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# Nucleotide Synthesis. I. Derivatives of Thymidine Containing *p*-Nitrophenyl Phosphate Groups[1a]

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The synthesis of a number of nucleotide derivatives of thymidine containing *p*-nitrophenyl phosphate groups which have utility as substrates of staphylococcal nuclease is described. Included in this group of compounds are thymidine 3′,5′-di(*p*-nitrophenyl phosphate), derivatives of thymidine 5′-(*p*-nitrophenyl phosphate) substituted at the 3′ position with phosphate and β-cyanoethyl phosphate groups, and derivatives of thymidine 3′-(*p*-nitrophenyl phosphate) substituted at the 5′ position with phosphate, β-cyanoethyl phosphate, methyl- and halomethylphosphonate, and sulfate. The proof of structure of the nucleotides prepared rests on characterization by uv, paper chromatography, and elemental analysis, supplemented by a semimicro method for the determination of p$K_a$-molecular weight data.

A large amount of data has been accumulated on the physiochemical properties of staphylococcal nuclease.[2] Knowledge of catalytic mechanisms and substrate specificity, however, has been limited by the unavailability of low-molecular-weight substrates of the enzyme. A recent paper[3] describes studies of staphylococcal nuclease with such low-molecular-weight substrates. The synthesis, physical properties, and proof of structure of a number of the nucleotides used in these studies form the subject matter of this paper. Furthermore, one of the nucleotides, thymidine 5′-phosphate-3′-(*p*-nitrophenyl phosphate) (**13**), on catalytic reduction of the *p*-nitrophenyl group, affords an intermediate which has been used in the purification of staphylococcal nuclease by affinity chromatography.[4] Since simple *p*-nitrophenyl compounds have been used successfully in the past as substrates for measuring the activity of many esterases, for example spleen and liver phosphodiesterase,[5] the more complex *p*-nitrophenyl nucleotides described herein may also have potential utility for probing the structure, specificity, and activity of other enzymes. Further, these compounds have activity as active-site-directed inhibitors (at *ca.* $2.5 \times 10^{-2}$ *M* concentration) or as intermediates in the synthesis of other active-site-directed inhibitors of a nuclear exoribonuclease isolated from mammalian cells.[6]

Thymidine 3′-(*p*-nitrophenyl phosphate) (**3**) and thymidine 5′-(*p*-nitrophenyl phosphate) (**5**) were used as starting materials in the synthesis of the 5′- and 3′-substituted *p*-nitrophenyl nucleotide derivatives. Compound **3** (Scheme I) was prepared from thymidine by tritylation of the 5′-hydroxyl group, followed by *p*-nitrophenyl phosphorylation of the 3′-hydroxyl group and hydrolysis of the 5′-*O*-trityl group. The *p*-nitrophenyl phosphorylation reaction was performed using *p*-nitrophenyl phosphorodichloridate according to the procedure of Turner and Khorana,[7] or using di-*p*-nitrophenyl phosphorochloridate, followed by a mild alkaline hydrolysis. The product, as were most of the nucleotide derivatives prepared, was purified by large-scale preparative paper chromatography. The preparative paper chromatography procedure outlined briefly in the Experimental Section has proved more efficient for gram quantity purification, in our hands, than column chromatography over anion-exchange resins and cellulose with buffer solutions. Compound **5** was prepared by the reaction of 3′-*O*-acetylthymidine (**4**) with di-*p*-nitrophenyl phosphorochloridate, followed by a mild alkaline hydrolysis to remove one *p*-nitrophenyl group and the 3′-*O*-acetyl protection. The product (**5**) was identical with material prepared by a lengthy, more difficult, procedure involving a DCC coupling reaction of excess *p*-nitrophenol with thymidine 5′-phosphate.[8]

(1) (a) A portion of this work has been reported: R. P. Glinski and C. L. Stevens, Abstracts, 155th National Meeting of the American Chemical Society, San Francisco, Calif., March 1968, N17. (b) To whom correspondence should be addressed: Ash Stevens, Inc. (c) Ash Stevens, Inc. (d) National Cancer Institute.

(2) For a review, see P. Cuatrecasas, H. Taniuchi, and C. B. Anfinsen, *Brookhaven Symp. Biol.*, **21**, 172 (1969).

(3) P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Biochemistry*, **8**, 2277 (1969).

(4) P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U. S.*, **61**, 636 (1968).

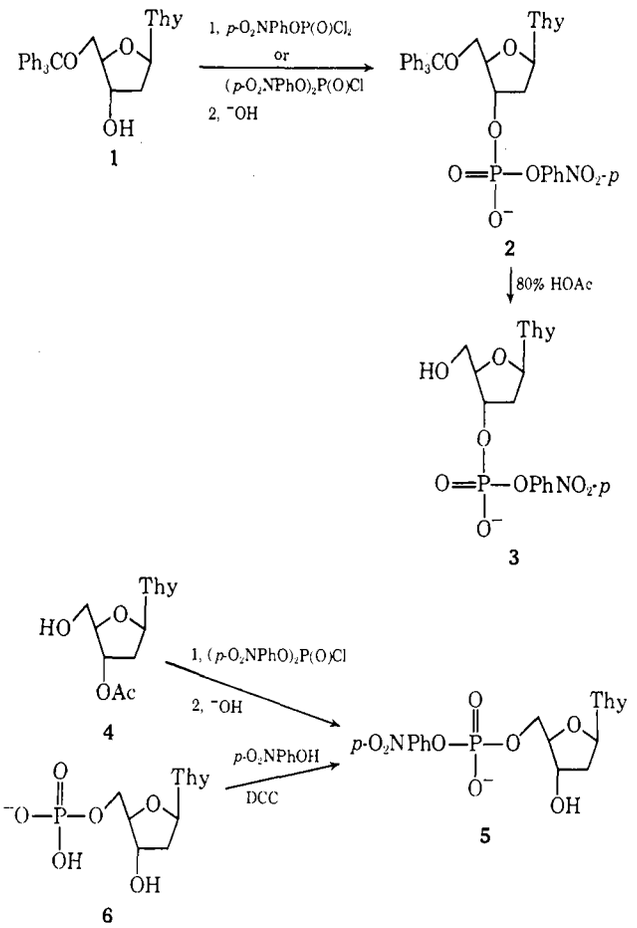
(5) H. G. Khorana, *Enzymes*, **5**, 79 (1961); W. E. Razzell, *Methods Enzymol.*, **6**, 236 (1963).

(6) For a review of the work performed on the isolation, properties, and inhibition of this enzyme, see M. B. Sporn, D. M. Berkowitz, R. P. Glinski, A. B. Ash, and C. L. Stevens, *Science*, **164**, 1408 (1969), and the references cited therein.

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

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

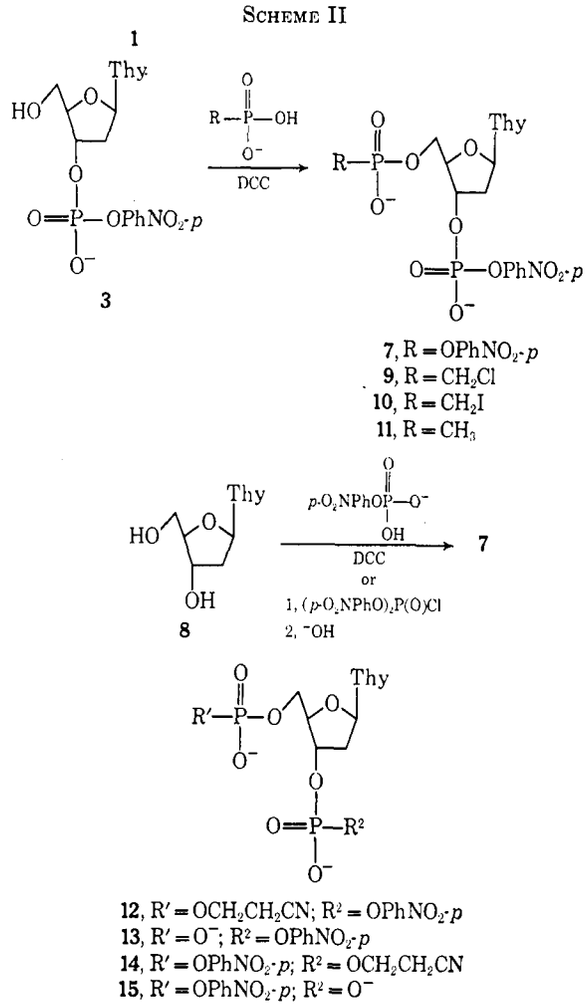
SCHEME I



DCC coupling reactions of both thymidine 3′-(*p*-nitrophenyl phosphate) (**3**) and 5′-(*p*-nitrophenyl phosphate) (**5**) were studied. Reaction of compound **3** with β-cyanoethyl phosphate, *p*-nitrophenyl phosphate, methyl-, chloromethyl-, and iodomethylphosphonate, and DCC gave compounds **12**, **7**, **11**, **9**, and **10**, respectively. Thymidine 3′,5′-di(*p*-nitrophenyl phosphate) (**7**) was synthesized also by either a DCC coupling of *p*-nitrophenyl phosphate with thymidine, or by phosphorylation of thymidine with di-*p*-nitrophenyl phosphorochloridate, followed by a basic hydrolysis (Scheme II). The latter procedure is the most convenient and has been used to prepare crystalline compound **7** on a 5–10-g scale, after large-scale preparative paper chromatography.

Thymidine 5′-(β-cyanoethyl phosphate)-3′-(*p*-nitrophenyl phosphate) (**12**) was prepared for use as an intermediate in the synthesis of thymidine 3′-(*p*-nitrophenyl phosphate)-5′-phosphate (**13**). Reaction of compound **12** with 1 *N* sodium hydroxide gave a selective β elimination of the β-cyanoethyl ester group in the presence of the 3′-(*p*-nitrophenyl phosphate) group. That this reaction was indeed selective was demonstrated by elemental analysis, p$K_a$–molecular weight determinations, and the fact that the liberation of *p*-nitrophenol (monitored at 400 mμ) was negligible during the reaction. Higher concentrations of sodium hydroxide or longer reaction times lead to cleavage of the *p*-nitrophenyl group in addition to the β-cyanoethyl group. Thymidine 5′-(*p*-nitrophenyl phosphate) (**5**) was converted into thymidine 3′-(β-cyanoethyl phosphate)-5′-(*p*-nitrophenyl phosphate) (**14**) and thymidine 5′-(*p*-

SCHEME II



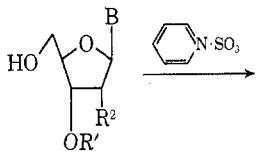
nitrophenyl phosphate)-3′-phosphate (**15**) by the same sequence of reactions used for the preparation of compound **13** with similar results.

Compound **3** was converted also into thymidine 3′-(*p*-nitrophenyl phosphate)-5′-sulfate (**19**). Two model reactions were attempted first. Uridine and thymidine were converted into uridine 2′,3′,5′-trisulfate (**18**) and thymidine 3′,5′-disulfate (**17**), using the pyridine–sulfur trioxide method[9] (Scheme III). The products were isolated in good yield as crystalline barium salts. Similarly, compound **3** afforded crystalline **19** as a barium salt in excellent yield, in which the 5′-hydroxyl group was sulfated in the presence of the 3′-*p*-nitrophenyl phosphate group. It is noteworthy that no difficulty was encountered with compounds containing a sulfatophosphate group;[10] presumably a product containing a sulfatophosphate group would be hydrolyzed to compound **19** during isolation.

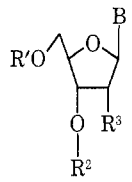
All of the new compounds reported herein were subjected to p$K_a$–molecular weight determinations (see Experimental Section). The method involves the quantitative conversion of a small, dried sample (7–15 mg) of nucleotide salt into the corresponding free acid by passage of an aqueous solution of the salt through a column of purified Dowex 50 ($H^+$). The acidic eluate (10 ml) is titrated with dilute sodium hydroxide (0.5–1.5 ml) using a Sargent pH-Stat. A plot of pH *vs.* ml

(9) P. W. Wigler and H. U. Choi, *J. Amer. Chem. Soc.*, **86**, 1636 (1964).
(10) J. Baddiley, J. G. Buchanan, R. Letters, and A. R. Sanderson, *J. Chem. Soc.*, 1731 (1959).

SCHEME III



**8**, $R' = R^2 = H$; B = thymine
**16**, $R' = H$; $R^2 = OH$; B = uridine
**3**, $R' = P(O)OPhNO_2\text{-}p$; $R^2 = H$; $R^3$ = thymine



**17**, $R' = R^2 = SO_3^-$; $R^3 = H$; B = thymine
**18**, $R' = R^2 = SO_3^-$; $R^3 = OSO_3^-$; B = uridine
**19**, $R' = SO_3^-$; $R^2 = P(O)OPhNO_2\text{-}p$; $R^3 = H$; B = thymine

of titrant gives curves from which $pK_a$'s and molecular weights can be calculated in the usual manner. The determined molecular weights are generally within 2% of the calculated values when 15–30 mg of sample is available or within 5% when 7–15 mg of sample is available.

The method establishes also the degree of substitution on phosphorus. Thus, disubstituted phosphate esters, such as thymidine 3′,5′-di-(*p*-nitrophenyl phosphate) (**7**), give curves with only one inflection and one $pK_a$. On the other hand, monosubstituted phosphate esters, or mixed monosubstituted and disubstituted esters, such as thymidine 5′-(*p*-nitrophenyl phosphate)-3′-phosphate (**15**), give curves with two inflections and two $pK_a$ values.

## Experimental Section

Paper chromatography (pc) was by the descending technique using the following solvent systems: A, 1-butanol–acetic acid–water (5:2:3, v/v); B, 2-propanol–water–concentrated ammonium hydroxide (7:1:2, v/v); C, 2-propanol–aqueous ammonium sulfate (1%) (5:2, v/v); D, ethanol–aqueous ammonium acetate (1%) (5:2, v/v). The nucleotides were detected with ultraviolet light. Analytical pc was performed using Whatman No. 1 and No. 7 paper. Unless stated otherwise, Whatman No. 1 paper was used. Whatman 3MM paper (1–2 g per 20 sheets) was used for the preparative paper chromatography (ppc). The paper was converted into a pulp suspension in water by use of a Waring Blendor. The pulp was washed thoroughly with water (2 l.) in a 2-l. sintered glass filter and was removed by filteration. The filtrate was concentrated to a small volume *in vacuo* below 50° using a Büchi Rotavap (Rinco Instruments). The remaining water was removed by lyophilization to afford a weighable solid.

Thin layer chromatography (tlc) analyses were performed using Eastman chromatogram sheets 6060 (silica gel) impregnated with a fluorescent indicator and Brinkman Instruments' MN-Polygram cel 300 PEI sheets (anion-exchange cellulose). The following tlc systems were used: A, chloroform–ammonium hydroxide (1%) in methanol (3:2, v/v); B, 1% sodium chloride in water. Uv spectra wre recorded using a Hatachi-Coleman 124 spectrophotometer using distilled water as solvent unless stated otherwise. Pyridine was distilled and stored over Linde Molecular Sieves (type 4A). Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind., on all new compounds.

**Thymidine 3′-(*p*-Nitrophenyl phosphate) Ammonium Salt (3).** A.—The title compound was prepared by a modified literature procedure[7] or by method B. In our hands, compound **3** required purification by ppc using pc solvent system A to remove a very minor faster-migrating impurity, relative to **3**. The yields of chromatographically homogeneous product were *ca.* 70%.

B.—**5′-*O*-Tritylthymidine**[7] (**1**, 562 mg) was dissolved in anhydrous pyridine (5 ml) and di-*p*-nitrophenyl phosphorochloridate[11] (653 mg) was added. The reaction mixture was allowed to stand at 1° for 16 hr. Water (4 ml) was added and the mixture was frozen (Dry Ice) and concentrated to a gum *in vacuo* (oil pump). Water (7 ml) was added to the gum and the pH of the mixture was adjusted to 8 (pH paper) by the dropwise addition of 1 *N* NaOH with vigorous shaking. The mixture became homogeneous at pH 5–6. The solution was allowed to stand at room temperature overnight. The pH of the reaction mixture was adjusted to 1–2 with 1 *N* hydrochloric acid. The solution was extracted with chloroform (three 10-ml portions). The chloroform extracts were combined and extracted with aqueous 1 *M* pyridine hydrochloride solution (five 10-ml portions). The chloroform solution was dried ($Na_2SO_4$) and was concentrated *in vacuo* to give a gum. The gum was dissolved in 80% acetic acid (11 ml). The solution was heated in a boiling water bath for 10 min. The reaction mixture was cooled to room temperature and water (15 ml) was added. The resulting heterogeneous mixture was frozen and lyophilized. The resulting solid was suspended in water and the mixture was lyophilized again. Water (20 ml) was added to the residue and the mixture was allowed to stand at 1° for 2 days. The suspension of trityl alcohol was removed by filtration and the solid was washed well with water. The filtrate and washings were combined and lyophilized. The resulting residue was dissolved in a minimum amount of water and the solution was applied to a Dowex 50 ($H^+$) column (10 ml). The column was eluted with water until the effluent was neutral to pH paper. The acidic effluent was neutralized to pH 7.0 with concentrated $NH_4OH$ and was lyophlized. The residue was purified by ppc using seven sheets of 3MM paper and solvent system A to afford 297 mg (64%) of chromatographically homogeneous product, identical with compound **3** ($NH_4^+$ salt) by ir (KBr), uv, and pc using four solvent systems.

**Thymidine 5′-(*p*-Nitrophenyl phosphate) Lithium Salt (5).**—Thymidine 3′-*O*-acetate[12] (**4**, 4.5 g) was dissolved in anhydrous pyridine (50 ml) and di-*p*-nitrophenyl phosphorochloridate[11] (7.5 g) was added. The mixture was stirred at room temperature overnight. The pH of the solution was adjusted to *ca.* 10–11 by the dropwise addition of 1 *N* sodium hydroxide (105 ml). After the addition was complete, the reaction mixture was allowed to stand at room temperature for 20 hr. The reaction mixture was cooled to 0° by the addition of ice and was added to an Amberlite IR-120 ($H^+$) column (100 ml) which was also cooled to 0° by preliminary elution with ice–water. The column was eluted with ice–water (*ca.* 1 l.). The effluent was concentrated *in vacuo* to *ca.* 200-ml volume. The heterogeneous aqueous phase was extracted with diethyl ether (three 200-ml portions). The resulting homogeneous aqueous phase was percolated through a small Amberlite IR-120 ($H^+$) column (20 ml). The column was eluted with water until the effluent was neutral. The pH of the total effluent was adjusted to 3.5 with lithium hydroxide solution and the solution was extracted with diethyl ether (three 100-ml portions) to remove additional *p*-nitrophenol. The pH of the aqueous phase was adjusted to 6.0 with lithium hydroxide solution. The neutralized solution was lyophilized to give 9.09 g of crude product **5**. The crude red solid was purified by ppc using pc system A and 120 sheets of 3MM paper to give chromatographically homogeneous compound **5** (4.32 g).

Compound **5** was prepared also on small scale by the method of Razzell and Khorana[8] which involved a DCC coupling of excess *p*-nitrophenol and thymidine 5′-phosphate. The products obtained by both routes were identical by pc and uv spectroscopy, and the analytical data were in agreement with reported values.[8]

**Thymidine 3′,5′-Di(*p*-nitrophenyl phosphate) Barium Salt (7).** A.—Thymidine 3′-(*p*-nitrophenyl phosphate) ammonium salt (**3**, 750 mg) and *p*-nitrophenylphosphoric acid (750 mg) were dissolved in anhydrous pyridine (15 ml). DCC (5 g) was added to the magnetically stirred solution. The reaction mixture was allowed to stand at room temperature for 24 hr. The pyridine was removed *in vacuo*. Water (25 ml) was added to the residue. The resulting heterogeneous mixture was stirred at room temperature for 2 hr. The solid was removed by filtration and was

(11) T. Ukita and H. Hayatsu, *J. Amer. Chem. Soc.*, **84**, 1879 (1962).
(12) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 951 (1953).

washed well with water. The filtrate and washings were combined and were passed through a Dowex 50 ($H^+$) column (50 ml). The column was eluted with water until the effluent was neutral. The effluent was neutralized with concentrated $NH_4OH$ and was lyophilized to afford crude compound **7**. The major nucleotide band migrating slightly slower than **3** was separated by ppc using pc systems A and C in succession to give chromatographically homogeneous compound **7** (250 mg, diammonium salt) as a hygroscopic solid. The diammonium salt was converted into the barium salt by passage through a Dowex 50 ($H^+$) column and neutralization with $Ba(OH)_2$ solution to give compound **7** (230 mg) as an amorphous yellow solid. The amorphous solid was fractionally precipitated from water–2-propanol mixtures. The first fractions yielded highly colored solids and gums. The latter fractions crystallized to afford analytically pure compound **7** (130 mg) after drying at 110° ($10^{-3}$ mm) for 2 hr: mp 264° dec; $R_f$ values in systems A, B, C, and D were 0.61, 0.69, 0.79, and 0.79, respectively: uv max ($H_2O$) 275 mμ (ε 21,000), 250–260 (0.64), 260–270 (0.77), 270–280 (0.99); $pK_a$ = 2.79; mol wt 798 (found) *vs.* 798 (calcd).

*Anal.* Calcd for $C_{22}H_{20}N_4O_{15}P_2 \cdot Ba \cdot H_2O$: C, 33.12; H, 2.78; N, 7.02; P, 7.77. Found: C, 33.11; H, 3.38; N, 7.23; P, 7.49.

Similarly, thymidine was coupled with excess *p*-nitrophenyl phosphate to give a low yield of product **7**.

**B.**—Thymidine (**8**, 5.0 g) was dissolved in anhydrous pyridine (125 ml). The solution was cooled to 0° and stirred. Di-*p*-nitrophenyl phosphorochloridate (22.5 g) was added portionwise. The reaction mixture was allowed to stand at 0° for 3 days. Sodium hydroxide, 1 *N* (175 ml), was added over 30 min with stirring. The reaction mixture was allowed to warm to room temperature and was allowed to stand at room temperature for 2 hr. Additional 1 *N* sodium hydroxide (5 ml) was added and the mixture was stirred at room temperature for an additional 16 hr. The reaction mixture was cooled with ice and added to a Dowex 50 ($H^+$) column (900 ml). The column was eluted with water. The total effluent was neutralized to pH 3.5 (pH paper) with 1 *N* sodium hydroxide. The solution (2 l.) was concentrated *in vacuo* to *ca.* 100-ml volume. The concentrated solution was extracted with ethyl ether (five 100-ml portions). The aqueous phase was adjusted to *ca.* pH 6.5 and lyophilized to yield 21.6 g of crude **7**. Compound **7** was purified by ppc (134 sheets of 3MM paper) using pc system A to give chromatographically homogeneous solid (9.8 g). The solid was dissolved in water and applied to a Dowex 50 ($H^+$) column (180 ml). The column was eluted with water until the effluent was neutral. The effluent was extracted with ethyl ether (twice) and stirred with a small amount of charcoal at room temperature for 1 hr. The charcoal treatment was repeated. The charcoal was removed by filtration. The pH of the filtrate was adjusted to pH 6.5 with $Ba(OH)_2$ solution. The solution was concentrated *in vacuo* and was adjusted to pH 7.0 with $Ba(OH)_2$ solution. The neutralized solution was stirred at room temperature for 3 hr with a small amount of charcoal. The charcoal was removed by filtration. The filtrate was lyophilized to yield amorphous compound **7** (9.64 g). The amorphous solid was dissolved in a minimum amount of warm water. The solution was cooled to room temperature and diluted with 2-propanol to the point of turbidity. The mixture was centrifuged. This treatment was repeated several times. The supernatant was transferred to a 125 ml flask, diluted to the point of turbidity, and seeded with crystalline compound **7**. A brown gum formed over a period of several hours. The supernatant was decanted away from the gum and the above procedure was repeated five times with similar results before crystallization occurred. 2-Propanol was added daily to the point of turbidity as crystallization continued. After *ca.* 1 week, 7.45 g (45%) of crystalline compound **7** was obtained. An additional 1.0 g (6%) of pure **7** was obtained by purification of the residue (obtained by evaporation of mother liquors) by ppc using pc system C and 30 sheets of 3MM paper. A sample was recrystallized from water–2-propanol mixtures and dried *in vacuo* as before for analysis.

*Anal.* Calcd for $C_{22}H_{20}N_4O_{15}P_2 \cdot Ba \cdot H_2O$: C, 33.12; H, 2.78; N, 7.02; P, 7.77. Found: C, 33.09; H, 3.03; N, 7.26; P, 8.25.

Preparations of **7** by both routes were identical by pc (four systems), uv, melting point, and mixture melting point determinations.

**Thymidine 5′-Chloromethylphosphonate-3′-(*p*-nitrophenyl phosphate) Diammonium Salt (9).**—Thymidine 3′-(*p*-nitrophenyl phosphate) ammonium salt (**3**, 300 mg) and anhydrous chloromethylphosphonic acid were dissolved in anhydrous pyridine (8 ml). DCC (1.3 g) was added and the heterogeneous mixture was stirred at room temperature for 16 hr. The solvent was removed *in vacuo*. Water (5 ml) was added to the residue. The mixture was stirred vigorously for 5 min. The dicyclohexylurea was removed by filtration and was washed thoroughly with water (four 5-ml portions). The brown filtrate and washings were combined and were passed through a Dowex 50 ($H^+$) column (25 ml). The column was eluted with water until the effluent was neutral. The yellow effluent was neutralized with dilute ammonium hydroxide and was lyophilized to give 350 mg of cride **9**. Compound **9** was purified by ppc (six 3MM sheets) using solvent systems C and A in succession to afford 213 mg (56%) of chromatographically homogeneous product **9**. An analytically pure sample was prepared by fractional precipitation from water–methanol–ethyl ether mixtures, followed by drying at room temperature ($5 \times 10^{-3}$ mm) over $P_2O_5$ for 48 hr: $R_f$ values (Whatman No. 7 paper) using solvent systems A, B, C, and D were 0.58, 0.62, 0.76, and 0.75, respectively; uv max 272 mμ (ε 14,300), 250–260 (0.63), 260–270 (0.82), 270–280 (1.09); $pK_a$ = 2.83; mol wt 558 (found) *vs.* 590 (calcd).

*Anal.* Calcd for $C_{17}H_{18}ClN_3O_{12}P_2$: Cl, 6.01. Found: Cl, 6.00.

**Thymidine 5′-Iodomethylphosphonate-3′-(*p*-nitrophenyl phosphate) Barium Salt (10).**—Thymidine 3′-(*p*-nitrophenyl phosphate) ammonium salt (**3**, 500 mg) and iodomethylphosphonic acid[13] were dissolved in anhydrous pyridine (5 ml). DCC (2.2 g) was added and the solution was stirred at room temperature for 24 hr. Using experimental procedures similar to those described for the preparation of compound **9**, 900 mg of crude **10** (barium salt) was obtained as a light yellow solid. The solid was dissolved in water (5 ml) and ethanol was added. A precipitate of barium iodomethylphosphonate (190 mg, identified by a $pK_a$–molecular weight determination) resulted immediately. The mother liquor was concentrated *in vacuo* to afford 700 mg of **10**. Compound **10** was chromatographically homogeneous at this point using four pc systems and Whatman No. 1 paper. Using Whatman No. 7 paper and solvent system A, however, a minor faster migrating impurity was evident. A sample (200 mg) was purified by ppc (Whatman No. 7 paper, 20 mg per sheet) using system A to give 105 mg of chromatographically homogeneous compound **10**. Compound **10** was purified further for analysis by fractional precipitation from water–ethanol mixtures. The first and last crops were discarded and the intermediate fractions were dried at 110° ($5 \times 10^{-3}$ mm) for 2 hr: $R_f$ values (Whatman No. 7 paper) in systems A, B, C, and D were 0.62, 0.63, 0.76, and 0.75, respectively; uv max 272 mμ (ε 15,200), 250–260 (0.65), 260–270 (0.82), 270–280 (1.09); $pK_a$ = 2.77; mol wt 690 (found) *vs.* 783 (calcd).

*Anal.* Calcd for $C_{17}H_{18}IN_3O_{12}P_2 \cdot Ba$: I, 16.21. Found: I, 16.50.

**Thymidine 5′-(Methylphosphonate)-3′-(*p*-nitrophenyl phosphate) Dilithium Salt (11).**—Thymidine 3′-(*p*-nitrophenyl phosphate) pyridinium salt (**3**), prepared from the diammonium salt (250 mg), was dissolved in anhydrous pyridine (6 ml) containing DCC (1.1 g). Methylphosphonic acid (80 mg) was added with stirring. The reaction mixture was allowed to stand at room temperature for 20 hr. Isolation, in a manner similar to that described for the preparation of compound **9**, gave crude compound **11** (lithium salt, 350 mg) which was essentially homogeneous by pc. Crude **11** was subjected to ppc using pc system A to give 191 mg of chromatographically homogeneous material. An analytically pure sample was obtained by fractional precipitation from methanol–diethyl ether mixtures, followed by drying at room temperature ($5 \times 10^{-3}$ mm) for 24 hr over $P_2O_4$: $R_f$ values in pc systems A, B, C, and D were 0.42, 0.46, 0.49, and 0.55; $pK_a$ = 2.9; mol wt 555 (found) *vs.* 597 (calcd); uv max 271 mμ (ε 15,250); uv max *ca.* 305 mμ, shoulder (ε 6900), 250–260 (0.63), 260–270 (0.84), 270–280 (1.11), 280–290 (1.35).

*Anal.* Calcd for $C_{17}H_{19}N_3O_{12}P_2 \cdot 2Li \cdot 2CH_3OH$: C, 38.21; H, 4.55; N, 7.04; P, 10.37. Found: C, 38.58; H, 4.72; N, 6.68; P, 10.42.

**Thymidine 5′-Phosphate-3′-(*p*-nitrophenyl phosphate) Trilithium Salt (13).**—Thymidine 3′-(*p*-nitrophenyl phosphate) pyridinium salt (**3**), prepared from the amonium salt (750 mg), was dissolved in pyridine (18 ml) containing DCC (3.35 g).

(13) P. C. Crofts and G. M. Kosolapoff, *J. Amer. Chem. Soc.*, **75**, 5738 (1953).

β-Cyanoethyl phosphate pyridine salt[14] (665 mg) was dissolved in anhydrous pyridine (9.3 ml) and the solution was added slowly, with stirring. The reaction mixture, processed in a manner similar to that described for the preparation of compound **9**, gave crude thymidine 5′-(β-cyanoethyl phosphate)-3′-(*p*-nitrophenyl phosphate) dilithium salt (**12**, 969 mg). Crude **12**, without purification by ppc, was dissolved in 1 *N* NaOH solution (2 ml) and the solution was allowed to stand at room temperature for 1 hr. The solution was applied to a Dowex 50 ($H^+$) column (15 ml). The column was eluted with water (250 ml). The effluent was stirred with charcoal (*ca.* 50 mg) at room temperature for 15 min. The charcoal was removed by filtration using a Celite bed. The filtrate was neutralized with lithium hydroxide solution, concentrated *in vacuo* (aspirator pressure) to a small volume, and lyophilized to yield crude compound **13** (870 mg). Analytical pc indicated that the hydrolysis was incomplete. The mixture was dissolved in 1 *N* sodium hydroxide (3 ml) and the solution was allowed to stand at room temperature for 1.5 hr. Processing the reaction mixture as before yielded essentially chromatographically homogeneous compound **13** (750 mg). Purification of compound **13** by ppc using pc system A afforded chromatographically homogeneous **13** (628 mg, 66%). Compound **13** was purified for elemental analysis by fractional precipitation from water–2-propanol mixtures. Six fractions were collected, the first five as gums. The sixth fraction, a solid (210 mg), was dried at room temperature ($5 \times 10^{-3}$ mm) for 24 hr over $P_2O_5$: $R_f$ values in systems A, B, C, and D were 0.42, 0.22, 0.39, and 0.48, respectively; uv max 270 mμ (ε 14,900), 300 shoulder (8100), 260–270 (0.87), 270–280 (1.12), 280–290 (1.32); $pK_{a^1}$ = 2.8, $pK_{a^2}$ = 7.0; mol wt$_1$ 6.06, mol wt$_2$ 617 (calcd 586).

*Anal.* Calcd for $C_{16}H_{16}N_3O_{13}P_2 \cdot 3Li \cdot 2.5H_2O$: C, 32.79; H, 3.61; N, 7.17; P, 10.57. Found: C, 32.87; H, 3.92; N, 6.81; P, 10.45.

**Thymidine 3′-Phosphate-5′-(*p*-nitrophenyl phosphate) Trilithium Salt** (**15**).—Thymidine 5′-(*p*-nitrophenyl phosphate) pyridinium salt (**5**, 362 mg) was dissolved in anhydrous pyridine (4 ml) containing DCC (0.715 mg). β-Cyanoethyl phosphate (290 mg),[14] dissolved in anhydrous pyridine (4 ml), was added slowly with stirring. The reaction mixture was processed in a manner similar to that described for the preparation of compound **13**. The yield of chromatographically homogeneous compound **15**, after ppc using pc system A, was 333 mg (76%). Compound **15** was purified for elemental analysis by fractional precipitation from wet methanol–2-propanol mixtures and drying at room temperature ($5 \times 10^{-3}$ mm) for 24 hr over $P_2O_5$: $R_f$ values in pc systems A, B, C, and D were 0.45, 0.19, 0.35, and 0.40, respectively; uv max 270 mμ (ε 15,420) *ca.* 305 mm, shoulder (7000), 260–270 (0.87), 270–280 (1.16), 280–290 (1.35); $pK_{a^1}$ = 3.0, $pK_{a^2}$ = 6.7; mol wt$_1$ 634, mol wt$_2$ 637 (calcd 637).

*Anal.* Calcd for $C_{16}H_{16}N_3O_{13}P_2 \cdot 3Li \cdot 3CH_3OH$: C, 35.81; H, 4.43; N, 6.59; P, 9.72. Found: C, 35.97; H, 4.80; N, 6.86; P, 9.69.

Alternatively, compound **15** (730 mg, purified by ppc) was suspended in methanol (20 ml). The insoluble material was removed by centrifugation. The supernatant was concentrated *in vacuo* to give a gel. The gel was dissolved in methanol (5 ml) and the solution was diluted with 2-propanol (5 ml) and *n*-pentane (10 ml). The resulting gelantinous precipitate was removed by centrifugation, ans washed with 2-propanol and *n*-pentane. The residue was dissolved in water and the water was removed *in vacuo* (water pump). This procedure was repeated three times. The residue was dissolved in water again and lyophilized to give 280 mg.

*Anal.* Calcd for $C_{16}H_{16}N_3O_{13}P_2 \cdot 3Li \cdot 4H_2O$: C, 31.34; H, 3.95; N, 6.85; P, 10.10. Found: C, 31.55; H, 3.84; N, 7.02; P, 9.93.

**Thymidine 3′,5′-Disulfate Barium Salt** (**17**).—Thymidine (1.21 g) and pyridine–sulfur trioxide complex[7] (4.0 g) were stirred in pyridine (90 ml) at room temperature for 48 hr. Water (90 ml) was added and the pH was adjusted to 10.5 (pH paper) with 1.3 *N* NaOH. The solvent was removed *in vacuo*, the residue was suspended in hot methanol (100 ml), and the suspension was cooled to 4°. The $Na_2SO_4$ was removed by filtration and the filtrate was concentrated *in vacuo* to yield a brown solid. The solid was dissolved in water and converted into the barium salt by passage of an aqueous solution through Dowex 50 ($H^+$), followed by neutralization of the water eluate with $Ba(OH)_2$ solution. The water solution was lyophilized. The resulting white salt was crystallized from aqueous ethanol to yield 2.21 g of the crystalline polyhydrate of **17** with mp 164–166° dec. A sample was dried at 110° ($5 \times 10^{-3}$ mm) for 2 hr to afford the dihydrate compound **17** with mp 200° dec: uv max ($H_2O$) 266.5 (ε 10,300),[15] 250–260 (0.65), 260–270 (0.94), 270–280 (1.55); $pK_a$ = 2.69; mol wt 591 (found) *vs.* 576 (calcd).

*Anal.* Calcd for $C_{10}H_{12}N_2O_{11}S_2Ba \cdot 2H_2O$: C, 20.94; H, 2.80; S, 11.18. Found: C, 21.09; H, 2.98; S, 11.00.

**Uridine 2′,3′,5′-Trisulfate Barium Salt** (**18**).—Title compound **18** was prepared from uridine (1.0 g) in the same manner that compound **17** was prepared from thymidine. The yield of chromatographically homogeneous compound **18** before crystallization was 2.0 g. A small sample (500 mg) was purified by crystallization from aqueous ethanol to give a polyhydrate of **18** (380 mg) with mp 173° dec. The sample was dried at 80° ($5 \times 10^{-3}$ mm) for 16 hr to give compound **18** monohydrate with mp 235° dec: $R_f$ values in systems A, B, C, and D were 0.11, 0.45, 0.53, and 0.72, respectively; uv max ($H_2O$) 260 mμ (ε 10,000), 250–260 (0.81), 260–270 (1.28), 270–280 (2.78); $pK_a$ = 2.84; mol wt 738 (found) *vs.* 705 (calcd).

*Anal.* Calcd for $C_9H_9N_2O_{15}S_3Ba_{1.5} \cdot H_2O$: C, 15.32; H, 1.57; N, 3.97; S, 13.64. Found: C, 15.33; H, 1.83; N, 4.30; S, 12.66.

**Thymidine 5′-Sulfate-3′-(*p*-nitrophenyl phosphate) Barium Salt** (**19**).—Thymidine 3′-(*p*-nitrophenyl phosphate) ammonium salt (**3**, 1.0 g) and pyridine–sulfur trioxide complex[7] (0.90 g) were stirred in dry pyridine at room temperature for 36 hr. After 24 hr, the reaction was complete by pc. The pyridine was removed *in vacuo* to afford a gum. The gum was dissolved in water and passed through a column of Dowex 50 ($H^+$), eluting with water. The highly acidic effluent was adjusted to pH 8 with $Ba(OH)_2$ and the insoluble $BaSO_4$ was removed by filtration. The filtrate was lyophilized to afford 1.3 g (theory, 1.43 g) of a white solid. Analytically pure, crystalline **19** monohydrate was obtained after four recrystallizations of a sample from water–alcohol mixtures and drying at 110° ($10^{-3}$ mm) for 2 hr: mp 235° dec; uv max ($H_2O$) 272 mμ (ε 15,300), 250–260 (0.63), 260–270 (0.82), 270–280 (1.1); $pK_a$ = 2.37; mol wt 687 (found) *vs.* 697 (calcd).

*Anal.* Calcd for $C_{16}H_{16}N_3O_{13}PS \cdot Ba \cdot H_2O$: C, 28.39; H, 2.68; N, 6.21; P, 4.58; S, 4.74. Found: C, 28.65; H, 3.06; N, 5.97; P, 4.70; S, 4.45.

**Registry No.**—**3**, 26886-08-8; **5**, 26886-09-9; **7**, 26886-10-2; **9**, 26886-11-3; **10**, 26886-12-4; **11**, 26963-85-9; **13**, 26886-13-5; **15**, 26886-14-6; **17**, 26886-15-7; **18**, 28594-70-9; **19**, 28594-71-0.

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(14) G. M. Tener, *J. Amer. Chem. Soc.*, **83**, 159 (1961).

(15) The uv data given for compound **17** (barium salt) are in agreement with that reported for the corresponding disodium salts: G. Kowollik and P. Langen, *Chem. Ber.*, **99**, 2725 (1966).