-nitrophenyl phosphate	0.53				
Guanosine-5′ phosphate	0.04		0.11		
Guanosine-3′,5′ cyclic phosphate	0.22	0.25	0.22		
Guanosine-5′ *p*-nitrophenyl phosphate	0.39				
Uridine-5′ phosphate	0.06		0.15		
Uridine-3′,5′ cyclic phosphate	0.29	0.31	0.25		
Uridine-5′ *p*-nitrophenyl phosphate	0.48				
Deoxyadenosine-5′ phosphate	0.08				
Deoxyadenosine-3′,5′ cyclic phosphate	0.41				
Deoxyadenosine-5′ *p*-nitrophenyl phosphate	0.63				
Deoxycytidine-5′ phosphate	0.10				
Deoxycytidine-3′,5′ cyclic phosphate	0.44				
Deoxycytidine-5′ *p*-nitrophenyl phosphate	0.58				
Deoxyguanosine-5′ phosphate	0.05				
Deoxyguanosine-3′,5′ cyclic phosphate	0.25				
Deoxyguanosine-5′ *p*-nitrophenyl phosphate	0.47				
Thymidine-5′ phosphate	0.12	0.20	0.36		
Thymidine-3′ phosphate	0.12				
Thymidine-3′,5′ cyclic phosphate	0.48	0.27	0.46		
Thymidine-5′ *p*-nitrophenyl phosphate	0.70	0.51	0.64		
Thymidine-3′ *p*-nitrophenyl phosphate	0.72	0.56	0.69		
Thymidine-5′ 2,4-dinitrophenyl phosphate	0.61				
Thymidine-5′ pyrophosphate	0.02				
P¹,P²-Dithymidine-5′ pyrophosphate	0.24				
Thymine	0.69				

Thymidine-3′,5′ Cyclic Phosphate from Thymidine-3′ *p*-Nitrophenyl Phosphate.—The ammonium salt of the *p*-nitrophenyl ester (20 μmoles)[1] in dimethyl sulfoxide (2.0 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (1.0 ml) at 20°. The solution became bright yellow immediately on addition of the base. After 5 min the reaction was neutralized with Dowex 50 W cation exchanger (pyridinium salt, 2.0 ml of wet resin). The resin was removed by filtration and washed thoroughly with water. The combined filtrate and washings were examined chromatographically in solvent 1, where only thymidine-3′,5′ cyclic phosphate and *p*-nitrophenol were detected, after which the reaction products were passed onto a column of diethylaminoethylcellulose (20 × 1.5 cm diameter). Products were eluted using a linear salt gradient with water (500 ml) in the mixing chamber and 0.1 *M* triethyl ammonium bicarbonate (500 ml) in the reservoir. Fractions were collected at 5-min intervals and the elution of products was followed spectrophotometrically. Thymidine-3′,5′ cyclic phosphate (18.5 μmoles, estimated spectrophotometrically) was eluted in fractions 20 to 25, *p*-nitrophenol in fractions 28 to 34, and what is presumably thymidine-3′ phosphate (1.5 μmoles) in fractions 40 to 46 (R_f 0.18 in solvent 1). No thymidine-3′ *p*-nitrophenyl phosphate was detectable.[1] The thymidine-3′,5′ cyclic phosphate was then chromatographed in solvent 1 and also in solvents 2 and 3. It was examined electrophoretically at pH 3.0 and 9.0. It was homogeneous under all conditions. The nucleotide was rapidly converted to thymine on treatment with 1 *M* hydrochloric acid at 50°.[31]

Thymidine-3′,5′ Cyclic Phosphate from Thymidine-5′ *p*-Nitrophenyl Phosphate.—The sodium salt of thymidine-5′ *p*-nitrophenyl phosphate (20 μmoles) in dimethyl sulfoxide (2.0 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (1.0 ml) at 20°. On addition of the base the solution turned yellow. After 30 min the reaction was neutralized with Dowex 50 W cation exchanger (pyridinium form) and the products were separated on diethylaminoethyl cellulose as in the reaction of thymidine-3′ *p*-nitrophenyl phosphate. Fractions 31 to 41 contained thymidine-3′,5′ cyclic phosphate (17 μmoles) and thymidine-5′ *p*-nitrophenyl phosphate (1.5 μmoles) which were separated by preparative chromatography in solvent 1 and estimated spectrophotometrically. Fractions 42 and 50 contained *p*-nitrophenol. Later fractions contained a trace of thymidine-5′ phosphate, together with some unidentified ultraviolet absorbing substances. Ion-exchange chromatography later showed that these were contaminants of the commercially obtained starting material. The thymidine-3′,5′ cyclic phosphate was homogeneous in solvents 1, 2, and 3 on electrophoresis at pH 3.0 and 9.0 and was hydrolyzed to thymine in 1 *M* hydrochloric acid at 50° at the same rate as authentic thymidine-3′,5′ cyclic phosphate.[31]

Reaction went to completion when the *p*-nitrophenyl ester (20 mmoles) in dimethyl sulfoxide (1.8 ml) was allowed to react with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) at 20° for 1 hr.

Deoxyadenosine-3′,5′ Cyclic Phosphate.—The ammonium salt of deoxyadenosine-5′ *p*-nitrophenyl phosphate (15 μmoles) in dimethyl sulfoxide (2.0 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (1.0 ml) at 20°. The solution became bright yellow on addition of base and some precipitate appeared. After 30 min the reaction was neutralized and the products were isolated on diethylaminoethylcellulose as in the preparation of thymidine-3′,5′ cyclic phosphate. Fractions 27 to 31 contained deoxyadenosine-3′,5′ cyclic phosphate (10 μmoles) and deoxyadenosine-5′ *p*-nitrophenyl phosphate (5 μmoles) which were separated chromatographically in solvent 1 and estimated spectrophotometrically. Fractions 32 to 36 contained *p*-nitrophenol and fractions 55 to 60 contained a trace of material, presumably deoxyadenosine-5′ phosphate. The deoxyadenosine-3′,5′ cyclic phosphate was characterized by chromatography in solvent 1, by electrophoresis at pH 3.0 and 9.0, by its spectral properties, by its stability in 1 *M* hydrochloric acid at 50°,[31] and by its rate of hydrolysis by adenosine-3′,5′ cyclic phosphate diesterase from brain.[31,63]

(63) Dr. G. I. Drummond kindly provided a preparation from rabbit brain. The enzyme hydrolyzed adenosine-3′,5′ cyclic phosphate at the rate of 10 μmoles/mg protein/hr.

In another experiment, where the *p*-nitrophenyl ester (10 μmoles) in dimethyl sulfoxide (1.8 ml) and 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.2 ml) were allowed to react at 20°, there was no detectable precipitate on addition of the base and the reaction to form deoxyadenosine-3′,5′ cyclic phosphate was complete, as judged by chromatography in solvent 1.

Deoxycytidine-3′,5′ Cyclic Phosphate.—The triethylammonium salt of deoxycytidine-5′ *p*-nitrophenyl phosphate (20 μmoles) in dimethyl sulfoxide (2.0 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (1.0 ml) at 20° as in the reaction of thymidine-5′ *p*-nitrophenyl phosphate. After neutralization and chromatography on diethylaminoethylcellulose, deoxycytidine-3′,5′ cyclic phosphate (13 μmoles) was eluted in fractions 10 to 15, and *p*-nitrophenol in fractions 30 to 35. The nucleotide was characterized by its spectral properties and its homogeneity on chromatography in solvent 1 and on electrophoresis at pH 3.0. These properties indicated that there was no contamination with deoxyuridine-3′,5′ cyclic phosphate which could have been a product if hydrolytic deamination had occurred.[20]

Deoxyguanosine-3′,5′ Cyclic Phosphate.—The ammonium salt of deoxyguanosine-5′ *p*-nitrophenyl phosphate (50 μmoles) in dimethyl sulfoxide (9.0 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (1.0 ml) at 20°. The solution became yellow immediately on addition of base, but no precipitate was produced. After 1 hr the reaction was neutralized with cation exchanger (Amberlite IR-120, ammonium form). The resin was washed well with water and the combined aqueous solutions were extracted thoroughly (five times) with diethyl ether to remove dinitrophenol. The products were then passed onto a diethylaminoethylcellulose column (25 × 2.5 cm diameter) and eluted using a linear gradient system with water (1.5 l.) in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (1.5 l.) in the reservoir. Fractions (20 ml) were collected every 10 mins. Deoxyguanosine-3′,5′ cyclic phosphate (37 μmoles) was eluted in fractions 42 to 47 and, presumably, deoxyguanosine-5′ phosphate (8 μmoles) in fractions 88 to 96. The nucleotides were characterized by their spectral properties and by chromatography in solvent 1. The triethylammonium salt of the residual deoxyguanosine-3′,5′ cyclic phosphate was converted to its ammonium salt by passage through Dowex 50 W, 2% cross-linked resin in the ammonium form, and isolated as a white powder after freeze drying. This procedure is the most convenient way of isolating nucleoside-3′,5′ cyclic phosphates in solid form.

Adenosine-3′,5′ Cyclic Phosphate.—Adenosine-5′ *p*-nitrophenyl phosphate (200 μmoles), as the sodium salt, in dimethyl sulfoxide (18 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (2 ml) at 20°. After 5 min the reaction was neutralized with a cation exchanger (excess Amberlite IR-120, ammonium form). The resin was washed thoroughly with dilute, aqueous ammonia. After removal of excess ammonia under reduced pressure, the products were passed onto a diethylaminoethylcellulose column and eluted as in the preparation of deoxyguanosine-3′,5′ cyclic phosphate. Adenosine-3′,5′ cyclic phosphate (181 μmoles) was eluted in fractions 36 to 44 followed by *p*-nitrophenol[64] in fractions 45 to 58 and by adenosine-5′ phosphate[65] (5 μmoles) in fractions 66 to 68. The adenosine-3′,5′ cyclic phosphate was estimated spectrophotometrically and the triethylammonium salt was obtained as a gum after removal of triethylammonium bicarbonate by evaporation. The nucleotide was dissolved in water and the optical rotation was determined, $[\alpha]_D$ − 43.0°. (The rotation of an equivalent amount of authentic triethylammonium adenosine-3′,5′ cyclic phosphate was also determined, $[\alpha]_D$ −48.0°.) The total preparation of adenosine-3′,5′ cyclic phosphate was transferred to deuterium oxide and the pmr spectrum was determined. It was identical with the spectrum of an authentic sample of the triethylammonium salt of adenosine-3′,5′ cyclic phosphate.[44,66] The nucleotide was also degraded to adenosine-5′ phosphate (characterized in solvents 1 and 4) by brain adenosine-3′,5′ cyclic phosphate diesterase at the same rate as authentic adenosine-3′,5′ cyclic phosphate[20,42,63] and was also degraded with barium hydroxide to a mixture of adenosine-3′ phosphate and adenosine-5′ phosphate (characterized in solvent 4).[20] The nucleotide was homogeneous on chromatography in solvents 1, 2, and 3 and on electrophoresis at pH 7.0 and 9.0. It had the same stability to 1 *M* hydrochloric acid at 100° as did authentic adenosine-3′,5′ cyclic phosphate and also crystallized readily from ethanolic hydrochloric acid.[20]

Guanosine-3′,5′ Cyclic Phosphate.—The ammonium salt of guanosine-5′ *p*-nitrophenyl phosphate (40 μmoles)[67] in dimethyl sulfoxide (4.5 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.5 ml) at 20°. The mixture became yellow on addition of the base and a precipitate appeared after a few seconds.[68] After 30 min the products were isolated by ion-exchange chromatography as in the synthesis of deoxyguanosine-3′,5′ cyclic phosphate. Guanosine-5′ *p*-nitrophenyl phosphate (10 μmoles) was eluted in fractions 54 to 61 and guanosine-3′,5′ cyclic phosphate (28 μmoles) in fractions 63 to 75. The cyclic phosphate was converted to the ammonium salt and isolated as a white powder after freeze drying (11 mg). It was homogeneous on chromatography in solvents 1, 2, and 3 and on electrophoresis at pH 3.0 and 9.0. The adenosine-3′,5′ cyclic phosphate diesterase[63] completely hydrolyzed the cyclic phosphate to guanosine-5′ phosphate.[20,42]

Cytidine-3′5′ Cyclic Phosphate.—Cytidine-5′ *p*-nitrophenyl phosphate (20 μmoles, sodium salt) in dimethyl sulfoxide (1.8 ml) was allowed to react with 1 *M* potassium *t*-butoxide (0.2 ml) at 20°. After 5 min the reaction was neutralized with cation exchanger as in the preparation of adenosine-3′,5′ cyclic phosphate and passed onto a column (15 × 1.5 cm diameter) of diethylaminoethylcellulose in the carbonate form. The products were eluted using a linear gradient system with water (500 ml) in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (500 ml) in the reservoir. Fractions (10 ml) were collected at 5-min intervals. Cytidine-3′,5′ cyclic phosphate (18 μmoles) was eluted in fractions 23 to 26, *p*-nitrophenol in fractions 39 to 44, and cytidine-5′ phosphate (1 μmole) in fractions 51 to 55. Cytidine-3′,5′ cyclic phosphate was characterized by its spectral properties, by its homogeneity on chromatography in solvents 1, 2, and 3, and on electrophoresis at pH 3.0 and 9.0. Uridine-3′,5′ cyclic phosphate was not detectable.

Uridine-3′,5′ Cyclic Phosphate.—The sodium salt of uridine-5′ *p*-nitrophenyl phosphate (20 μmoles) in dimethyl sulfoxide (1.8 ml) was treated with 1 *M* potassium *t*-butoxide (0.2 ml) as in the preparation of cytidine-3′,5′ cyclic phosphate. After work-up and ion-exchange chromatography as in that preparation, uridine-3′,5′ cyclic phosphate was obtained together with uridine-5′ *p*-nitrophenyl phosphate and *p*-nitrophenol in fractions 19 to 28 followed by a trace of uridine-5′ phosphate in fractions 45 to 47. After removal of triethylammonium bicarbonate under reduced pressure, the combined fractions 19 to 28 were chromatographed in solvent 1 and the uridine-3′,5′ cyclic phosphate was isolated after elution with water. The preparation was homogeneous and identical with authentic uridine-3′,5′ cyclic phosphate in solvents 1, 2, and 3 and on electrophoresis at pH 3.0 and 9.0.

Rates of Reaction of Nucleoside *p*-Nitrophenyl Phosphates with Potassium *t*-Butoxide in Dimethyl Sulfoxide.—The *p*-nitrophenyl esters of thymidine-3′, thymidine-5′, deoxyadenosine-5′, deoxycytidine-5′, deoxyguanosine-5′, adenosine-5′, cytidine-5′, guanosine-5′, and uridine-5′ phosphates (approximately 10 μmoles) in dimethyl sulfoxide (1.9 ml) were treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) at 20°. At intervals, aliquots (100 μl) were removed and diluted into water (5 ml). The *p*-nitrophenoxide was estimated spectrophotometrically at 400 mμ. Results are recorded in Figures 1 and 2.

Reaction of Thymidine-5′ *p*-Nitrophenyl Phosphate with Potassium *t*-Butoxide in Various Solvents.—The nucleotide as its sodium salt (10 μmoles) in the appropriate solvent (1.9 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) at 20°. At intervals, aliquots (100 μl) were removed and diluted into water (5 ml); *p*-nitrophenoxide was estimated at 400 mμ. The nucleotide did not appear to dissolve to any extent in acetonitrite and there was no release of *p*-nitrophenoxide

(64) Extraction of the *p*-nitrophenol with ether prior to ion-exchange chromatography, as in the synthesis of deoxyguanosine-3′,5′ cyclic phosphate, is a more generally useful method for purification.

(65) In the case of the ribonucleotides it was possible to show by chromatography that this fraction was free of nucleoside-3′ phosphate. Consequently it must have arisen from the nucleoside-5′ *p*-nitrophenyl phosphate rather than the nucleoside-3′,5′ cyclic phosphate.[20]

(66) We are indebted to Dr. L. D. Hall for this determination.

(67) Although triethylammonium, sodium, or ammonium salts have been used in this work, the latter appear to be the most generally convenient form in which to store nucleotide *p*-nitrophenyl esters.[1]

(68) Presumably this is the potassium salt of the *p*-nitrophenyl ester and the precipitation could be avoided by using the conditions described for the synthesis of deoxyguanosine-3′,5′ cyclic phosphate.

90 min after addition of the base. The nucleotide was completely soluble in formamide, but there was no release of *p*-nitrophenoxide 90 min after addition of the base. Chromatography in solvent 1 confirmed that no reaction had taken place. The nucleotide was completely soluble in dimethylformamide and on addition of base the solution immediately turned yellow. A slight precipitate was produced at the same time. When the release of *p*-nitrophenoxide was complete, the product was examined chromatographically in solvent 1. The major product was thymidine-3′,5′ cyclic phosphate; however, some *p*-nitrophenyl ester was present. Presumably this was precipitated on addition of the base. Rates of reactions are depicted in Figure 3.

Attempted Reaction of Adenosine-5′ *p*-Nitrophenyl Phosphate with Triethylamine in Dimethyl Sulfoxide.—The nucleotide as its sodium salt (5 μmoles) in dimethyl sulphoxide (1.0 ml) containing triethylamine (1.0 mmole) was heated at 100° for 15 min. There was no release of *p*-nitrophenoxide.

Treatment of Thymidine-5′ 2,4-Dinitrophenyl Phosphate, P¹,P²-Dithymidine-5′ Pyrophosphate, Thymidine-5′ Pyrophosphate, and Thymidylyl-(5′→3′)-thymidine with Potassium *t*-Butoxide in Dimethyl Sulfoxide.—The nucleotide (approximately 10 μmoles) in dimethyl sulfoxide (1.9 ml) was treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) at 20°. With thymidine-5′ 2,4-dinitrophenyl phosphate[1] the release of 2,4-dinitrophenoxide was followed spectrophotometrically at 400 mμ and was complete in 1 min (Figure 4). Chromatography in solvent 1 showed that the product was thymidine-3′,5′ cyclic phosphate together with a lesser amount of thymidine-5′ phosphate.

After 30 min, chromatography in solvent showed that P¹,P²-dithymidine-5′ pyrophosphate, thymidine-5′ pyrophosphate, and thymidylyl-(5′→3′)-thymidine were completely unaffected.

Treatment of Adenosine-5′ 2,4-Dinitrophenyl Phosphate, P¹-Diphenyl-P²-Adenosine-5′ Pyrophosphate, and Adenosine-5′ Phosphorofluoridate with Potassium *t*-Butoxide in Dimethyl Sulfoxide.—The nucleotides (approximately 10 μmoles) in dimethyl sulfoxide (1.9 ml) were treated with 1 *M* potassium *t*-butoxide in *t*-butyl alcohol (0.1 ml) at 20°. Aliquots were removed at intervals, neutralized with Dowex 50 W (ammonium form), and examined chromatographically in solvents 1 and 2 except with P¹-diphenyl-P²-adenosine-5′ pyrophosphate[55] where solvent 5 was used. Reaction was complete in less than 5 min for the first two nucleotides (first aliquot) the products being adenosine-3′,5′ cyclic phosphate together with traces of adenosine-5′ phosphate and, except in the reaction of the phosphorofluoridate, P¹,P²-diadenosine-5′ pyrophosphate. With adenosine-5′ phosphorofluoridate[61] conversion to adenosine-3′,5′ cyclic phosphate was complete in 1 hr (Figure 4).

Studies on Geometric Isomerism by Nuclear Magnetic Resonance. III. Stereochemistry of α-Cyanocinnamic Esters[1]

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The nmr technique has revealed that the Cope–Knoevenagel reaction of aromatic ketones with cyanoacetic esters gives a stereoisomeric mixture of α-cyanocinnamic esters. Configurational assignments have been made by the analysis of the chemical shifts of the carboaluxy group; the signal for the *trans* isomer appears at lower field than that for the *cis* isomer. It has been found that, as the size of a β substituent becomes larger, the amount of the *cis* isomer increases. Further, on the basis of some assumption the angle of twist of the benzene ring from coplanarity has been calculated.

In the preceding paper[1] it has been established that the Cope–Knoevenagel reaction of unsymmetrical aliphatic ketones with cyanoacetic esters leads to the preferential formation of the isomers in which the bulkier alkyl group and the carboalkoxy group are on opposite sides of the carbon–carbon double bond. On the other hand, previous investigators[2,3] have shown that the condensation of aromatic aldehydes with cyanoacetic esters gives only α-cyano-*trans*-cinnamic esters. These facts agree with what would be expected on steric grounds, because the bulkiest groups are at a distance. In the course of study, however, it was found that the condensation of some aromatic ketones with cyanoacetic esters leads to the predominant formation of the *cis* isomers.[4] This appeared not to agree with the steric considerations. Therefore, it is of interest to determine which of two stereoisomers would be preferentially produced.

Since, however, no stereoisomer of a known configuration was available, assignments should be made according to the same method as had been used in the previous papers.[1,5] However, in the present paper we wish to show that it is much better to assign configurations by the analysis of the chemical shifts of the carboalkoxy protons. Further, we have discussed the conformations of these esters on the basis of the nuclear magnetic resonance and ultraviolet spectroscopic studies.

Results and Discussion

Proton resonance data for the esters studied in the present work are given in Tables I and II, together with the stereoisomeric composition. Representative nmr spectra of the esters are shown in Figures 1 and 2.

As seen from the tables, the chemical shifts for the alkyl and aryl groups on the β position are practically unchanged on going from the carbomethoxy group to the carboethoxy group. This fact was often useful not only in differentiating the signals of the ester alkyl protons from those of other alkyl groups, but also in assigning the signals superimposed upon by those of either the carbomethoxy group or the carboethoxy group.

Assignments.—In the preceding papers[1,5] the author has determined the geometric configurations of the compounds of the $R_1R_2C{=}C(CN)\ COOR_3$ type on the basis of some assumption, which states that the chemical shift for the β-methyl group occurs at a higher field in the *trans* than in the *cis* configuration with respect to the carboalkoxy group, whereas the reverse is true for the γ-methyl. However, it is doubtful whether

(1) Part II: T. Hayashi, M. Igarashi, S. Hayashi, and H. Midorikawa, *Bull. Chem. Soc. Japan*, **38**, 2063 (1965). Presented partly at the 17th Annual Meeting of the Chemical Society of Japan, Tokyo, April 1964.

(2) W. Baker and C. S. Howes, *J. Chem. Soc.*, 119 (1953).

(3) J. Zabicky, *ibid.*, 683 (1961).

(4) In the present paper the isomer, in which the phenyl and carboalkoxy groups are on opposite sides of the carbon–carbon double bond, is referred to as the *trans* isomer.

(5) T. Hayashi, I. Hori, H. Baba, and H. Midorikawa, *J. Org. Chem.*, **30**, 695 (1965).