for **16**. However, on the basis of the demonstrated structure of **17** and the known course of the Diels–Alder reaction, there is no apparent reason to challenge the postulated structures.

The bicyclic structures showed typical norbornene spectra.[17] Compound **23** showed the following signals: vinylic, τ = 3.55 and 3.83 ppm (broadened AB, J_{AB} = 8 cps); C*H*Cl, τ = 5.3 ppm (broad); bridgehead, τ = 6.83 ppm (broad); and ring protons at τ = 7.95–8.5 ppm. Similar parameters were observed for **18**, although the vinylic AB (τ = 3.92 ppm) was not resolved. Ester absorptions were normal in each case and were doubled in the case of **18** due to the presence of *endo* and *exo* isomers.

Similarly, the postulated structures of reduction products were supported by their pmr spectra. The spectrum of the mixture of **10** and **11** is typical: $POCH_2CH_3$, τ = 5.98 and 8.72 ppm; $CHCH_3$, two doublets, τ = 8.96 and 9.08 ppm, J_{HH} = 6.8 cps; ring hydrogens, featureless multiplet, τ = 7.8–9.0 ppm.

The infrared spectra of the adducts showed the expected absorptions in the normal ranges: ν_{PO} 1228–1258, ν_{POC} 1022–1028, 1048–1060, ν_{POEt} 1147–1163, $\nu_{C=C}$ 1641–1655, and a band at 741–756 cm^{-1} tentatively assigned as the *cis*-olefinic out-of-plane C–H deformation.

Acknowledgment.—We are indebted to Drs. M. Gordon and M. P. Williamson and Mr. W. E. Byrne for the determination of pmr spectra.

Nucleotide Synthesis. II. Nucleotide *p*-Nitrophenyl and 2,4-Dinitrophenyl Esters[1,2]

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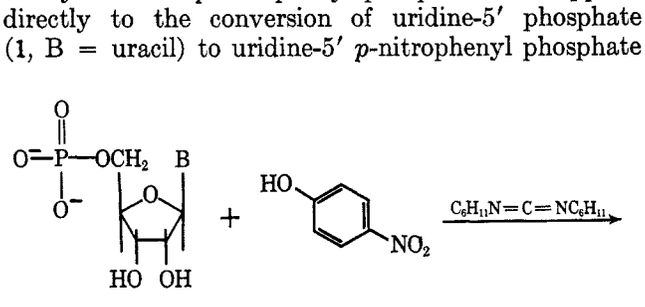
The syntheses of deoxyribonucleoside-5′ and ribonucleoside-5′ *p*-nitrophenyl phosphates by reaction of nucleoside-5′ phosphates with dicyclohexylcarbodiimide and *p*-nitrophenol are described. The reaction of *p*-nitrophenol phosphate and dicyclohexylcarbodiimide with suitably protected nucleosides proves a convenient new route to both nucleoside-5′ *p*-nitrophenyl phosphates and deoxyribonucleoside-3′ *p*-nitrophenyl phosphates. Nucleoside-5′ 2,4-dinitrophenyl phosphates are produced by reaction of nucleoside-5′ phosphates with dicyclohexylcarbodiimide and 2,4-dinitrophenol in the absence of pyridine. Where pyridine is present, the P^1,P^2-dinucleoside-5′ pyrophosphate is produced. The reactions of 2,4-dinitrofluorobenzene and related compounds with nucleoside-5′ phosphates are discussed.

Nucleoside-5′ and nucleoside-3′ *p*-nitrophenyl phosphates are effective and specific substrates for phosphodiesterases.[4–6] Recently it has been shown that both classes of diester are readily converted to nucleoside-3,′5′ cyclic phosphates on reaction with potassium *t*-butoxide in anhydrous solvent.[7] This reaction, which has been applied to the synthesis of both deoxyribonucleoside-3′,5′ and ribonucleoside-3′,5′ cyclic phosphates, also takes place when nucleoside-5′ 2,4-dinitrophenyl esters are treated with potassium *t*-butoxide in anhydrous solvent.[8]

A number of procedures have been applied to the synthesis of nucleotide *p*-nitrophenyl esters. Guanosine-5′ and uridine-5′ *p*-nitrophenyl phosphates have been prepared from suitably protected nucleosides using tetra-*p*-nitrophenyl pyrophosphate as phosphorylating agent.[9,10] Di-*p*-nitrophenyl phosphorochloridate has been used in the synthesis of uridine-5′ *p*-nitrophenyl phosphate[11] and the related reagent, *p*-nitrophenyl phosphorodichloridate,[12] has been used very successfully in the synthesis of deoxyadenosine-3′, deoxycytidine-3′, deoxyguanosine-3′, and thymidine-3′ *p*-nitrophenyl phosphates.[6,13] Thymidine-5′ *p*-nitrophenyl phosphate has been prepared by reaction of thymidine-5′ phosphate with dicyclohexylcarbodiimide *p*-nitrophenol in pyridine in the presence of triethylamine.[14] Because of the ready availability of nucleoside-5′ phosphates this last approach has been extended to other nucleoside derivatives in the present study. Thus, the reaction conditions employed in the synthesis of thymidine-5′ *p*-nitrophenyl phosphate were applied directly to the conversion of uridine-5′ phosphate (1, B = uracil) to uridine-5′ *p*-nitrophenyl phosphate



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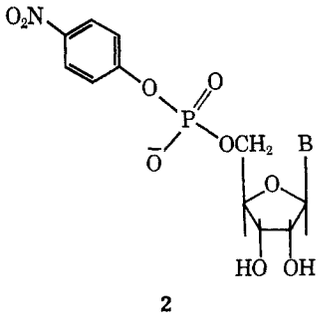

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(1) Part I: G. I. Drummond, M. W. Gilgan, E. J. Reiner, and M. Smith, *J. Am. Chem. Soc.*, **86**, 1626 (1964).

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

(5) W. E. Razzell and H. G. Khorana, *ibid.*, **236**, 1144 (1961).

(6) W. Fiers and H. G. Khorana, *ibid.*, **238**, 2780 (1963).

(7) M. Smith, *J. Am. Chem. Soc.*, **86**, 3586 (1964).

(8) R. K. Borden and M. Smith, *J. Org. Chem.*, **31**, 3247 (1966).

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

(10) R. W. Chambers, J. G. Moffatt, and H. G. Khorana, *ibid.*, **79**, 3747 (1957).

(11) Y. Anraku, *J. Biol. Chem.*, **239**, 3412 (1964); T. Ukita and H. Hayatsu, *J. Am. Chem. Soc.*, **84**, 1879 (1962).

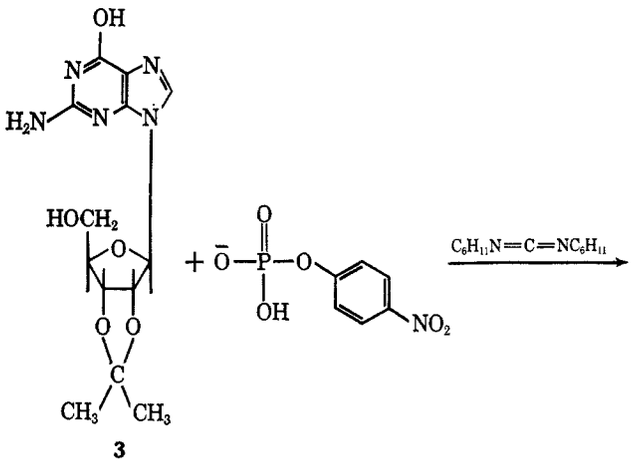
(12) G. R. Cebrian, *Anales Real. Soc. Espan. Fis. Quim.* (Madrid), **47B**, 841 (1951); *Chem. Abstr.*, **46**, 11140 (1952).

(13) A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4651 (1959).

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

(2, B = uracil). In the reaction of adenosine-5′ phosphate, the nucleotide was suspended in a mixture of pyridine and dimethylformamide[15] containing 2 equiv of triethylamine,[16,17] 10 equiv of *p*-nitrophenol, and 10 equiv of dicyclohexylcarbodiimide and shaken in the dark for 7 days. After work-up and ion-exchange chromatography on diethylaminoethyl cellulose, adenosine-5′ *p*-nitrophenyl phosphate (2) was isolated as its ammonium salt[18] in 41% yield the other product of the reaction being P^1,P^2-diadenosine-5′ pyrophosphate. When the triethylamine was replaced by tributylamine, a homogeneous solution was obtained. Chromatography indicated that the adenosine-5′ phosphate had reacted completely in 48 hr; however, the yield of *p*-nitrophenyl ester was somewhat lower.[19] Synthesis of deoxyadenosine-5′ *p*-nitrophenyl phosphate using the heterogeneous system in the presence of triethylamine gave a 40% yield of the isolated ammonium salt of the ester. Cytidine-5′ and deoxycytidine-5′ *p*-nitrophenyl phosphates were prepared by the same procedure and both were isolated as their triethylammonium salts in 55% and 46% yield, respectively. However, when the same reaction conditions were applied to the synthesis of guanosine-5′ and deoxyguanosine-5′ *p*-nitrophenyl phosphates, the conversion to the *p*-nitrophenyl ester was low for both esters, presumably owing to the insolubility of the nucleoside-5′ phosphates. In the case of deoxyguanosine-5′ phosphate, the yield was improved by carrying out the reaction in dimethylformamide in the presence of tri-*n*-butylamine where deoxyguanosine-5′ phosphate was completely soluble.

Phosphorylation with *p*-Nitrophenyl Phosphate and Dicyclohexylcarbodiimide.—It is possible that the above reaction conditions can be modified to achieve greater yields of *p*-nitrophenyl esters. However, the results encouraged an investigation of the possibility of the phosphorylation of suitably protected nucleosides using *p*-nitrophenyl phosphate and dicyclohexylcarbodiimide. Because *p*-nitrophenyl phosphate is insoluble in pyridine, the usual medium for carbodiimide reactions,[20] the more recently developed technique for using dimethylformamide was employed.[21,22] Because of its known inertness to phosphorylation, 2′,3′-O-isopropylidineguanosine (3)[10] was chosen as an appropriate test case for the procedure. When the nucleoside was brought into reaction with *p*-nitrophenyl phosphate and dicyclohexylcarbodiimide in dimethylformamide, 2′,3′-O-isopropylidineguanosine-5′ *p*-nitrophenyl phosphate (4)[10] was obtained in 70% yield.



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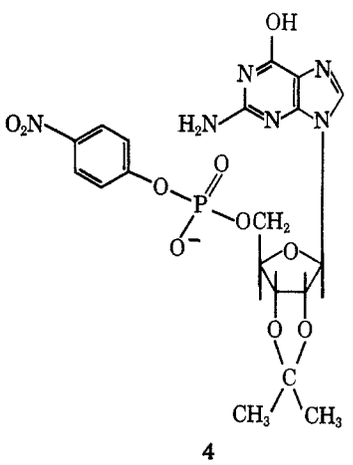

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Mild acid readily removed the protecting group to provide a convenient route to guanosine-5′ *p*-nitrophenyl phosphate. The 3′-hydroxyl group in a deoxyribonucleoside, like the 5′-hydroxyl group in guanosine, is difficult to phosphorylate.[13] Consequently the preparation of thymidine-3′ *p*-nitrophenyl phosphate by the new method was next investigated. 5′-O-Dimethoxytritylthymidine[23,24] on reaction with *p*-nitrophenyl phosphate and dicyclohexylcarbodiimide in dimethyl formamide followed by isolation by ion-exchange chromatography in aqueous alcohol[23] gave 5′-O-dimethoxytritylthymidine-3′ *p*-nitrophenyl phosphate in 77% yield. This in turn was readily converted to thymidine-3′ *p*-nitrophenyl phosphate by reaction with acid. Thus, phosphorylation with *p*-nitrophenyl phosphate and dicyclohexylcarbodiimide in dimethylformamide provides a convenient alternate route to *p*-nitrophenyl esters of nucleotides.

Esterification of Nucleotides with 2,4-Dinitrophenol and Dicyclohexylcarbodiimide.—Two general procedures have been described for the preparation of 2,4-dinitrophenyl esters of nucleotides. Adenosine-5′ phosphoromorpholidate [5, X = $N(CH_2CH_2)_2O$] on reaction with 2,4-dinitrophenol yields the 2,4-dinitrophenyl ester (6)[25] which is also obtained by reaction of the phenol with P^1-diphenyl-P^2-adenosine-5′ pyrophosphate [5, X = $(C_6H_5O)_2PO_2$].[26] In the present study the phosphoromorpholidate method has been

(15) Whereas the triethylammonium salt of thymidine-5′ phosphate is soluble in pyridine, the nucleotides derived from adenine, cytosine, and guanine are much less soluble. Therefore, the pyridine–dimethyl formamide mixture was employed in an effort to increase solubility.

(16) The mechanism of formation of esters using the carbodiimide reaction in the presence of trialkylamine has been discussed previously.[17]

(17) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

(18) Simple phospho diesters are often most conveniently isolated in solid form as their ammonium salts.[17]

(19) Presumably the higher yield of ester in the heterogeneous reaction results from the greater ratio of *p*-nitrophenol to nucleotide present in the reaction medium. A higher yield of deoxycytidine-5′ *p*-nitrophenyl phosphate was obtained when its synthesis was carried out in pyridine rather than in a mixture of pyridine and dimethylformamide (see the Experimental Section).

(20) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958).

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

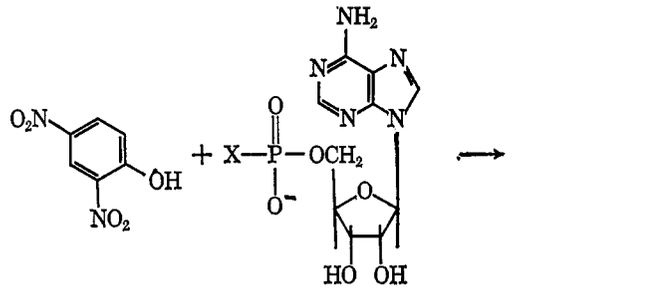
(22) In this procedure an anhydrous cation exchanger is added to the reaction medium to remove any amines present.

(23) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962).

(24) H. Schaller, G. Wiemann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963).

(25) J. G. Moffatt and H. G. Khorana, *ibid.*, **83**, 649 (1961).

(26) A. M. Michelson, *Biochim. Biophys. Acta*, **91**, 1 (1964).



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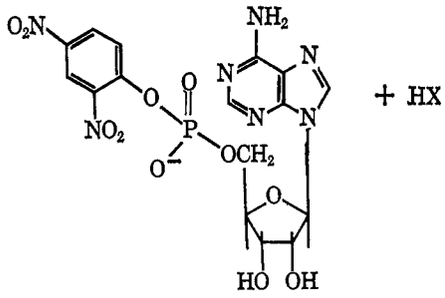

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applied to the synthesis of thymidine-5′ 2,4-dinitrophenyl phosphate.

An alternate route to the 2,4-dinitrophenyl esters of nucleotides would appear to be provided by the reaction of the nucleotide, dicyclohexylcarbodiimide, and 2,4-dinitrophenol. However, when this reaction was attempted on thymidine-5′ phosphate in pyridine in the presence of trialkylamine, the sole product of the reaction was P¹,P²-dithymidine-5′ pyrophosphate. Closer examination of the reaction, in the case of both thymidine-5′ and adenosine-5′ phosphates, showed that in fact the desired 2,4-dinitrophenyl esters were produced initially but that they then reacted further to produce the P¹,P²-dinucleoside-5′ pyrophosphate. The effect of solvent on this reaction of 2,4-dinitrophenyl esters was next investigated since it is known that pyridine will catalyze exchange reactions in phosphate derivatives.[25,27] With dimethylformamide replacing pyridine as solvent, the conversion of 2,4-dinitrophenyl ester to pyrophosphate did not occur and these reaction conditions provided a convenient route to both adenosine-5′ and thymidine-5′ 2,4-dinitrophenyl phosphates.[28,29]

Reaction of Nucleoside-5′ Phosphates with 2,4-Dinitrofluorobenzene.—It has been reported that 2,4-dinitrofluorobenzene reacts specifically with the terminal nucleoside-5′ phosphate in amino acid transfer ribonucleate.[30] Also the reagent converts ribonucleoside-2′(3′) phosphates to ribonucleoside-2′,3′ cyclic phosphates and nucleoside-5′ phosphates to P¹,P²-dinucleoside-5′ pyrophosphates in pyridine and to nucleoside-5′ phosphorofluoridates in other solvents.[31,32] Because of the possibility that 2,4-dinitrophenyl esters could be isolated as intermediates in some of these reactions some further investigations have been carried out. Adenosine-5′ phosphate was allowed to react with 2 equiv of 2,4-dinitrofluorobenzene in the presence of tributylamine in dimethyl formamide at 20° and the reaction was examined periodically by chromatography. Initially a product with the same spectral and chromatographic properties as adenosine-5′ 2,4-dinitrophenyl phosphate was detected. Subsequently, in 2 to 4 hr, adenosine-5′ phosphorofluoridate appeared. All the adenosine-5′ phosphate had disappeared in 8 hr and at this time there appeared a substance which was not further characterized but which, from its spectrum, chromatographic mobility, and resistance to periodic acid oxidation, could be 2′(3′)-O-(2,4-dinitrophenyl)adenosine-5′ phosphorofluoridate. After 48 hr all the material with the properties of adenosine-5′ 2,4-dinitrophenyl phosphate had disappeared from the reaction medium, the final products, isolated by ion-exchange chromatography on diethylaminoethylcellulose, being adenosine-5′ phosphorofluoridate and what is believed to be 2′-O-(2,4-dinitrophenyl)adenosine-5′ phosphorofluoridate. Comparison of different solvents indicated that the reaction followed a similar patten in dimethylformamide and dioxane, although the latter was a less effective solvent. In dimethyl sulfoxide the reaction occurred more slowly than in dimethylformamide and in methanol a number of additional products were detected. Further experiments using different proportions of 2,4-dinitrofluorobenzene in reaction with thymidine-5′ phosphate in dimethylformamide containing tri-*n*-butylamine did not provide conditions where reaction of the nucleoside-5′ phosphate was complete prior to the formation of phosphorofluoridate.

The demonstration that 2,4-dinitrophenyl esters can be obtained by reaction of 2,4-dinitrofluorobenzene with nucleoside-5′ phosphates encouraged the investigation of other nitrobenzene derivatives as potential agents for the formation of nucleotide esters. However, *p*-nitrofluorobenzene and *p*-nitrophenyl trifluoracetate did not react with nucleoside-5′ phosphates nor did 2,4-dinitrochlorobenzene. Picryl chloride, which in pyridine solution has been used to obtain phosphodiester linkages,[33] reacted with adenosine-5′ phosphate to produce P¹,P²-diadenosine-5′ pyrophosphate in dimethylformamide containing tri-*n*-butylamine. Picrylsulfonic acid[34] did not react with the nucleotide under these conditions.

Experimental Section

Reaction solvents were dried over calcium hydride or Linde 4-A Molecular Sieves. Other chemicals were commercially available reagent grade. Chromatography, on Whatman 40 paper, was carried out using the descending technique in solvent 1, isopropyl alcohol–concentrated ammonia–water (7:1:2); solvent 2, butyl alcohol–acetic acid–water (5:2:3); solvent 3, isobutyric acid–concentrated ammonia–water (66:1:33). Nucleotides were detected by viewing under a short-wavelength (emission peak 265 mμ) ultraviolet light and *p*-nitrophenyl esters by viewing under both short-wavelength and long-wavelength (emission peak 300 mμ) ultraviolet light. The *p*-nitrophenyl

(27) D. L. M. Verheyden, W. E. Wehrli, and J. G. Moffatt, *J. Am. Chem. Soc.*, **87**, 2257 (1965).

(28) The formation of P¹,P²-dinucleoside-5′ pyrophosphates by direct reaction of nucleoside-5′ phosphate with dicyclohexylcarbodiimide takes place to a much lesser degree than it does in the preparation of *p*-nitrophenyl esters by this route.

(29) The inherent instability of 2,4-dinitrophenyl esters remains a problem in their isolation.[25,26]

(30) H. Ping-Cheng, L. Cheng-pin, W. Hui, and L. Chih-chuan, *Acta Biochim. Biophys. Sinica*, **4**, 24 (1964).

(31) J. Stockx, *Bull. Soc. Chim. Belges*, **70**, 125 (1961); **70**, 595 (1961); J. Stockx and M. van Montagu, *ibid.*, **71**, 634 (1962).

(32) R. Wittmann, *Chem. Ber.*, **96**, 771 (1963).

(33) F. Cramer, R. Wittmann, K. Daneck, and G. Weimann, *Angew. Chem.*, **75**, 92 (1963); K. L. Agarwal and M. M. Dhar, *Ind. J. Chem.*, **2**, 493 (1964).

(34) T. Okuyama and K. Satake, *J. Biochem.* (Tokyo), **47**, 454 (1960).

esters on chromatograms turned brown on storage at 20°. The *p*-nitrophenol was detected by its yellow color when exposed to ammonia vapor. Chromatographic mobilities (R_f values) are recorded in Table I. Anhydrous pyridinium salts were prepared by passing an aqueous solution of the nucleotide (or *p*-nitrophenylphosphate) through Amberlite IR-120 (or Dowex 50) cation exchanger in the hydrogen form (or pyridinium form if the nucleotide was unstable in acid, for example the purine deoxyribonucleotides) followed by repeated additions of pyridine followed by evaporation under reduced pressure to remove last traces of water by entrainment. Solutions were concentrated to dryness at 30 to 40° using either a rotary evaporator or, when anhydrous conditions were required, an oil pump equipped with Dry Ice trap. Phosphate was determined by King's method.[35]

TABLE I

R_f VALUES OF NUCLEOTIDES

Compound	Solvent 1	Solvent 2	Solvent 3
Adenosine-5′ phosphate	0.08	0.15	0.27
Adenosine-5′ *p*-nitrophenyl phosphate	0.53	0.49	0.45
P¹,P²-Diadenosine-5′ pyrophosphate	0.08	0.05	0.15
Adenosine-5′ 2,4-dinitrophenyl phosphate	0.55	0.51	
Adenosine-5′ phosphorofluoridate	0.26	0.44	
Cytidine-5′ phosphate	0.07	0.25	0.17
Cytidine-5′ *p*-nitrophenyl phosphate	0.53	0.49	0.35
P¹,P²-Dicytidine-5′ pyrophosphate	0.07	0.15	
Guanosine-5′ phosphate	0.04	0.21	
Guanosine-5′ *p*-nitrophenyl phosphate	0.39	0.37	
2′,3′-O-Isopropylidineguanosine-5′ *p*-nitrophenyl phosphate	0.70		
Uridine-3′,5′ cyclic phosphate	0.33	0.30	
Uridine-5′ phosphate	0.06	0.25	
Uridine-5′ *p*-nitrophenyl phosphate	0.48	0.46	
P¹,P²-Diuridine-5′ pyrophosphate		0.10	
Deoxyadenosine-5′ phosphate	0.08	0.27	
Deoxyadenosine-5′ *p*-nitrophenyl phosphate	0.63	0.52	
P¹,P²-Deoxyadenosine-5′ pyrophosphate	0.20	0.15	
Deoxycytidine-5′ phosphate	0.10	0.29	
Deoxycytidine-5′ *p*-nitrophenyl phosphate	0.58	0.60	
Deoxyguanosine-5′ phosphate	0.05	0.07	0.21
Deoxyguanosine-5′ *p*-nitrophenyl phosphate	0.47	0.41	0.44
P¹,P²-Deoxyguanosine-5′ pyrophosphate	0.06	0.02	0.16
Thymidine-5′ phosphate	0.12	0.20	0.36
Thymidine-5′ *p*-nitrophenyl phosphate	0.58	0.51	0.64
P¹,P²-Dithymidine-5′ pyrophosphate	0.24		
Thymidine-5′ 2,4-dinitrophenyl phosphate	0.70	0.45	
Thymidine-5′ phosphorofluoridate	0.50		
Thymidine-5′ phosphoromorpholidate	0.45		
5′-O-Dimethoxytritylthymidine-3′ *p*-nitrophenyl phosphate	0.81		
Thymidine-3′ *p*-nitrophenyl phosphate	0.72	0.56	0.69
p-Nitrophenol	0.73	0.90	0.80
2,4-Dinitrophenol	0.81	0.89	
p-Nitrophenyl phosphate	0.38		
P¹,P²-Di(*p*-nitrophenyl) pyrophosphate	0.70		

Thymidine-5′ *p*-Nitrophenyl Phosphate.—Thymidine-5′ phosphate (1 mmole of the ammonium salt) in water was converted to the acid by passage through a column (10 × 1.0 cm diameter) of a cation exchanger (Amberlite IR-120) in the acid form. Pyridine (2 ml) was added to the eluate and the solution was then concentrated to dryness. Pyridine (15 ml), *p*-nitrophenol (10 mmoles), and triethylamine (2 mmoles) were added and the solution was again concentrated to dryness. Addition of pyridine (10 ml) and concentration to dryness was repeated twice to ensure complete removal of water. Finally the mixture was dissolved in pyridine (10 ml) together with dicyclohexylcarbodiimide (10 mmoles) and kept in a stoppered flask at 20° for 3 days. The pyridine was removed under reduced pressure and the residue was suspended in water (100 ml) and ether (50 ml). Insoluble dicyclohexylurea was removed by filtration. The aqueous layer was washed three times with ether (50 ml) to remove *p*-nitrophenol and then passed onto a column of diethylaminoethylcellulose (30 × 3.0 cm diameter) in the carbonate form. Products were eluted using a linear gradient system with water (2 l.) in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (2 l.) in the reservoir. Fractions (20 ml) were collected at 5-min intervals. Thymidine-5′ *p*-nitrophenyl phosphate (0.35 mmole estimated spectrophotometrically) was eluted in fractions 50 to 65. Triethylammonium bicarbonate and water were removed by evaporation and the nucleotide was isolated as its sodium salt (150 mg).[14,36]

Uridine-5′ *p*-Nitrophenyl Phosphate.—Uridine-5′ phosphate, as the pyridinium salt (1 mmole), was allowed to react with dicyclohexylcarbodiimide (10 mmoles) and *p*-nitrophenol (10 mmoles) in the presence of triethylamine (2 mmoles) as in the preparation of thymidine-5′ *p*-nitrophenyl phosphate. The yield of uridine-5′ *p*-nitrophenyl phosphate[9] isolated after diethylaminoethyl cellulose chromatography was 0.25 mmole (estimated spectrophotometrically). It was isolated as its sodium salt,[36] but was subsequently converted to the ammonium salt by treatment with Dowex 50-W resin in the ammonium form and isolated as the anhydrous salt after freeze drying.[18] The nucleotide was homogeneous on chromatography in solvents 1 and 2. Ultraviolet absorptions were found at λ_{max} 261 mμ (ε 15,000), λ_{infl} 292 mμ (ε 8200), λ_{min} 233 mμ (ε 4500) at pH 7.0; λ_{max} 261 mμ (ε 12,500), λ_{infl} 292 mμ (ε 8200), λ_{min} 239 mμ (ε 7300) at pH 12.0.

Anal. Calcd for $C_{15}H_{15}N_3O_{11}P \cdot NH_4$: P, 6.7. Found: P, 6.7.

A similar yield (0.25 mmole) of the *p*-nitrophenyl ester was obtained when 1 mmole of triethylamine was used and also when the reaction was carried out in a larger volume of pyridine (50 ml). In the absence of triethylamine, the conversion to *p*-nitrophenyl ester was more efficient (0.50 mmole). However, the ester was contaminated with uridine-3′,5′ cyclic phosphate (characterized by its chromatographic mobility in solvents 1 and 2).

Adenosine-5′ *p*-Nitrophenyl Phosphate.—Adenosine-5′ phosphate as the acid (1 mmole) was suspended in pyridine (5 ml) and dimethylformamide (5 ml) containing triethylamine (2 moles). To this was added *p*-nitrophenol (10 mmoles) followed by dicyclohexylcarboiimide (10 mmoles), and the mixture was shaken in the dark at 20° for 4 days. The nucleotide was purified as in the preparation of thymidine-5′ *p*-nitrophenyl phosphate. Adenosine-5′ *p*-phosphate, obtained as the triethylammonium salt from the diethylaminoethylcellulose column, was converted to the ammonium salt and isolated as an anhydrous, white powder after freeze drying (200 mg, 0.41 mmole). It was homogeneous on chromatography in solvents 1 and 2. Ultraviolet absorption was found at λ_{max} 259 mμ (ε 17,200), λ_{infl} 290 mμ (ε 7300), λ_{min} 232 mμ (ε 5600) at pH 7.0; λ_{max} 257 mμ (ε 16,500), λ_{infl} 292 mμ (ε 7700), λ_{min} 232 mμ (ε 5500) at pH 2.0.

Anal. Calcd for $C_{16}H_{16}N_6O_9P \cdot NH_4$: P, 6.4. Found: P, 6.1.

The other product of the reaction was P¹,P²-diadenosine-5′ pyrophosphate characterized by its mobility in solvent 2. In another preparation of the *p*-nitrophenyl ester carried out in dimethylformamide (10 ml) in the presence of tri-*n*-butylamine (2 mmoles) the yield of the ammonium salt was 150 mg (0.31 mmole).

Deoxyadenosine-5′ *p*-Nitrophenyl Phosphate.—Deoxyadenosine-5′ phosphate, as its pyridinium salt (1 mmole), was treated with dicyclohexylcarbodiimide (10 mmoles) and *p*-nitrophenol (10 mmoles) in the presence of triethylamine (2 mmoles) in pyridine (5 ml) and dimethylformamide (5 ml) at 20° as in the preparation of adenosine-5′ *p*-nitrophenyl phosphate. Deoxyadenosine-5′ *p*-nitrophenyl phosphate was isolated as its ammonium salt (190 mg, 0.40 mole) and was homogeneous in solvents 1 and 2. Ultraviolet absorptions were found at λ_{max} 259 mμ (ε 16,600), λ_{infl} 288 mμ (ε 7200), λ_{min} 232 mμ (ε 5400)

(35) E. J. King, *Biochem. J.*, **26**, 292 (1932). We are indebted to Mrs. T. G. Oikawa for phosphate analyses.

(36) The nucleotide in methanol (5 ml) was treated with 1 *M* sodium iodide in acetone (2 ml) followed by ether (20 ml). The precipitate was washed with ether and dried.

at pH 7.0; λ_{max} 258 mμ (ε 15,100), λ_{infl} 292 mμ (ε 7300), λ_{min} 231 mμ (ε 5500) at pH 12.0.

Anal. Calcd for $C_{16}H_{16}N_6O_8 \cdot NH_4$: P, 6.6. Found: P, 6.5.

Cytidine-5′ *p*-Nitrophenyl Phosphate.—Cytidine-5′ phosphate, as the acid (1 mmole) was treated with dicyclohexylcarbodiimide (10 mmoles) and *p*-nitrophenol (10 mmoles) in the presence of triethylamine (1.0 mmole) in pyridine (5.0 ml) and dimethylformamide (5.0 ml) at 20° and the *p*-nitrophenyl ester was then purified on diethylaminoethylcellulose as in the preparation of adenosine-5′ *p*-nitrophenyl phosphate. After removal of water and triethylammonium bicarbonate by evaporation, the nucleotide was dissolved in ethanol and isolated as its triethylammonium salt by precipitation with acetone (301 mg, 0.55 mmole). The nucleotide was stored as its ammonium salt, isolated as a white powder after freeze drying from water. It was homogeneous in solvents 1 and 2. Ultraviolet absorptions were found at λ_{max} 275 mμ (ε 16,500) and λ_{min} 245 mμ (ε 7500) at pH 7.0; λ_{max} 280 mμ (ε 20,800) and λ_{min} 242 mμ (ε 3500) at pH 2.0.

Anal. Calcd for $C_{15}H_{16}N_4O_{10}P \cdot NH_4$: P, 6.7. Found: P, 6.6.

Deoxycytidine-5′ *p*-Nitrophenyl Phosphate.—Deoxycytidine-5′ phosphate, as the acid (1 mmole) was treated with dicyclohexylcarbodiimide (10 mmoles) and *p*-nitrophenol (10 mmoles) in the presence of triethylamine (1 mmole) in pyridine (10 ml) at 20° and the resultant *p*-nitrophenyl ester was purified on diethyl aminoethylcellulose as in the preparation of cytidine-5′ *p*-nitrophenyl phosphate. The yield of triethylammonium deoxycytidine-5 *p*-nitrophenyl phosphate was 285 mg (0.54 mmole). The nucleotide was stored as its ammonium salt, isolated as a white powder after freeze drying from water. It was homogeneous in solvents 1 and 2. Ultraviolet absorptions were found at λ_{max} 275 mμ (ε 16,200) and λ_{min} 245 mμ (ε 7300) at pH 7.0; λ_{max} 280 mμ (ε 21,200) and λ_{min} 242 mμ (ε 3200) at pH 12.0.

Anal. Calcd for $C_{15}H_{16}N_4O_9P \cdot NH_4$: P, 7.0. Found: P, 7.0.

When the reaction was carried out in a mixture of pyridine (5 ml) and dimethylformamide (5 ml) the yield of *p*-nitrophenyl ester was 0.46 mmole (estimated spectrophotometrically).

Guanosine-5′ *p*-Nitrophenyl Phosphate.—Guanosine-5′ phosphate pyridinium salt (1 mmole) was allowed to react with dicyclohexylcarbodiimide (10 mmoles) and *p*-nitrophenol (10 mmoles) in the presence of triethylamine (1 mmole) in pyridine (5 ml) and dimethylformamide (5 ml) at 20° as in the preparation of adenosine-5′ *p*-nitrophenyl phosphate. The *p*-nitrophenyl ester was isolated as the triethylammonium salt (54 mg, 0.09 mmole) after ion-exchange chromatography. The guanosine-5′ *p*-nitrophenyl phosphate[10] was stored as its ammonium salt and was homogeneous in solvents 1 and 2. Ultraviolet absorptions were found at λ_{max_1} 258 mμ (ε 15,200), λ_{max_2} 275 mμ (ε 14,100), λ_{infl} 301 mμ (ε 7200), λ_{min_1} 230 mμ (ε 7200), λ_{min_2} 266 mμ (ε 14,000) at pH 7.0; λ_{max_1} 258 mμ (ε 14,850), λ_{max_2} 275 mμ (ε 14,350), λ_{min_1} 233 mμ (ε 5900), λ_{min_2} 266 mμ (ε 14,250) at pH 2.0; λ_{max} 268 mμ (ε 16,600), λ_{infl} 300 mμ (ε 7500) λ_{min} 232 mμ (ε 6800) at pH 12.0.

Anal. Calcd for $C_{16}H_{16}N_6O_{10}P \cdot NH_4 \cdot H_2O$: P, 5.96. Found: P, 5.9.

Deoxyguanosine-5′ *p*-Nitrophenyl Phosphate.—Deoxyguanosine-5 phosphate, pyridinium salt (1 mmole) in dimethylformamide (10 ml) containing tri-*n*-butylamine (2 mmoles) was reacted with dicyclohexylcarbodiimide (10 mmoles) and *p*-nitrophenol (10 mmoles) at 20° for 24 hr. The *p*-nitrophenyl ester was isolated as its triethylammonium salt (200 mg, 0.35 mmole) after removal of water and triethylammonium bicarbonate by evaporation and freeze drying. The deoxyguanosine-5′ *p*-nitrophenyl phosphate was stored as its ammonium salt and was homogeneous in solvents 1 and 2. Ultraviolet absorptions were found at λ_{max_1} 256 mμ (ε 15,100), λ_{max_2} 275 mμ (ε 13,750), λ_{infl} 301 mμ (ε 6800), λ_{min_1} 231 mμ (ε 7000), λ_{min_2} 266 mμ (ε 13,650) at pH 7.0; λ_{max_1} 258 mμ (ε 14,600), λ_{max_2} 275 mμ (ε 13,850), λ_{min_1} 233 mμ (ε 5660), λ_{min_2} 266 mμ (ε 13,700) at pH 2.0; λ_{max} 268 mμ (ε 16,500), λ_{infl} 302 mμ (ε 7300), λ_{min} 235 mμ (ε 6600) at pH 12.0.

Anal. Calcd for $C_{16}H_{16}N_6O_9P \cdot NH_4 \cdot H_2O$: P, 6.2. Found: P, 6.1.

In a reaction carried out in a mixture of dimethylformamide and pyridine, as in the preparation of guanosine-5′ *p*-nitrophenyl phosphate, the yield of deoxyguanosine-5′ *p*-nitrophenyl phosphate was 0.24 mmole.

2′,3′-O-Isopropylideneguanosine-5′ *p*-Nitrophenyl Phosphate.—2′,3′-O-Isopropylidineguanosine (1 mmole) and *p*-nitrophenyl phosphate as its pyridinium salt (2 mmoles) in dimethylformamide (4 ml) and pyridine (2 ml) were treated with dicyclohexylcarbodiimide (5 mmoles) together with anhydrous Dowex 50-W, hydrogen form, (100 mg) at 20° for 3 days. Further dicyclohexylcarbodiimide (5 mmoles) was added, the reaction being allowed to proceed for 5 days when water (1.0 ml) was added. After an additional 24 hr the reaction mixture was diluted with water (100 ml), filtered to remove insoluble dicyclohexylurea, and partially concentrated by evaporation to remove pyridine. The products were passed onto a diethylaminoethylcellulose column (22 × 3.0 cm diameter) in the carbonate form and eluted using a linear gradient system with water (2 l.) in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (2 l.) in the reservoir. Fractions (20 ml) were collected at 5-min intervals. 2′,3′-O-Isopropylideneguanosine-5′ *p*-nitrophenyl phosphate (0.70 mmole, estimated spectrophotometrically)[10] was eluted in fractions 90 to 100. It was isolated as the triethylammonium salt after removal of triethylammonium bicarbonate and water by evaporation. The nucleotide was homogeneous in solvent 1 and its ultraviolet absorption spectrum was identical with that of guanosine-5′ *p*-nitrophenyl phosphate.

The nucleotide (0.7 mmole) in acetic acid (8 ml) containing water (2 ml) was heated in a boiling water bath for 2 hr. The acetic acid was removed by evaporation and the residue in water was passed onto a diethylaminoethylcellulose column (22 × 3.0 cm diameter) in the carbonate form. The products were eluted using a linear gradient system with water (2 l.) in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (2 l.) in the reservoir. Fractions (20 ml) were collected at 5-min intervals. Guanosine-5 *p*-nitrophenyl phosphate (0.70 mmole, estimated spectrophotometrically)[10] was eluted in fractions 70 to 90 and was isolated as its triethylammonium salt after removal of triethylammonium bicarbonate and water by evaporation.

5′-O-Dimethoxytritylthymidine-3′ *p*-Nitrophenyl Phosphate.—*p*-Nitrophenyl phosphate, as the anhydrous pyridinium salt (4 mmoles) was dissolved in dimethylformamide (10 ml) and pyridine (5 ml) containing dry Dowex 50-W, hydrogen form (250 mg), followed by 5′-O-dimethoxytritylthymidine (2 mmoles) and dicyclohexylcarbodiimide (10 mmoles). The mixture was shaken at 20° for 2 days and more dicyclohexylcarbodiimide (2 mmoles) was added and the shaking continued for 2 days. Water (2 ml) was then added. After an additional 24 hr the solution was concentrated by evaporation to remove pyridine. Ethanol (100 ml) was added to the residue which was filtered to remove insoluble material and then passed onto a diethylaminoethylcellulose column (35 × 3.5 cm diameter, carbonate form) which had been packed in 50% ethyl alcohol. The column was washed successively with 50% ethyl alcohol (1 l.), water (500 ml), and 0.1 *M* triethylamonium bicarbonate in water (1 l.). The column was then eluted using a linear gradient system with 50% ethyl alcohol (2 l.) and 0.1 *M* triethylammonium bicarbonate in 50% ethanol (2 l.). Fractions (20 ml) were collected at 5-min intervals. 5′-O-Dimethoxytritylthymidine-3′ *p*-nitrophenyl phosphate was eluted in fractions 41 to 90 and was isolated as its triethylammonium salt (1.30 g, 1.55 mmoles) after freeze drying. The nucleotide was homogeneous in solvent 1 and was converted to thymidine-3′ *p*-nitrophenyl phosphate quantitatively on treatment with 80% acetic acid at 20° for 4 hr.

Anal. Calcd for $C_{37}H_{35}N_3O_{12}P \cdot C_6H_{16}N$: P, 3.7. Found: P, 3.7.

Reaction of Thymidine-5′ Phosphate with Dicyclohexylcarbodiimide in Pyridine in Presence of 2,4-Dinitrophenol.—Thymidine-5′ phosphate, as the acid (1 mmole), and triethylamine (2 mmoles) in pyridine (25 ml) was treated with dicyclohexylcarbodiimide (10 mmoles) and 2,4-dinitrophenol (10 mmoles) at 20° for 7 days. Examination of the product in solvent 1 showed that only thymidine-5′ phosphate, P^1,P^2-dithymidine-5′ pyrophosphate and 2,4-dinitrophenol were present.

Reaction of Thymidine-5′ Phosphoromorpholidate with Dinitrophenol.—Thymidine-5′ phosphoromorpholidate, as the N^1,N^1-dicyclohexylcarboxamidinium salt (0.25 mmole)[25] was treated under anhydrous conditions with 2,4-dinitrophenol (0.75 mmole) in pyridine (2.5 ml) at 20° for 6 hr. The nucleotide was precipitated by addition of ether (50 ml) and the precipitate was washed thoroughly with ether to remove 2,4-dinitrophenol. The resulting thymidine-5′ 2,4-dinitrophenyl phosphate, N^1,N^2-dicyclohexylcarboxamidinium salt, (140 mg, 0.18 mmole) was examined in solvent 1 and solvent 2 and found to be free of

thymidine-5′ phosphate, although a trace of 2,4-dinitrophenol was present.

Anal. Calcd for $C_{16}H_{16}N_4O_{12}P \cdot C_{17}H_{32}N_3O$: P, 4.0. Found: P, 4.5.

Adenosine-5′ 2,4-Dinitrophenyl Phosphate.[26]—A solution of P^1-diphenyl-P^2-adenosine-5′ pyrophosphate (0.5 mmole) in dimethylformamide (1 ml) was allowed to react with 2,4-dinitrophenol (1.5 mmoles) in pyridine (2 ml) for 14 hr at 20°. Ether (50 ml) was added and the resultant precipitate was collected and dissolved in methyl alcohol (5 ml). A small amount of insoluble material was removed and then ether (50 ml) was added to precipitate adenosine-5′ 2,4-dinitrophenyl phosphate as a white powder (presumably the tri-*n*-butylamine salt). Chromatography in solvents 1 and 2 indicated contamination with traces of adenosine-5′ phosphate and P^1,P^2-diadenosine-5′ pyrophosphate.

Reaction of Adenosine-5′ Phosphate with Dicyclohexylcarbodiimide and 2,4-Dinitrophenol. A.—Adenosine-5′ phosphate, as the acid (0.5 mmole) and tri-*n*-butylamine (1 mmole) in dimethylformamide (5 ml) and pyridine (5 ml) together with 2,4-dinitrophenol (10 mmoles) and dicyclohexylcarbodiimide (10 mmoles) were kept at 20° in the dark and the reaction was followed chromatographically in solvents 1 and 2. After 2 hr, the products were predominantly adenosine-5′ 2,4-dinitrophenyl phosphate, identified by chromatographic mobility and ultraviolet absorption (λ_{max} 256 mμ and λ_{infl} 290 mμ at pH 7.0) and P^1,P^2-diadenosine-5′ pyrophosphate together with a trace of adenosine-5′ phosphate. After 48 hr the product was predominantly P^1,P^2-diadenosine-5′ pyrophosphate and after 72 hr all the 2,4-dinitrophenyl ester had disappeared.

B.—The same reactants were dissolved in dimethylformamide (10 ml) in the absence of pyridine. Again reaction of the adenosine-5′ phosphate was complete in 2 hr, the major product being the 2,4-dinitrophenyl ester together with appreciable P^1,P^2-diadenosine-5′ pyrophosphate. However, after 72 hr the proportions of these products were still the same, there being no detectable conversion of adenosine-5′ 2,4-dinitrophenyl phosphate to the P^1,P^2-dinucleoside pyrophosphate.

Reaction of Thymidine-5′ Phosphate with Dicyclohexylcarbodiimide and 2,4-Dinitrophenol in Dimethylformamide.—Thymidine-5′ phosphate, triethylammonium salt (1 mmole), in dimethylformamide (20 ml) containing 2,4-dinitrophenol (10 mmoles) was treated with dicyclohexylcarbodiimide (20 mmoles) at 20°. The reaction was followed chromatographically in solvent 2. Formation of thymidine-5′ 2,4-dinitrophenyl phosphate (λ_{max} 258 mμ and λ_{infl} 295 mμ at pH 7.0) was complete and essentially quantitative in 1 hr, the product being homogeneous in solvents 1 and 2.

Reaction of Adenosine-5′ Phosphate with 2,4-Dinitrofluorobenzene.—Adenosine-5′ phosphate, as the acid (1 mmole), in dimethylformamide (2 ml) and tri-*n*-butylamine (4 mmoles) was treated with 2,4-dinitrofluorobenzene (2 mmoles) at 20°. The mixture was shaken until all the nucleotide was in solution (5 to 10 min); it turned yellow soon after addition of the 2,4-dinitrofluorobenzene. Aliquots (0.2 ml) were removed at intervals and diluted into ether (20 ml). The precipitate was collected by centrifugation, washed with ether, and then dissolved in water prior to chromatography in solvents 1 and 3. Initially, the product was adenosine-5′ 2,4-dinitrophenyl phosphate, characterized by its chromatographic mobility in solvents 1 and 2 and by its ultraviolet absorption (λ_{max} 256 mμ and λ_{infl} 290 mμ at pH 7.0). After 2 hr, adenosine-5′ phosphorofluoridate appeared. After between 4 and 8 hr, the reaction of adenosine-5′ phosphate was complete, the products being ithe 2,4-dinitrophenyl ester and the phosphorofluoridate in approximately equal amounts. After 8 hr there appeared a new substance, tentatively assigned the structure 2′(3′)-O-(2,4-dinitrophenyl)-adenosine-5′ phosphorofluoridate on the basis of its resistance to periodic acid oxidation, its chromatographic mobility in solvent 1 (R_f 0.73) and solvent 2 (R_f 0.65), and its ultraviolet absorption spectrum (λ_{max} 258 mμ and λ_{infl} 290 mμ at pH 7.0). After 48 hr all the adenosine-5′ 2,4-dinitrophenyl phosphate had disappeared, the major product being adenosine-5′ phosphorofluoridate.[32] The nucleotide was isolated as its triethylammonium salt after ion-exchange chromatography on diethylaminoethylcellulose and precipitation from ethyl alcohol with ether.

When the reaction was carried out using the same reactants in the same concentrations with dioxane, dimethyl sulfoxide, or methyl alcohol as solvents, essentially the same products were obtained. However, in dimethyl sulfoxide the reaction was slower than in dioxane or methanol where the rate was similar to that in dimethylformamide. In methanol, there were a number of side products.

Reaction of Thymidine-5′ Phosphate with 2,4-Dinitrofluorobenzene.—Thymidine-5′ phosphate, as the acid (0.5 mmole), triethylamine (2.0 mmoles), and 2,4-dinitrofluorobenzene (0.5 mmole) in dimethylformamide (2 ml) were kept at 20°. Aliquots (0.2 ml) were removed at intervals and diluted into ether (5 ml). The precipitate was collected by centrifugation and dissolved in ethyl alcohol prior to examination by chromatography in solvents 1 and 2. After 1 hr, the compounds present were thymidine-5′ phosphate and thymidine-5′ 2,4-dinitrophenyl phosphate in approximately equal amounts together with a trace of thymidine-5′ phosphorofluoridate.[32] When 0.5, 2.0, and 4.0 mmoles of 2,4-dinitrofluorobenzene were employed in the reaction, essentially the same pattern of products was obtained. Thymidine-5′ phosphorofluoridate was the major product after 24 hr.

Reaction of Adenosine-5′ Phosphate with Picryl Chloride in Dimethylformamide.—Adenosine-5′ phosphate, as the acid (1 mmole), and tri-*n*-butylamine (4 mmoles) in dimethylformamide (2 ml) containing picryl chloride (2 mmoles) were kept at 20°, the progress of the reaction being followed chromatographically in solvents 1 and 2. Conversion to P^1,P^2-adenosine-5′ pyrophosphate was complete in 4 to 8 hr.

When adenosine-5′ phosphate was treated with picrylsulfonic acid (2 mmoles) under the same conditions or with *p*-nitrophenyl trifluoroacetate (4 mmoles), 2,4-dinitrochlorobenzene (1 mmole), or *p*-nitrofluorobenzene (2 mmoles), there was no reaction detectable by chromatography in solvent 2 after 2 days.