2′,5′-Oligoadenylates and Related 2′,5′-Oligonucleotide Analogues. 1. Substrate Specificity of the Interferon-Induced Murine 2′,5′-Oligoadenylate Synthetase and Enzymatic Synthesis of Oligomers†

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ABSTRACT: The substrate specificity of the interferon-induced mouse L-cell enzyme, 2′,5′-oligoadenylate synthetase, was determined with a number of nucleoside 5′-triphosphate analogues. Selected nucleoside 5′-triphosphates were converted to 2′,5′-oligonucleotides with the following order of efficiency for the nucleoside: 8-azaadenosine > adenosine = 2-chloroadenosine > sangivamycin > toyocamycin > formycin > 3-ribosyladenine > ribavirin > tubercidin > adenosine 1-oxide > 2-β-D-ribofuranosylthiazole-4-carboxamide > inosine = 1,N^6-ethenoadenosine > guanosine > 8-bromoadenosine = uridine > cytidine. Adenosine 5′-(β,γ-imidotriphosphate) did not seem to be a recognizable substrate since no detectable product resulted. Either the 2′,5′-oligoadenylate synthetase is not as specific as had been previously thought, or there may be more than one 2′,5′-oligonucleotide synthetase. The 2′,5′-oligonucleotide analogue products in which the adenosine of ppp($A_{2'p5'}$)$_n$A was replaced by the various nucleoside analogues were separated by DEAE-cellulose column chromatography and the chain length and number of 5′-phosphate residues analyzed by a rapid, efficient high-performance liquid chromatographic (HPLC) system involving ion-pairing C_{18} reversed-phase column chromatography. Separation of the 5′-mono-, 5′-di-, and 5′-triphosphorylated forms of the 2′,5′-oligonucleotide analogue dimers, trimers, tetramers, and pentamers was readily achieved by this useful HPLC system. No 5′-nonphosphorylated forms were detected for any of the 2′,5′-oligonucleotide analogue products.

The compound 5′-triphosphooligo[(2′–5′)adenylyl]adenosine, ppp($A_{2'p5'}$)$_n$A, is the product of the synthesis by the enzyme 2′,5′-oligoadenylate synthetase [ATP:(2′–5′)oligo(A) adenyltransferase (EC 2.7.7.19)], from 5′-ATP[1] and was first isolated from interferon-treated mouse L cells by Kerr & Brown (1978). ppp($A_{2'p5'}$)$_n$A (2′,5′-A) has been implicated in the antiviral action of interferon (Williams et al., 1979a,b; Hovanessian & Wood, 1980; Panet et al., 1981) and may also be a natural growth regulator since it has been found in a wide variety of cells (Stark et al., 1979; Etienne-Smekens et al., 1981) and varies with the hormonal status (Stark et al., 1979; Krishnan & Baglioni, 1980) and state of growth of the cells (Kimchi et al., 1981a,b). 2′,5′-A is known to activate a latent endoribonuclease that degrades viral RNA in vitro (Ball & White, 1979a; Nilsen et al., 1980), cellular rRNA (Hovanessian et al., 1979; Wreschner et al., 1981a), and mRNA (Clemens & Williams, 1978) and is responsible for the inhibition of protein synthesis. 2′,5′-A is known to bind to the endoribonuclease (Slattery et al., 1979), leading to specific cleavage patterns in the viral RNAs (Floyd-Smith et al., 1981; Wreschner et al., 1981b). 2′,5′-A is also known to inhibit in vitro capping methylation of vaccinia virus mRNA (Sharma & Goswami, 1981).

In the present study, a number of new 2′,5′-oligonucleotide analogues were synthesized with the mouse L-cell enzyme 2′,5′-oligoadenylate synthetase. These oligonucleotides were characterized as to chain length and number of 5′-phosphate residues by a very efficient and rapid HPLC system involving ion-pairing reversed-phase column chromatography. These newly synthesized 2′,5′-oligonucleotide analogues represent a group of potential antiviral and antitumor agents. In addition, these studies have further defined the substrate specificity requirements for the interferon-induced murine 2′,5′-oligoadenylate synthetase.

Materials and Methods

Materials. Commercially available high-purity reagent-grade chemicals and solvents were used. Solvents were freshly distilled before use. Pyridine and dimethylformamide were refluxed over calcium hydride, distilled through a short column, and stored over molecular sieves (Aldrich, 3 Å, 8–12 mesh). Tri-*n*-octylamine (Aldrich), tri-*n*-butylamine (Aldrich), and triethylamine (Sigma) were purified by passage through a neutral alumina column. Triethylammonium bicarbonate (0.8 N) was prepared by bubbling CO_2 into 0.8 M aqueous triethylamine at 0 °C until the pH reached approximately 7.5. Dowex 50W-X8, H^+ form (Baker), was 20–50 mesh, and DEAE-Sephadex (Sigma) beads were 40–120 μm.

The tri-*n*-butylammonium salt of pyrophosphate was prepared in different quantities by varying the amounts of the reagents used according to the ratio in the following procedure. Anhydrous pyrophosphate tetrasodium salt (266 mg, 1 mmol) was added to a mixture of cold water (20 mL) and Dowex 50W-X8 H^+-form resin (10 mL) and swirled until dissolved. This mixture was applied to a Dowex 50W-X8 H^+-form column (1 cm × 10 cm) and washed with cold water. The acidic fractions were collected dropwise in a rapidly stirred, cold mixture of water (10 mL) and tri-*n*-butylamine (1.9 mL,

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[1] Abbreviations: rRNA, ribosomal ribonucleic acid; mRNA, messenger ribonucleic acid; tRNA, transfer ribonucleic acid; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PEI, poly(ethylenimine); BAP, bacterial alkaline phosphatase (EC 3.1.3.1); VSV, vesicular stomatitis virus; 5′-AMP, adenosine 5′-monophosphate; 5′-ADP, adenosine 5′-diphosphate; 5′-ATP, adenosine 5′-triphosphate; p_4A, adenosine 5′-tetraphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/83/0422-2116$01.50/0

8 mmol). The solution was extracted with ether, and the water portion was evaporated in vacuo at 35 °C and coevaporated twice with dry dimethylformamide. The resulting syrup was used immediately or stored over nitrogen at −10 °C.

Toyocamycin and sangivamycin had been previously prepared in our laboratory (Tolman et al., 1968, 1969). Tubercidin and formycin were obtained from Calbiochem-Behring Corp. 2-Chloroadenosine was prepared from 2,6-dichloro-9-β-D-ribofuranosylpurine (Gerster & Robins, 1966) and methanolic ammonia at room temperature. 8-Azaadenosine was prepared according to our previously published procedure (Hutzenlaub et al., 1972). 3-β-D-Ribofuranosyladenine (3-ribosyladenine) was prepared from 8-bromoadenine as previously reported from our laboratory (Tindall et al., 1972). 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) (Witkowski et al., 1972) and 2-β-D-ribofuranosylthiazole-4-carboxamide (Srivastava et al., 1977) were prepared respectively according to the published procedures. 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide 5′-phosphate and 5′-triphosphate were prepared by procedures previously reported from our laboratory (Allen et al., 1978). 2-β-D-Ribofuranosylthiazole-4-carboxamide 5′-phosphate was synthesized via the treatment of the corresponding nucleoside with a mixture of phosphoryl chloride, pyridine, water, and acetonitrile as reported by Srivastava & Robins (1983).

Adenosine 1-oxide 5′-triphosphate, inosine 5′-triphosphate, 1,N^6-ethenoadenosine 5′-triphosphate, guanosine 5′-triphosphate, 8-bromoadenosine 5′-triphosphate, uridine 5′-triphosphate, cytidine 5′-triphosphate, adenosine 5′-(β,γ-imidotriphosphate), 5′-AMP, 5′-ADP, 5′-ATP, and p_4A were from Sigma Chemical Co. 2′,5′-ApA, 2′,5′-ApApA, 2′,5′-ApApApA, 2′,5′-pApApA, and 2′,5′-pppApApA were from P-L Biochemicals, Inc. 2′,5′-ppp$(Ap)_{2-4}$A were also obtained as a generous gift from Dr. Peter Lengyel's laboratory at Yale University.

Synthesis of Nucleoside 5′-Monophosphates. Method A. The nucleoside (5 mmol) was added to a cold (0 °C) solution of phosphoryl chloride (0.93 mL, 10 mmol) and trimethyl phosphate (15 mL) under anhydrous conditions. The resulting solution was protected from moisture and stirred at 0 °C for 2–3 h. Additional phosphoryl chloride (0.42 mL, 4.5 mmol) was added, and the solution was stirred at 0 °C for an additional 2–4 h. The resulting reaction solution was poured slowly into 250 mL of cold anhydrous ethyl ether with rapid stirring.[2] The precipitate was allowed to settle, and the ether was decanted.[3] The solid was washed well with ether 3 more times. The residue was then dissolved at 0 °C in a minimum of cold water and adjusted to pH 2 with cold 10% NaOH. The solution was cooled to allow crystallization to occur completely. The crystals were collected, washed with a small amount of cold water, and dried in vacuo at 80 °C over phosphorus pentoxide for 3 h to give the free acid form of the nucleoside 5′-monophosphate. The nucleoside 5′-phosphate remaining in the filtrate or from the pH 2 solution, if crystallization did not occur, was isolated as follows: The solution was applied to a Dowex 50W-X8, H^+ column (2.8 cm × 20 cm), and the column was washed with water until free of inorganic acids and nucleoside bisphosphate impurities. The column was then successively eluted with 30, 40, 50, 60, and 70% acetic acid until the eluant showed no UV absorption (approximately 300 mL of each). The appropriate fractions containing the 5′-phosphate (TLC) were collected and evaporated to a syrup in vacuo at 35 °C. The syrup was dissolved in a minimum of water and poured into 500 mL of absolute ethanol. The precipitate was filtered, washed with ethanol, and dried in vacuo at 80 °C over phosphorus pentoxide for 3 h to give the free acid form of the nucleoside 5′-monophosphate.

Method B. Sangivamycin 5′-Monophosphate. Sangivamycin 5′-monophosphate was prepared from toyocamycin 5′-monophosphate (1.11 g, 3 mmol), which was added to cold water (20 mL) and adjusted to pH 9 with 3 N ammonium hydroxide. Hydrogen peroxide (30%, 0.6 mL, 6 mmol) was added, and the solution was stirred at room temperature for 2 h. The pH was then adjusted to 2 with 3 N HCl, and the resulting crystals were filtered, washed with cold water, and dried in vacuo over phosphorus pentoxide at 80 °C for 3 h to give sangivamycin 5′-monophosphate (free acid form) (1.04 g).

Synthesis of Nucleoside 5′-Triphosphates. Method C. To a solution of tri-*n*-butylamine (0.24 mL, 1 mmol) and dimethylformamide (10 mL) was added 1 mmol of nucleoside 5′-monophosphate (free acid form). The mixture was warmed to 35 °C, and water was added in 0.5-mL portions until the solid dissolved. The solution was evaporated at 35 °C in vacuo to a syrup, which was coevaporated twice with dimethylformamide, and the anhydrous syrup was then dissolved in dimethylformamide (15 mL). *N,N′*-Carbonyldiimidazole (0.81 g, 5 mmol) was added and the flask gently shaken until solution occurred. The reaction mixture was maintained under anhydrous conditions at room temperature for 12 h. Dry methanol (0.16 mL, 4 mmol) was added to the reaction mixture, and it was kept under anhydrous conditions for 30 min. A solution of dry dimethylformamide (10 mL) and the tri-*n*-butylammonium salt of pyrophosphate (5 mmol) were added, and the reaction mixture was maintained under anhydrous conditions for 20 h. Any solid was filtered, and the reaction mixture was evaporated in vacuo at 35 °C. The syrup was cooled to 0 °C, dissolved in a minimum of cold water, and adjusted to pH 8 with cold 0.5 N ammonium hydroxide. This solution was extracted with ether, and then the aqueous portion was applied to a bicarbonate-form DEAE-Sephadex column (2.5 cm × 15 cm). Elution with a linear gradient of water to 0.8 M triethylammonium bicarbonate (1 L each) at 4 °C separated the 5′-triphosphate from inorganic salts, 5′-diphosphate, and unreacted 5′-monophosphate. The proper fractions were identified by comparison with 5′-AMP, 5′-ADP, 5′-ATP, and the respective 5′-monophosphate on TLC (PEI–cellulose), and the purity was determined by HPLC. The fractions of triphosphate were collected, evaporated in vacuo at 30 °C, and coevaporated 10 times with methanol (or until the odor of triethylamine was not detected). The resulting syrup was dissolved in a minimum of methanol and poured into a solution of sodium iodide (0.60 g, 4 mmol) and acetone (20 mL). The precipitate was separated by centrifugation and washed 5 times with acetone and twice with ether. The resulting solid was dried at room temperature over phosphorus pentoxide in vacuo for 2 h to give the nucleoside 5′-triphosphate tetrasodium salt.

Method D. One millimole of nucleoside 5′-monophosphate (free acid form) was refluxed with absolute methanol (10–15 mL) and tri-*n*-octylamine (0.44 mL, 1 mmol) until the solid dissolved. The solution was evaporated in vacuo at 35 °C, and the resulting syrup was coevaporated 3 times with dimethylformamide and then dissolved in dimethylformamide (5 mL). To this solution was added diphenyl chlorophosphate (0.3 mL,

[2] With some compounds, precipitation did not occur completely. Instead, a gum formed. This gum could be triturated with ether at 0 °C, the ether decanted, and then the gum dissolved in water at 0 °C.

[3] Centrifugation was often necessary in order to collect the microcrystalline precipitate.

1.5 mmol) and tri-*n*-butylamine (0.47 mL, 2 mmol). The mixture was maintained under anhydrous conditions at room temperature for 2 h, after which it was evaporated to a syrup, swirled with anhydrous ether, and then kept at 0 °C for 20 min. The ether was decanted, and excess ether was removed in vacuo at 35 °C. The syrup was dissolved in dimethylformamide (5 mL) and added dropwise, over a 10-min period and under anhydrous conditions, to a stirred solution of the tri-*n*-butylammonium salt of pyrophosphate (1.2 mmol) in dimethylformamide (5 mL) and pyridine (15 mL). The reaction mixture was kept at room temperature for 30 min, after which solvents were removed in vacuo at 30 °C. The resulting syrup was swirled with anhydrous ether (50 mL) and kept at 0 °C for 20 min. The ether was decanted and excess ether was removed in vacuo at 30 °C. The syrup was dissolved in cold water (25 mL) and adjusted to pH 8 with dilute ammonium hydroxide. This solution was extracted with ether and then applied to a bicarbonate-form DEAE-Sephadex column (2.5 cm × 15 cm). Separation, collection, and precipitation of the nucleoside 5′-triphosphate were carried out as described for method C.

Method E. Sangivamycin 5′-Triphosphate. Sangivamycin 5′-triphosphate was prepared from sangivamycin 5′-monophosphate by method C except that after warming the mixture of 5′-monophosphate, dimethylformamide (10 mL), and tri-*n*-butylamine (0.24 mL, 1 mmol), additional tributylamine (0.24 mL) and dimethyl sulfoxide (5 mL) were added to aid in dissolving the solid.

Method F. Sangivamycin 5′-Triphosphate. Sangivamycin 5′-triphosphate was also prepared from toyocamycin 5′-triphosphate by the following procedure. Toyocamycin 5′-triphosphate tetrasodium salt (310 mg, 0.5 mmol) was added to cold water (5 mL), and the pH was adjusted to 9 with cold 0.5 N ammonium hydroxide. Hydrogen peroxide (30%, 15 mL, 1.5 mmol) was added, and the solution was maintained at 5 °C for 12 h. The solution was evaporated at 30 °C in vacuo to a volume of 2 mL and then poured into 20 mL of cold acetone. The resulting solid was dried at room temperature over phosphorus pentoxide in vacuo for 2 h to give an 85% yield of sangivamycin 5′-triphosphate tetrasodium salt.

Interferon Treatment of Cell Cultures. Mouse L cells (American Type Culture Collection CCL 1 clone 929) were grown as monolayers in Eagle's minimal essential medium with Earle's salts in 10% bovine serum. Plates were seeded in 100-mm plastic plates at 5.1×10^6 cells/plate, and mouse fibroblast interferon (from Lee BioMolecular Research Laboratories, Inc., or from Kurt Paucker's laboratory) (200 units/mL of 1.1×10^7 units/mg) was added in fresh medium (5 mL) 1 day later.

Mouse interferon titers were determined on mouse L cells by a plaque-reduction assay against vesicular stomatitis virus (Stewart, 1979). Titers were expressed in International Reference Units, on the basis of NIH mouse interferon reference G-002-904-511.

Preparation of Cell Extracts. Cell extracts were prepared 24 h after the addition of interferon according to Kimchi et al. (1979) and were stored at −75 °C except that the cell washing and lysing solutions were those used by Derynck et al. (1980) and plates were shaken on a reciprocal shaker at 120 rpm for 9 min at 4 °C to lyse the cells.

Assay for 2′,5′-Oligoadenylate Synthetase. The 2′,5′-oligoadenylate synthetase was assayed according to Derynck et al. (1980) with the omission of fructose 1,6-bisphosphate, followed by incubation at 30 °C for 1 h. The reactions (9.5 μL/tube) were stopped by heating at 93 °C for 3 min, centrifuged 2 min at 10000*g*, and 4 μL of the supernatant was treated with 8 μL of Worthington *Escherichia coli* bacterial alkaline phosphatase (BAP) (dialyzed in 0.1 M Hepes–KOH, pH 7.5) (53 units/mL) at 37 °C for 1 h. An aliquot (2 μL) was spotted on PEI–cellulose thin-layer plates (Schleicher and Schuell) and chromatographed in 1 (Ball & White, 1978) or 4 M acetic acid. 2′,5′-ApA, 2′,5′-ApApA, 2′,5′-ApApApA, and 2′,5′-ApApApApA moved as a single spot (R_f = 0.84) in 4 M acetic acid, which facilitated quantitation. A total of 5 μL was also spotted on Whatman 3MM paper, and electrophoresis was carried out according to Zilberstein et al. (1978) except a Camag high-voltage electrophoresis apparatus at 2500 V was used. Autoradiography was carried out and the incorporation of [^{32}P]ATP (ICN Chemical & Radioisotope Division) into 2′,5′-A determined. Protein determinations were made by the Bradford (1976) method with reagent obtained from Bio-Rad Laboratories with bovine serum albumin as a standard. The specific activity of the enzyme was calculated in nanomoles of AMP incorporated per milligram of protein per hour.

Synthesis of 2′,5′-Oligonucleotides by 2′,5′-Oligoadenylate Synthetase. The synthesis of the 2′,5′-oligonucleotides was carried out with the L-cell enzyme, 2′,5′-oligoadenylate synthetase, free in solution according to Derynck et al. (1980) (5 mM nucleoside 5′-triphosphate, omitting the fructose 1,6-bisphosphate) for 4 or 8 h at 30 °C or bound to a solid support [poly(riboinosinic acid)–poly(ribocytidylic acid) covalently linked to agarose from P-L Biochemicals, Inc.] according to modifications in the procedure of Hovanessian & Kerr (1978) with 3 mM nucleoside 5′-triphosphate in buffer B of Kimchi et al. (1979), which was replaced every 12 h for 5 days at 30 °C. The 2′,5′-oligonucleotide products were heated to 93 °C for 3 min and centrifuged for 3 min at 2000*g*, and the supernatant was either precipitated with acetone (Martin et al., 1979) or put directly on a DEAE-cellulose DE-52 (Whatman) column (1.3 × 47 cm) equilibrated in 10 mM Hepes–KOH, pH 7.4, 50 mM KCl, and 2.5 mM magnesium acetate at 4 °C. A new DEAE-cellulose column was used for each 2′,5′-oligonucleotide synthesis reaction. The unreacted nucleoside 5′-triphosphate was removed by extensive washing with the equilibration buffer, and the 2′,5′-oligonucleotide product eluted with a linear gradient of 10–450 mM ammonium bicarbonate (1 L:1 L) after a modification of Samanta et al. (1980). Yields were calculated on the basis of UV absorbance (UV absorbance for products/UV absorbance total) without a correction for hyperchromicity for the 2′,5′-oligonucleotides. Oligonucleotide peaks were collected and lyophilized repeatedly with at least six successive additions of water to rid of any residual ammonium bicarbonate.

High-Performance Liquid Chromatographic Analysis of Nucleoside 5′-Triphosphates. A Beckman high-performance liquid chromatograph (Model 332) equipped with a Hitachi variable-wavelength spectrophotometer and Model C-R1A peak integrator was used to determine the retention times and states of purity of the nucleoside 5′-triphosphates studied as substrates for the 2′,5′-oligoadenylate synthetase. High-performance liquid ion-pairing chromatography was performed on a reversed-phase Ultrasphere C_{18} ODS column (Beckman, 4.6 × 25 cm) with an attached RP-18 Lichrosorb precolumn (Rheodyne, 10 μm, 3-cm size). A parabolic gradient consisting of solvent A (0.1 M KH_2PO_4, 5 mM tetrabutylammonium phosphate from Waters, pH 5.0) and solvent B (methanol from Burdick and Jackson) at 1 mL/min and ambient temperature was used. Solvent A was filter sterilized through a 0.22-μm filter. Column effluents were monitored at the wavelength

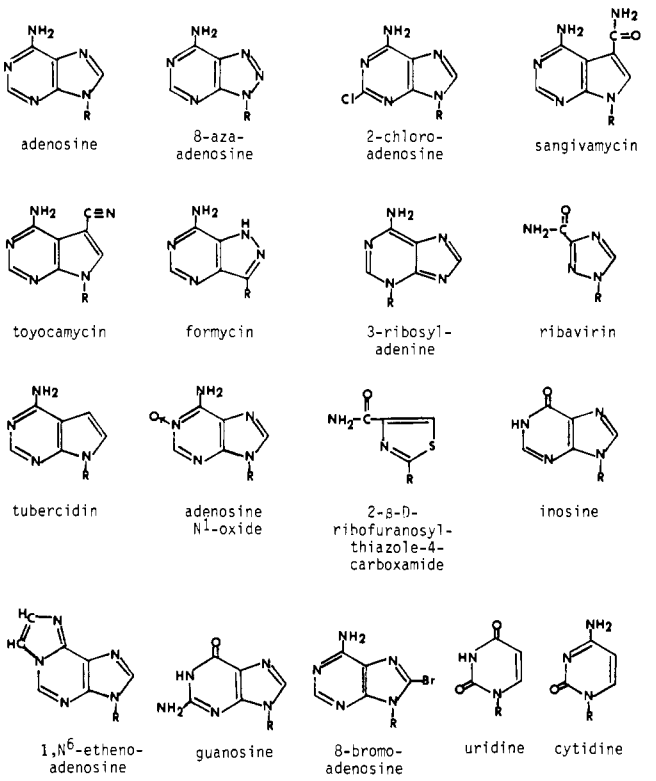

FIGURE 1: Chemical structures of nucleoside moieties of nucleoside 5′-triphosphates or 2′,5′-oligonucleotides. R = β-D-ribofuranosyl moiety.

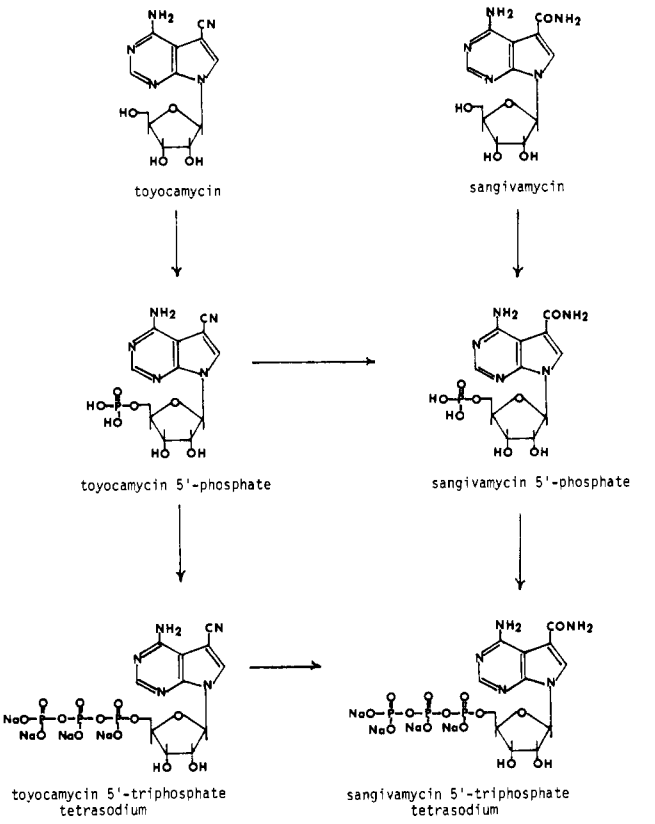

FIGURE 2: Synthesis of toyocamycin 5′-triphosphate and sangivamycin 5′-triphosphate.

of maximum absorption for the particular nucleoside 5′-triphosphate being characterized. The columns were equilibrated for 30 min with 5% solvent B, and samples (20 μL) were injected simultaneously with the beginning of the parabolic gradient (of the form $Y = |X^{1/2}|$) shown in Table III. After the injection of typically six samples per day, the columns were washed in 100% methanol and stored overnight in methanol–water (1:1).

High-Performance Liquid Chromatographic Analysis of 2′,5′-Oligonucleotides. The 2′,5′-oligonucleotide analogues were analyzed as to their states of purity, chain length, and number of 5′-phosphates by the same parabolic-gradient HPLC system described in the preceding section.

Results

The nucleoside analogues (Figure 1) were converted to the corresponding 5′-phosphates shown in Table I by method A, involving modification of the general procedure of Yoshikawa et al. (1967) employing phosphoryl chloride and trimethyl phosphate. The purification of these 5′-phosphates on a Dowex 50, H^+ column was found to be very convenient. The inorganic acids (HCl and H_3PO_4) and nucleoside bisphosphates contaminating the product were easily washed out of the column with water. The pure adenosine analogue 5′-phosphate, which is substantially retarded on the cation-exchange column, can then be eluted with acetic acid. The yields of the nucleoside 5′-phosphates are recorded in Table I. Sangivamycin 5′-phosphate was also prepared via the oxidation of the nitrile group of toyocamycin 5′-phosphate with hydrogen peroxide in the presence of ammonium hydroxide (pH 9) to provide the product in 90% yield (Table I), which was superior to the direct phosphorylation of sangivamycin (Figure 2).

The preparation of the requisite nucleoside 5′-triphosphates from the corresponding 5′-phosphates was accomplished by either method C or method D. Method C involved the use of *N,N′*-carbonyldiimidazole and pyrophosphate and is a modified procedure of Hoard & Ott (1965). Method D involved the use of diphenyl chlorophosphate and pyrophosphate and is a modified procedure of Mickelson (1964). The yields of the nucleoside 5′-triphosphate and the procedure employed are listed in Table I.

The direct conversion of toyocamycin 5′-triphosphate to sangivamycin 5′-triphosphate with hydrogen peroxide and dilute ammonium hydroxide provided the product in an 85% yield, which was superior to the conversion of sangivamycin 5′-phosphate to the corresponding triphosphate by method E (Figure 2). The yields and methods used for the synthesis of the nucleoside 5′-triphosphates are shown in Table I. The triphosphates were isolated as the tetrasodium salts and were stored at −20 °C. The 5′-triphosphates of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) and the related 2-β-D-ribofuranosylthiazole-4-carboxamide were selected for substrate characterization of the 2′,5′-oligoadenylate synthetase since ribavirin has been shown to be converted to the 5′-phosphate by adenosine kinase (Streeter et al., 1974; Willis et al., 1978) and in this regard may be considered a pseudoanalogue of adenosine.

Retention times and purity of nucleoside 5′-triphosphates (Table II) were determined by the HPLC ion-pairing reversed-phase system on a parabolic gradient, which gave efficient separation from nucleoside 5′-mono-, 5′-di-, or 5′-tetraphosphates.

The enzymatic synthesis of the 2′,5′-oligonucleotides was achieved under conditions that gave optimum yields for the 2′,5′-A trimer. The dimer was not quantitatively recovered from the DEAE-cellulose columns since most of it came off in the equilibration-buffer wash step along with the unreacted 5′-ATP. The total yield for the 2′,5′-A product was 30%, including all of the dimer (Table IV), while that obtained from the peaks separated by the gradient was 16%. Figure 3 shows that the 2′,5′-A oligonucleotides were eluted with the gradient

Table I: Synthetic Procedures and Characterization of Nucleoside 5′-Mono- and 5′-Triphosphates

nucleotide	yield from precursor (%)	method of synthesis	R_f[a] W	R_f[a] X	R_f[a] Y	R_f[a] Z	pH	UV[b] λ_{max}	UV[b] ϵ
toyocamycin			0.62	0.82					
5′-monophosphate	72	A	0.43	0.32	0.45	0.48			
5′-triphosphate	62	C			0.14	0.02	1	233	17 100
								273	11 700
							7	224	11 500
								275	13 600
							11	224	11 400
								277	14 500
tubercidin			0.49	0.76					
5′-monophosphate	34	A	0.27	0.22	0.36	0.46			
5′-triphosphate	34	C			0.20	0.02	1	237	24 000
								280	6 100
							7	224	15 700
								278	6 700
							11	226	15 300
								278	6 700
formycin			0.49	0.64					
5′-monophosphate	74	A	0.27	0.12	0.39	0.45			
5′-triphosphate	43	C			0.26	0.02	1	214	15 500
								213	8 700
								265	6 900
								313	6 900
							7	233	11 000
								303	9 300
							11	213	17 900
								235	13 800
								314	8 100
2-chloroadenosine			0.67	0.82					
5′-monophosphate	85	A	0.43	0.26	0.53	0.43			
5′-diphosphate					0.37				
5′-triphosphate	13	D			0.22	0.04	1	210	19 700
								265	10 900
							7	208	21 100
								263	12 200
							11	210	20 800
								263	12 700
8-azaadenosine			0.74	0.82					
5′-monophosphate	26	A	0.41	0.32	0.42	0.57			
5′-diphosphate					0.31				
5′-triphosphate	34	D			0.18	0.02	1	261	11 100
							7	279	9 300
							11	208	14 900
								276	10 200
3-ribosyladenine			0.49	0.24					
5′-monophosphate	48	A	0.27	0.04	0.45	0.49			
5′-triphosphate	38	D			0.27	0.06	1	275	16 300
							7	213	16 300
								275	12 100
							11	212	18 300
								274	12 000
2-β-D-ribofuranosylthiazole-4-carboxamide			0.56	0.76					
5′-monophosphate			0.32	0.32	0.54	0.73			
5′-diphosphate					0.43				
5′-triphosphate	28	D			0.30	0.06	1	236	5 700
							7	235	6 000
							11	236	5 600
sangivamycin			0.49	0.50					
5′-monophosphate	90	B	0.27	0.10	0.34	0.27			
5′-triphosphate	85	E, F			0.09	0.02	1	232	11 400
								274	10 100
							7	230	9 300
								278	12 400
							11	231	9 000
								277	12 100

[a] For method A, TLC was carried out on silica gel $60F_{254}$ precoated TLC sheets (EM-Reagents, 0.22 mm) with two systems: W = concentrated ammonia–1-butanol–water (2:3:4) and X = acetonitrile–0.1 N ammonium chloride (7:3). For method B, TLC was carried out on PEI-cellulose sheets (Schleicher and Schuell) with two systems: Y = 0.5 N ammonium bicarbonate and Z = 0.3 N ammonium bicarbonate and 1.0 N LiCl for 1 and 5 min, respectively. [b] UV spectra were performed on a Cary 15 spectrophotometer.

Table II: HPLC Retention Times and Purity of Nucleoside 5′-Triphosphates

nucleoside 5′-triphosphate	retention time (min)	% purity
adenosine 5′-triphosphate	27.23	99
8-azaadenosine 5′-triphosphate	24.92	93
2-chloroadenosine 5′-triphosphate	38.04	87
sangivamycin 5′-triphosphate	37.68	92
toyocamycin 5′-triphosphate	32.95	99
formycin 5′-triphosphate	30.47	84
3-ribosyladenine 5′-triphosphate	25.00	92
ribavirin 5′-triphosphate	19.63	69
tubercidin 5′-triphosphate	34.74	87
adenosine 1-oxide 5′-triphosphate[a]	20.43	84
2-β-D-ribofuranosylthiazole-4-carboxamide 5′-triphosphate	24.77	82
inosine 5′-triphosphate	22.03	96
1,N^6-ethenoadenosine 5′-triphosphate	30.20	85
guanosine 5′-triphosphate	22.04	93
8-bromoadenosine 5′-triphosphate	31.18	77
uridine 5′-triphosphate	22.43	94
cytidine 5′-triphosphate	20.23	97
adenosine 5′-(β,γ-imidotriphosphate)	25.83	93

[a] Contained 6% 5′-ATP as a contaminant.

Table III: HPLC Parabolic Gradient Program for Analysis of Nucleoside 5′-Triphosphates and 2′,5′-Oligonucleotides

time (min)	% solvent B	duration (min)
0	7.4	1
1	9.1	1
2	10.6	1
3	11.9	1
4	13.1	1
5	14.1	1
6	15.1	1
7	16.1	1
8	17.0	1
9	17.8	1
10	19.4	2
12	21.5	3
15	24.7	5
20	26.9	4
24	28.5	3
27	30.0	3
60	5.0	

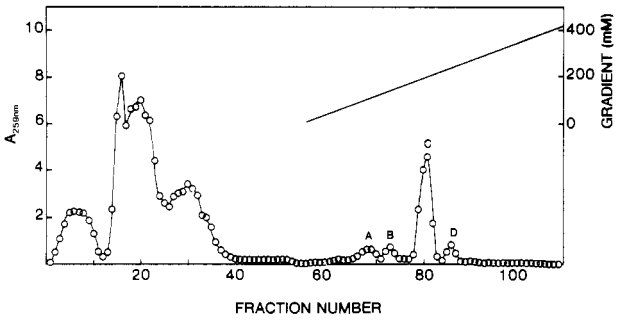

FIGURE 3: DEAE-cellulose chromatographic profile for enzymatic synthesis of 2′,5′-A by the in-solution method (Derynck et al., 1980) for 4 h: (A) 8.8% pApA, 88% ppApA; (B) 0.04% pApA, 1.2% ppApA, 88% pppApA, less than 0.4% of any trimers; (C) 2.0% pppApA, 1.4% pApApA, 9.2% ppApApA, 85% pppApApA, less than 0.5% of any tetramers; (D) 2.1% pApApA, 2.9% ppApApA, 4.0% pppApApA, 0.52% pApApApA, 6.6% ppApApApA, 78% pppApApApA, 0.38% pApApApApA, 1.7% ppApApApApA, 3.6% pppApApApApA.

as expected on the basis of their increasing negative charge. Peak A contained mostly ppApA, peak B pppApA, peak C pppApApA, and peak D pppApApApA, respectively. Figure 3 shows that the 5′-triphosphorylated forms predominate in the product with little of the 5′-monophosphorylated forms

Table IV: Yields Obtained for 2′,5′-Oligonucleotide Syntheses

substrate	method: in solution[a]	method: solid support
adenosine 5′-triphosphate	16%[b,d]	8.7%
8-azaadenosine 5′-triphosphate	21%[c]	
2-chloroadenosine 5′-triphosphate	16%[c]	
sangivamycin 5′-triphosphate	12%	
toyocamycin 5′-triphosphate	10%	
formycin 5′-triphosphate	7.3%[c]	7.2%
3-ribosyladenine 5′-triphosphate	6.2%[c]	
ribavirin 5′-triphosphate	5.8%[c]	
tubercidin 5′-triphosphate	4.3%	
adenosine 1-oxide 5′-triphosphate	3.8%[c]	
2-β-D-ribofuranosylthiazole-4-carboxamide 5′-triphosphate	2.9%[c]	
inosine 5′-triphosphate	1.5%[c]	
1,N^6-ethenoadenosine 5′-triphosphate	1.5%[d]	
guanosine 5′-triphosphate	1.4%[d]	
8-bromoadenosine 5′-triphosphate	1.1%[d]	
uridine 5′-triphosphate	1.1%[c]	
cytidine 5′-triphosphate	0.95%[c]	
adenosine 5′-(β,γ-imidotriphosphate)	0%[c]	

[a] Reaction mixtures contained 1.0–1.9 mg/mL protein with a specific activity of 50–130 nmol/(mg·h) for the 2′,5′-oligoadenylate synthetase. [b] Quantitation of the yield by the paper electrophoresis, TLC, and HPLC systems described under Materials and Methods showed that the yield including all of the dimer product was actually 30%. The lower yield reported above (16%) is for the product eluted from the gradient that was subsequently lyophilized and does not include most of the dimer since it was eluted in the equilibration-buffer wash step. [c] 8-h reaction time. [d] A similar yield resulted for either a 4- or 8-h reaction time.

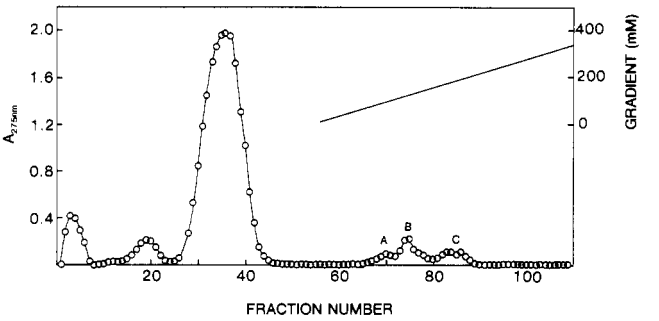

FIGURE 4: DEAE-cellulose chromatographic profile for enzymatic synthesis of 2′,5′-oligotoyocamycin (T) by the in-solution method (Derynck et al., 1980) for 4 h: (A) 0.43% pTpT, 38% ppTpT, 26% pppTpT, 7.5% pTpTpT, 10% ppTpTpT; (B) 12% ppTpT, 56% pppTpT, 6.8% pTpTpT, 13% ppTpTpT; (C) 10% ppTpT, 13% pppTpT, 3.1% pTpTpT, 3.5% ppTpTpT, 54% pppTpTpT, 1.7% pTpTpTpT, 1.7% ppTpTpTpT, 0.32% pppTpTpTpT.

being synthesized and that no detectable amounts of 2′,5′-A's without 5′-phosphate groups ("core" 2′,5′-A's) were obtained. The products were identified by the thin-layer chromatography (TLC) and paper electrophoresis systems described under Materials and Methods after the BAP treatment as well as by the HPLC methodology both with and without the BAP treatment with known 2′,5′-A standards. All of the methods gave similar ratios for the dimer, trimer, tetramer, and pentamer forms of the 2′,5′-A. The 2′,5′-A trimer from peak C was also tested from biological activity in inhibition of HeLa- and L-cell growth (Hovanessian & Wood, 1980), inhibition of [^{35}S]methionine incorporation into protein (Hovanessian & Wood, 1980), inhibition of translation in the rabbit reticulocyte lysate protein synthesizing system (Pelham & Jackson, 1976), degradation of rRNA (Wreschner et al., 1981a), and antiviral activity against VSV (Hovanessian & Wood, 1980) with similar results to those obtained in other labora-

tories. Mixed 2′,5′–3′,5′ linkages seemed unlikely since HPLC analyses appeared to be devoid of such peaks.

A typical DEAE-cellulose chromatographic profile resulting for the synthesis of a 2′,5′-oligonucleotide analogue of adenosine is shown in Figure 4. The yield obtained for the 2′,5′-oligotoyocamycin product was 10% (Table IV) and, hence, was lower than that obtained similarly for 2′,5′-A (16%). Figure 4 shows that mostly dimer is eluted first with the gradient (peaks A and B), followed by trimer mixed with a small amount of tetramer (peak C).

Table IV shows the relative yields that were obtained for the various 2′,5′-oligonucleotides. The yield obtained for the 2′,5′-A product was generally greater than that obtained for any of the other 2′,5′-oligonucleotides synthesized by the in-solution method. Only the 2-chloroadenosine and 8-azaadenosine 5′-triphosphates gave similar or slightly better yields than 5′-ATP. The in-solution method gave a better yield for the 2′,5′-A than the solid-support method, and since it also required only 4–8 h compared to 5 days for the solid-support method, it was generally used. The various yields obtained show that mouse L-cell enzyme, 2′,5′-oligoadenylate synthetase, will accept a number of different 5′-triphosphate substrates. Particularly interesting is the fact that four other naturally occurring nucleotides, inosine, guanosine, uridine, and cytidine 5′-triphosphate, all functioned as substrates for the enzyme in vitro. The yields obtained were lower for 5′-ITP (1.5%), 5′-GTP (1.4%), 5′-UTP (1.1%), and 5′-CTP (0.95%) than for 5′-ATP (16%), however. This raises the obvious question as to whether these nucleoside 5′-triphosphates are also converted to the 2′,5′-oligonucleotides in vivo. It also seems possible from these data that more than one 2′,5′-oligonucleotide synthetase may exist since homogeneous enzyme was not used in these syntheses and two peaks of enzyme activity after gel filtration have been reported for HeLa and SV80 cells (Lengyel, 1982). The possible natural occurrence of 2′,5′-oligo(inosinic acid), 2′,5′-oligo(guanylic acid), 2′,5′-oligo(uridylic acid), and 2′,5′-oligo(cytidylic acid) or their mixed heterooligomers and clarification as to the actual number of 2′,5′-oligonucleotide synthetases await further investigation.

The substrate-recognition data shown by the relative yields obtained for the various nucleoside 5′-triphosphates in Table IV implicate at least two sites in enzyme binding at the active site. There are four potential binding sites on the purine ring of adenosine, i.e., the 1-, 3-, and 7-nitrogens and the 6-amino group. The 7-nitrogen does not seem to be required for 2′,5′-oligoadenylate synthetase since it is absent in sangivamycin, toyocamycin, and tubercidin, and the yields for their 2′,5′ products are still quite high (12, 10, and 4.3%, respectively). Neither does the 3-nitrogen seem to be required since the presence of the ribose moiety in this position in the 3-ribosyladenine 5′-triphosphate substrate still gave a 6.2% yield and its absence in ribavirin resulted in a 5.8% yield. The 1-nitrogen does seem to be rather important in binding since the yield dropped to 3.8% for adenosine 1-oxide and bridging as in 1,N^6-ethenoadenosine resulted in a greatly lowered yield (1.5%). However, both ribavirin and 2-β-D-ribofuranosylthiazole-4-carboxamide, which do not have an 1-nitrogen, still gave quite good yields (5.8 and 2.9%, respectively). The 6-amino group seems to be quite important since substitution by a keto group in inosine or guanosine markedly lowered the yield to 1.5 and 1.4%, respectively, and bridging at this position in 1,N^6-ethenoadenosine also resulted in a substantially lower yield (1.5%). Ribavirin and the related 2-β-D-ribofuranosylthiazole-4-carboxamide seem to have the equivalent of a 6-amino group since ribavirin resembles adenosine in some of its structural features and is also known to function as a substrate for adenosine kinase (Streeter et al., 1974; Willis et al., 1978). Both of these nucleotides gave intermediate yields.

Table V: Position for Gradient Elution of 2′,5′-pppNpNpN's from DEAE-cellulose Chromatographic Columns

N	apparent molarity (mM)[a]	N	apparent molarity (mM)[a]
adenosine	200	adenosine 1-oxide	155
8-azaadenosine	145	2-β-D-ribofuranosyl-thiazole-4-carboxamide	162
2-chloroadenosine	170		
sangivamycin	358	inosine	211
toyocamycin	198	1,N^6-ethenoadenosine	270
formycin	216	guanosine	232
3-ribosyladenine	200	8-bromoadenosine	62
ribavirin	182	uridine	162
tubercidin	132	cytidine	152

[a] The apparent molarity was determined from a graph of the linear gradient (10–450 mM ammonium bicarbonate) vs. fraction number. Refer to Figures 3 and 4 for examples.

The anti conformation seems to be important in enzyme substrate recognition since 8-bromoadenosine, which assumes predominantly the syn conformation, gave only a 1.1% yield.

Electron-withdrawing groups or atoms, e.g., the ring 8-nitrogen in 8-azaadenosine, the 2-chloro in 2-chloroadenosine, the cyano group in toyocamycin, and the amide group in sangivamycin, all seemed to favorably increase yields for the 2′,5′-oligonucleotide products. These should make the ring nitrogens and 6-amino group less basic and may lead to more rapid product release from the enzyme and, hence, to the increased yields demonstrated. The lower yield for formycin may be explained by the extra hydrogen binding allowable at the 7-nitrogen, which may then lead to altered binding to the enzyme active site.

Substitution of the 5′-β,γ-imidotriphosphate group for the 5′-triphosphate in the substrate did not result in a detectable product as shown in Table IV. As little as 10 ng of 2′,5′-oligonucleotide product out of 100 mg of adenosine 5′-(β,γ-imidotriphosphate) in the reaction mixture could have been detected by the sensitive HPLC system.

The relative placement of the 5′-triphosphorylated 2′,5′-trimer analogues upon gradient elution from DEAE-cellulose columns is shown in Table V. The trimers were identified by HPLC analysis in combination with the DEAE-cellulose chromatography data. Chromatograms similar to those shown in Figures 3 and 4 resulted for the other 2′,5′-oligonucleotides synthesized (data not shown).

HPLC analysis of the 2′,5′-oligonucleotides was carried out with the same parabolic gradient used to determine the retention times and purity of the nucleoside 5′-triphosphates (Table III). Standardization of the HPLC columns with a series of adenosine 5′-phosphates (5′-AMP, 5′-ADP, 5′-ATP, and p_4A) is shown in Figure 5. The information obtained for the retention time for 5′-ATP (Figure 5) compared to the retention time for the other nucleoside 5′-triphosphates (Table II) was used to predict the positions for the 2′,5′-oligonucleotides relative to those known for the 2′,5′-A's. This information was used in combination with the successive gradient positions obtained for the 2′,5′-oligonucleotides on DEAE-cellulose column chromatography and allowed identification of the 2′,5′-oligonucleotides as to chain length and number of 5′-phosphates. Figure 6 shows the retention times obtained for the combination of 2′,5′-A peaks (A–D) obtained from gradient elution like that in Figure 3. Figure 6 shows

Table VI: HPLC Retention Times (min) for 2′,5′-Oligonucleotides[a]

N	adenosine[b]	8-aza-adenosine[c]	2-chloro-adenosine[d]	sangiva-mycin	toyocamycin	formycin	3-ribosyl-adenine	ribavirin	tubercidin
pNpN	25.73	22.90	36.32	30.80	31.70	29.38	23.29	17.91	31.89
ppNpN	28.43	25.90	39.82	32.98	33.88	31.58	25.98	20.18	32.99
pppNpN	31.40	28.86	43.29	34.51	35.41	33.78	29.02	22.91	33.96
pNpNpN	32.91	30.13	44.05	35.27	37.07	36.18	30.22	24.64	35.89
ppNpNpN	35.03	32.37	45.48	36.60	39.01	38.82	32.59	27.14	37.49
pppNpNpN	36.77	33.93	48.28	38.57	41.15	40.02	34.14	28.84	39.02
pNpNpNpN	38.50	36.09	49.28	40.10	42.86	41.89	36.09	30.03	41.12
ppNpNpNpN	40.20	37.69	51.22	41.47	44.36		37.76	32.23	42.99
pppNpNpNpN	41.23	38.42	54.22	43.14	45.29		38.86	33.03	43.99
pNpNpNpNpN	42.76	40.00	55.48		48.29		40.40		
ppNpNpNpNpN	44.46	41.47	57.42						
pppNpNpNpNpN	45.29	42.07	61.08						

N	adenosine 1-oxide	2-β-D-ribofuranosyl-thiazole-4-carboxamide	inosine	1,N^6-etheno-adenosine	guanosine	8-bromo-adenosine	uridine	cytidine
pNpN	19.36	23.42	20.33	29.62	23.98	33.71	20.18	18.28
ppNpN	21.99	25.52	23.28	31.59	25.11	35.08	23.52	22.34
pppNpN	24.99	28.75	25.72	33.85	27.68	36.28	26.42	25.04
pNpNpN	26.51	30.58	27.32	35.60	28.61	37.81	27.67	26.42
ppNpNpN	28.63	31.88	29.35	37.27	31.18	38.31	29.35	28.76
pppNpNpN	30.33	32.92	31.92	39.71	32.68	39.21	31.64	30.52
pNpNpNpN	32.10	34.85	33.15	41.14	35.28	39.98	32.88	32.26
ppNpNpNpN	33.81	36.98	35.21	42.85	36.51		34.41	33.28
pppNpNpNpN	34.82	38.98	36.56		37.51		35.42	34.79
pNpNpNpNpN		40.37			39.20		36.96	36.34
ppNpNpNpNpN		41.47			40.31		38.77	37.97
pppNpNpNpNpN		42.54			41.87		39.64	39.05

[a] HPLC conditions were the same as those shown for Figure 5. [b] Similar retention times resulted for 2′,5′-A's synthesized in our laboratory and for 2′,5′-A's obtained from Dr. Peter Lengyel's laboratory or P-L Biochemicals, Inc. [c] Retention times for $p(Np)_5N$, $pp(Np)_5N$, and $ppp(Np)_5N$ for N = 8-azaadenosine were 43.80, 45.53, and 46.40 min, respectively. [d] Retention times for $p(Np)_5N$, $pp(Np)_5N$, $ppp(Np)_5N$, $ppp(Np)_6N$, and $ppp(Np)_7N$ for N = 2-chloroadenosine were 63.19, 64.53, 69.69, 81.42, and 92.86 min, respectively.

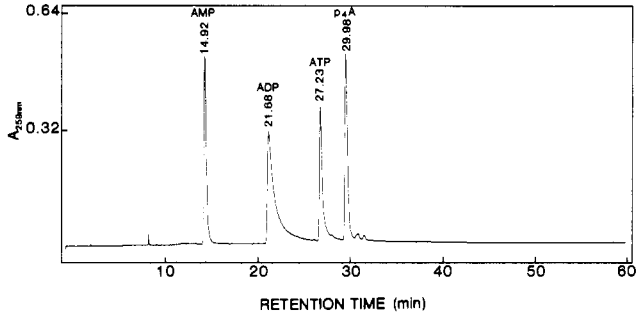

FIGURE 5: HPLC separation of 5′-AMP, 5′-ADP, 5′-ATP, and p_4A. Column: Ultrasphere C_{18} ODS with an attached RP-18 Lichrosorb precolumn. Gradient: as shown in Table II. Flow rate = 1.0 mL/min. Temperature: ambient.

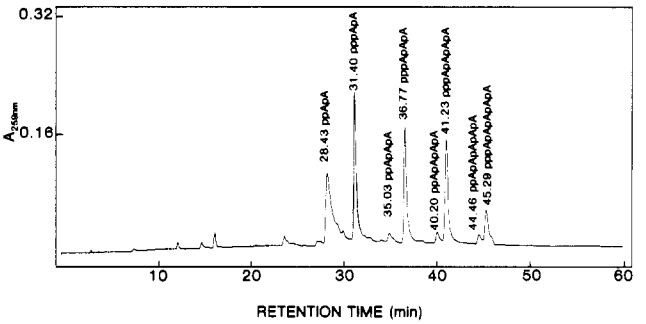

FIGURE 6: HPLC separation of 2′,5′-ppApA, 2′,5′-pppApA, 2′,5′-ppApApA, 2′,5′-pppApApA, 2′,5′-ppApApApA, 2′,5′-pppApApApA, 2′,5′-ppApApApApA, and 2′,5′-pppApApApApA. The conditions were the same as those shown in Figure 5.

that this HPLC system is very efficient for separating dimers, trimers, tetramers, and pentamers. It also allows for efficient separation of 5′-mono-, 5′-di-, and 5′-triphosphorylated forms as shown in Table VI. Retention times were confirmed by using 2′,5′-A's synthesized in our laboratory and also by 2′,5′-A's obtained from Dr. Peter Lengyel and P-L Biochemicals, Inc. The retention times for the various 2′,5′-oligonucleotides reported in Table VI were determined on two different C_{18} reversed-phase columns. The retention times were normalized to those obtained for the first column since each new column required standardization with 5′-ATP and 2′,5′-A's. Changing the precolumn had little effect on the retention times.

No nonphosphorylated (core) forms were detected in the resultant enzymatically synthesized 2′,5′-oligonucleotide products. Figure 7 shows the HPLC retention times that would have resulted for the 2′,5′-A series with known standards.

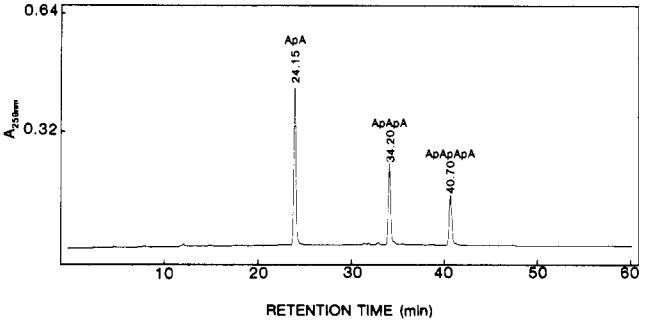

FIGURE 7: HPLC separation of 2′,5′-ApA, 2′,5′-ApApA, and 2′,5′-ApApApA. The conditions were the same as those shown for Figure 5.

Discussion

We have succeeded in synthesizing a number of new 2′,5′-oligonucleotide analogues with the mouse L-cell en-

zyme(s) 2′,5′-oligoadenylate synthetase(s). The oligonucleotides contain (1) closely related analogues of adenosine, (2) 2-β-D-ribofuranosylthiazole-4-carboxamide or ribavirin, or (3) the naturally occurring inosine, guanosine, uridine, or cytidine as the base substitutions. Particularly interesting is the fact that 5′-ITP, 5′-GTP, 5′-UTP, and 5′-CTP were all found to be converted into their respective 2′,5′-oligonucleotide products. Either the 2′,5′-oligoadenylate synthetase is less specific than previously thought, or since a homogeneous enzyme was not used in these studies, it is possible that more than one 2′,5′-oligonucleotide synthetase exists. Up until now, only 1,N^6-ethenoadenosine 5′-triphosphate (with HeLa-cell enzyme) (Minks et al., 1980) and 3′-deoxyadenosine 5′-triphosphate (with the rabbit reticulocyte enzyme) (Doetsch et al., 1981) had been found to function as substrates to form 2′,5′-oligonucleotides without either the addition of 5′-ATP, which forms a 2′,5′-A primer, or the addition of the 2′,5′-A primer itself (Ball & White, 1979b; Justesen et al., 1980; Ferbus et al., 1981). 5′-ITP was not recognized as a substrate by the reticulocyte cell free 2′,5′-oligoadenylate synthetase (Suhadolnik et al., 1982). The use of the sensitive HPLC system described in this paper has now made possible the detection of even low yields for some of these newly synthesized 2′,5′-oligonucleotides.

Using a number of nucleoside 5′-triphosphates in addition to 5′-ATP as substrates for the 2′,5′-oligoadenylate synthetase(s) gave the following order of efficiency for formation of the 2′,5′-oligonucleotide products: 8-azaadenosine > adenosine = 2-chloroadenosine > sangivamycin > toyocamycin > formycin > 3-ribosyladenine > ribavirin > tubercidin > adenosine 1-oxide > 2-β-D-ribofuranosylthiazole-4-carboxamide > inosine = 1,N^6-ethenoadenosine > guanosine > 8-bromoadenosine = uridine > cytidine. On the basis of the substrate specificity requirements shown, it seems apparent that (1) the 3- and 7-nitrogens of the purine ring are not essential to enzyme binding and recognition at the active site, (2) the 1-nitrogen probably plays a minor role in enzyme substrate recognition, (3) the 6-amino group is quite important although some product still resulted when it was replaced with a 6-keto group, (4) the anti conformation for the nucleoside 5′-triphosphate is preferred, (5) electron-withdrawing groups such as chloro, cyano, and amido led to increased product yields, and (6) the 5′-triphosphate form is required since no detectable product resulted with adenosine 5′-(β,γ-imidotriphosphate) as a substrate. A more rigorous description of substrate specificity requirements awaits further definition of the kinetics of the reactions for the various 5′-NTP's used.

A number of primers for 2′,5′-oligoadenylate synthetase have been found that will function as acceptors in the formation of 2′-linked products. Ball & White (1979b) have shown that the enzyme from interferon-treated chicken cells will use short-chain 2′,5′-oligoadenylates, diribonucleoside monophosphates (linked either 2′–5′ or 3′–5′) containing a 3′-adenosine residue, nicotinamide adenine dinucleotide (NAD), ADP-ribose, or diadenosine tetraphosphate (AppppA) as acceptors for 2′-adenylation. Justesen et al. (1980) have shown that nucleosides (C, G, U, T, dC, dG, dA, dT) other than adenosine can be incorporated into a primer, resulting in $ppp(A_{2'}p_{5'})_nA_{2'}p_{5'}N$, but that such incorporation resulted in chain termination for the rabbit reticulocyte enzyme. Ferbus et al. (1981) have reported that NAD acts as an acceptor for A, U, C, G, or dA by the enzyme isolated from mouse L cells, rabbit reticulocytes, or human spleen cells and also that tRNA functions as a primer for 2′-adenylation. On the basis of the information known for functional primers as well as the variety of nucleoside 5′-triphosphates now shown to form 2′,5′-oligonucleotides reported in this paper, it is evident that it will be possible to prepare a number of new 2′,5′-linked products with the 2′,5′-oligoadenylate synthetases.

The newly synthesized 2′,5′-oligonucleotides reported here were efficiently separated according to chain length by DEAE-cellulose column chromatography. An HPLC ion-pairing C_{18} reversed-phase system was used to further characterize the 2′,5′-oligonucleotides since it gave fast, efficient separation of 2′,5′-oligonucleotides as well as the 5′-tri-, 5′-di-, and 5′-monophosphorylated forms. Triphosphorylated forms predominated with small amounts of diphosphorylated forms and little of the monophosphorylated derivatives. No non-phosphorylated forms were detected for any of the oligonucleotides.

These 2′,5′-linked oligonucleotides represent a new series of potential antiviral and antitumor agents. Some of their biological activities are described in the succeeding paper (Hughes & Robins, 1983). These synthetic 2′,5′-oligonucleotides may be able to exhibit directly certain selective desirable properties of interferon without some of the known side effects.

Acknowledgment

We are grateful for the excellent technical assistance of M. Joleen Glassett.

Registry No. 2′,5′-Oligoadenylate synthetase, 69106-44-1; toyocamycin, 606-58-6; toyocamycin 5′-monophosphate, 15742-78-6; toyocamycin 5′-triphosphate, 15676-18-3; tubercidin, 69-33-0; tubercidin 5′-monophosphate, 16719-46-3; tubercidin 5′-triphosphate, 10058-66-9; formycin, 6742-12-7; formycin 5′-monophosphate, 13270-66-1; formycin 5′-triphosphate, 16409-13-5; 2-chloroadenosine, 146-77-0; 2-chloroadenosine 5′-monophosphate, 21466-01-3; 2-chloroadenosine 5′-diphosphate, 16506-88-0; 2-chloroadenosine 5′-triphosphate, 49564-60-5; 8-azaadenosine, 10299-44-2; 8-azaadenosine 5′-monophosphate, 55612-44-7; 8-azaadenosine 5′-diphosphate, 84894-76-8; 8-azaadenosine 5′-triphosphate, 59652-91-4; 3-ribosyladenine, 2273-78-1; 3-ribosyladenine 5′-monophosphate, 2862-19-3; 3-ribosyladenine 5′-triphosphate, 2273-79-2; 2-β-D-ribofuranosylthiazole-4-carboxamide, 60084-10-8; 2-β-D-ribofuranosylthiazole-4-carboxamide 5′-monophosphate, 83161-83-5; 2-β-D-ribofuranosylthiazole-4-carboxamide 5′-diphosphate, 84894-77-9; 2-β-D-ribofuranosylthiazole-4-carboxamide 5′-triphosphate, 84894-78-0; sangivamycin, 18417-89-5; sangivamycin 5′-monophosphate, 17210-78-5; sangivamycin 5′-triphosphate, 17210-68-3; adenosine 5′-triphosphate, 56-65-5; ribavirin 5′-triphosphate, 63142-71-2; adenosine 1-oxide 5′-triphosphate, 17670-17-6; inosine 5′-triphosphate, 132-06-9; 1,N^6-ethenoadenosine 5′-triphosphate, 37482-17-0; guanosine 5′-triphosphate, 86-01-1; 8-bromoadenosine 5′-triphosphate, 23567-97-7; uridine 5′-triphosphate, 63-39-8; cytidine 5′-triphosphate, 65-47-4; adenosine 5′-(β,γ-iminotriphosphate), 25612-73-1; pNpN (N = adenosine), 20307-28-2; ppNpN (N = adenosine), 81410-37-9; pppNpN (N = adenosine), 65954-94-1; pNpNpN (N = adenosine), 61172-40-5; ppNpNpN (N = adenosine), 76991-64-5; pppNpNpN (N = adenosine), 65954-93-0; pNpNpNpN (N = adenosine), 66048-58-6; ppNpNpNpN (N = adenosine), 81410-35-7; pppNpNpNpN (N = adenosine), 65954-95-2; pNpNpNpNpN (N = adenosine), 66048-59-7; ppNpNpNpNpN (N = adenosine), 81410-36-8; pppNpNpNpNpN (N = adenosine), 65954-96-3; pNpN (N = 8-azaadenosine), 84894-79-1; ppNpN (N = 8-azaadenosine), 84894-80-4; pppNpN (N = 8-azaadenosine), 84894-81-5; pNpNpN (N = 8-azaadenosine), 84894-82-6; ppNpNpN (N = 8-azaadenosine), 84811-33-6; pppNpNpN (N = 8-azaadenosine), 84811-32-5; pNpNpNpN (N = 8-azaadenosine), 84894-83-7; ppNpNpNpN (N = 8-azaadenosine), 84894-84-8; pppNpNpNpN (N = 8-azaadenosine), 84894-85-9; pNpNpNpNpN (N = 8-azaadenosine), 84894-86-0; ppNpNpNpNpN (N = 8-azaadenosine), 84894-87-1; pppNpNpNpNpN (N = 8-azaadenosine), 84894-88-2; pNpN (N = 2-chloroadenosine), 84894-89-3; ppNpN (N = 2-chloroadenosine), 84894-90-6; pppNpN (N = 2-chloroadenosine), 84894-91-7; pNpNpN (N = 2-chloroadenosine), 84894-92-8; ppNpNpN (N = 2-chloroadenosine), 84811-51-8; pppNpNpN (N = 2-chloroadenosine),

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