Nucleoside S-Alkyl Phosphorothioates. II. Preparation and Chemical and Enzymatic Properties[1]

Alan F. Cook, Michael J. Holman, and Alexander L. Nussbaum

Contribution from the Chemical Research Department, Hoffmann-La Roche Inc., Nutley, New Jersey 07110. *Received August 20, 1968*

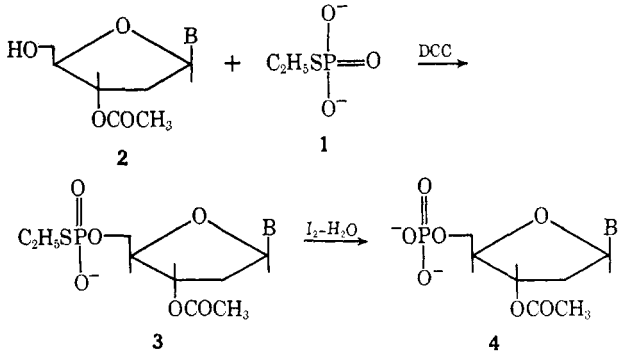
Abstract: Nucleoside 5′-S-ethyl phosphorothioates, prepared by condensation of pyridinium S-ethyl phosphorothioate with a variety of suitably protected nucleosides using dicyclohexylcarbodiimide (DCC) as the condensing agent, have been converted into the corresponding nucleotides by treatment with aqueous iodine or sodium periodate. The protecting groups employed were found to be stable under these conditions. This procedure represents an extremely mild method for the phosphorylation of nucleosides. The nucleoside 5′-S-ethyl phosphorothioates have been used as intermediates in the preparation of a wide variety of nucleotide derivatives, including methyl, β,β,β-trichloroethyl, and *p*-nitrophenyl esters, dinucleoside monophosphates, a morpholidate, and di- and triphosphates. All of these reactions involve the cleavage of the P–S bond using iodine and the appropriate nucleophile. The enzymatic susceptibilities of the nucleoside S-ethyl phosphorothioates have also been studied.

Much of the difficulty with the chemical synthesis of compounds containing several functional groups lies in the design of appropriate protecting groups. Differential blockade of complex molecules is particularly difficult where sensitivity to a variety of chemical and physical agents is an important factor, for instance in nucleic acid chemistry. The phosphorylation of nucleosides is a case in point.[2] In order to prevent the formation of di- and triesters as well as anhydrides, reversible partial blockade of the phosphoric acid molecule is necessary. The selective removal of the protecting group(s) must be possible under mild conditions, since the sensitivity of nucleotides to acid, base, and reducing agents imposes severe limits upon the choice of reagents available for unmasking. A list of some of the more widely used phosphorylating agents, illustrating the variety of conditions used for unmasking, is displayed in Table I.

It had previously been found that S-alkyl phosphorothioates were quite stable toward hydrolytic fission near neutral and at higher pH, but that the P–S bond could be labilized by the action of halogens.[3] In this work we wish to report the use of S-ethyl phosphorothioate (**1**, Scheme I) for the phosphorylation of nucleosides.

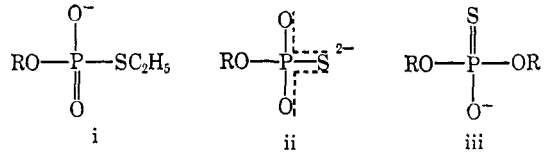
S-Ethyl phosphorothioate (**1**) was prepared by Åkerfeldt's method.[4] The crude material was converted into its pyridinium salt by passage through a column of Dowex 50 resin (pyridinium form) and condensed with 3′-O-acetylthymidine (**2a**), using DCC as the condensing agent. The mixture of products was purified by chromatography on a DEAE ion-exchange cellulose column (acetate form) and 3′-O-acetylthymidine 5′-S-ethyl phosphorothioate (**3a**)[5] was obtained in a yield of 50%.[8] This compound was isolated in solid form as its dihydrated sodium salt. In addition small amounts of three by-products were obtained. These were identified as 3′-O-acetylthymidine 5′-phosphate (**4a**), the symmetrical pyrophosphate **5**, and the 5′,5′-dinucleoside monophosphate **6**. These by-products were presumably formed by condensation of **2a** with orthophosphate or phosphorothioate which were present in the crude dilithium S-ethyl phosphorothioate.[9] Compounds **4a** and **5** were identified by

Scheme I



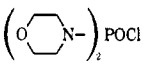
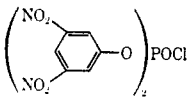
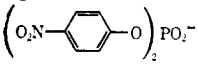
a, B = thymine
b, B = 5-fluorouracil
c, B = N^4-benzoylcytosine
d, B = N^6-benzoyladenine

(1) A preliminary communication by A. L. Nussbaum and R. Tiberi, *J. Am. Chem. Soc.*, **87**, 2513 (1965) is paper I in this series.
(2) D. M. Brown, *Advan. Org. Chem.*, **3**, 75 (1963).
(3) S. Åkerfeldt, *Svensk Kem. Tidskr.*, **75**, 231 (1963).
(4) S. Åkerfeldt, *Acta Chem. Scand.*, **16**, 1897 (1962).
(5) It is important to differentiate the compounds described herein [nucleoside S-ethyl phosphorothioates (i)] from the nucleoside phosphorothioate monoesters[6] (ii) and the diesters[7] (iii) which might properly be called phosphorothionates.

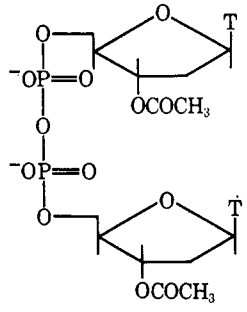


(6) F. Eckstein, *J. Am. Chem. Soc.*, **88**, 4292 (1966).
(7) F. Eckstein, *Tetrahedron Letters*, 1157 (1967).
(8) Attempts were made to prepare the S-ethyl derivative **3a** by condensation of 3′-O-acetylthymidine 5′-phosphate with ethanethiol, but no condensation occurred when either DCC or triisopropylbenzenesulfonyl chloride was used as the condensing agent.
(9) M. Mikolajczyk, *Chem. Ber.*, **99**, 2083 (1966), has shown that during the reactions of phosphonothioates with DCC, the sulfur atom attacks the DCC to form an intermediate which subsequently undergoes P–S fission to give dicyclohexylthiourea. Thus in this case both phosphorothioate and orthophosphate would be expected to give rise to the same by-products **4a**, **5**, and **6**.

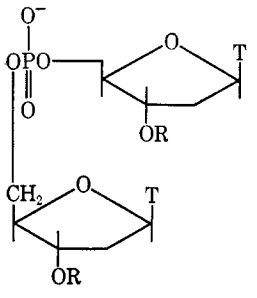
Table I. Common Phosphorylating Agents

Phosphorylating agent	Conditions for unmasking
$NCCH_2CH_2OPO_3^{2-}$ + condensing agent	Base[a]
	Acid[b]
$t\text{-}C_4H_9OPO_3^{2-}$ + condensing agent	Acid[c]
	Light[d]
$CCl_3CH_2OPO_3^{2-}$ + condensing agent	Zinc[e]
	Platinum–hydrogen or base + enzyme[f]

[a] G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961). [b] H. A. C. Montgomery and J. H. Turnbull, *J. Chem. Soc.*, 1963 (1958). [c] F. Cramer, H. Neunhoeffer, K. H. Scheit, G. Schneider, and J. Tennigkeit, *Angew. Chem. Intern. Ed. Engl.*, **1**, 331 (1962). [d] A. J. Kirby and A. G. Varvoglis, *Chem. Commun.*, 406 (1967). [e] F. Eckstein, *Chem. Ber.*, **100**, 2228 (1967). [f] J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 3741 (1957), R. W. Chambers, J. G. Moffatt, and H. G. Khorana, *ibid.*, **79**, 3747 (1957).



5



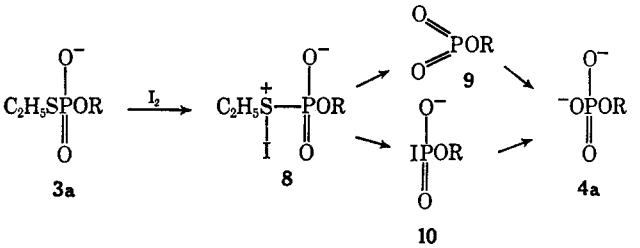
6, R = $COCH_3$
7, R = H

chromatographic comparison with authentic samples.[10,11] The dinucleoside monophosphate **6** was chromatographically identical with a sample prepared by condensation of **4a** with 3′-O-acetylthymidine (**2a**). Its structure was also confirmed by deacetylation to **7** which was resistant to the action of bacterial alkaline phosphatase and spleen phosphodiesterase, thus indicating the absence of phosphomonoester and 3′-phosphodiester linkages. Compound **7** was degraded by venom phosphodiesterase, however,[12] with the formation of thymidine and thymidine 5′-phosphate.

3′-O-Acetylthymidine 5′-S-ethyl phosphorothioate (**3a**) was smoothly converted into 3′-O-acetylthymidine 5′-phosphate (**4a**) in 87% yield by treatment with iodine in aqueous acetone. This procedure provides an efficient and mild method for the phosphorylation of nucleosides.

The mechanism for the conversion of the S-ethyl compound **3a** into the nucleotide **4a** using iodine probably involves an intermediate iodosulfonium species such as **8** (Scheme II).[13] This species can undergo P–S cleavage to give metaphosphate **9** (or its equivalent) followed by attack of water to give the monoester **4a**. Metaphosphates have frequently been postulated as intermediates in phosphorylation reactions[14] including oxidative phosphorylations,[15] but their intermediacy has not been conclusively demonstrated. Alternatively, **8** could undergo displacement by iodide ion to give a phosphoroiodidate **10** which would be subsequently hydrolyzed to **4a**. Brown, *et al.*,[16] have postulated this kind of species as an intermediate during the iodine oxidation of phosphorohydrazidate diesters, and Kirby[17] has suggested a similar intermediate in the reactions of phosphites with iodine and alcohols.

Scheme II



3a **8** **9** **10** **4a**

R = 3′-O-acetylthymidine 5′-

The labilization of the P–S bond was also accomplished in another manner. Compound **3a** was treated with an aqueous solution of sodium periodate overnight at room temperature, and complete conversion to **4a** was achieved. The S-ethyl group is presumably labilized by oxidation at sulfur to give a species which can readily undergo P–S cleavage with attack by water, metaphosphate also being a possible intermediate in this reaction. This method provides an efficient and mild alternative to the iodine procedure. A similar principle has been employed for the activation of 4-(methylthio)phenyl esters of peptides.[18]

The new phosphorylation technique was applied to a variety of other partially protected nucleosides. 3′-O-Acetyl-5-fluorodeoxyuridine (**2b**)[19] was converted into its 5′-S-ethyl phosphorothioate **3b**, which was subsequently treated with iodine in aqueous acetone to give 3′-O-acetyl-5-fluorodeoxyuridine 5′-phosphate (**4b**). In the deoxycytidine series, 3′-O-acetyl-N^4-benzoyldeoxycytidine (**2c**)[20] was easily converted into the S-ethyl nucleotide **3c** by the same method, and this material was isolated as the sodium salt. Conversion of **3c** into the nucleotide **4c** was accomplished in the usual manner, both the N-benzoyl and O-acetyl groups being stable under these conditions.

(10) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958).
(11) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **81**, 4660 (1959).
(12) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).
(13) See ref 9 for a more detailed discussion on the reactions of S-alkyl phosphorothioates with halogens.
(14) G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 4329 (1962).
(15) V. M. Clark, D. W. Hutchinson, G. W. Kirby, and A. R. Todd, *J. Chem. Soc.*, 715 (1961).
(16) D. M. Brown, J. A. Flint, and N. K. Hamer, *ibid.*, 326 (1964).
(17) A. J. Kirby, *Chem. Ind.* (London), 1877 (1963).
(18) B. J. Johnson and P. M. Jacobs, *Chem. Commun.*, 73 (1968).
(19) H. J. Thomas and J. A. Montgomery, *J. Med. Pharm. Chem.*, **5**, 24 (1962).
(20) A. F. Cook, *J. Org. Chem.*, **33**, 3589 (1968).

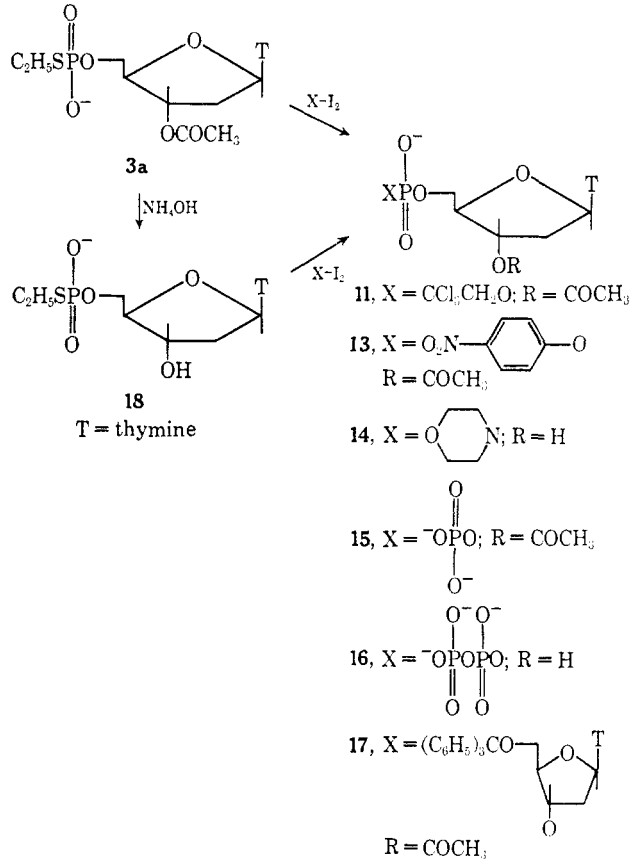
3′-O-Acetyl-N⁶-benzoyldeoxyadenosine (**2d**) was used as the starting material for phosphorylation in the deoxyadenosine series.[21] This compound was prepared from N⁶-benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine[22] by a published procedure. Condensation of **2d** with **1** gave the S-ethyl nucleotide **3d** and this material was converted into the nucleotide **4d** by treatment with iodine in aqueous acetone. Although the N⁶-benzoyladenine moiety is extremely sensitive to acid, no cleavage of the anomeric bond was detected in these experiments, thus demonstrating the mildness of the phosphorylation procedure. Compound **4d** was chromatographically identical with a sample prepared by another route,[23] and its structure was also confirmed by deacylation to deoxyadenosine 5′-phosphate.

The S-ethyl phosphorothioates, then, are efficiently hydrolyzed to the nucleotides upon activation with iodine. Can nucleophiles other than water discharge the reactive intermediate? Schemes III and IV illustrate that this is so; a variety of derivatives were prepared by reaction of the nucleoside S-ethyl phosphorothioates with the appropriate nucleophile in the presence of iodine. Thus, nucleotide β,β,β-trichloroethyl (**11**), methyl (**12**), and *p*-nitrophenyl (**13**) esters, a phosphoroamidate (**14**), and phosphate anhydrides (the diphosphate **15** and the triphosphate **16**) were prepared by this procedure. Wieland and Lambert[24] have used an approach similar to this for the oxidative phosphorylation of a variety of substrates, and yields of up to 50% were achieved in favorable cases. Inorganic pyrophosphate has also been prepared by a similar method.[25] These reactions bear resemblance to the oxidation of quinol phosphates with bromine,[15] and the oxidation of phosphorohydrazidates with iodine.[16]

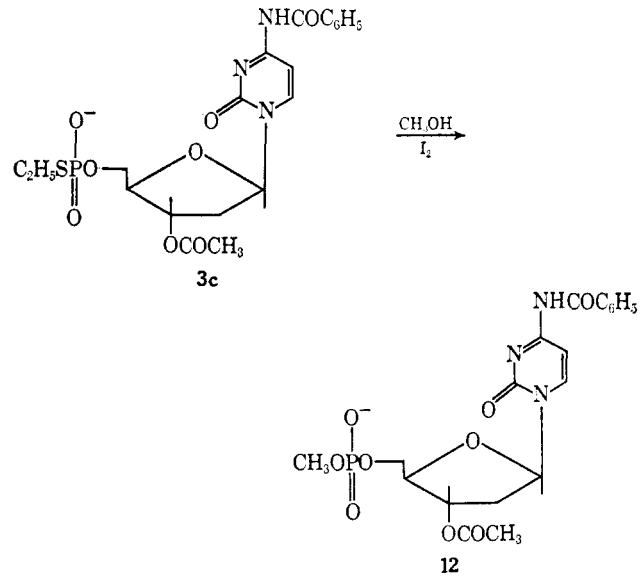
Attention was also focused upon the reactions of **3a** with nucleoside hydroxyl groups. Treatment of compound **3a** with 4.5 equiv of 3′-O-acetylthymidine in the presence of iodine in pyridine, gave the dinucleoside phosphate **6** in 53% yield. This material was chromatographically identical with the corresponding side product isolated during the preparation of **3a**, and displayed the same enzymatic susceptibilities. When 10 equiv of 3′-O-acetylthymidine were employed, the yield of **6** was increased to 60%. The reaction of **3a** with the relatively hindered 3′-hydroxyl group of 5′-O-tritylthymidine also proceeded satisfactorily. Compound **17** was obtained in 42% yield when 15 equiv of the nucleoside with iodine in pyridine were employed. Ammonia treatment of the product liberated 5′-O-tritylthymidylyl-(3′,5′)-thymidine, which was identified by comparison with an authentic sample.[26] Treatment of **17** with acid followed by ammonia gave the free dinucleoside phosphate thymidylyl-(3′,5′)-thymidine. These experiments demonstrate that phosphodiesters can readily be prepared by this route, and the method offers an acceptable alternative to conventional procedures.

While the foregoing experiments illustrate the utility of the labilized P–S bond, the stability of the nucleoside S-ethyl phosphorothioates in the absence of activators is also worth notice. Thus, **3a** could be easily deacetylated to the free 3′ alcohol **18** using aqueous am-

Scheme III. Reactions of Thymidine 5′-S-Ethyl Phosphorothioates



Scheme IV



(21) This material was kindly prepared by Mr. E. Heimer of these laboratories.
(22) M. W. Moon, S. Nishimura, and H. G. Khorana, *Biochemistry*, **5**, 937 (1966).
(23) H. Weimann, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3835 (1963).
(24) T. Wieland and R. Lambert, *Chem. Ber.*, **89**, 2476 (1956).
(25) D. C. Dittmer and V. O. Silverstein, *J. Org. Chem.*, **26**, 4706 (1961).
(26) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 368 (1965).

monia or 1 *N* sodium hydroxide, and compound **18** could be reacetylated to **3a** using acetic anhydride in pyridine. Compound **3a** was also quite stable in water, pyridine, or β,β,β-trichloroethanol. Treatment of **18** with 1 *N* hydrochloric acid at 100°, however, liberated thymidine 5′-phosphate, ethanethiol and other unidentified products. The stability of the S-ethyl phosphorothioate group in the absence of iodine forms the basis of a method for oligonucleotide synthesis which will be reported at a later date.

In several of these experiments, the symmetrical pyrophosphate **5** was isolated as a by-product. This compound was presumably formed by the action of traces of water on the active intermediate to give the monoesterified phosphate **4a**, which could then act as a nucleophile by attack of the active intermediate to give **5**. Alternatively, **5** could be formed by attack of the S-ethyl derivative **3a** on the active species to give a sulfur-containing pyrophosphate triester which would then undergo P–S fission. In an attempt to rationalize the formation of pyrophosphate, **3a** was treated with iodine in pyridine, followed by treatment with water to remove any unreacted starting material. Analysis of the product showed that the pyrophosphate **5** was present to a minor extent. When the reaction was repeated in the presence of an excess of 3′-O-acetylthymidine 5′-phosphate (**4a**), the pyrophosphate **5** was formed in almost quantitative yield. These experiments indicate that the presence of 3′-O-acetylthymidine 5′-phosphate is required for pyrophosphate formation. The ease of formation of pyrophosphate in the presence of **4a** suggests that this method would be useful for the synthesis of nucleotide coenzymes containing pyrophosphate linkages.

The enzymatic susceptibilities of the nucleoside S-ethyl phosphorothioates have also been investigated. The 5′-S-ethyl phosphorothioates of thymidine, 5-fluorodeoxyuridine, and deoxycytidine were all resistant to bacterial alkaline phosphatase, and to spleen phosphodiesterase, but were all split by a sample of snake venom phosphodiesterase with the formation of ethanethiol and the corresponding nucleotide. Experiments with a sample of a phosphodiesterase from carrot[27] gave the same results as with snake venom diesterase. In contrast, a dinucleoside 3′,5′-phosphorothionate has been reported to be resistant to venom diesterase.[7]

Experimental Section

General Procedures. Paper chromatography was performed by the descending technique using Whatman No. 1 paper. The following systems were used: (a) ethanol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v); (b) 1-butanol–acetic acid–water (5:2:3 v/v/v); (c) isobutyric acid–0.5 *N* ammonium hydroxide (60:36, v/v); (d) acetonitrile–0.1 *M* ammonium acetate, pH 7.5 (70:30, v/v). Visualization of sulfur-containing spots on paper chromatograms was carried out as described by Wieland and Lambert.[24] Paper electrophoresis was performed with Whatman No. 1 paper at 1500 V using 0.1 *M* triethylammonium bicarbonate buffer at pH 8. In all reactions involving pyridine, dried material was employed. This was obtained by distillation over potassium hydroxide and storage over Linde Molecular Sieve Type 4A. Ultraviolet absorption measurements were carried out using a Zeiss PMQ II or a Carey Model 14 instrument, and infrared spectra were obtained with a Beckman IR-5 or IR-9. Ion-exchange cellulose column chromatography was carried out using Whatman DE23 cellulose. Unless otherwise stated the acetate form of the cellulose was employed, with triethylammonium acetate at pH 6.0 as the buffer. Samples were adjusted to pH 6.5 before application to the column, and a linear gradient was employed.

Preparation of 3′-O-Acetylthymidine 5′-S-Ethyl Phosphorothioate (3a). Dilithium S-ethyl phosphorothioate (2.17 g, 14 mmol)[4] was dissolved in 10% aqueous pyridine by gently grinding the mixture in the presence of Dowex 50 resin (pyridinium form). The slurry was applied to a Dowex 50 column (pyridinium form) which was eluted with 10% aqueous pyridine. The eluate was evaporated to dryness, 3′-O-acetylthymidine (**2a**, 2.0 g, 7 mmol) and pyridine (15 ml) were added, and the solution was dried by repeated evaporation of added pyridine (three 15-ml portions). The mixture was dissolved in pyridine (30 ml) and treated with DCC (4.4 g), and the solution was shaken for 14 hr at room temperature. Water (50 ml) was added, and after storage overnight at 0° the solution was filtered. The filtrate and washings were evaporated to dryness and dissolved in water. The solution was extracted twice with chloroform, and the aqueous portion was applied to a DEAE cellulose column (88 × 4.5 cm). Elution was carried out using 4 l. of 0.005 *M* buffer in the mixing vessel and 4 l. of 0.05 *M* buffer in the reservoir, and 25-ml fractions were collected. The fractions containing the product (tubes 450–530) were pooled, evaporated to dryness, dissolved in water, and converted into the sodium salt by passage through a Dowex 50 column (sodium form). The eluate was evaporated to dryness and dried by distillation of ethanol over the residue. The syrup was dissolved in a mixture of dry methanol (50 ml) and acetone (10 ml), and addition of dry ether (500 ml) precipitated a white solid. This material was collected by centrifugation, washed with ether, and dried *in vacuo* to give 1.58 g (50%) of **3a**: $\lambda_{max}^{H_2O}$ 265 mμ (ε 9300); ν_{max}^{KBr} 1690 cm^{-1} (C=O).

Anal. Calcd for $C_{14}H_{20}N_2NaO_8PS \cdot H_2O$: C, 37.51; H, 4.94; P, 6.91; S, 7.15. Found: C, 37.93; H, 4.86; P, 5.82; S, 7.04.

A portion of the product was converted into the pyridinium form and stored in anhydrous pyridine for use in subsequent experiments. In addition, **4a**, 4030 OD_{267} units (6%), the symmetrical pyrophosphate **5**, 4100 OD_{267} units (6%), and the dinucleoside phosphate **6**, 2000 OD_{267} units (3%) were obtained as by-products. Compounds **4a** and **5** were identified by paper chromatographic comparison with authentic materials.[10,11]

3′-O-Acetyl-5-fluorodeoxyuridine 5′-S-Ethyl Phosphorothioate (3b). Pyridinium S-ethyl phosphorothioate (14 mmol) and 3′-O-acetyl-5-fluorodeoxyuridine (**2b**, 2.05 g, 7.1 mmol)[19] were dried as before, and pyridine (40 ml) and DCC (4.12 g) were added. The solution was shaken for 14 hr at room temperature, and water (50 ml) was added. After storage for 24 hr at 0°, the product was purified and isolated as the sodium salt 1.55 g (50%) by the procedure described in the previous experiment, $\lambda_{max}^{H_2O}$ 265 mμ (ε 8300); ν_{max}^{KBr} 1710 cm^{-1} (C=O).

Anal. Calcd for $C_{13}H_{17}FN_2NaO_8PS$: C, 35.93; H, 3.93; P, 7.14; S, 7.38. Found: C, 36.35; H, 3.97; P, 7.35; S, 6.79.

3′-O-Acetyl-N⁴-benzoyldeoxycytidine 5′-S-Ethyl Phosphorothioate (3c). Pyridinium S-ethyl phosphorothioate (3.3 mmol) and 3′-O-acetyl-N⁴-benzoyldeoxycytidine (608 mg, 1.6 mmol)[20] were dried by the usual procedure. Pyridine (15 ml) and DCC (1.08 g) were added, and the reaction was shaken for 23 hr at room temperature. The mixture was purified in the usual way by DEAE-cellulose column (30 × 4.5 cm) chromatography, and **3c** 527 mg (60%) was isolated as the sodium salt: $\lambda_{max}^{H_2O}$ 259 mμ (ε 21,000) and 302 (11,600); $\lambda_{max}^{0.1\,N\,HCl}$ 257 mμ (ε 12,900) and 315 (19,800); ν_{max}^{KBr} 1655, 1695, and 1735 cm^{-1} (C=O).

Anal. Calcd for $C_{20}H_{23}N_3NaO_8PS \cdot H_2O$: C, 44.69; H, 4.69; P, 5.76. Found: C, 44.29; H, 4.60; P, 5.50.

3′-O-Acetyl-N⁶-benzoyldeoxyadenosine 5′-S-Ethyl Phosphorothioate (3d). A solution of **2d** (77 μmol)[22] and pyridinium S-ethyl phosphorothioate (182 μmol) were dried by the usual method, and pyridine (2 ml) and DCC (100 mg) were added. The solution was shaken for 42 hr at room temperature, and water (25 ml) was added. After storage overnight at 0°, the product was treated in the usual manner. After purification on a DEAE-cellulose column, using the gradient as employed for the preparation of **3a**, the product was evaporated to dryness. Portions of pyridine were added at intervals during this procedure in order to prevent a fall in pH. The product **3d**, 59 μmol (77%), $\lambda_{max}^{H_2O}$ 280–281 mμ, was directly converted into **4d** as subsequently described.

Conversion of 3a into 3′-O-Acetylthymidine 5′-Phosphate (4a). A solution of **3a** (66 μmol, pyridinium form) in water (2 ml) was treated with a solution of iodine (126 mg) in acetone (2 ml). After 4 hr the solution was diluted with water (20 ml) and extracted with

(27) C. Harvey, L. Malsman, and A. L. Nussbaum, *Biochemistry*, **6**, 3689 (1967).

ether (three 20-ml portions), and the aqueous solution was applied to a DEAE-cellulose column (50 × 2.3 cm). Elution with a gradient of 2 l. of 0.005 *M* buffer in the mixing vessel and 2 l. of 0.2 *M* buffer in the reservoir gave **4a**, 553 OD_{267} units (87%). This material was chromatographically identical with an authentic sample[10] in several paper systems.

3′-O-Acetyl-5-fluorodeoxyuridine 5′-Phosphate (4b). A solution of **3b** (100 mg, 230 μmol, sodium salt) in water (5 ml) was treated with a solution of iodine (86 mg) in acetone (5 ml) for 2 hr. The product, 1740 OD_{268} units (85%), was isolated by the procedure described for **4a**. The product was identified by paper chromatographic comparison with an authentic sample.[19]

3′-O-Acetyl-N⁴-benzoyldeoxycytidine 5′-Phosphate (4c). A sample of **3c** (100 mg, 204 μmol, sodium salt) in water (4 ml) was treated with iodine (200 mg) in acetone (4 ml) for 6 hr. The product, 2840 OD_{259} units (70%), was isolated by the procedure outlined for **4a**. A sample was converted into the sodium form, dissolved in dry methanol (1 ml), and precipitated with dry ether (50 ml). The product was collected by centrifugation and dried *in vacuo*; $\lambda_{max}^{H_2O}$ 257 mμ (ε 21,900), 303 (12,200); $\lambda_{max}^{0.1\ N\ HCl}$ 257 mμ (ε 13,650) and 315 (20,800); ν_{max}^{KBr} 1650, 1700, and 1735 cm^{-1} (C=O).

Anal. Calcd for $C_{16}H_{18}N_3Na_2O_9P \cdot 5H_2O$: C, 36.82; H, 4.80; P, 5.28. Found: C, 36.56; H, 3.86; P, 5.30.

3′-O-Acetyl-N⁶-benzoyldeoxyadenosine 5′-Phosphate (4d). A solution of **4c** (59 μmol) in water (6 ml) was treated with iodine (73 mg) in acetone (5 ml) for 2 hr. The product was extracted in the usual way, and the aqueous solution was purified by DEAE-cellulose column chromatography. The appropriate fractions were pooled and evaporated to dryness in the presence of added pyridine. The product **4a**, 710 OD_{280} units (59%), was chromatographically identical with an authentic sample of **4d** which had been prepared by the standard method.[23] Deacylation of **4d** gave deoxyadenosine 5′-phosphate which was identified by comparison with an authentic sample.

3′-O-Acetylthymidine 5′-β,β,β-Trichloroethyl Phosphate (11). A solution of **3a** (61 mg, 142 μmol, sodium salt) in dry β,β,β-trichloroethanol (5 ml) was treated with a solution of iodine (55 mg) in β,β,β-trichloroethanol (5 ml). After 5 hr, water (25 ml) was added and the solution was extracted with ether (three 20-ml portions). The aqueous layer was evaporated to dryness, and the residue was dissolved in dry methanol (3 ml) and acetone (1 ml). Addition of dry ether (70 ml) gave a precipitate which was collected by centrifugation and dried *in vacuo* to give 62.4 mg, (80%) of **11** as a white solid: $\lambda_{max}^{H_2O}$ 267 mμ (ε 9400); ν_{max}^{KBr} 1680 cm^{-1} (C=O). The product was chromatographically identical with a sample of **11** which had been prepared by a published procedure.[28]

3′-O-Acetyl-N⁴-benzoyldeoxycytidine 5′-Methyl Phosphate (12). A solution of the S-ethyl derivative **3c** (252 mg, 0.47 mmol, sodium salt) and iodine (514 mg) in dry methanol (35 ml) was allowed to stand at room temperature for 2.5 hr. The solvent was removed by evaporation, and the residue was dissolved in water (20 ml) and extracted with ether (three 20-ml portions). The aqueous layer was applied to a DEAE-cellulose column (30 × 4.5 cm) which was eluted with a gradient of 2 l. of 0.005 *M* buffer in the mixing vessel and 2 l. of 0.1 *M* buffer in the reservoir. The appropriate fractions were pooled and evaporated to dryness. The residue was dissolved in water, converted into the sodium form, evaporated to dryness, and dissolved in dry methanol (10 ml). Addition of dry ether (200 ml) gave a precipitate which was collected and dried *in vacuo* to give 145 mg (60%) of **12** as a white solid: $\lambda_{max}^{H_2O}$ 258 mμ (ε 21,350) and 302 (11,850); $\lambda_{max}^{0.1\ N\ HCl}$ 257mμ (ε 13,650) and 315 (19,900); ν_{max}^{KBr} 1660, 1710, and 1750 cm^{-1} (C=O).

Anal. Calcd for $C_{19}H_{21}N_3NaO_9P \cdot 1.5H_2O$: C, 44.20; H, 4.69; P, 6.00. Found: C, 44.16; H, 4.85; P, 5.38.

This material was chromatographically identical with a sample prepared by the method of Khorana.[29]

Reaction of 18 with Morpholine. A solution of **18** (208 μmol) pyridinium salt) and iodine (200 mg) in morpholine (5 ml) was stored at room temperature overnight. The crystalline precipitate was filtered and washed sparingly with morpholine, and the filtrate and washings were evaporated to dryness. The residue was dissolved in water (20 ml) and extracted with ether (three 20-ml portions). The aqueous layer was applied to a DEAE-cellulose column (bicarbonate form, 50 × 2.3 cm) and eluted with a gradient of 2 l. of 0.005 *M* triethylammonium bicarbonate, pH 8.0, in the mixing vessel and 2 l. of 0.1 *M* triethylammonium bicarbonate, pH 8.0, in the reservoir. The appropriate fractions were pooled, evaporated to dryness, converted into the sodium form, and dissolved in dry methanol (1.5 ml). Addition of ether (30 ml) gave a precipitate which was collected and dried *in vacuo* to give **14** as the trihydrate, 83 mg (83%): $\lambda_{max}^{H_2O}$ 267–268 mμ (ε 9150).

Anal. Calcd for $C_{14}H_{21}N_3NaO_8P \cdot 3H_2O$: C, 35.97; H, 5.78; N, 8.99; P, 6.64. Found: C, 36.37; H, 5.07; N, 8.60; P, 6.62.

This material was chromatographically identical with a commercial sample. Hydrolysis in 0.5 *N* sulfuric acid for 30 min liberated thymidine 5′-phosphate.

Reaction of 3a with *p*-Nitrophenol. A mixture of *p*-nitrophenol (1.39 g) and **3a** (132 μmol, pyridinium salt) was dried by evaporation of added pyridine (three 10-ml portions). Pyridine (4 ml) and iodine (254 mg) were added, and the solution was stored at room temperature for 17 hr. Water (10 ml) was added, and after 2 hr the solution was extracted with ether (three 10-ml portions), and the aqueous layer was applied to a DEAE-cellulose column (50 × 3.5 cm). The column was eluted with a gradient of 2 l. of 0.005 *M* buffer in the mixing vessel and 2 l. of 0.15 *M* buffer in the reservoir, and the required fractions were pooled and evaporated to dryness. The residue was converted into the sodium form, dried, and dissolved in dry methanol (5 ml). Addition of ether (50 ml) precipitated **13** as the tetrahydrate, 46.7 mg (58%): $\lambda_{max}^{0.01\ N\ HCl}$ 270 mμ (ε 15,800).

Anal. Calcd for $C_{15}H_{19}N_3NaO_{11}P \cdot 4H_2O$: P, 5.36. Found: P, 5.75.

This material was chromatographically identical with a sample obtained by acetylation of commercial thymidine 3′-*p*-nitrophenyl phosphate. Degradation with venom diesterase gave *p*-nitrophenol and 3′-O-acetylthymidine 5′-phosphate.[12]

Reaction of 3a with Orthophosphate. A solution of tri-*n*-butylamine (1.2 ml, 5 mmol) in 85% orthophosphoric acid (0.34 ml, 5 mmol) was added to a solution of **3a** (66 μmol, pyridinium salt) in pyridine. The mixture was dried by evaporation of added pyridine (three 10-ml portions), dissolved in pyridine (4 ml), and treated with iodine (131 mg) for 17 hr at room temperature. The mixture was purified by the procedure described in the previous experiment. The required fractions from the column were pooled and evaporated to dryness to give 401 OD_{267} units (73%) of **15.** A sample was deacetylated using concentrated ammonium hydroxide, and the product was chromatographically identical wth commercial thymidine 5′-diphosphate. Degradation of the deacetylated material with bacterial alkaline phosphatase yielded thymidine, while treatment with venom diesterase gave thymidine 5′-phosphate.

Reaction of 3a with Pyrophosphate. An aqueous solution of sodium pyrophosphate decahydrate (446 mg, 1 mmol) was converted into the pyridinium salt by passage over a Dowex 50 resin (pyridinium form), and the eluate was evaporated to dryness and dissolved in 80% aqueous pyridine (5 ml). Tri-*n*-butylamine (0.95 ml, 4 mmol) and **3a** (196 μmol, pyridinium salt) were added, and the mixture was evaporated and dried by evaporation of 2-picoline (three 10-ml portions). 2-Picoline (10 ml) and iodine (250 mg) were added, and the mixture was shaken for 16 hr at room temperature. The product was treated with water (10 ml) for 15 min and then evaporated to dryness. The residue was dissolved in water (20 ml) and extracted with ether (three 20-ml portions). The aqueous layer was evaporated to dryness and treated with concentrated aqueous ammonia (40 ml). After 3 hr the precipitate was removed, and the filtrate concentrated to 10 ml, and applied to a DEAE-cellulose column (50 × 2 cm, bicarbonate form) which was eluted with a gradient of 2 l. of 0.005 *M* triethylammonium bicarbonate at pH 7.5 in the mixing vessel and 2 l. of 0.5 *M* bicarbonate buffer in the reservoir. The appropriate fractions were combined to give 126 μmol (64%) of thymidine 5′-triphosphate (**16**). This material was chromatographically identical with a commercially obtained sample of **16.**

Reaction of 3a with 3′-O-Acetylthymidine. A mixture of 3′-O-acetylthymidine (129 mg, 454 μmol) and **3a** (100 μmol, pyridinium salt) was dried and dissolved in pyridine (3 ml). Iodine (129 mg) was added, and the solution was stored for 17 hr at room temperature. Water (20 ml) was added, and after 1 hr the solution was extracted with ether (three 20-ml portions). The aqueous layer was applied to a DEAE-column (50 × 2.3 cm) and eluted with the buffer system described in the preparation of **4a**. The fractions containing the product were evaporated to dryness to give 1020 OD_{267} units (53%) of **6**: $\lambda_{max}^{H_2O}$ 267 mμ (ε 19,300).

(28) See footnote *e*, Table I.

(29) H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4657 (1959).

Anal. Calcd for $C_{24}H_{30}N_4NaO_{14}P \cdot 4H_2O$: C, 39.80; H, 5.29; N, 7.73, P, 4.28. Found: C, 40.00; H, 4.92; N, 7.36; P 4.25.

In addition, the pyrophosphate **5** was obtained, 272 OD_{267} units (14%). When the reaction was carried out using 10 equiv of 3'-O-acetylthymidine, the yield of **6** increased to 60%. Deacetylation of **6** with concentrated ammonium hydroxide gave **7**. Venom diesterase degraded **7** to thymidine and thymidine 5'-phosphate,[12] whereas no degradation occurred during incubation with spleen diesterase.

Reaction of 3a with 5'-O-Tritylthymidine. 5'-O-Tritylthymidine (1.405 g, 2.5 mmol) and **3a** (165 μmol, pyridinium salt) were dried by repeated evaporation of added pyridine (three 20-ml portions), and dissolved in pyridine (5 ml). Iodine (254 mg) was added and the solution was stored at room temperature for 72 hr. Water (1 ml) was added, and after 1.5 hr the solution was evaporated to dryness. The residue was dissolved in 0.005 *M* buffer containing 50% ethanol (20 ml), and some 5'-O-tritylthymidine crystallized at this stage. The crystals were filtered and the filtrate was applied to a DEAE-cellulose column (50 × 2.3 cm). Elution was carried out with 2 l. of 0.005 *M* buffer containing 50% ethanol in the mixing vessel and 2 l. of 0.15 *M* buffer containing 50% ethanol in the reservoir. The appropriate fractions were combined, evaporated to dryness, and dissolved in methanol (3 ml). Addition of ether (100 ml) gave a precipitate which was collected by centrifugation, washed with ether, and dried *in vacuo* to give 70 mg (42%) of **17** as the triethylammonium salt hexahydrate, $\lambda_{max}^{CH_3OH}$ 266 mμ (ε 18,750).

Anal. Calcd for $C_{46}H_{68}N_4O_{13}P \cdot 6H_2O$: P, 3.06. Found: P, 3.16.

The symmetrical pyrophosphate **5** was also isolated in 21% yield. A sample of **17** (3 mg) was treated with 80% acetic acid (0.5 ml) for 2 hr, evaporated to dryness and then treated with concentrated ammonium hydroxide overnight. A second sample (3 mg) was treated with concentrated ammonium hydroxide overnight. The samples of thymidylyl-(3',5')-thymidine and 5'-O-tritylthymidylyl-(3',5')-thymidine obtained in this manner were chromatographically identical with authentic materials.[26]

Deacetylation of 3a. A solution of **3a** (1.0 g, sodium salt) in concentrated ammonium hydroxide was allowed to stand at room temperature for 2 hr, and then evaporated to dryness. The residue was converted into the pyridinium form and stored in dry pyridine. A sample was converted into the sodium salt and dissolved in methanol (3 ml) containing a few drops of acetone. Addition of ether (60 ml) gave a white precipitate of **18** which was collected by centrifugation and dried *in vacuo:* $\lambda_{max}^{H_2O}$ 266 mμ (ε 9100); ν_{max}^{KBr} 1690 cm^{-1} (C=O).

Anal. Calcd for $C_{12}H_{18}N_2NaO_7PS \cdot 0.5H_2O$: C, 36.28; H, 4.82; P, 7.80. Found: C, 36.85; H, 4.89; P, 7.47.

Treatment of 3a with Iodine in Pyridine. A solution of **3a** (132 μmol, pyridinium salt) in pyridine was dried and treated with iodine (254 mg) and pyridine (3 ml) for 17 hr. The products were isolated by the procedure described for the preparation of **6** and were identified by paper chromatography as **4a**, 793 OD_{267} units (63%), and **5**, 267 OD_{267} units (22%). This experiment was repeated in the presence of 3 equiv of **4a**. Under these conditions the yield of **5** was almost quantitative.

Enzymatic Experiments. a. Venom Phosphodiesterase. The substrate (0.3 μmol) in water (20 μl) was incubated with 1 *M* Tris buffer pH 8 (20 μl) and venom diesterase (1 μl, 2.5 mg/ml, Worthington Biochemical Corp.) for 3 hr at 37°.

b. Carrot Phosphodiesterase. The substrate (0.3 μmol) in water (80 μl) was incubated with carrot diesterase (1 μl, 1.38 units),[27] 1 *M* Tris buffer pH 9 (10 μl), and 1 *M* magnesium chloride (10 μl) for 4 hr at 37°.

c. Spleen Phosphodiesterase. The substrate (0.3 μmol) in water (10 μl) was treated with spleen diesterase (5 μl, 10–15 units/ml, Worthington) and 1 *M* ammonium acetate pH 5.9 (10 μl) for 1.5 hr at 37°.

d. Bacterial Alkaline Phosphatase. The substrate (0.3 μmol) in water (20 μl) was incubated with 1 *M* Tris buffer pH 8 (20 μl) and bacterial alkaline phosphatase (1 μl, 10 mg/ml, Worthington) for 3 hr at 37°.

In all cases the incubation mixtures were analyzed by paper chromatography.

Acknowledgments. Thanks are extended to Dr. V. Toome for ultraviolet spectra, to Mr. S. Traiman for infrared spectra, and to Dr. F. J. Scheidl for microanalyses.