Nucleotide Synthesis. Derivatives of Thymidine Containing *m*- and *p*-Haloacetamidophenyl Phosphate Groups*

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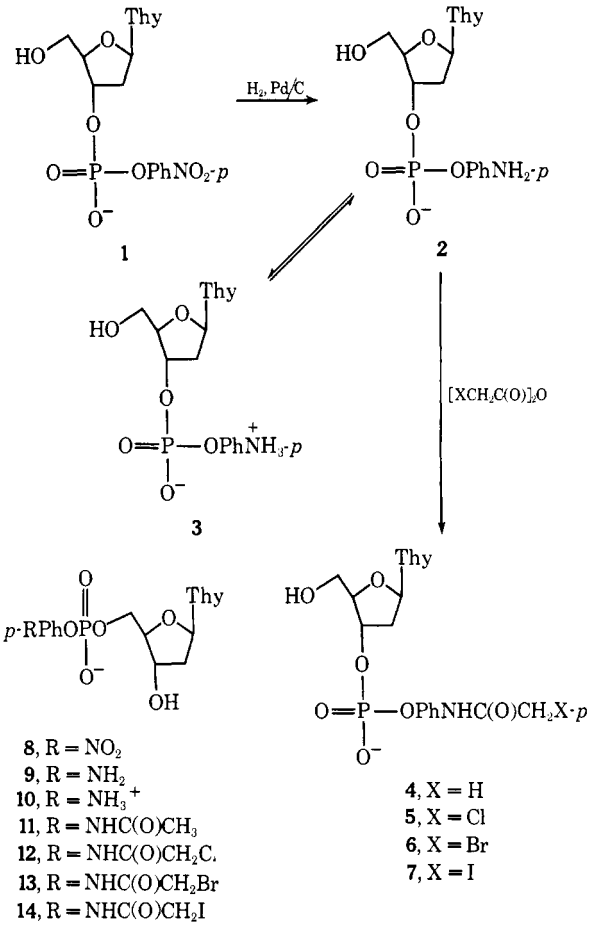
ABSTRACT: Mono- and dinucleotide derivatives of thymidine containing acetamidophenyl and haloacetamidophenyl phosphate esters were synthesized as part of a program to design active-site-directed irreversible inhibitors of a nuclear exoribonuclease isolated from mammalian cell nuclei. The general synthetic pathway involved treatment of the appropriate nucleoside or dinucleotide with bis(*m*- or bis(*p*-nitrophenyl) phosphorochloridate. The resulting intermediate, having one or two dinitrophenyl phosphate groups, was not isolated, but was allowed to react with mild base to give compounds having one or two mononitrophenyl phosphate groups. The nitro groups of these compounds were reduced by catalytic hydrogenation to yield the aminophenyl analogs, which, in turn, were allowed to react with acetic anhydride or various haloacetic anhydrides in methanol solution. Thymidine 3′-(*p*-acetamidophenyl and *p*-haloacetamidophenyl phosphates), the corresponding 5′isomers, thymidine 3′,5′-bis(*p*-acetamidophenyl and *m*- and *p*-haloacetamidophenyl phosphates) and 5′-*p*-iodoacetamidophenyl phosphorylthymidylyl-(3′→5′)-thymidine 3′-(*p*-iodoacetamidophenyl phosphate) were synthesized in this manner.

The concept of designing active-site-directed irreversible inhibitors for investigating the structure, mechanism of action, and cellular function of enzymes has received increased attention (Wofsy *et al.*, 1962; Singer, 1967; Baker, 1967, Shaw, 1970). We report herein the synthesis of mono- and dinucleotides containing *p*-acetamido- and *m*- or *p*-haloacetamidophenyl groups attached to phosphorus as part of the investigation of this general concept. The utilization of these inhibitors, as applied to a nuclear exoribonuclease (isolated from mammalian cell nuclei), which destroys single-stranded or rapidly labeled RNA (Lazarus and Sporn, 1967; Sporn *et al.*, 1969b), has been reported elsewhere (Sporn *et al.*, 1969a). In addition, since the appearance of our preliminary publications (Glinski and Stevens, 1968b; Sporn *et al.*, 1969a), the utilization of thymidine 3′- and 5′-(*p*-bromoacetamidophenyl phosphate) (**6** and **13**), respectively), as affinity labels of the active site of staphylococcal nuclease, has appeared in the literature (Cuatrecasas *et al.*, 1969). It is not unreasonable, therefore, to predict that a number of the compounds reported herein may have potential utility for studying other enzymes, in addition to nuclear exoribonuclease and staphylococcal nuclease.

Thymidine 3′-(*p*-nitrophenyl phosphate) (**1**) (Turner and Khorana, 1959) and thymidine 5′-(*p*-nitrophenyl phosphate) (**8**) (Razzell and Khorana, 1959; Glinski and Stevens, 1968a; Glinski *et al.*, 1971) were converted into the respective nucleotides containing 3′- and 5′-(*p*-acetamidophenyl and *p*-haloacetamidophenyl phosphate) groups by essentially the same procedures. The nitro groups of compounds **1** and **8** were reduced by catalytic hydrogenation to give thymidine 3′-(*p*-aminophenyl phosphate) (**2**) and thymidine 5′-(*p*-aminophenyl phosphate) (**9**), respectively, in yields which were generally above 90%. The products were essentially homogeneous by paper chromatography and were sufficiently pure for further transformations, although analytically pure samples were prepared. Treatment of compounds **2** and **9** with acetic anhydride, or various haloacetic anhydrides, at



1
2
3

8, R = NO_2
9, R = NH_2
10, R = NH_3^+
11, R = NHC(O)CH_3
12, R = NHC(O)CH_2C.
13, R = NHC(O)CH_2Br
14, R = NHC(O)CH_2I

4, X = H
5, X = Cl
6, X = Br
7, X = I

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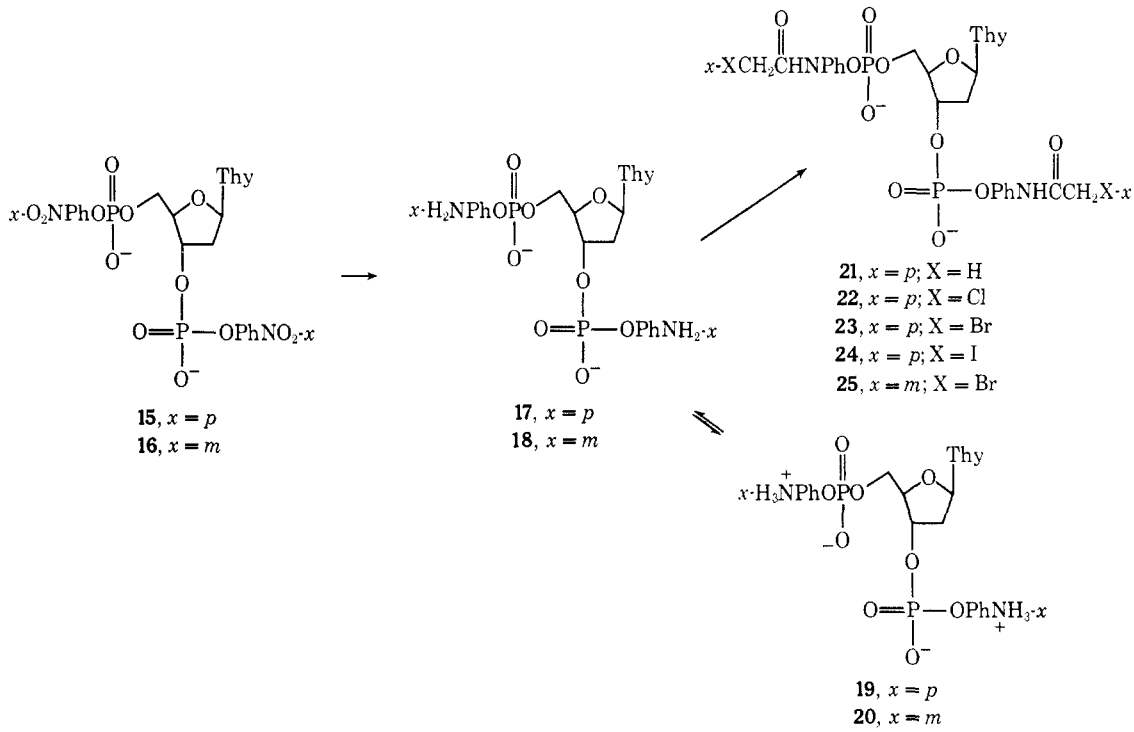


0° in methanol solvent, gave **4–7** and **11–14**, respectively, in excellent yields. The crude products were purified by preparative paper chromatography and fractional precipitation. The structures of the purified products were supported by elemental analyses and pK_a–molecular weight data, determined according to published procedures (Glinski and Stevens, 1968a; Glinski *et al.*, 1971), when the stability of the products permitted. Thus, the pK_a's of **2** and **9** were *ca.* 5.0, whereas the pK_a's of the products (**4–7** and **11–14**) were *ca.* 3.0, confirming acetylation of the amino group. Further, only slightly more than 1 equiv of acetic or haloacetic anhydride was required in most cases for complete conversion into products as evidenced by tlc and paper chromatography.

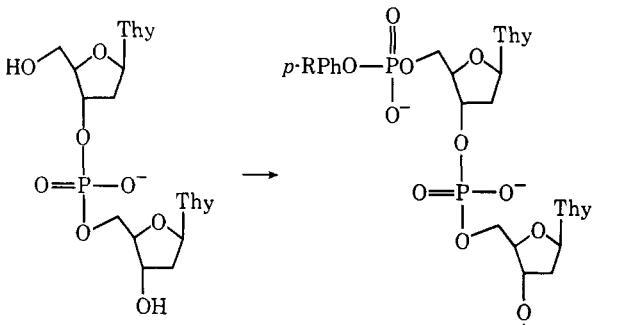
Compounds **2** and **9** were converted also into the corresponding crystalline inner salts **3** and **10** by treatment with acid. Compound **3** was prepared most conveniently by the addition of several equivalents of perchloric or trifluoroacetic acid to an aqueous solution of compound **2**; the product crystallized on standing. Compound **10** was obtained by treatment of a methanol solution of compound **9** with trifluoroacetic acid, followed by fractional precipitation and crystallization. Both products gave the expected pK_a–molecular weight values and the expected uv spectral data. Compounds **3** and **10** could be used for the preparation of target inhibitors **4–7** and **11–14** by conversion into compounds **2** and **9** *in situ* with lithium carbonate or sodium bicarbonate.

Crystalline thymidine 3′,5′-bis(*p*-nitrophenyl phosphate) barium salt (**15**) (Glinski and Stevens, 1968a; Glinski *et al.*, 1971) was converted into thymidine 3′,5′-bis(*p*-aminophenyl phosphate) barium salt (**17**), by catalytic reduction in aqueous solution. Compound **17** had the expected pK_a–molecular weight data, elemental analysis, and uv spectrum. Also, on treatment of an aqueous solution of **17** (barium salt) with several equivalents of trifluoroacetic acid, the inner salt **19** crystallized. Again, elemental analysis, pK_a–molecular weight, and uv data were as expected. Either **17** (barium salt) or inner salt **19** was converted into the dilithium salt of **17** before conversion into **21–24** containing acetamido and haloacetamido groups. Compound **17** (barium salt) was converted into the dilithium salt by use of a Dowex 50 (Li^+) column and the product was isolated by lyophilization and characterized. Inner salt **19** was converted into **17** (dilithium salt) by stirring a suspension of **19** and lithium carbonate in methanol. As dilithium salt formation occurred, the solution became homogeneous. The solution was used directly for the formation of compounds **21–24** by reaction with acetic anhydride or haloacetic anhydrides at 0°. Compound **17**, as a dilithium salt, was used in preference to the barium salt in these reactions because the solubility characteristics of the dilithium salt were more suitable for the acetylation reactions, as well as for recovery of the products from paper after purification by preparative paper chromatography.

One nucleotide containing a *m*-haloacetamidophenyl group was prepared to test the effect of moving the haloacetyl group from the para position (**23**) to the meta position (**25**) on enzyme inhibition and selectivity. The reaction sequence to prepare thymidine 3′,5′-bis(*m*-bromoacetamidophenyl phosphate) (**25**) was similar to that used for the preparation of **23**. Thus, thymidine was allowed to react with excess bis(*m*-nitrophenyl)phosphorochloridate in pyridine solution to form thymidine 3′,5′-bis(bis-*m*-nitrophenyl phosphate), which was not isolated. Reaction of this intermediate with base cleaved two of the four nitrophenyl ester linkages to give thymidine 3′,5′-bis(*m*-nitrophenyl phosphate) (**16**). Compound **16** was purified by large-scale preparative paper chromatography and was isolated and characterized as an amorphous barium salt and as a crystalline dimorpholine salt. Reduction of compound **16** (dimorpholine salt) in the presence of 10% palladium on carbon gave thymidine 3′,5′-bis(*m*-aminophenyl phosphate) dimorpholine salt (**18**). Compound **18** (dimorpholine salt) was converted into the corresponding disodium salt of **18** and the inner salt **20**. Compound **20** was not obtained in crystalline form, in contrast to the other inner salts prepared thus far. Compound **18** (disodium salt) was allowed to react with bromoacetic anhydride in methanol at 0° to give the target compound, thymidine 3′,5′-bis(*m*-bromoacetamidophenyl phosphate) disodium salt (**25**).

One dinucleotide bis(*p*-haloacetamidophenyl phosphate)

was prepared. Thymidylyl-(3′→5′)-thymidine (**26**), prepared by a recent literature procedure (Letsinger and Ogilvie, 1967, 1969), was allowed to react with bis(*p*-nitrophenyl) phosphorochloridate, followed by reaction with mild base to afford *p*-nitrophenyl 5′-phosphorylthymidylyl-(3′→5′)-thymidine 3′-(*p*-nitrophenyl phosphate) (**27**). Compound **27** was reduced in the presence of 10% palladium on carbon to give *p*-aminophenyl 5′-phosphorylthymidylyl-(3′→5′)-thymidine 3′-(*p*-aminophenyl phosphate) (**28**). Compound **28** was contaminated with a minor impurity by paper chromatography, but was sufficiently pure for further reactions. No attempt was made to convert **28** into the inner salt. Compound **28** (barium salt) was insoluble in methanol and thus a methanol–water mixture was used for the reaction with iodoacetic anhydride to yield *p*-iodoacetamidophenyl 5′-phosphorylthymidylyl-(3′→5′)-thymidine 3′-(*p*-iodoacetamidophenyl phosphate) (**29**). Compound **29**, the most effective inhibitor prepared to date, was purified by preparative paper chromatography and fractional precipitation.



27, R = NO_2
28, R = NH_2
29, R = $NHC(O)CH_2I$

All of the nucleotides containing *p*-acetamidophenyl and *p*-haloacetamidophenyl phosphate groups were tested as active-site-directed irreversible inhibitors (Sporn *et al.*, 1969a) of the nuclear exoribonuclease isolated from mammalian cell nuclei (Lazarus and Sporn, 1967, 1971; Sporn *et al.*, 1969b). In general, it was found that the *p*-acetamidophenyl analogs did not result in significant irreversible inhibition at concentrations at which the *p*-bromo- and iodoacetamidophenyl nucleotides were effective. The increase in chain length and negative charge, progressing from compounds **4–7** and **11–14**, to **21–25**, to **29**, greatly increased the ability of the haloacetyl group to inactivate the exoribonuclease. This finding was in accord with earlier work (Lazarus *et al.*, 1968) which had shown that an increase in chain length and negative charge of a homologous series of reversible oligonucleotide inhibitors increased the binding affinity of reversible inhibitors for the enzyme. Thus, **29** with the more potent iodoacetyl inactivating group and three negatively charged phosphate groups, exhibited the greatest amount of irreversible inhibition (28%) at the lowest concentration (1×10^{-5} M) of all the compounds tested (Sporn *et al.*, 1969a). From these data, it is possible to predict that increasing the chain length of **29** (by inserting additional mononucleotide units) should increase the extent of irreversible inhibition considerably.

The specific location, on the enzyme, of the site which is alkylated by the above inhibitors has not yet been determined. It is apparent that the inactivation of nuclear exoribonuclease by a bromoacetyl or iodoacetyl group is dependent on the affinity of this enzyme for the nucleotide ligand to which the inactivating group is attached (Sporn *et al.*, 1969a). However, it cannot be assumed that the catalytically active nucleophile on the enzyme is alkylated by these inhibitors since their alkylating groups are somewhat distant from the phosphodiester bond. Since the active site of the enzyme is large enough to bind an oligonucleotide containing six mononucleotide units (Lazarus *et al.*, 1968), it would seem reasonable to suppose that there may be several amino acid residues within the active-site area, other than the catalytically active nucleophile, which might be capable of reacting with these alkylating inhibitors.

Recent test results (Sporn *et al.*, unpublished data) have shown that changing the position of the inactivating group from the para position, as in **23**, to the meta position, as in **25**, roughly doubles the amount of inactivation of nuclear exoribonuclease when the compounds are tested at the same concentration. This fact, along with other data, such as increasing chain length being proportional to the magnitude of enzyme inhibition, form the basis for the future design of more efficient nucleotide inhibitors of the nuclear exoribonuclease.

Experimental Section

Paper chromatography 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 (7:2:1), v/v); C, 2-propanol–aqueous 1% ammonium sulfate (5:2, v/v); and D, ethanol–aqueous 1% ammonium acetate (5:2, v/v). The nucleotides were detected with ultraviolet light. Whenever R_F values for system B are omitted, the compound in question showed extensive streaking or multiple spots due to decomposition in this system. Analytical paper chromatography was performed using Whatman No. 1 paper. Whatman 3MM paper and solvent system A were used for the large-scale preparative paper chromatography (1–2 g of compound per 20 sheets) as outlined earlier (Glinski *et al.*, 1971).[1] Tlc was performed using plastic foil silica gel sheets impregnated with a fluorescent indicator and cellulose anion-exchange plastic sheets (MN-Polygram Cel 300 PEI) purchased from the Brinkmann Instrument Company. The following tlc systems were used: A, chloroform–1% ammonium hydroxide in methanol (3:2, v/v) and B, 1% sodium chloride in water. R_F's for all nucleotide derivatives are listed in Table I.

Uv spectra (distilled water) were recorded using a Hatachi-Coleman 124 spectrophotometer. Pyridine was distilled and stored over Linde Molecular Sieves (Type 4A). Determinations of pK_a–molecular weight were performed according to procedures outlined earlier (Glinski *et al.*, 1971), unless indicated otherwise.

Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind. The samples were prepared for ele-

[1] This procedure allowed purification of 1- to 2-g quantities of compounds per 24-hr period, requiring approximately 3 hr of technician time per purification. One disadvantage to the use of preparative paper chromatography, however, was the contamination of the "one-spot" products with colloidal cellulose and/or other non-uv-absorbing impurities from the paper sheets. This fact necessitated performing a fractional precipitation to obtain elemental analyses and other physical constants. Washing or soaking the sheeets beforehand helped but did not eliminate the problem.

TABLE I: Paper Chromatography of Nucleotide Derivatives.

Compound	R_F's in Systems			
	A	B	C	D
2 and **3**	0.33	0.47	0.51	0.62
4	0.41	0.58	0.63	0.71
5	0.52		0.66	0.73
6	0.44		0.58	0.77
7	0.51		0.62	0.73
9 and **10**	0.37	0.59	0.48	0.59
11	0.47		0.39	0.67
12	0.51		0.43	0.68
13	0.53		0.43	0.71
14	0.53		0.43	0.71
16	0.59	0.50	0.31	0.72
17 and **19**	0.15	0.26	0.27	0.47
18 and **20**	0.15	0.34	0.28	0.50
21	0.44		0.37	0.77
22	0.50		0.41	0.76
23	0.52		0.44	0.76
24	0.57		0.41	0.77
25	0.43		0.48	0.70
27	0.42	0.44	0.37	0.62
29	0.38		0.40	

mental analyses by fractional precipitation from various mixed solvent systems and drying *in vacuo*. Fractions from the fractional precipitations were collected by centrifugation. Elemental analyses were complicated in several cases by the retention of organic solvent (methanol or 2-propanol) in the product where mixed solvent systems were employed for fractional precipitation or crystallization. For example, crystalline **3** analyzed correctly for C, H, N, and P as a methanol solvate after crystallization from methanol followed by recrystallization from water. In a parallel experiment, **3** was prepared and crystallized from aqueous solutions only; this material analyzed correctly for C, H, N, and P as a monohydrate. Thymidine 3′,5′-bis(*p*-acetamidophenyl phosphate) dilithium salt (**21**) analyzed correctly for C, H, N, and P as 2-propanol solvate–1.5-hydrate after fractional precipitation from water–2-propanol mixtures. A sample of this material was lyophilized (4 times) and dried; the product then analyzed correctly for C, H, N, and P as a dihydrate. Similarly, thymidine 3′,5′-bis(*p*-iodoacetamidophenyl phosphate) dilithium salt (**24**) was analyzed for C, H, I, N, and P, both as a hexamethanolate and a monohydrate. In these cases, the new compounds had the expected N:P:halogen (when present) ratios.

Thymidine 3′-(p-Aminophenyl phosphate) Lithium Salt (**2**). Thymidine 3′-(*p*-nitrophenyl phosphate) sodium salt (**1**, 5.0 g) (Turner and Khorana, 1959), dissolved in water (60 ml), was stirred under hydrogen at atmospheric pressure in the presence of prereduced 10% Pd/C (210 mg) for 8 hr. At the end of this time, the hydrogen uptake was constant at 100% of the theoretical amount. The catalyst was removed by filtration (celite) and was washed thoroughly with water. The filtrate and washings were combined and lyophilized to yield 4.22 g of compound **2** (sodium salt): uv max 265 mμ (ε 10,000), 250/260 0.79, 260/270 0.98, 270/280 1.42. A small sample of this hygroscopic material was converted into the lithium salt in *ca.* 90% yield *via* passage through a column of Dowex 50 (Li^+ form, tenfold excess). This product was less hygroscopic: pK_a = 5.0,[2] 4.6; mol wt 421,[2] 420 *vs.* 420 (calcd). A sample was purified further by fractional precipitation from methanol–2-propanol–anhydrous ethyl ether mixtures. Four fractions were collected. The third fraction was reprecipitated from a methanol–2-propanol mixture, air-dried, and dried at 5 × 10^{-3} mm (room temperature) over P_2O_5 for 24 hr for analysis. *Anal.* Calcd for $C_{16}H_{19}N_3O_8P \cdot Li \cdot CH_3OH$: C, 45.25; H, 5.14; N, 9.31; P, 6.86. Found: C, 45.68; H, 5.36; N, 9.16; P, 7.08.

Thymidine 3′-(p-Aminophenyl phosphate) Inner salt (**3**). Thymidine 3′-(*p*-aminophenyl phosphate) sodium salt (**2**, 500 mg) was dissolved in methanol and the solution was cooled to 0°. Trifluoroacetic acid (0.20 ml) was added dropwise over a period of several minutes. The precipitate which resulted was removed by filtration and air-dried. The product (410 mg) was dissolved in hot water (50 ml), seeded, and allowed to cool to 1°. Cubic crystals deposited which were collected by filtration and air-dried to give pure compound **3** (250 mg). A sample was dried for analysis at room temperature (5 × 10^{-3} mm) over P_2O_5 for 48 hr: uv max 265 mμ (ε 10,400), 250/260 0.74, 260/270 1.00, 270/280 1.54. *Anal.* Calcd for $C_{16}H_{20}N_3O_8P \cdot CH_3OH$: C, 45.95; H, 5.22; N, 9.46; P, 6.97. Found: C, 45.69; H, 5.78; N, 9.25; P, 7.06.

Compound **3** was more conveniently prepared by direct crystallization from water solution; moreover, the product analyzed free of methanol. Thus, compound **2** (200 mg) was dissolved in aqueous 0.054 N $HClO_4$ acid (8.5 ml). The solution (pH ~3 using pH paper) was seeded with a sample of **3** to induce crystallization. Alternately, the acidification could be accomplished by addition of several equivalents of trifluoroacetic acid or any other strong acid (*e.g.*, HCl) to an aqueous solution of **2**. The reaction mixture was set aside at 1° for 20 hr. The resulting dense crystalline product was removed by filtration, washed thoroughly with water, and air-dried to afford 150 mg of **3** with mp *ca.* 195° dec. A sample was dried at room temperature (5 × 10^{-3} mm) over P_2O_5 for 24 hr: pK_a = 5.0; mol wt 475 *vs.* 430 (calcd); uv max 265 mμ (ε 9,000), 250/260 0.78, 260/270 1.02, 270/280 1.48. *Anal.* Calcd for $C_{16}H_{20}N_3O_8P \cdot H_2O$: C, 44.66; H, 4.92; N, 9.76; P, 7.20. Found: C, 44.75; H, 5.20; N, 9.47; P, 7.36.

Thymidine 3′-(p-Acetamidophenyl phosphate) Lithium salt (**4**). Thymidine 3′-(*p*-aminophenyl phosphate) lithium salt (**2**, 500 mg) was dissolved in methanol (10 ml) and the solution was cooled to 0°. Acetic anhydride (185 mg) was added dropwise to the stirred solution. After stirring at 0° for 25 min, the course of the reaction was monitored by tlc using a silica gel plastic foil and tlc system A. The reaction mixture gave one spot at R_F 0.37; starting material **2** had an R_F of 0.25. The methanol was removed *in vacuo* to yield a gum. The gum was triturated with anhydrous ethyl ether to afford a solid (660 mg). The solid was purified by preparative paper chromatography (six sheets 3MM paper) to afford 492 mg of chromatographically homogeneous **4**. Compound **4** was purified further

[2] This pK_a–mol wt value (5.0, 421) was obtained by the addition of 0.1 N $HClO_4$ acid to an aqueous solution of compound **2** to adjust the pH to *ca.* 3.0, and by back titration with base. The first inflection in the pH *vs.* milliliters of titrant curve was due to excess acid and the second inflection was due to neutralization of the resultant inner salt (**3**); the milliliters of titrant needed to progress from one inflection point to the next gave the base uptake for the compound. The second value (4.6, 420) was obtained in the usual manner by titration of compound **3**, obtained by passage of a solution of compound **2** through a Dowex 50 (H^+) column (Glinski *et al.*, 1971). The pK_a value for aniline is 4.6 (Albert and Serjeant, 1962).

by fractional precipitation from wet methanol–2-propanol mixtures. Six fractions were collected. Fraction six (360 mg) was dried at room temperature (5×10^{-3} mm) over P_2O_5 for 2 days for analysis: uv max 245 (ϵ 14,800), 230/240 0.81, 240/250 0.99, 250/260 1.16; pK_a = 2.7 and mol wt 468 *vs.* 479 (calcd). *Anal.* Calcd for $C_{19}H_{21}N_3O_9P \cdot Li \cdot H_2O$: C, 45.11; H, 4.84; N, 8.77; P, 6.46. Found: C, 45.47; H, 5.34; N, 9.28; P, 6.34.

Thymidine 3′-(p-Chloroacetamidophenyl phosphate) Lithium Salt (**5**). Thymidine 3′-(*p*-aminophenyl phosphate) lithium salt (**2**, 500 mg), dissolved in methanol (15 ml) at 0°, was treated with chloroacetic anhydride (214 mg) as was described for the preparation of compound **4**. The yield of chromatographically homogeneous compound **5** after preparative paper chromatography (seven 3MM sheets) was 416 mg. A sample was purified for analysis by fractional precipitation from methanol–2-propanol–ethyl ether mixtures. Six fractions were collected. Fraction six (185 mg) was reprecipitated from methanol–2-propanol mixtures. Two fractions were collected. The second fraction (115 mg after drying in a stream of dry nitrogen) was dried at room temperature (5×10^{-3} mm) over P_2O_5: uv max 256 mμ (ϵ 15,200), 240/250 0.86, 250/260 0.99, 260/270 1.19; pK_a = 3.4; mol wt 540 *vs.* 546 (calcd). *Anal.* Calcd for $C_{18}H_{20}ClN_3O_9P \cdot Li \cdot CH_3OH \cdot H_2O$: C, 41.81; H, 4.80; Cl, 6.50; N, 7.70; P, 5.67. Found: C, 41.96; H, 4.88; Cl, 6.49; N, 7.78; P, 5.28.

Thymidine 3′-(p-Bromoacetamidophenyl phosphate) Lithium Salt (**6**). Thymidine 3′-(*p*-aminophenyl phosphate) lithium salt (**2**, 3.69 g), dissolved in methanol (100 ml) at 0°, was allowed to react with bromoacetic anhydride (2.7 g) as was described for the preparation of compound **4**. The yield of chromatographically homogeneous compound **6** after preparative paper chromatography (46 sheets of 3MM paper) was 3.45 g. Compound **6** was purified by fractional precipitation from methanol–2-propanol–water mixtures in two batches (1.96 g and 1.49 g). Six fractions were collected in each case. The sixth fractions were combined, dissolved in water (20 ml), lyophilized, and dried at room temperature (5×10^{-3} mm) over P_2O_5 for 24 hr to give a total of 2.28 g of compound **6**: pK_a = 3.0; mol wt 503 *vs.* 558 (calcd); uv max 257 mμ (ϵ 16,200), 240/250 0.765, 250/260 0.94, 260/270 1.16. Compound **6** is somewhat unstable and decomposes on prolonged drying, when developed in paper chromatography system B, and on standing in the dry state or in solvents for long periods of time. The low molecular weight (−10%) may be due to decomposition during passage through the Dowex 50 (H^+) column for the pK_a–molecular weight titration. *Anal.* Calcd for $C_{18}H_{20}BrN_3O_9P \cdot Li \cdot H_2O$: C, 38.73; H, 3.97; Br, 14.32; N, 7.53; P, 5.55. Found: C, 39.31; H, 4.29; Br, 12.98; N, 6.88; P, 6.23.

Thymidine 3′-(p-Iodoacetamidophenyl phosphate) Lithium Salt (**7**). Thymidine 3′-(*p*-aminophenyl phosphate) lithium salt (**2**, 640 mg), dissolved in methanol (10 ml) at 0°, was allowed to react with iodoacetic anhydride (600 mg, Abderhalden and Guggenheim, 1908) as was described for the preparation of compound **4**. The yield of homogeneous **7** after preparative paper chromatography (10 sheets of 3MM paper) was 865 mg. Compound **7** was purified further by fractional precipitation from methanol–2-propanol and methanol–2-propanol–ethyl ether mixtures. Twelve fractions were collected. Fraction 12 (290 mg after drying in a stream of N_2) was dried at room temperature (5×10^{-3} mm) over P_2O_5 for 48 hr: pK_a = 3.1; mol wt 603 *vs.* 637 (calcd); uv max 263 mμ (ϵ 15,500), 250/260 0.856, 260/270 1.04, 270/280 1.36. *Anal.* Calcd for $C_{18}H_{20}IN_3O_9P \cdot Li \cdot CH_3OH \cdot H_2O$: C, 35.81, H, 4.11; I, 19.91; N, 6.59; P, 4.86. Found: C, 36.02; H, 4.06; I, 19.91; N, 6.95; P, 4.19.

Thymidine 5′-(p-Aminophenyl phosphate) Lithium Salt (**9**). Thymidine 5′-(*p*-nitrophenyl phosphate) lithium salt (**8**, 2.5 g, Razzell and Khorana, 1959; Glinski *et al.*, 1971) was stirred at room temperature in water (10 ml) in the presence of prereduced 10% Pd/C (300 mg) for 24 hr. The hydrogen uptake was constant at 98% of the theoretical amount. Processing of the reaction mixture, as was described for compound **2**, gave 2.18 g (94%) of homogeneous **9**: pK_a = 5.0; mol wt 431 *vs.* 425 (calcd for monohydrate), sufficiently pure for further transformations. A portion of the solid was purified for analysis by fractional precipitation from methanol–ethyl ether mixtures. Seven fractions were collected. Fraction six, which was a gum initially, was solidified by trituration under ethyl ether, air-dried, and dried at room temperature (5×10^{-3} mm for 14 hr, followed at 110° (5×10^{-3} mm) for 3 hr. This fraction had the highest ϵ value of the seven collected: uv max 267.5 mμ (ϵ 9,600), 260/270 0.95, 270/280 1.31. *Anal.* Calcd for $C_{16}H_{19}N_3O_8P \cdot Li \cdot CH_3OH$: C, 45.25; H, 5.14; N, 9.31; P, 6.86. Found: C, 45.81; H, 5.22; N, 8.77; P, 7.22.

Thymidine 5′-(p-Aminophenyl phosphate) Inner salt (**10**). Crude thymidine 5′-(*p*-aminophenyl phosphate) lithium salt (**9**, 300 mg) was dissoved in methanol (2 ml). An insoluble impurity was removed by centrifugation. Trifluoroacetic acid (0.06 ml) was added dropwise to the supernatant. A gum formed which was removed by centrifugation. 2-Propanol was added to the supernatant to the point of turbidity and the mixture was centrifuged. Repetition of this treatment removed more brown material. On prolonged standing, compound **10** crystallized (long needles) from the supernatant to give 195 mg with mp 197° dec. A sample was dissolved in water (3 drops), diluted with methanol (5 ml), and centrifuged. The light yellow supernatant was diluted with 2-propanol (*ca.* 5 ml) to the point of turbidity and seeded. Crystallization commenced immediately. The crystalline material was removed to give pure **10** with mp *ca.* 205° dec. The product was dried at room temperature (5×10^{-3} mm) for 20 hr, and at 100° (5×10^{-3} mm) for 2 hr for analysis: pK_a = 4.85; mol wt 428 *vs.* 425 (calcd). *Anal.* Calcd for $C_{16}H_{20}N_3O_8P \cdot 0.75H_2O$: C, 45.13; H, 4.85; N, 9.86; P, 7.27. Found: C, 45.05; H, 5.37; N, 9.69; P, 7.43.

Thymidine 5′-(p-Acetamidophenyl phosphate) Lithium Salt (**11**). Thymidine 5′-(*p*-aminophenyl phosphate) lithium salt (**9**, 500 mg), dissolved in methanol at 0°, was allowed to react with acetic anhydride (2×0.17 ml) as was described for compound **4**. After the reaction was complete, as indicated by tlc using system A and silica gel sheets (the sheet required three successive developments to obtain separation between compounds **9** and **11**), the reaction mixture was diluted with water and was applied directly to 20 sheets of 3MM paper. The yield of homogeneous **11** was 531 mg. Compound **11** was purified further by fractional precipitation from methanol–2-propanol and methanol–2-propanol–*n*-pentane mixtures. Five fractions were collected. Fractions three and four were combined (220 mg) and dried at room temperature (5×10^{-3} mm) for 20 hr, followed at 110° (5×10^{-3} mm) for 20 hr for analysis: uv max 246 mμ (ϵ 15,300), 230/240 0.81, 240/250 1.00, 250/260 1.25. *Anal.* Calcd for $C_{18}H_{21}N_3O_9P \cdot Li \cdot CH_3OH \cdot H_2O$: C, 44.63; H, 5.32; N, 8.22; P, 6.06. Found: C, 45.00; H, 5.05; N, 8.10; P, 6.21.

Thymidine 5′-(p-Chloroacetamidophenyl phosphate) Lithium Salt (**12**). Thymidine 5′-(*p*-aminophenyl phosphate) lithium salt (**9**, 500 mg), dissolved in methanol (10 ml), was allowed to react with chloroacetic anhydride (250 mg) as was

described for the preparation of compound **4**. The yield of homogeneous **12** was 587 mg after preparative paper chromatography. Compound **12** was purified for analysis by fractional precipitation from wet methanol–2-propanol mixtures and was dried at room temperature (5×10^{-3} mm) for 20 hr: uv max 252 mμ (ε 15,000), 240/250 0.85, 250/260 1.05, 260/270 1.24. *Anal.* Calcd for $C_{18}H_{22}ClN_3O_{10}P \cdot Li \cdot H_2O$: C, 42.08; H, 4.32; Cl, 6.90; N, 8.17; P, 6.03. Found: C, 42.01; H, 4.72; Cl, 6.70; N, 7.88; P, 6.03.

Thymidine 5′-(p-Bromoacetamidophenyl phosphate) Lithium Salt (**13**). Thymidine 5′-(*p*-aminophenyl phosphate) lithium salt (**9**, 500 mg), dissolved in methanol (10 ml) at 0°, was allowed to react with bromoacetic anhydride (375 mg) as was described in the preparation of compound **4**. The yield of homogeneous **13** was 483 mg after ppc (18 sheets of 3MM paper). Compound **13** was purified for analysis by fractional precipitation from water–2-propanol mixtures. Six fractions were collected. Fractions four and five (110 mg) were dried at room temperature (5×10^{-3} mm) for 15 hr, followed at 100° (5×10^{-3} mm) for 3 hr: uv max (H_2O) 255 mμ (ε 15,900), 240/250 0.78, 250/260 0.98, 260/270 1.15. *Anal.* Calcd for $C_{18}H_{20}BrN_3O_9P \cdot Li \cdot H_2O$: C, 38.73; H, 3.97; Br, 14.32; N, 7.53; P, 5.55. Found: C, 38.90; H, 4.25; Br, 12.96; N, 7.57, P, 5.63.

Thymidine 5′-(p-Iodoacetamidophenyl phosphate) Lithium Salt (**14**). Thymidine 5-(*p*-aminophenyl phosphate) lithium salt (**9**, 500 mg), dissolved in methanol (10 ml) at 0°, was allowed to react with iodoacetic anhydride (Abderhalden and Guggenheim, 1908) as was described for the preparation of compound **4**. Purification and isolation of compound **14** by preparative paper chromatography (20 sheets of 3MM paper) gave 712 mg of material containing several trace impurities, as evidenced by analytical paper chromatography. The impurities apparently arose during concentration of the preparative paper chromatography extracts (2 l. of water) under aspirator vacuum at 40° (bath temperature). The product was purified again by preparative paper chromatography (seven sheets of 3MM paper). The preparative paper chromatography extracts were lyophilized to yield 430 mg of homogeneous **14**. A sample was prepared for analysis by fractional precipitation from wet methanol–2-propanol mixtures and by drying at room temperature (5×10^{-3} mm) for 20 hr: uv max 263 mμ (ε 17,000), 250/260 0.84, 260/270 1.04, 270/280 1.35. *Anal.* Calcd for $C_{18}H_{20}IN_3O_9P \cdot Li \cdot 2CH_3OH \cdot 0.5H_2O$: C, 36.38; H, 4.43; I, 19.22; N, 6.36; P, 4.69. Found: C, 36.29; H, 4.07; I, 19.32; N, 6.17; P, 4.87.

Thymidine 3′,5′-Bis(p-aminophenyl phosphate) Barium Salt (**17**). Crystalline thymidine 3′,5′-bis(*p*-nitrophenyl phosphate) barium salt (**15**, 5.0 g, Glinski *et al.*, 1971), dissolved in water (20 ml), was stirred under hydrogen at atmospheric pressure in the presence of 10% Pd/C (400 mg) for 7 hr. The reaction mixture was processed as was described for compound **2** to yield 4.70 g of homogeneous **17**. A small portion was fractionally precipitated from water–2-propanol mixtures. Four fractions were collected as gums. The gums were solidified by trituration under 2-propanol. The fourth fraction was dried at room temperature (5×10^{-3} mm) for 17 hr for analysis: pK_a = 5.3; mol wt 760 *vs.* 747 (calcd); uv max 267 mμ (ε 9,800), 260/270 0.98, 270/280 1.29. *Anal.* Calcd for $C_{22}H_{24}N_4O_{11}P_2 \cdot Ba \cdot 1.5H_2O$: C, 35.38; H, 3.65; N, 7.50; P, 8.30. Found: C, 35.34; H, 3.76; N, 7.22; P, 8.40.

Compound **17** (barium salt, 1.26 g) was converted into the corresponding dilithium salt *via* passage through a Dowex 50 (Li^+) column (60 ml). The column was eluted with water (400 ml). The effluent was lyophilized to yield 782 mg of **8** (lithium salt), which was used directly in other preparations. Alternately, crystalline **19** gave **17** (dilithium salt) on treatment of a suspension of **19** in methanol with Li_2CO_3. The dilithium salt of **17** is methanol soluble and this solution was used directly on occasion in the formation of several preparations of compounds **21–24** (not described).

Thymidine 3′,5′-Bis(p-aminophenyl phosphate) Inner Salt (**19**). (a) Thymidine 3′,5′-bis(*p*-aminophenyl phosphate) lithium salt (**17**, 1.0 g) was dissolved in wet methanol (50 ml) and trifluoroacetic acid (0.26 ml) was added dropwise with vigorous stirring. A white precipitate formed immediately. The heterogeneous mixture was stirred for 15 min. The precipitate was removed by centrifugation, washed well with methanol, 2-propanol, and *n*-pentane and was dried in a nitrogen stream. After air-drying for 1 hr, the yield of product **19** was 975 mg. A sample was dried for analysis at room temperature (5×10^{-3} mm) for 40 hr, and at 110° (5×10^{-3} mm) for 2 hr: uv and paper chromatography essentially identical with compound **17**; pK_a = 4.95; mol wt 621 *vs.* 602 (calcd). *Anal.* Calcd for $C_{22}H_{26}N_4O_{11}P_2 \cdot H_2O$: C, 43.86; H, 4.68; N, 9.30; P, 10.28. Found (analyzed as received): C, 44.12; H, 5.07; N, 9.10; P, 10.30.

(b) Two years after the original preparation, compound **19** crystallized from an aqueous solution during another reaction. In a subsequent preparation of **19**, compound **17** (barium salt, 1.36 g) was dissolved in water (10 ml) and trifluoroacetic acid (420 mg) was added. The solution was seeded with crystalline **19** and, after standing at room temperature for several hours, 910 mg of crystalline product was obtained. A sample (140 mg) was recrystallized from hot water (100 ml) to give 100 mg of pure **19**, after drying at room temperature (5×10^{-3} mm) and at 110° (5×10^{-3} mm) for 16 and 2 hr, respectively: uv max 267 mμ (ε 10,700), 260/270 0.94, 270/280 1.42; pK_a = 5.1; mol wt 602 *vs.* 611 (calcd). *Anal.* Calcd for $C_{22}H_{26}N_4O_{11}P_2 \cdot 1.5H_2O$: C, 43.21; H, 4.78; N, 9.17; P, 10.14. Found: C, 43.24; H, 4.63; N, 9.33; P, 9.89. *Anal.* Calcd for $C_{22}H_{26}N_4O_{11}P_2 \cdot 0.5H_2O$: C, 44.54; H, 4.59; N, 9.44. Found, after block drying at 170°: C, 44.90; H, 4.82; N, 9.69.

Thymidine 3′,5′-Bis(p-acetamidophenyl phosphate) Dilithium Salt (**21**). Thymidine 3′,5′-bis(*p*-aminophenyl phosphate) dilithium salt (**17**, 400 mg), dissolved in methanol (5 ml) at 0°, was allowed to react with acetic anhydride (2×0.125 ml) until tlc (silica gel sheets; tlc system A) indicated the absence of starting material **17**. The reaction mixture was processed as was described for the preparation of compound **4** to afford 550 mg of crude **21**. The crude product was purified by preparative paper chromatograpny (eight sheets of 3MM paper) to afford 460 mg. Compound **21** was purified further by fractional precipitation from water–2-propanol mixtures. Six fractions were collected. The sixth fraction (285 mg) was fractionally precipitated again and two fractions were collected. The second fraction (90 mg) was dried over P_2O_5 at room temperature (5×10^{-3} mm) for 48 hr: (ε 24,800), shoulder 270 mμ (ε 10,300), 230/240 0.79, 240/250 1.09; pK_a = 2.8 mol wt 777 *vs.* 767 (calcd). *Anal.* Calcd for $C_{26}H_{28}N_4O_{13}P_2 \cdot 2Li \cdot (CH_3)_2CHOH \cdot 1.5H_2O$: C, 45.38; H, 5.12; N, 7.30; P, 8.07. Found: C, 45.47; H, 5.32; N, 7.38; P, 7.77.

A portion of the analytical sample was freed of 2-propanol by lyophilization (four times) and drying over P_2O_5 at room temperature (5×10^{-3} mm) for 4 hr; uv max 242.5 mμ (ε 25,400), shoulder 270 mμ (ε 10,700), 230/240 0.80, 240/250 1.08. *Anal.* Calcd for $C_{26}H_{28}N_4O_{13}P_2 \cdot 2Li \cdot 2H_2O$: C, 43.59; H, 4.50; N, 7.82; P, 8.65. Found, after block drying at 125°: C, 43.62; H, 4.59; N, 8.19; P, 8.61.

Thymidine 3′,5′-Bis(p-chloroacetamidophenyl phosphate) Dilithium Salt (**22**). Thymidine 3′,5′-bis(*p*-aminophenyl phos-

phate dilithium salt (**17**, 300 mg), dissolved in methanol (5 ml) at 0°, was allowed to react with chloroacetic anhydride (200 mg) as was described for the synthesis of compound **4**. The yield of crude **22** was 400 mg. Purification of **22** by preparative paper chromatography (eight sheets of 3MM paper) gave 386 mg. Compound **22** was purified further by fractional precipitation from water–2-propanol mixtures. Four fractions were collected. The fourth fraction was reprecipitated and two fractions were collected. The second fraction (115 mg), a hygroscopic white solid, was dried at room temperature (5×10^{-3} mm) over P_2O_5 for 48 hr for analysis: uv max/247 mμ (ε 24,500), 230/240 0.77, 240/250 0.95, 250/260 1.24; pK_a = 2.9; mol wt 781 *vs.* 785 (calcd). *Anal.* Calcd for $C_{26}H_{26}Cl_2N_4O_{13}P_2 \cdot Li_2 \cdot 2H_2O$: C, 39.77; H, 3.85; Cl, 9.03; N, 7.13; P, 7.89. Found: C, 39.84; H, 4.11; Cl, 9.22; N, 6.89; P, 7.76.

Thymidine 3′,5′-Bis(p-bromoacetamidophenyl phosphate) Dilithium Salt (**23**). Thymidine 3′,5′-bis(*p*-aminophenyl phosphate) dilithium salt (**17**, 300 mg), dissolved in methanol (5 ml) at 0°, was allowed to react with bromoacetic anhydride (300 mg), as described for the preparation of compound **13**. The yield of crude **23** was 500 mg. Purification of **23** by preparative paper chromatography (eight sheets of 3MM paper) yielded 370 mg of chromatographically homogeneous solid. The solid was purified further by fractional precipitation from water–2-propanol and water–2-propanol–ethyl ether mixtures. Five fractions were collected as gums. The gums were solidified by trituration under anhydrous ethyl ether. The fifth fraction (250 mg) was reprecipitated and two fractions were collected. The second fraction (190 mg) was dried at room temperature (5×10^{-3} mm) over P_2O_5 for 48 hr: uv max 253 mμ (ε 23,400), 240/250 0.826, 250/260 1.03, 260/270 1.21; pK_a = 2.9; mol wt 834 *vs.* 865 (calcd). *Anal.* Calcd for $C_{26}H_{26}Br_2N_4O_{13}P_2 \cdot 2Li \cdot 1.5H_2O$: C, 36.09; H, 3.38; Br, 18.47; N, 6.48; P, 7.16. Found: C, 36.53; H, 3.89; Br, 17.94; N, 6.12; P, 7.25.

Thymidine 3′,5′-Bis(p-iodoacetamidophenyl phosphate) Dilithium Salt (**24**). Thymidine 3′,5′-bis(*p*-aminophenyl phosphate) dilithium salt (**17**, 300 mg), dissolved in methanol (5 ml) at 0°, was allowed to react with iodoacetic anhydride (430 mg, Abderhalden and Guggenheim, 1908) as was described for the synthesis of compound **4**.

Purification of **24** by preparative paper chromatography (eight sheets of 3MM paper) afforded 440 mg of chromatographically homogeneous solid. The solid was purified further by fractional precipitation from methanol–2-propanol mixtures. Six fractions were collected. Fraction six (220 mg) was dried at room temperature (5×10^{-3} mm) over P_2O_5 for 20 hr to give analytically pure **24**: uv max 262 mμ (ε 28,000), 250/260 0.89, 260/270 1.05, 270/280 1.32; pK_a = 2.9; mol wt 1030 *vs.* 1124 (calcd). *Anal.* Calcd for $C_{26}H_{26}I_2N_4O_{13}P_2 \cdot 2Li \cdot 6CH_3OH$: C, 34.18; H, 4.48; I, 22.57; N, 4.98; P, 5.51. Found: C, 34.28; H, 4.12; I, 22.84; N, 5.00; P, 5.76.

In another experiment, 300 mg of compound **17** gave 341 mg of compound **24** after preparative paper chromatography. Fractional precipitation from water–2-propanol mixtures, instead of methanol–2-propanol mixtures, and drying as before gave material analyzing without methanol of solvation. *Anal.* Calcd for $C_{26}H_{26}I_2N_4O_{13}P_2 \cdot 2Li \cdot H_2O$: C, 32.87; H, 2.97; I, 26.71; N, 5.90; P, 6.52. Found: C, 33.06; H, 3.15; I, 26.00; N, 5.72; P, 6.59.

Bis(m-nitrophenyl) Phosphorochloridate. The title compound was prepared according to the procedure outlined for the preparation of bis(*p*-nitrophenyl) phosphorochloridate (Ukita and Hayatsu, 1962) and had mp 88.5–89.5°. *Anal.* Calcd for $C_{12}H_8ClN_2O_7P$: C, 40.19; H, 2.24; Cl, 9.89; N, 7.81. Found: C, 40.37; H, 2.28; Cl, 10.06; N, 7.58.

Thymidine 3′,5′-Bis(m-nitrophenyl phosphate) Barium and Dimorpholine Salts (**16**). Thymidine (1.0 g), dissolved in anhydrous pyridine (5 ml), was allowed to react with bis(*m*-nitrophenyl) phosphorochloridate (4.5 g) as was described for the preparation of thymidine 3′,5′-bis(*p*-nitrophenyl phosphate) barium salt (Glinski *et al.*, 1971). The yield of **16** (dilithium salt) after preparative paper chromatography (40 sheets of 3MM paper) was 2.45 g. Compound **16** (dilithium salt), dissolved in a minimum amount of water, was converted into the corresponding free acid by passage through an Amberlite IR 120 (H^+) column (20 ml). The column was eluted with water (200 ml). The eluate was lyophilized. The hygroscopic residue was dissolved in water (*ca.* 25 ml) and the solution was neutralized with morpholine to pH 7. 2-Propanol (400 ml) and diethyl ether (50 ml) were added and the solution was set aside at 1°. Two crops of the crystalline dimorpholine salt of compound **16** were obtained: 1.35 g (mp 167–172°) and 0.50 g (mp 170–172°). A sample was recrystallized (twice) from water–2-propanol mixtures (mp 173–175°) and was dried at 110° (5×10^{-3} mm) for 4 hr for analysis: uv max 266 mμ (ε 22,500), 250/260 0.69, 260/270 0.96, 270/280 1.35; pK_a = 2.9; mol wt 842 *vs.* 819 (calcd). *Anal.* Cacld for $C_{22}H_{20}N_4O_{15}P_2 \cdot 2C_4H_{10}NO$: C, 44.02; H, 4.93; N, 10.27; P, 7.57. Found: C, 44.05; H, 4.94; N, 9.99; P, 7.33.

A sample of the dimorpholine salt of **16** was converted into the barium salt by passage through Dowex 50 (H^+), followed by neutralization of the effluent with $Ba(OH)_2$ solution, and the product was purified by fractional precipitation from water–2-propanol mixtures. Six fractions were collected. The sixth fraction was dried for ananlysis at room temperature (5×10^{-3} mm) for 24 hr, followed at 110° (5×10^{-3} mm) for 5 hr: R_F's and uv identical with those of the dimorpholine salt of **16**; pK_a = 3.0; mol wt 785 *vs.* 780 (calcd). *Anal.* Calcd for $C_{22}H_{20}N_4O_{15}P_2 \cdot Ba$: C, 33.98; H, 2.33; N, 7.20; P, 7.97. Found: C, 33.71; H, 2.74; N, 7.42; P, 7.69.

Thymidine 3′,5′-Bis(m-aminophenyl phosphate) Dimorpholine and Disodium Salts (**18**). Crystalline thymidine 3′,5′-bis(*m*-nitrophenyl phosphate) dimorpholine salt (**16**, 2.0 g), dissolved in water (10 ml), was hydrogenated in the presence of 10% Pd/C (300 mg) to give compound **18** (dimorpholine salt, 1.86 g, 96% yield): uv max 268 mμ (ε 10.400), 260/270 0.89, 270/280 1.25; pK_a = 4.3 (aminophenyl), 8.3 (morpholine); mol wt 792, 797 *vs.* 795 (calcd).[2] *Anal.* Calcd for $C_{22}H_{24}N_4O_{11}P_2 \cdot 2C_4H_{10}NO \cdot 2H_2O$: C, 45.34; H, 6.09; N, 10.58; P, 7.80. Found: C, 45.18; H, 5.91; N, 10.76; P, 7.69.

Compound **18** (dimorpholine salt) was converted into the corresponding disodium salt by passage through a Dowex 50 (Na^+) column. Compound **18** (disodium salt) was purified by precipitation from a water–2-propanol mixture (yielded a gum) followed by fractional precipitation from methanol–ethanol and methanol–ethanol–2-propanol mixtures. A sample was dried for analysis at room temperature (5×10^{-3} mm) for 24 hr and 110° (5×10^{-3} mm) for 3 hr. *Anal.* Calcd for $C_{22}H_{24}N_4O_{11}P_2 \cdot 2Na$: C, 42.05; H, 3.85; N, 8.92; P, 9.86. Found, after block drying at 160° by the analyst: C, 42.32; H, 4.72; N, 9.43; P, 10.09.

Thymidine 3′,5′-Bis(m-aminophenyl phosphate) Inner Salt (**20**). Thymidine 3′,5′-bis(*m*-aminophenyl phosphate) dimorpholine salt (**18**, 1.37 g) was dissolved in methanol (25 ml) and trifluoroacetic acid (0.27 ml) was added with vigorous stirring. A precipitate resulted. The precipitate was removed by centrifugation and was dried at 110° (5×10^{-3} mm) for 2 hr to give product **20** (1.1 g): R_F's and uv were essentially

identical with those of compound **18**; pK_a = 4.30; mol wt 679 *vs.* 649 (calcd). *Anal.* Calcd for $C_{22}H_{26}N_4O_{11}P_2 \cdot 2CH_3OH$: C, 44.45; H, 5.28; N, 8.64; P, 9.55. Found: C, 44.70; H, 5.23; N, 8.75; P, 9.47.

Thymidine 3′,5′-Bis(m-bromoacetamidophenyl phosphate) Disodium Salt (**25**). Thymidine 3′,5′-bis(aminophenyl phosphate) disodium salt (**18**, 300 mg), dissolved in methanol (5 ml) at 0°, was allowed to react with bromoacetic anhydride (290 mg) as was described for the preparation of compound **4**. Isolation after preparative paper chromatography (16 sheets of 3MM paper) gave homogeneous **25** (438 mg). The product, as a solid, was extracted with ethanol (3 × 40 ml). The extracts were concentrated *in vacuo* to *ca.* 2-ml volume and excess 2-propanol was added to precipitate the product. The product was partially dissolved in hot ethanol. The ethanol was decanted while hot and allowed to cool slowly. A precipitate resulted. Excess 2-propanol was added to complete the precipitation of compound **25**. A sample (extremely hygroscopic) was dried at room temperature (5×10^{-3} mm) 16 hr and at 110° (5×10^{-3} mm) for 2 hr: uv max 253 mμ (ε 25,000), 250/260 1.03, 260/270 1.20. *Anal.* Calcd for $C_{26}H_{26}Br_2N_4O_{13}P_2 \cdot 2Na \cdot 2(CH_3)_2CHOH$: C, 38.80; H, 4.06; Br, 16.14; N, 5.66; P, 6.25. Found, after block drying at 170° (some decomposition may have occurred): C, 38.81; H, 4.06; Br, 15.20; N, 6.48; P, 6.89.

p-Nitrophenyl Phosphorothymidylyl-(3′→5′)-thymidine 3′-(p-Nitrophenyl phosphate)-1.5-barium Salt (**27**). Thymidylyl-(3′→5′)-thymidine pyridinium salt (**26**, 1.14 g, Letsinger and Ogilvie, 1967, 1969) was dissolved in anhydrous pyridine (10 ml) and Linde Molecular Sieves (Type 4A, *ca.* 10 g) were added. The mixture was stirred at room temperature for 1.5 hr. The reaction mixture was cooled to 0° and bis(*p*-nitrophenyl) phosphorochloridate (3.75 g, Ukita and Hayatsu, 1962) was added. Tlc (tlc system B, cellulose anion-exchange sheets) showed that the reaction was complete after 3 hr. Sodium hydroxide (1 N-60 ml) was added dropwise over a period of 1.5 hr. The reaction was set aside at 1° overnight. The reaction mixture was poured onto an Amberlite IR 120 (H^+) column (130 ml) and the column was eluted slowly with ice water initially to prevent the column from warming excessively. The effluent (*ca.* 600 ml) was extracted with diethyl ether (6 × 200 ml) and the extracts were discarded. The aqueous solution was treated with charcoal (*ca.* 100 mg) at room temperature for a few minutes. The resulting yellow solution was neutralized (pH 6.5–7.0) with concentrated LiOH solution. The solution was concentrated *in vacuo* to *ca.* 100-ml volume and lyophilized to afford 3.70 g of crude product **27**. The crude product was a three-component mixture as evidenced by paper chromatography and tlc. Homogeneous **27** (1.37 g, lithium salt) was obtained by preparative paper chromatography (60 sheets of 3MM paper). Compound **27** was converted into the barium salt by passage through Dowex 50 (H^+), followed by neutralization of the effluent with $Ba(OH)_2$ solution and lyophilization, to give 1.28 g of white solid. The solid was purified by fractional precipitation from water–2-propanol mixtures. Seven fractions were collected as gums. The gums were solidified by trituration under 2-propanol and were dried at 110° (5×10^{-3} mm) for several hours. The fifth fraction (630 mg) had the highest ε value and a sample was sent for analysis: uv max 270 mμ (ε 31,000), uv max shoulder) 304 mμ (ε 13,000), 260/270 0.88, 270/280 1.14; pK_a = 2.8; mol wt 1240 *vs.* 1224 (calcd). *Anal.* Calcd for $C_{32}H_{32}N_6O_{22}P_3 \cdot 1.5Ba \cdot 4H_2O$: C, 31.41; H, 3.30; N, 6.87; P, 7.59. Found: C, 31.37; H, 3.46; N, 7.36; P, 7.34.

5′-p-Aminophenyl Phosphorylthymidylyl-(3′→5′)-thymidine 3′-(p-Aminophenyl phosphate)-1.5-Barium Salt (**28**). Compound **27** (850 mg), dissolved in water (10 ml), was hydrogenated for 22 hr at atmospheric pressure in the presence of 10% Pd/C (200 mg) to give crude **28** (743 mg). Paper chromatography indicated the presence of a minor component, migrating faster than the product but slower than starting material. Compound **28** was used in the preparation of compound **29** without further purification.

5′-p-Iodoacetamidophenyl Phosphorylthymidylyl-(3′→5′)-thymidine 3′-(p-Iodoacetamidophenyl phosphate)-1.5-Barium Salt (**29**). Compound **28** (300 mg) was dissolved in a mixture of methanol (5 ml) and water (7 ml). Iodoacetic anhydride (230 mg, Abderhalden and Guggenheim, 1908) was added to the stirred mixture at 0°. The iodoacetic anhydride did not dissolve immediately. Additional methanol (1 ml) was added and the iodoacetic anhydride dissolved within 15 min. The major product was isolated by preparative paper chromatography (six sheets of 3MM paper) as was described for the preparation of compound **4** to give 285 mg of homogeneous **29**. Compound **29** was purified further by fractional precipitation from water–2-propanol mixtures. Seven fractions (gums) were collected and discarded. The supernatant of the seventh fraction was diluted with excess 2-propanol. The resulting solid was removed by centrifugation and dried to give 130 mg of white solid. The solid was again fractionally precipitated from water–2-propanol mixtures and two fractions were collected. The second fraction (70 mg) was dried at room temperature (5×10^{-3} mm) for 16 hr and at 110° (5×10^{-3} mm) for 4 hr over P_2O_5: uv max 262 mμ (ε 40,000), 250/260 0.82, 260/270 1.05, 270/280 1.38. *Anal.* Calcd for $C_{36}H_{38}I_2N_6O_{20}P_3 \cdot 1.5Ba \cdot 2CH_3CH(OH)CH_3$: C, 32.59; H, 3.52; I, 16.40; N, 5.43; P, 6.00. Found: C, 32.26; H, 3.55; I, 16.32; N, 6.37; P, 6.59.

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Mie Scattering by Optically Active Particles*

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ABSTRACT: A method, based on classical Mie theory, is presented for calculating the effects of scattering on the circular dichroism (CD) and optical rotatory dispersion (ORD) of suspensions of optically active spherical particles. The method is illustrated for model solid spheres with the known intrinsic optical constants of a helical polypeptide. Significant size-dependent effects of two types are predicted in the ultraviolet, CD, and ORD spectra of this model. (1) Unequal scattering of left and right circularly polarized light distorts these spectra and gives rise to substantial red shifts (*e.g.*, 2–3 nm for spheres of radius 0.03 μ). (2) At wavelengths where absorption is high, the CD and ORD spectra exhibit flattening, which is increasingly severe as particle size is increased. Similar significant effects are also predicted for a spherical shell model. Red shifts and distortions similar to those calculated here have been observed experimentally in the CD and ORD of suspensions of biological particles, such as membranes and viruses; scattering is a likely basis of these observed effects.

Ultraviolet circular dichroism (CD)[1] and optical rotatory dispersion (ORD) have been widely applied as structural probes of biological molecules and macromolecules in solution. In the past 5 years, these probes have been extended to particulate systems, such as plasma membranes (Wallach and Zahler, 1966; Lenard and Singer, 1966; Gordon *et al.*, 1969; Glaser *et al.*, 1970), mitochondria (Urry *et al.*, 1967; Steim and Fleischer, 1967; Wrigglesworth and Packer, 1968), and viruses (Maestre and Tinoco, 1967), with the goal of obtaining information about the structure of their component macromolecules. In these studies, models of membrane or virus structure were based on CD and ORD spectra without full appreciation of the potentially significant effect of scattering on these measurements; such effects were either neglected or dismissed on the basis of inadequate experimental tests.

Recent experimental studies (Ji and Urry, 1969; Urry *et al.*, 1970; Schneider *et al.*, 1970) demonstrate that CD and ORD measurements on particulate systems do in fact exhibit substantial dependence on particle size, for sizes comparable to or exceeding the wavelength of light. Such effects distort the CD and ORD spectra and obscure their informational content; from the biologist's point of view, they are therefore artifacts.

Experimental evidence alone is insufficient to unambiguously distinguish information from artifact; for this purpose, a quantitative theoretical understanding of the physical origins of the effect of particle size upon CD and ORD spectra is essential. Previous theoretical calculations of these artifacts (Urry and Ji, 1968; Ottaway and Wetlaufer, 1970; Gordon and Holzwarth, 1971a; Glaser and Singer, 1971) have been based on the absorption flattening formulation of Duysens (1956), in which scattering is neglected, and on Rayleigh scattering theory, which is valid only for particles much smaller than the wavelength of light. As Schneider (1971) has indicated, these treatments also share the fundamental flaw of treating absorption and refraction as independent phenomena, and not as the imaginary and real parts of a single complex analytic function of wavelength. A calculation of the size-dependent artifacts in the CD and ORD, based on classical Mie scattering theory, in which these objections are removed, is presented in the present paper.

In this paper, only suspensions of identical, discrete, optically active, spherically symmetric particles are considered. It is assumed that the suspensions are sufficiently dilute that the waves scattered by individual particles are not coherently related; multiple scattering effects are neglected. A scattering matrix formulation of the Mie theory is applied to this model to derive expressions for CD, ORD, optical density (OD), and refractive index of a suspension from the corresponding intrinsic spectral properties of the scatterers. The relative roles of absorption and scattering in the calculated CD and OD spectra are analyzed. The nature and magnitude of the size-dependent artifacts predicted by the model are illustrated for two simple spherical geometries: (1) the solid sphere, which is perhaps a useful representation of viruses and other solid particles of biological interest, and (2) the spherical shell, which approximates the shape of a solvent-filled membrane vesicle. For the solid sphere, CD, ORD, and OD spectra are calculated for suspensions of several sizes of spheres having the assumed optical properties of a synthetic helical polypeptide; substantial artifacts, not unlike those observed ex-

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[1] Abbreviations used are: CD, circular dichroism; ORD, optical rotatory dispersion; OD, optical density; PGA, poly-L-glutamic acid.