Preparation of the 2,4-Dinitrophenyl Esters of Thymidine 3′- and Thymidine 5′-Phosphate and Their Use as Substrates for Phosphodiesterases*

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ABSTRACT: Thymidine 3′-(2,4-dinitrophenyl)phosphate and thymidine 5′-(2,4-dinitrophenyl)phosphate were prepared by reacting thymidine 3′-phosphate or thymidine 5′-phosphate with 2,4-dinitrophenol and dicyclohexylcarbodiimide. The 2,4-dinitrophenylphosphates were isolated in high yield after column chromatography using DEAE-cellulose. The anhydrous products were obtained by flash evaporating and lyophilizing the solutions at pH 4. Some physical and chemical characteristics of thymidine 3′- and thymidine 5′-(2,4-dinitrophenyl)phosphate are reported. Snake venom phosphodiesterase hydrolyses thymidine 5′-(2,4-dinitrophenyl)phosphate and thymidine 5′-(*p*-nitrophenyl)phosphate at identical rates. In the case of spleen phosphodiesterase, the specific activity is 30% higher with thymidine 3′-(2,4-dinitrophenyl)-phosphate than with thymidine 3′-(*p*-nitrophenyl)phosphate. The 2,4-dinitrophenyl esters are useful substrates especially for phosphodiesterases with an acid pH optimum since the reaction can be followed directly at 360 nm above pH 5.

Nucleoside *p*-nitrophenylphosphates are useful substrates for phosphodiesterases (Razzell and Khorana, 1959, 1961; Fiers and Khorana, 1963; Razzell, 1967) and for spleen acid deoxyribonuclease (Bernardi and Griffé, 1964) because of the absorbance of the anion of *p*-nitrophenol which is released on hydrolysis. Direct spectrophotometric assay, however, is only possible at alkaline pH (*p*-nitrophenol; pK = 7.2). The use of nucleoside 2,4-dinitrophenylphosphates as enzyme substrates would, therefore, have an advantage over the use of nucleoside *p*-nitrophenylphosphates, since hydrolysis could be followed directly under both acid and alkaline conditions (Stockx, 1961) (2,4-dinitrophenol; pK = 4.0). This would be especially convenient in work with spleen phosphodiesterase and other phosphodiesterases which have a pH optimum between pH 5 and 8.

The preparation of adenosine 5′-(2,4-dinitrophenyl)phosphate by reaction of 2,4-dinitrophenol with adenosine 5′-phosphomorpholidate (Moffat and Khorana, 1961) or with P^1-adenosine 5′-(P^2-diphenyl)pyrophosphate (Michelson, 1964) and of thymidine 5′-(2,4-dinitrophenyl)phosphate by reaction of 2,4-dinitrophenol and thymidine 5′-phosphate with dicyclohexycarbodiimide in dimethylformamide (Borden and Smith, 1966a) have been previously reported. In each of these procedures the phosphodiester was formed in good yield. However, it had not been possible to isolate pure products (Moffatt and Khorana, 1962; Borden and Smith, 1966a).

Because of our interest in the 2,4-dinitrophenyl esters of nucleotides as potential intermediates in the chemical synthesis of nucleoside 3′,5′-cyclic phosphates and polynucleotides, and also because of their possible utility as substrates for phosphodiesterases, we have now further investigated the isolation of nucleoside 2,4-dinitrophenylphosphates formed by the reaction of nucleoside phosphates and 2,4-dinitrophenol with dicyclohexylcarbodiimide in dimethylformamide. This study has provided a simple and general procedure for the preparation of pure nucleoside 2,4-dinitrophenylphosphates. This communication reports the synthesis and isolation of 2,4-dinitrophenyl esters of thymidine 3′- and thymidine 5′-phosphates together with some of their physical and chemical characteristics. Additionally, the hydrolysis of these compounds by snake venom phosphodiesterase and spleen phosphodiesterase is described and compared with the hydrolysis of the corresponding thymidine *p*-nitrophenylphosphates. The results indicate that the 2,4-dinitrophenyl esters are useful phosphodiesterase substrates.

Materials and Methods

Reaction solvents were redistilled and dried over calcium hydride or Linde 4-A Molecular Sieve. Thymidine 5′-phosphate and thymidine were purchased from Calbiochem. Thymidine 3′-(*p*-nitrophenyl)phosphate and thymidine 5′-(*p*-nitrophenyl)phosphate were prepared according to the methods of Borden and Smith (1966a). All other chemicals and the ion exchangers were obtained commercially. Snake venom phosphodiesterase (phosphodiesterase I) and spleen phosphodiesterase (phosphodiesterase II) were purchased from Worthington Biochemical Corp.

Phosphodiesterase activity was measured according to the method of Razzell (1963), except for minor modifications. 2,4-Dinitrophenolate and *p*-nitrophenolate released from 2,4-dinitrophenyl esters and *p*-nitrophenyl esters were measured at 360 and 400 nm, respectively.

The half-life of 2,4-dinitrophenyl esters in alkaline solutions at 37° was determined from the increase in absorbance at 360 nm.

All spectrophotometric measurements were carried out with a Unicam SP. 800 ultraviolet spectrophotometer.

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Descending paper chromatography on Whatman 40 paper was performed in the following solvent systems: 1, isobutyric acid–1 M NH_4OH (5:3, v/v); 2, 0.1 M sodium phosphate (pH 6.8)–$(NH_4)_2SO_4$–1-propanol (100:60:2, v/wt/v); and 3, isopropyl alcohol–NH_4OH–H_2O (7:1:2, v/v/v). Nucleotides were detected under ultraviolet light. Thymidine 3′,5′-cyclic phosphate was also characterized by the liberation of thymine during hydrolysis in 1 M hydrochloric acid at 50° for 2 hr (Drummond *et al.*, 1963). Thymidine 3′-phosphate was distinguished from thymidine 5′-phosphate by its resistance to the 5′-nucleotidase in crude snake venom (Turner and Khorana, 1959).

All manipulations involved in the synthesis of 2,4-dinitrophenyl esters, including the work-up, were carried out under reduced light conditions at 25°.

Phosphate analysis was carried out according to the method of Ames (1966). Protein was determined using the method of Lowry *et al.* (1951).

Results

Preparation of Thymidine 5′-(2,4-Dinitrophenyl)phosphate. Thymidine 5′-phosphate (triethylammonium salt, 1 mmole) and 2,4-dinitrophenol (10 mmoles) in 20 ml of dimethylformamide were reacted with dicyclohexylcarbodiimide (20 mmoles) at 25° for 2 hr. The solvent was removed by flash evaporation. The residue was suspended in 80 ml of water and extracted with ether until most of the 2,4-dinitrophenol was removed from the water layer. After brief flash evaporation to remove dissolved ether the water solution was applied to a 25 × 350 mm column of DEAE-cellulose in the carbonate form. Products were eluted with 460 ml of water followed by a linear gradient consisting of 1000 ml of water and 1000 ml of 0.1 M ammonium bicarbonate. Fractions (12 ml) were collected at a flow rate of 4 ml/min. The product, thymidine 5′-(2,4-dinitrophenyl)phosphate, was eluted in fractions 53–64 in 90% yield (estimated spectrophotometrically). The combined fractions were concentrated by rotary evaporation. During this operation the pH of the solution was maintained at pH 4 using a cation exchanger (Bio-Rad AG 50-X2, H^+ form). After removal of the exchanger and lyophilization of the concentrated solution, 440 mg of a nearly white powder was obtained, corresponding to an 87% yield of the anhydrous ammonium salt. The ultraviolet absorption showed λ_{max} 263 nm (ϵ 18,800), λ_{infl} 295 nm (ϵ 7400), and λ_{min} 232 nm (ϵ 11,900) at pH 7.

Anal. Calcd for $C_{16}H_{16}N_4O_{12}P \cdot NH_4$: P, 6.14. Found: P, 6.03.

Preparation of Thymidine 3′-(2,4-Dinitrophenyl)phosphate. Thymidine 3′-phosphate, the starting material for this synthesis, was prepared from 5′-monomethoxytritylthymidine (Schaller *et al.*, 1963) by phosphorylation with 2-cyanoethylphosphate (Tener, 1961). It was homogeneous when chromatographed in solvent systems 1, 2, and 3 and it was not dephosphorylated by crude snake venom, indicating insignificant contamination by thymidine 5′-phosphate. Thymidine 3′-(2,4-dinitrophenyl)phosphate was prepared as outlined above using thymidine 3′-phosphate (1.28 mmoles), 2,4-dinitrophenol (12.8 mmoles), and dicyclohexylcarbodiimide (25.6 mmoles). The anhydrous ammonium salt was isolated as a nearly white powder (479 mg) in 74% yield. The ultraviolet absorption showed λ_{max} 265 nm (ϵ 20,200), λ_{infl} 295 nm (ϵ 7500), and λ_{min} 232 nm (ϵ 11,800).

Anal. Calcd for $C_{16}H_{16}N_4O_{12}P \cdot NH_4$: P, 6.14. Found: P, 5.96.

Paper Chromatography. Thymidine 3′-(2,4-dinitrophenyl)-phosphate and thymidine 5′-(2,4-dinitrophenyl)phosphate were homogeneous in solvent systems 1 and 2. In system 1 the R_F values were 0.76 and 0.69, respectively. In system 2, after 48-hr chromatography, the migration distances were 4.9 and 13.0 cm, respectively. Chromatography in system 3 led to extensive breakdown of both esters due to the alkalinity of the solvent.

Alkaline Hydrolysis. Hydrolysis of the thymidine 2,4-dinitrophenylphosphates was followed spectrophotometrically at 360 nm, the λ_{max} of 2,4-dinitrophenolate ion. Above pH 5.0, only insignificant changes of the absorption of 2,4-dinitrophenol (ϵ 14,450) (Hormann and Endres, 1955) were observed at different hydrogen ion concentrations. No breakdown of thymidine 2,4-dinitrophenylphosphates was observed in 0.25 M ammonium acetate (pH 5.9) or 0.1 M Tris-HCl (pH 9.0) at 37° (the two buffer systems used in enzymatic hydrolysis) during a period of at least 10 min.

The half-lives of the thymidine 3′ and the thymidine 5′ esters in 0.1 M sodium hydroxide at 37° were 2.7 hr and 5.5 hr. In 0.5 M sodium hydroxide at 37° the half-lives were 0.38 and 0.90 hr, respectively. Upon hydrolysis in 0.5 M sodium hydroxide at 37°, thymidine 3′-(2,4-dinitrophenyl)phosphate yielded (in addition to 2,4-dinitrophenol) thymidine 3′-phosphate (73%) and thymidine 3′,5′-cyclic phosphate (27%). Thymidine 5′-(2,4-dinitrophenyl)phosphate yielded thymidine 5′-phosphate (92%), thymidine 3′,5′-cyclic phosphate (4%), and an unidentified product (4%).

A value of 14,500 for the molecular absorptivity, ϵ, of 2,4-dinitrophenolate was calculated from the absorption at 360 nm after complete hydrolysis in 0.1 M sodium hydroxide and after phosphate analysis of the thymidine 2,4-dinitrophenylphosphates. This value is in accordance with that of Hormann and Endres (1955) but differs from that of Stockx (1961) (ϵ 12,400) and of Jutisz and Penasse (1952) (ϵ 19,950).

Enzymatic Hydrolysis. The rates of hydrolysis of thymidine 2,4-dinitrophenylphosphates and thymidine *p*-nitrophenylphosphates by snake venom phosphodiesterase and spleen phosphodiesterase were compared. The reaction mixtures used had the following composition per milliliter of solution at pH 9.0: Tris-HCl (pH 9.0), 100 μmoles; $MgCl_2$, 10 μmoles; and substrate, 0.5 μmole. At pH 5.9 the reaction mixture contained per milliliter of solution: ammonium acetate (pH 5.9), 250 μmoles; sodium EDTA, 1 μmole; Tween-80, 0.05%; and substrate, 1 μmole. Enzyme solution, usually 10 μl, containing snake venom phosphodiesterase (0.44 μg of protein) or spleen phosphodiesterase (26 μg of protein), was added after equilibration of the reaction mixture to 37°. The reactions were followed continuously at 360 nm for 2,4-dinitrophenyl esters and at 400 nm for *p*-nitrophenyl esters, except when *p*-nitrophenyl esters were used at pH 5.9. In this case 100-μl samples were removed from the reaction mixture at 1, 2, 3, 5, 10, 15, and 20 min when thymidine 3′-(*p*-nitrophenyl)phosphate was the substrate and at 30, 60, 120, 180, and 240 min when thymidine 5′-(*p*-nitrophenyl)phosphate was the substrate. The absorbance was measured after addition of alkali. Specific activities were based on ϵ 14,500 for 2,4-dinitrophenolate and ϵ 18,000 for *p*-nitrophenolate and are shown in Table I.

TABLE I: Hydrolysis of 2,4-Dinitrophenyl and *p*-Nitrophenyl Esters by Snake Venom Phosphodiesterase and Spleen Phosphodiesterase[a] at 37°.

Substrate	Rate of Hydrolysis (μmoles/hr mg of Protein)	
	Snake Venom Phosphodiesterase	Spleen Phosphodiesterase
Specific Activities at pH 9.0		
Thymidine 5′-(*p*-nitrophenyl)phosphate	2840	
Thymidine 5′-(2,4-dinitrophenyl)phosphate	2840	<0.5
Thymidine 3′-(*p*-nitrophenyl)phosphate	<30	
Thymidine 3′-(2,4-dinitrophenyl)phosphate	<30	12.9
Specific Activities at pH 5.9		
Thymidine 5′-(*p*-nitrophenyl)phosphate		<0.5
Thymidine 5′-(2,4-dinitrophenyl)phosphate	<30	3.9
Thymidine 3′-(*p*-nitrophenyl)phosphate		78.5
Thymidine 3′-(2,4-dinitrophenyl)phosphate	<30	112.0

[a] Snake venom phosphodiesterase and spleen phosphodiesterase were the products of Worthington Biochemical Corp. Incubation conditions are described in the text.

Discussion

Previous studies have shown that the reaction of a nucleoside 5′-phosphate as its trialkylammonium salt, with dicyclohexylcarbodiimide and 2,4-dinitrophenol, provides a potentially rapid and efficient route to the nucleotide 2,4-dinitrophenyl ester if pyridine is not present in the reaction solvent (Borden and Smith, 1966a). This present study was directed at extending the reaction to a nucleoside 3′-phosphate and at the isolation of nucleotide 2,4-dinitrophenyl esters in pure form. Previous methods of isolation had led to impure products, due either to incomplete removal of contaminants or decomposition of the 2,4-dinitrophenyl ester during isolation (Moffatt and Khorana, 1962; Borden and Smith, 1966a). The most probable causes of decomposition are base-catalyzed or photocatalyzed hydrolysis of the 2,4-dinitrophenyl ester (Kirby and Varvoglis, 1967). Examination of the products immediately after ion-exchange chromatography indicated that 2,4-dinitrophenyl esters were pure at this stage. However, hydrolysis occurred during isolation of the nucleotides from the eluate. Consequently, the nucleotide solution was adjusted to pH 4.0 with a cation exchanger prior to rotary evaporation and freeze drying. Additionally, all the isolation steps were carried out in subdued light. Using these precautions it was possible to prepare in good yield the 2,4-dinitrophenyl esters of thymidine 3′- and thymidine 5′-phosphates as their anhydrous ammonium salts after reaction of 2,4-dinitrophenol and thymidine 5′- or thymidine 3′-phosphate with dicyclohexylcarbodiimide in dimethylformamide.

Some of the physical properties of the two esters are of interest. The molecular absorptivity (20,200), at the absorbance maximum (265 nm) of the thymidine 3′ ester is significantly higher than that (18,800) at the absorbance maximum (263 nm) of the thymidine 5′ ester. Additionally, the chromatographic mobilities of the thymidine 5′ ester differ from those of the thymidine 3′ ester. Both the spectral and the chromatographic properties of the esters suggest that there may be a more marked interaction between the 2,4-dinitrophenyl group and thymidine in the thymidine 5′-phosphate derivative.

Chemically, the two esters differ in stability to alkali, thymidine 5′-(2,4-dinitrophenyl)phosphate being more stable. Both diesters yielded their parent nucleoside phosphate as the major product of alkaline hydrolysis in 0.5 M sodium hydroxide. However, there was much more transesterification in the case of thymidine 3′-(2,4-dinitrophenyl)phosphate, which gave thymidine 3′,5′-cyclic phosphate in 27% yield whereas the yield of cyclic phosphate from the thymidine 5′ ester was only 4%. These results confirm earlier evidence of the more facile production of nucleoside 3′,5′-cyclic phosphates from derivatives of nucleoside 3′-phosphates (Borden and Smith, 1966b).

The results of the experiments with the two exonucleases, snake venom phosphodiesterase and spleen phosphodiesterase, show that thymidine 5′- and thymidine 3′-(2,4-dinitrophenyl)-phosphates are as efficient substrates as are the corresponding *p*-nitrophenyl esters (Table I). The fact that 2,4-dinitrophenol is ionized at the acid pH optimum of spleen phosphodiesterase makes thymidine 3′-(2,4-dinitrophenyl)phosphate an especially useful substrate for this class of enzymes, since substrate hydrolysis can now be followed directly. In this connection the substrate has already proved useful in the characterization of an analogous phosphodiesterase isolated from salmon testes (K. M. J. Menon and M. Smith, unpublished results). For similar reasons, thymidine 5′-(2,4-dinitrophenyl)-phosphate should be a useful substrate for nucleotide pyrophosphatase which is optimally active at pH 7.0 (Razzell, 1966).

In the present study, there was noted an activity in commercial spleen phosphodiesterase which hydrolyzed thymidine 5′-(2,4-dinitrophenyl)phosphate at pH 5.9 (Table I). This was

not due to contaminating phosphodiesterase I since there was no hydrolysis at pH 9.0. Contamination of the substrate with thymidine 3′-(2,4-dinitrophenyl)phosphate was ruled out because the reaction went to completion in the presence of excess enzyme and with prolonged incubation. Although a lack of specificity of spleen phosphodiesterase, only evident with the 2,4-dinitrophenyl esters, cannot be ruled out, it appears possible that the activity is due to another enzyme contaminating commercial spleen phosphodiesterase. A similar enzyme activity, hydrolying thymidine 5′-(*p*-nitrophenyl)phosphate under acidic conditions, has been detected in liver extracts and is suspected to be due to a pyrophosphatase (Razzell, 1967).

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