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Phosphorothioate Analogues of 2′,5′-Oligoadenylate. Enzymatically Synthesized 2′,5′-Phosphorothioate Dimer and Trimer: Unequivocal Structural Assignment and Activation of 2′,5′-Oligoadenylate-Dependent Endoribonuclease†

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ABSTRACT: In continued studies to elucidate the requirements for binding to and activation of the 2′,5′-oligoadenylate-dependent endoribonuclease (RNase L), chirality has been introduced into the 2′,5′-oligoadenylate (2-5A, p_3A_n) molecule to give the R_P configuration in the 2′,5′-internucleotide backbone and the S_P configuration in the α-phosphorus of the pyrophosphoryl moiety of the 5′-terminus. This was accomplished by the enzymatic conversion of (S_P)-ATPαS to the 2′,5′-phosphorothioate dimer and trimer by the 2-5A synthetase from lysed rabbit reticulocytes. The most striking finding reported here is the ability of the 2′,5′-phosphorothioate *dimer* 5′-triphosphate (i.e., $p_3A_2\alpha S$) to *bind to and activate* RNase L. $p_3A_2\alpha S$ displaces the $p_3A_4[^{32}P]pCp$ probe from RNase L with an IC_{50} of 5×10^{-7} M, compared to an IC_{50} of 5×10^{-9} M for authentic p_3A_3. Further, $p_3A_2\alpha S$ activates RNase L to hydrolyze poly(U)-3′-$[^{32}P]pCp$ (20% at 2×10^{-7} M), whereas authentic p_3A_2 is unable to activate the enzyme. Similarly, the enzymatically synthesized $p_3A_2\alpha S$ at 10^{-6} M activated RNase L to degrade 18S and 28S rRNA, whereas authentic p_3A_2 was devoid of activity. $p_3A_3\alpha S$ was as active as authentic p_3A_3 in the core–cellulose and rRNA cleavage assays. The absolute structural and configurational assignment of the enzymatically synthesized $p_3A_2\alpha S$ and $p_3A_3\alpha S$ was accomplished by high-performance liquid chromatography, charge separation, enzymatic hydrolyses, and comparison to fully characterized chemically synthesized (R_P)- and (S_P)-2′,5′-phosphorothioate dimer and trimer cores. The absolute structural assignment for $p_3A_2\alpha S$ is 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)adenosine, and for $p_3A_3\alpha S$ it is 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)-(R_P)-*P*-thioadenylyl(2′–5′)adenosine. These assignments confirm the previous suggestion of an R_P configuration at the 2′,5′-internucleotide linkages of enzymatically synthesized $p_3A_3\alpha S$ [Lee, C., & Suhadolnik, R. J. (1985) *Biochemistry 24*, 551–555].

The 2′,5′-oligoadenylate [2′,5′-ppp(Ap)$_n$A; 2-5A][1] system is widely accepted to be involved in the antiviral mechanism of interferon [consult Johnston and Torrence (1984) and Lengyel (1982) for recent reviews] and may also be involved in the regulation of cell growth and differentiation (Etienne-Smekens et al., 1983; Ferbus et al., 1984; Jacobsen et al., 1983; Bayard et al., 1986; Wells & Mallucci, 1985; Zullo et al., 1985). 2-5A synthesized from ATP by 2′,5′-oligoadenylate synthetase [ATP:(2′–5′)oligo(A) adenylyltransferase (EC 2.7.7.19)] exerts its biological effects by binding to and activating its only known target enzyme, the unique 2-5A-dependent endoribonuclease (RNase L, EC 3.1.27), which cleaves viral and cellular mRNA or rRNA, thereby inhibiting protein synthesis (Hovanessian & Kerr, 1979; Kerr & Brown, 1978). 2-5A, however, is metabolically unstable and is degraded by a cellular 2′-phosphodiesterase (2′-PDE) and phosphatases (Knight et al., 1981; Minks et al., 1979; Williams et al., 1978).

The literature is replete with structurally modified 2-5A molecules with modifications in the adenyl or ribosyl moiety designed to explore the biological role of the 2-5A synthetase/RNase L system. Our strategy for the examination of the requirements for binding to and activation of RNase L has involved backbone modification of the 2-5A molecule,

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employing phosphorothioate substitution in the 2′,5′-internucleotide linkages and a pyrophosphoryl moiety at the 5′-terminus of 2-5A (Lee & Suhadolnik, 1985; Suhadolnik & Lee, 1985; Karikó et al., 1987). Our studies focused on the internucleotide linkages because they are recognized as the critical determinant in the interaction between the 2-5A molecule and RNase L. The primary source of conformational flexibility in the 2-5A molecule is in the backbone, similar to 3′,5′-linked RNA and DNA (Srinivasan & Olson, 1986). However, theoretical and experimental analyses have revealed that the conformation of 2′,5′-linked dinucleotides and polynucleotide chains are significantly different than that of 3′,5′-linked nucleotides (Anukanth & Ponnuswamy, 1986; Dhingra & Sarma, 1978; Parthasarathy et al., 1982; Srinivasan & Olson, 1986). The ribose phosphate backbone of 2-5A has also been demonstrated to be the major antigenic determinant in the molecule (Johnston et al., 1983).

Few reports have appeared on the synthesis of 2-5A analogues with backbone modifications. Core analogues containing methylphosphonate and methylphosphotriester groups have been synthesized (Eppstein et al., 1982; Jager & Engels, 1981). However, with the "uncharged" methylphosphotriester analogues, complete loss of activity was observed (Eppstein et al., 1982). Substitution of the 2′,5′-phosphodiester linkages with 3′,5′-linkages also led to substantial decrease in biological activity (Lesiak et al., 1983); replacement of only one 2′,5′-internucleotide linkage resulted in at least 1 order of magnitude loss of activity, and nearly complete loss of biological activity was observed when both 2′,5′-phosphodiester linkages in 2-5A trimer were replaced with 3′,5′ bonds. One report has appeared on the chemical synthesis of the phosphorothioate dimer and the diastereomeric pairs of the trimer core analogues of 2-5A (Nelson et al., 1984). Eppstein et al. (1986) have reported on the metabolic stabilities and antiviral activities of the R_PR_P/S_PR_P and R_PS_P/S_PS_P racemic mixtures.

In addition to their wide use in the stereochemical analysis of enzyme reaction mechanisms [for reviews, see Eckstein (1985), Eckstein et al. (1982), and Frey et al. (1982)], phosphorothioate analogues of nucleotides and nucleic acid polymers have proven to be extremely valuable probes in studies of DNA conformation, DNA/protein interactions, and antiviral agents. For example, Zon and co-workers have shown that substitution of one of the diastereotopic oxygens with sulfur in the 3′,5′-internucleotide linkages in DNA results in two phosphorothioate-containing diastereomers which possess different structural and electronic features as well as altered physicochemical properties. Furthermore, recent molecular models and computer simulations reveal that the P–S bond axis for the R_P configuration is oriented into the major groove, while in the S_P configuration this bond axis points away from the helix (Suggs & Taylor, 1985; Cosstick & Eckstein, 1985; Zon, personal communication).

In an earlier account of our investigations, the enzymatic synthesis, preliminary characterization, and biological activities of the phosphorothioate analogues of 2-5A synthesized from (S_P)-ATPαS [but not (R_P)-ATPαS] by the 2-5A synthetase from L929 cell extracts were described (Lee & Suhadolnik, 1985). On the basis of enzyme degradations and known preference of snake venom phosphodiesterase (SVPD) for the R_P configuration (Burgers & Eckstein, 1978; Bryant & Benkovic, 1979; Nelson et al., 1984), it was suggested that the 2-5A synthetase proceeds by inversion of configuration of (S_P)-ATPαS to yield the R_P configuration at the 2′,5′-internucleotide linkages. However, absolute proof of configuration of these phosphorothioate analogues of 2-5A has awaited the availability of chemically synthesized phosphorothioate diastereomers because only one of the four possible trimer diasteromers can be prepared enzymatically. As the result of rigorous chemical synthesis and characterization, we describe here the unequivocal assignment of the structure and configuration of the phosphorothioate analogues of 2-5A synthesized from (S_P)-ATPαS.

In this paper and the two following papers, we introduce a new concept to the study of the binding process and the activation process of RNase L by specific stereochemical modification of the backbone of the 2-5A molecule via the chiral/achiral 2′,5′-phosphorothioates (this report; Karikó et al., 1987; Suhadolnik et al., 1987). This new class of chemically and enzymatically synthesized biological modifier molecules has contributed dramatic new information to the analysis of the 2-5A pathway that can be used to assess the role of the 2-5A synthetase/RNase L system in virus infection, antineoplastic chemotherapy, cell growth, and cell differentiation. A preliminary report of these studies was presented as a plenary lecture (Karikó et al., 1986).

Experimental Procedures

Materials. (R_P)-ATPαS and (S_P)-ATPαS were generous gifts from Dr. P. Frey. 2-5A core–cellulose was a generous gift from Dr. R. Silverman. All other chemicals, enzymes, and radioisotopes were as previously described from this laboratory.

Enzymatic Synthesis of 2-5A and 2′,5′-Phosphorothioates. The synthesis of 2′,5′-oligonucleotides was accomplished with the 2-5A synthetase from rabbit reticulocyte lysates (Green Hectares, Oregon, WI) bound to poly(rI)·poly(rC)–agarose essentially as described (Doetsch et al., 1981; Suhadolnik et al., 1983; Lee & Suhadolnik, 1985). The syntheses were at 30 °C for 3–17 h with optimum yields at 17 h. The synthetic products were analyzed by DEAE-cellulose column chromatography and high-performance liquid chromatography (HPLC) (solvent system 3, see below) (Doetsch et al., 1981). Peak fractions in the 350 mM KCl buffer wash (as determined by radioactive content and ultraviolet measurement) were pooled, dialyzed, and lyophilized. The concentration of the 2′,5′-oligonucleotides was calculated on the basis of known specific activities of [α-^{32}P]ATP and (S_P)-[α-^{35}S]ATPαS. The 5′-monophosphorothioate of the enzymatically synthesized

[1] Abbreviations: AMPS, adenosine 5′-*O*-phosphorothioate; 2-5A, 2′,5′-oligoadenylates (p_3A_n); p_3A_2, p_3A_3, and p_3A_4, dimer, trimer, and tetramer, respectively, of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-triphosphate; pA_3, trimer of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-monophosphate; A_2, A_3, and A_4, 5′-dephosphorylated p_3A_2, p_3A_3, and p_3A_4; (R_P)- and (S_P)-ATPαS, R_P and S_P diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); 2′,5′-phosphorothioate dimer ($p_3A_2\alpha S$), 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)adenosine; 2′,5′-phosphorothioate trimer ($p_3A_3\alpha S$), 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)-(R_P)-*P*-thioadenylyl(2′–5′)adenosine; $pA_3\alpha S$, 5′-*O*-[1-*P*-thiomonophosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)-(R_P)-*P*-thioadenylyl(2′–5′)adenosine; R_P and S_P dimer core, phosphorothioate analogues of A_2 with R_P and S_P stereoconfigurations in the internucleotide linkages; R_PR_P, S_PR_P, R_PS_P, and S_PS_P trimer cores, phosphorothioate analogues of A_3 with R_P and S_P stereoconfigurations in the two chiral centers with assignment of configuration from the 5′-terminus to the 2′-terminus; BAP, bacterial alkaline phosphatase; DEAE, diethylaminoethyl; 2′-PDE, 2′-phosphodiesterase; PEI, poly(ethylenimine); RNase L, 2-5A-dependent endoribonuclease; SCP, specific cleavage products; SVPD, snake venom phosphodiesterase; T_2 RNase, ribonuclease T_2; IFN, interferon; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

2′,5′-phosphorothioate trimer (i.e., $pA_3\alpha S$) was prepared by hydrolysis with bacterial alkaline phosphatase (90 min, 37 °C).

Chemical Synthesis of 2′,5′-Phosphorothioate Cores and Their 5′-Monophosphates. 2′,5′-Phosphorothioate dimer cores (R_P, S_P), trimer cores (R_PR_P, S_PR_P, R_PS_P, S_PS_P), and trimer 5′-monophosphates (pR_PR_P, pS_PR_P, pR_PS_P, pS_PS_P) were chemically synthesized by the phosphotriester approach and separated by preparative thin-layer chromatography on silica gel (Pfleiderer & Charubala, 1987; unpublished results). ^1H NMR analyses of the blocked dimer and trimer cores have been completed, and the assignment for each proton is in agreement with the structures proposed (Pfleiderer & Charubala, 1987).

Cell Culture. HeLa and L929 cells were maintained in monolayer culture in Dulbecco's modified Eagle medium supplemented with 5% bovine serum (Gibco). Extracts of cells were prepared as described by Karikó and Ludwig (1985) from semiconfluent monolayer cultures with or without treatment with mouse fibroblast interferon (200 units/mL, 20 h) and were used as the source of RNase L for all biological assays.

Structural Elucidation of the 2′,5′-Phosphorothioates. The structural elucidation and stabilities of the enzymatically and chemically synthesized 2′,5′-phosphorothioates were determined by enzymatic hydrolyses with ribonuclease T_2 (T_2 RNase), snake venom phosphodiesterase (SVPD), and bacterial alkaline phosphatase (BAP) as described previously from this laboratory (Doetsch et al., 1981; Suhadolnik et al., 1983; Lee & Suhadolnik, 1985). Stability of the 2′,5′-phosphorothioates to 2′-phosphodiesterase (2′-PDE) in IFN-treated L929 cell extracts was as described (Lee & Suhadolnik, 1985). Stability to hydrolysis by serum phosphodiesterases was determined as previously described (Eppstein et al., 1986). Enzymatic hydrolyses with nuclease S1 and P1 (Sigma) were as described by Eckstein and co-workers (Potter et al., 1983a,b). Hydrolysis products were initially identified by direct comparison of R_f values with those of authentic samples of oligonucleotides, mononucleotides, and nucleosides on PEI–cellulose (Brinkman), cellulose (Eastman Chromagram, 13254), or DEAE-cellulose (Brinkman, CEL 300 DEAE) thin-layer chromatography plates developed with three different solvent systems: 0.75 M KH_2PO_4, pH 3.5, for PEI, 1-propanol/NH_4OH/H_2O (60:10:30 v/v/v) for cellulose, and 0.1 M ammonium formate/9 M urea/1 mM Na_2-EDTA after a short prerun in water for DEAE-cellulose. Charge determination of the enzymatically synthesized 2′,5′-phosphorothioates was accomplished as described (Suhadolnik et al., 1983).

High-Performance Liquid Chromatography. HPLC was carried out with two Waters Associates Model 6000 pumps controlled by a Model 660 solvent programmer. Reverse-phase chromatography was performed with a Waters C_{18} μBondapak analytical column (3.9 mm × 30 cm) (flow rate 1 mL/min). The column was eluted with one of the following solvent systems, with solvent A = 50 mM ammonium phosphate, pH 7.0, and solvent B = methanol/H_2O (1:1 v/v): system 1, isocratic gradient (t = 0 min, 30% B; t = 20 min, 30% B) followed by a linear gradient (t = 20 min, 30% B; t = 50 min, 40% B); system 2, linear gradient (beginning at 1 min) (t = 1 min, 10% B; t = 31 min, 40% B) followed by maintenance at 40% B for 20 min; system 3, linear gradient (t = 1 min, 10% B; t = 31 min, 20% B) followed by maintenance at 20% B for 10 min.

^{31}P NMR Spectroscopy. Samples for NMR spectroscopy (2 mM) were dissolved in 0.5 mL of ^2H_2O. The probe temperature was 20–21 °C in 5-mm tubes; a Bruker WH-300 spectrometer operating at 121.09637 MHz (for coupled spectra) and a Nicolet NTC 200 FT NMR spectrometer operating at 80.99055 MHz (for decoupled spectra) were used. Spectra were referenced to 85% H_3PO_4 in a tube that contained ^2H_2O coaxially. The decoupled spectra were obtained courtesy of Dr. P. Frey. The coupled spectra were performed by the Shared Magnetic Resonance Facility, Temple University School of Medicine.

Radiobinding Assays. Radiobinding assays were performed according to Knight et al. (1981) with $p_3A_4[^{32}P]pCp$ of specific activity 3000 Ci/mmol (Amersham) and L929 cell extracts as the source of RNase L.

Core–Cellulose Assays. The core–cellulose assay for RNase L activation was performed as previously described by Silverman (1985). Briefly, this technique consists of immobilizing and partially purifying RNase L from L929 cell extracts by using 2-5A_4 core–cellulose. Activation of RNase L is measured by the conversion of poly(U)-3′-[^{32}P]pCp to acid-soluble fragments.

Ribosomal RNA Cleavage Assays. rRNA cleavage assays were performed according to the procedure described by Wreschner et al. (1981). Extracts of L929 cells were prepared as described (Karikó & Ludwig, 1985) and incubated for 1 h at 30 °C in the presence or absence of 2-5A or 2′,5′-phosphorothioates. The total RNA was extracted, denatured, and analyzed by electrophoresis on 1.8% agarose gels. The gels were stained with ethidium bromide and the RNA bands visualized under ultraviolet light.

Radioactive measurement was determined on a Beckman LS-100C liquid scintillation spectrometer with counting efficiencies of 99%, 90%, and 50% for ^{32}P, ^{35}S, and ^3H, respectively.

Results

Characterization of the Chemically Synthesized (R_P)- and (S_P)-2′,5′-Phosphorothioate Dimer and Trimer Cores. The structures of the four chemically synthesized 2′,5′-phosphorothioate trimer cores are shown in Figure 1. The 2′,5′-phosphorothioate dimer cores (R_P and S_P) and trimer cores (with R_PR_P, S_PR_P, R_PS_P, and S_PS_P internucleotide configurations) were characterized by ^{31}P NMR, UV, fast atom bombardment (FAB) mass spectrometry, and enzymatic digestions. HPLC was used to separate the 2′,5′-phosphorothioate dimer and trimer cores and the enzyme digestion products. Assignment of the absolute configurations was made according to the following literature observations. SVPD preferentially cleaves (R_P)-3′,5′- or 2′,5′-phosphorothioate linkages from the 2′/3′-terminus (Burgers & Eckstein, 1978; Bryant & Benkovic, 1979; Nelson et al., 1984; Eppstein et al., 1986; Lee & Suhadolnik, 1985). SVPD hydrolysis of the chemically synthesized R_P dimer core yielded adenosine and AMPS in a molar ratio of 1:1, respectively; the half-life was 3 h (Table I). The S_P dimer core was not a substrate for SVPD under these conditions. Trimer core 1 (see Figure 1 and Table I) has the R_PR_P configuration as determined by 30-min hydrolysis by SVPD to yield AMPS plus R_P dimer core in a molar ratio of 1:1, respectively (Figure 2); further hydrolysis for 24 h resulted in complete hydrolysis. Similarly, SVPD hydrolysis of trimer core 2 yielded S_P dimer core and AMPS, thus identifying trimer core 2 as having the S_PR_P configuration (Table I). Trimer cores 3 and 4 were not substrates for SVPD (Table I), revealing the presence of the S_P configuration in the internucleotide linkage adjacent to the 2′/3′-termini.

The four 2′,5′-phosphorothioate trimer cores were further characterized by hydrolysis with 2′-PDE, an exoribonuclease

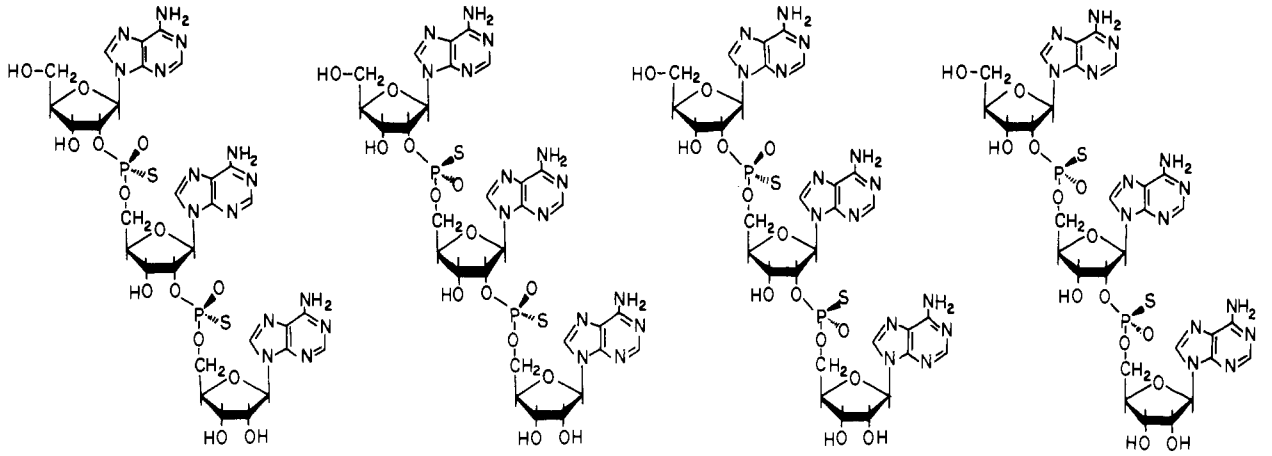

FIGURE 1: Structures of the four diastereomers of the 2′,5′-phosphorothioate trimer cores.

Table I: Analytical Data on Dimer and Trimer 2′,5′-Phosphorothioate Cores

			hydrolysis by										
			SVPD		L cell extract		serum phosphodiesterases		S1 nuclease		P1 nuclease		stereoconfiguration assigned
2′,5′-phosphorothioate	^{31}P NMR (ppm)[a]	R_T (min)[d]	dimer core isolated	half-life	dimer core isolated	half-life	dimer core isolated	half-life	dimer core isolated	half-life	dimer core isolated	half-life	
dimer cores													
1	57.63	19.5		3 h	not cleaved		not cleaved		not cleaved		not cleaved		R_P
2	56.13	24.2	not cleaved		not cleaved		not cleaved		not cleaved		not cleaved		S_P
trimer cores													
1	57.45, 57.71	30.5	R_P[e]	1 h	R_P	18 h	R_P	8 h	not cleaved		not cleaved		R_PR_P[b]
2	57.55, 56.62	33.0	S_P	8 h	S_P	15 h	not cleaved		not cleaved		not cleaved		S_PR_P[c]
3	56.34, 57.54	35.2	not cleaved		R_P	20 days	not cleaved		not cleaved		not cleaved		R_PS_P[c]
4	56.50, 56.26	39.5	not cleaved		not cleaved		not cleaved		not cleaved		not cleaved		S_PS_P[b]
A_3 core		14.5		5 min		10 min		10 min		24 h		24 h	
A_2 core			ND[f]			10 min	ND		ND		ND		

[a] Decoupled spectra. [b] Assignment confirmed by coupled and decoupled ^{31}P NMR. [c] Assignment confirmed by coupled ^{31}P NMR and enzymatic hydrolyses. [d] HPLC retention times in solvent system 2. [e] After 1-h incubation; after 24-h incubation, no dimer can be isolated. [f] ND, not determined.

which cleaves from the 2′/3′-terminus (Schmidt et al., 1978). Whereas authentic A_2 and A_3 cores were hydrolyzed to adenosine and AMP with a half-life of 10 min, the R_P and S_P dimer cores were *not* substrates for 2′-PDE (Table I). Eppstein et al. (1986) reported similar findings. The fact that the 2′,5′-phosphorothioate dimer cores were not substrates for 2′-PDE (unlike authentic 2-5A) greatly assisted in the assignment of the stereoconfigurations of the 2′,5′-phosphorothioate trimer cores. Trimer core 1 was a substrate for 2′-PDE; the products of hydrolysis were R_P dimer core and AMPS. Trimer core 2 was a substrate for 2′-PDE, yielding S_P dimer core and AMPS; trimer core 3 was a substrate, yielding R_P dimer core and AMPS; trimer core 4 was not a substrate for 2′-PDE. Previous studies have demonstrated that the (S_P)-3′,5′-phosphorothioate bond is a substrate for nuclease S1 and P1 (Potter et al., 1983a,b). However, neither the (R_P)- nor the (S_P)-2′,5′-phosphorothioate was a substrate (Table I).

^{31}P NMR spectroscopy has revealed that S_P diastereomers of phosphorothioates resonate to higher field than R_P diastereomers. Further, the S_P diastereomers have a longer retention time on reverse-phase HPLC than R_P diastereomers (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982; Cosstick & Eckstein, 1985). The ^{31}P NMR decoupled spectra of the chemically synthesized 2′,5′-phosphorothioate dimer and trimer cores are shown in Figure 3. The S_P dimer core resonates upfield from the R_P dimer core (Figure 3A,B; Table I); similarly, the two singlets observed for the S_PS_P trimer core resonate upfield from the two singlets for the R_PR_P trimer core (Figure 3C,D; Table I). Assignment of the absolute configurations of trimer cores 1 (R_PR_P) and 4 (S_PS_P) was based on the two singlets which resonate at the same frequency as the singlets observed for the R_P and S_P dimer cores. Assignment of configurations for trimer cores 2 and 3 was made in combination with the enzyme degradations and HPLC analyses (Table I). The ^{31}P NMR spectra revealed that the δ between the two singlets for the S_PR_P trimer core is 1.2 ppm, whereas the two singlets for the R_PS_P trimer core have a δ of 0.8 ppm (assignment is 5′- to 2′/3′-terminus) (Figure 3E,F). The configurations assigned to the chemically synthesized 2′,5′-phosphorothioate dimer and trimer cores are shown in Figure 1.

The fast atom bombardment (FAB) mass spectrum [m/e 978 (MNa^+)] for the S_PS_P trimer core was completed to verify the molecular weight of the core and is in agreement with the empirical formula assigned. The separation of the chemically synthesized (R_P)- and (S_P)-2′,5′-phosphorothioate dimer and trimer cores was accomplished by reverse-phase HPLC in solvent system 1 (Figure 4B,C). Thus, the combination of

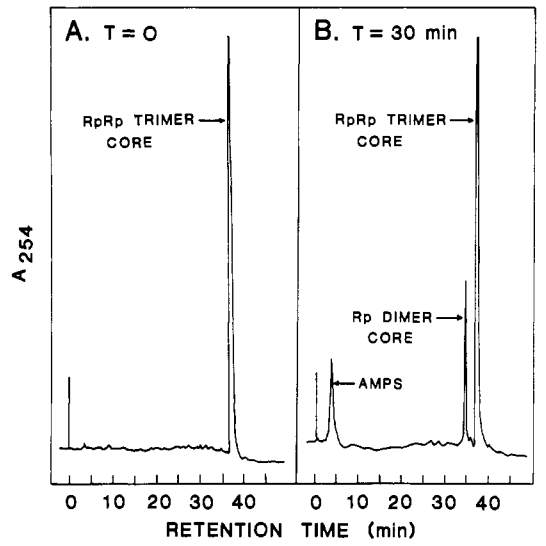

FIGURE 2: HPLC analysis of the SVPD digest of R_PR_P trimer core. SVPD hydrolysis was for 30 min followed by HPLC analysis in solvent system 2. R_P dimer core and AMPS were detected in the enzyme digest in a molar ratio of 1:1.

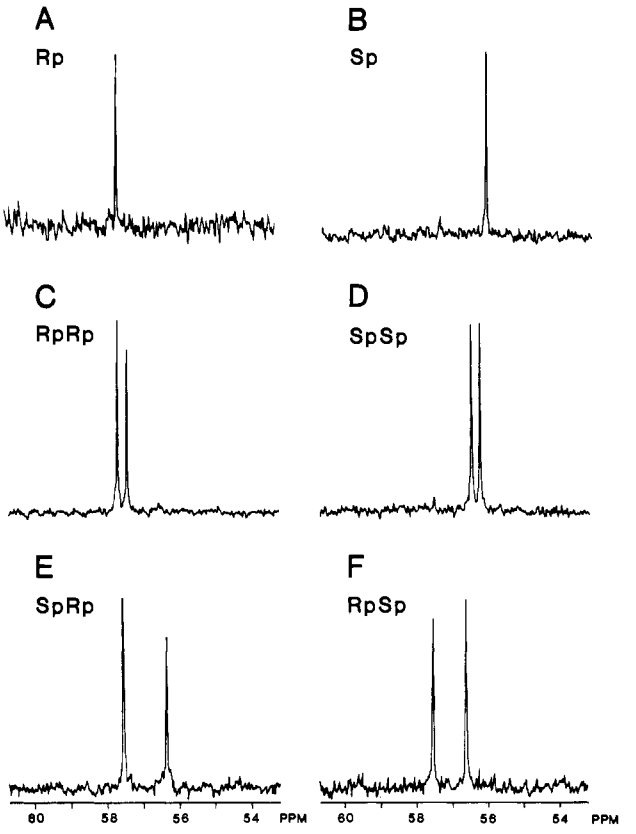

FIGURE 3: Decoupled ^{31}P NMR spectra of the 2',5'-phosphorothioate dimer and trimer cores: (A) R_P dimer core, (B) S_P dimer core, (C) R_PR_P trimer core, (D) S_PS_P trimer core, (E) S_PR_P trimer core, and (F) R_PS_P trimer core. Samples contained 1 μmol dissolved in 500 μL of 50% ^2H_2O, 100 mM Tris-HCl, pH 8.5, 1 mM EDTA, and 10 mM NaCl. Samples were referenced to 85% H_3PO_4.

physicochemical methods and enzyme hydrolyses has established structures, configurations, and empirical formulas of the chemically synthesized dimer and trimer cores.

Enzymatic Synthesis and Characterization of 2',5'-Phosphorothioate Dimer and Trimer 5'-Triphosphates. Earlier studies from this laboratory reported the enzymatic synthesis of the 2',5'-phosphorothioate trimer and tetramer 5'-triphosphate from (S_P)-[α-^{35}S]ATPαS with 2-5A synthetase

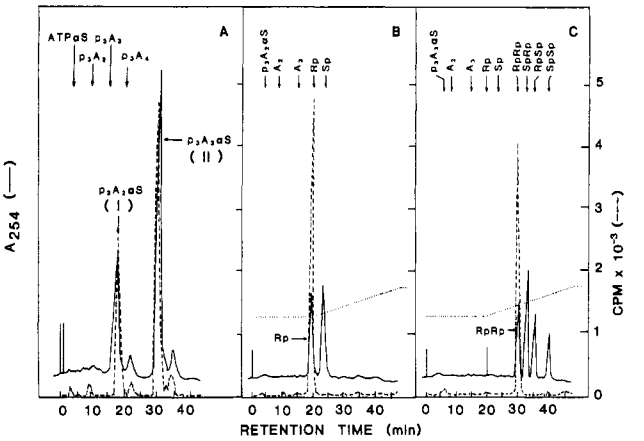

FIGURE 4: HPLC analyses of 2',5'-phosphorothioates synthesized from (S_P)-ATPαS by the 2-5A synthetase from lysed rabbit reticulocytes. (A) HPLC analysis (solvent system 3) of the product (250-μL sample injected) synthesized from (S_P)-[α-^{35}S]ATPαS displaced from DEAE-cellulose columns with 350 mM KCl buffer was as described under Experimental Procedures. Radioactivity in each 500-μL fraction was determined. The elution times of standard (S_P)-ATPαS, p_3A_2, p_3A_3, and p_3A_4 are indicated by arrows. Peak I, putative 2',5'-phosphorothioate dimer 5'-triphosphate ($p_3A_2\alpha S$); peak II, putative 2',5'-phosphorothioate trimer 5'-triphosphate ($p_3A_3\alpha S$). HPLC analysis (solvent system 1) of the products following BAP hydrolysis (24 h) of enzymatically synthesized $p_3A_2\alpha S$ (B) and $p_3A_3\alpha S$ (C). Elution times of standard $p_3A_3\alpha S$, A_2, A_3, R_P dimer core, S_P dimer core, and R_PR_P, S_PR_P, R_PS_P, and S_PS_P trimer cores are indicated by arrows. The dashed line indicates the elution profile of the BAP hydrolysis product of $p_3A_2\alpha S$ (B) and $p_3A_3\alpha S$ (C).

from L929 cell extracts (Lee & Suhadolnik, 1985; Suhadolnik & Lee, 1985). The same incubation procedure was used for the enzymatic synthesis and isolation, except that the source of the 2-5A synthetase was lysed rabbit reticulocytes. (S_P)-[α-^{35}S]ATPαS was converted to two radioactive species (Figure 4A). The yield of 2',5'-phosphorothioates was 7.6%, which compares to an 11.3% yield for authentic 2-5A. HPLC separation of the enzymatically synthesized 2',5'-phosphorothioates revealed two ultraviolet absorbing peaks coinciding with two radioactive peaks, tentatively assigned as $p_3A_2\alpha S$ (peak I) and $p_3A_3\alpha S$ (peak II), with a molar ratio of 1:1.6 (Figure 4). With the chemically synthesized (R_P)- and (S_P)-2',5'-phosphorothioate dimer and trimer cores, it was possible to unequivocally establish the stereochemical configuration of the internucleotide linkages in these 2',5'-phosphorothioates as follows. Hydrolysis of the putative $p_3A_2\alpha S$ (peak I) with BAP for 24 h followed by HPLC analysis revealed that all of the radioactivity was eluted at the same time as chemically synthesized (R_P)-2',5'-phosphorothioate dimer core (Figure 4B). Twenty-four-hour BAP treatment of the putative $p_3A_3\alpha S$ (peak II, Figure 4A) revealed that all of the radioactivity was eluted with authentic R_PR_P trimer core (Figure 4C), whereas 90-min BAP treatment of $p_3A_2\alpha S$ and $p_3A_3\alpha S$ resulted in the accumulation of their respective 5'-monophosphorothioate derivatives. The charge of the enzymatically synthesized dimer and trimer was determined by PEI–cellulose TLC and DEAE-cellulose TLC. The 2',5'-phosphorothioate dimer ($p_3A_2\alpha S$) migrated with a charge of 6– which was equivalent to that of authentic trimer (p_3A_3). $p_3A_3\alpha S$ migrated with a charge of >7–. Enzymatic digestions with SVPD and T_2 RNase were performed as previously described (Lee & Suhadolnik, 1985). SVPD hydrolysis of $p_3A_2\alpha S$ and $p_3A_3\alpha S$ resulted in the isolation of (S_P)-ATPαS, thus demonstrating that the 5'-*O*-(1-thiotriphosphoryl) group had the S_P configuration. Therefore, these results support the structural and configurational assignment

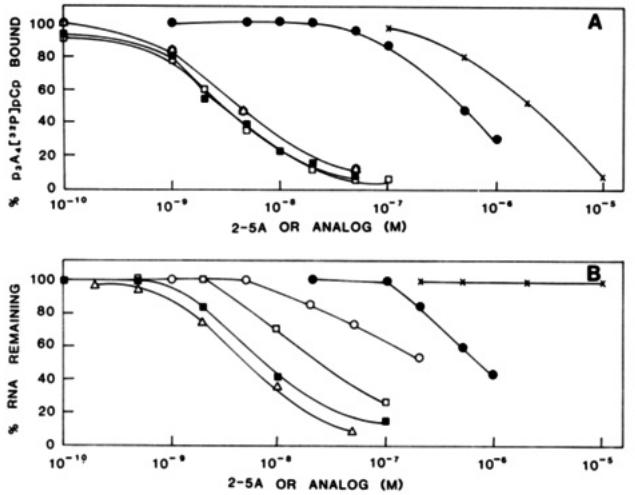

FIGURE 5: (A) Ability of 2′,5′-phosphorothioates to compete with p_3A_4[^{32}P]pCp probe for binding to the RNase L in L929 cell extracts in the radiobinding assay (Knight et al., 1981). Approximately 60% of the p_3A_4[^{32}P]pCp was bound in the absence of added oligonucleotide (total dpm = 23000). (B) Ability of 2′,5′-phosphorothioates to activate the partially purified RNase L from L929 cell extracts as determined by the hydrolysis of poly(U)-3′-[^{32}P]pCp in the core–cellulose assay (Silverman, 1985). Activation of RNase L was determined by conversion of poly(U)-3′-[^{32}P]pCp to acid-soluble fragments after incubation. One hundred percent represents 15000 dpm of labeled poly(U)-3′-[^{32}P]pCp bound to glass fiber filters. p_3A_2 (×); p_3A_3 (△); pA_3 (○); $p_3A_2\alpha S$ (●); $p_3A_3\alpha S$ (■); $pA_3\alpha S$ (□).

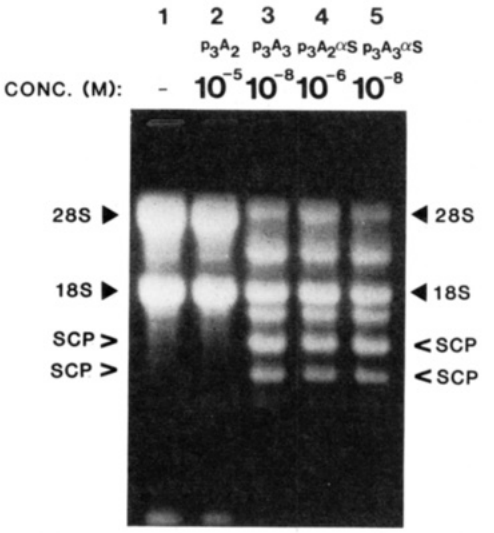

FIGURE 6: Ribosomal RNA cleavage assay with 2′,5′-phosphorothioates. L929 cell extracts were incubated in the absence (lane 1) or presence of p_3A_2 at 10^{-5} M (lane 2), p_3A_3 at 10^{-8} M (lane 3), enzymatically synthesized $p_3A_2\alpha S$ at 10^{-6} M (lane 4), or $p_3A_3\alpha S$ at 10^{-8} M (lane 5). The positions of 28S and 18S rRNA are shown; the arrows indicate the positions of the well-characterized specific cleavage products (SCP) of RNase L.

of the dimer as 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-*P*-thioadenylyl(2′–5′)adenosine ($p_3A_2\alpha S$, peak I, Figure 4A) and of the trimer as 5′-*O*-[(S_P)-1-*P*-thiotriphosphoryl]-(R_P)-thioadenylyl(2′–5′)-(R_P)-*P*-thioadenylyl(2′–5′)adenosine ($p_3A_3\alpha S$, peak II, Figure 4A).

Binding of 2′,5′-Phosphorothioate Analogues to RNase L. The ability of the enzymatically synthesized 2′,5′-phosphorothioate dimer and trimer to bind to RNase L was examined in radiobinding assays (Knight et al., 1981); the 2′,5′-phosphorothioates were added to RNase L in mouse L929 cell extracts to which p_3A_4[^{32}P]pCp had been previously bound. Displacement curves are illustrated in Figure 5A. $p_3A_2\alpha S$ (●) binds to RNase L with an IC_{50} of 5×10^{-7} M; $p_3A_3\alpha S$ (■) has an IC_{50} of 5×10^{-9} M. This compares with IC_{50}'s of 5×10^{-6} M and 5×10^{-9} M for authentic p_3A_2 (×) and p_3A_3 (△), respectively. The 2′,5′-phosphorothioate trimer 5′-monophosphorothioate ($pA_3\alpha S$, □) displaced the radioactive probe equally as well as both $p_3A_3\alpha S$ (■) and authentic pA_3 (○).

Activation of Partially Purified RNase L by 2′,5′-Phosphorothioates As Determined by Hydrolysis of Poly(U)-3′-[^{32}P]pCp. The recently developed core–cellulose assay, which involves the immobilization and partial purification of RNase L on 2-$5A_4$ core–cellulose and determination of the percent hydrolysis of poly(U)-3′-[^{32}P]pCp (Silverman, 1985), was used to measure the ability of the 2′,5′-phosphorothioates to activate RNase L. The 2′,5′-phosphorothioate dimer ($p_3A_2\alpha S$, ●) activated RNase L such that 20% hydrolysis of poly(U)-3′-[^{32}P]pCp was detected at 2×10^{-7} M. Authentic p_3A_2 (×) did not activate RNase L even at 10^{-5} M (Figure 5B) in agreement with a previous report (Silverman, 1985). The 2′,5′-phosphorothioate trimer ($p_3A_3\alpha S$, ■) was capable of activating RNase L to degrade poly(U)-3′-[^{32}P]pCp as well as authentic p_3A_3 (△). A dramatic decrease in activation of RNase L by pA_3 (○) compared to p_3A_3 (△) was observed; however, there is little difference between RNase L activation by $pA_3\alpha S$ (□) and $p_3A_3\alpha S$ (■) at 1×10^{-8} M. Finally, pA_3 (○) showed a greatly reduced activation of RNase L compared to $pA_3\alpha S$ (□).

Activation of RNase L by 2′,5′-Phosphorothioate Dimer and Trimer to Cleave Ribosomal RNA in L929 Cell Extracts. In view of the ability of the 2′,5′-phosphorothioate dimer and trimer 5′-triphosphates and the trimer 5′-monophosphorothioate to activate the partially purified RNase L to degrade the homopolyribonucleotide poly(U)-3′-[^{32}P]pCp (Figure 5B) and the ability of the 2′,5′-phosphorothioate trimer and tetramer 5′-triphosphates to inhibit cellular protein synthesis (Lee & Suhadolnik, 1985), it was deemed essential to determine if the enzymatically synthesized 2′,5′-phosphorothioates were capable of activating RNase L to degrade 28S and 18S ribosomal RNA (rRNA). The rRNA cleavage assay is the most specific functional assay for 2-5A, based on the observation that RNase L degrades rRNA to highly characteristic specific cleavage products (SCP) following activation by 2-5A (Wreschner et al., 1981). The activation of RNase L by the 2′,5′-phosphorothioate dimer and trimer in the rRNA cleavage assays was consistent with the core–cellulose assays (Figure 5B). Significant, dose-dependent rRNA cleavage was observed with the 2′,5′-phosphorothioate *dimer and trimer* analogues. A representative gel is shown in Figure 6. The $p_3A_2\alpha S$ (Figure 6, lane 4) activated RNase L to cleave 28S and 18S rRNA at 10^{-6} M, while authentic p_3A_2 (lane 2) failed to induce any degradation even at 10^{-5} M. $p_3A_3\alpha S$ (lane 5) was as potent as authentic p_3A_3 (lane 3) in activating RNase L, with significant cleavage observed at 10^{-8} M.

Discussion

The activation of the unique 2-5A-dependent endoribonuclease, RNase L, by 2′,5′-oligoadenylates and the hydrolysis of rRNA, tRNA, and cellular and viral mRNA is important in the inhibition of viral replication and regulation of cell growth. Although RNase L is known to be the substrate target for 2-5A and one of the chief functional enzymes of the interferon-induced biological cascade, the mechanism of the binding and activation processes of RNase L is not well understood; the enzyme has not yet been highly purified, and many of the structurally modified 2-5A analogues are not

metabolically stable or lack biological activity. Our aim in the studies described here and in the following two papers (Karikó et al., 1987; Suhadolnik et al., 1987) has been to analyze the stereodynamics involved in the binding and activation processes of RNase L by using the 2′,5′-phosphorothioates, a new class of biological modifiers. The presence of the phosphorothioate group in the 2′,5′-internucleotide linkage and/or in the α-, β-, or γ-pyrophosphoryl groups at the 5′-triphosphate terminus of 2-5A induces physical, chemical, and biochemical modifications in the 2-5A molecule including (i) R_P/S_P chirality, (ii) lowered pK_a, (iii) altered metal ion chelation, (iv) charge modulation, (v) increased bond length, (vi) altered degree of hydration, and (vii) increased metabolic stabilities (see following two papers for a further discussion of these parameters).

Our hypothesis was that the nonbridging oxygens of the 2′,5′-phosphodiester linkages in 2-5A become asymmetric once the 2-5A molecule binds to RNase L. Precedents for the asymmetry of the phosphate oxygens of phosphodiester linkages was the enhanced asymmetry of the 3′,5′-phosphodiester bonds in the highly ordered three-dimensional environment of helical DNA. This is based on computer analysis of double-stranded DNA which suggests that the diastereotopic oxygens of the 3′,5′-phosphodiester bond are not equivalent. In the findings reported here, several interesting aspects to RNase L binding and activation are revealed following the introduction of sulfur into the 2′,5′-internucleotide linkages and the pyrophosphoryl moiety of 2-5A via the phosphorothioate groups.

The enzymatic conversion of (S_P)-ATPαS to 2′,5′-phosphorothioates differs with the source of the 2-5A synthetase. The 2-5A synthetase from L929, HeLa, and Daudi cell extracts has previously been shown to convert (S_P)-ATPαS [but not (R_P)-ATPαS] to the 2′,5′-phosphorothioate trimer and tetramer, with no dimer formation (Lee & Suhadolnik, 1985). With the 2-5A synthetase from lysed rabbit reticulocytes, the products formed are exclusively the dimer ($p_3A_2\alpha S$) and trimer ($p_3A_3\alpha S$) (Figure 4). Lysed rabbit reticulocytes were chosen for this study because the reticulocyte 2-5A synthetase converts ATP predominantly to 2-5A trimer with some 2-5A dimer (Martin et al., 1979).

We have also demonstrated that the 2′,5′-phosphorothioates have substantially greater negative charges than their corresponding 2′,5′-oligoadenylates. $p_3A_2\alpha S$ has a net charge of 6– with three P–O^- groups (charge 3–) and two P–S^- groups (charge 3–). Authentic p_3A_2 and p_3A_3 have charges of 5– and 6–. Therefore, the P–S^- charge of 3– in $p_3A_2\alpha S$ could be attributed to the increased anionic properties of the two P–S^- groups such that each P–S^- group would have a minimum net charge of 1.5– (compared to 1.0– for the P–O^- group). This observation is in agreement with the report of Frey and Sammons (1985). The importance of increased negative charge is also observed when a P–S^- group is substituted for a P–O^- group at the β-phosphorus in the pyrophosphoryl group at the 5′-triphosphate terminus of 2-5A [see third of three papers in this issue (Suhadolnik et al., 1987)]. The lowered pK_a of the 2′,5′-phosphorothioates with a concomitant decrease in metal ion chelation would provide a 2-5A molecule with increased ionic charge and/or a change in charge modulation (Suggs & Taylor, 1985; Plaut et al., 1979; Jaffe & Cohn, 1978).

We were able to confirm the absolute configuration of the internucleotide linkages of the 2′,5′-phosphorothioate dimer (R_P) and trimer (R_PR_P) 5′-triphosphates (see Figures 1 and 3) through the use of chemically synthesized and fully characterized (R_P)- and (S_P)-2′,5′-phosphorothioate dimer and trimer cores. We had tentatively assigned the R_P configuration for the internucleotide linkages of the enzymatically synthesized 2′,5′-phosphorothioate trimer and tetramer on the basis of enzyme hydrolyses (Lee & Suhadolnik, 1985). Our initial assignment of structure and configuration has proven correct.

The formation of a productive complex between the 2′,5′-phosphorothioate dimer ($p_3A_2\alpha S$), RNase L, and RNA may be facilitated by a more direct interaction of the P–S^- group with the basic amino acid residues of RNase L (i.e., arginyl, histidinyl, lysinyl residues) which could result in the necessary conformational change in RNase L for the activation of the enzyme. Other structural changes in 2-5A dimer may also be sufficient for activation of RNase L. For example, Krause et al. (1986) reported that a 5′-capped 2-5A dimer (i.e., AppppA2′p5′A) was able to activate RNase L, although at a 1000-fold higher concentration than p_3A_3. However, Watling et al. (1985) reported that a 2-5A dimer analogue, with a 5′-(methylthiomonophosphoryl) cap (i.e., $CH_3SpA2'pA2'pp3'OCH_3$), cannot bind to nor activate RNase L. Alkylation of 2-5A to form *n*-decyl-NHp_3A_3, where a 10-carbon cap is present, results in an analogue that does activate RNase L (Karikó & Ludwig, 1985).

Other important evidence is reported here with respect to the structure/stability/activity relationship of the 2′,5′-phosphorothioates. The metabolic stability of the 2′,5′-phosphorothioate dimer and trimer cores is markedly greater than that of authentic 2-5A. The rate of hydrolysis of the trimer cores by SVPD (in order of decreasing stability) is $S_PR_P > R_PR_P \gg A_3$; R_PS_P and S_PS_P trimer cores are not substrates (Table I). These results are in agreement with reports on the phosphorothioate analogues of oligodeoxyribonucleotides having two adjacent P–S centers, as this 3′-exonuclease did not hydrolyze R_PS_P or S_PS_P diastereomers (Stec et al., 1984). Apparently, the 3′ → 5′ direction of stepwise cleavage by SVPD is blocked by an S_P configuration, preventing cleavage of the upstream, adjacent phosphorothioate linkage. With 2′-PDE, the R_PR_P, S_PR_P, and R_PS_P trimer cores, but not the S_PS_P trimer core, were substrates. With both SVPD and 2′-PDE, the dimer cores (either R_P or S_P) accumulate following hydrolysis of the R_PR_P, S_PR_P, and R_PR_P trimer cores. Hydrolysis of the 2-5A molecule by SVPD and 2′-PDE proceeds from the 2′/3′-terminus; therefore, introduction of the phosphorothioate group into the trimer cores results in the accumulation of the R_P or S_P dimer cores from the 5′-terminus and the accumulation of AMPS from the 2′/3′-terminus (Figure 2, Table I). With authentic A_3, there is no detectable accumulation of A_2 following hydrolysis by SVPD or 2′-PDE.

Because it is known that the (S_P)-3′,5′-phosphodiester linkage is cleaved by nuclease S1 and P1 (Potter et al., 1983a,b), it was of utmost interest to establish the stability of the (S_P)-2′,5′-phosphorothioate linkages with these nucleases. Neither the (S_P)- nor the (R_P)-2′,5′-phosphorothioates were hydrolyzed by S1 or P1 nuclease. These results reveal new substrate specificity for the S1 and P1 nucleases.

^{31}P NMR, HPLC analyses, and hydrolyses by SVPD and 2′-PDE permit the assignment of the absolute configuration of the four trimer cores (Figure 1). ^{31}P NMR spectroscopy of the 2′,5′-phosphorothioate dimer and trimer cores has provided a new line of evidence to support assignments of chirality at phosphorus in the phosphorothioates previously based on enzymatic procedures. ^{31}P NMR spectroscopy has revealed that the S_P diasteromer of 3′,5′-dinucleoside phosphorothioates always resonates upfield from the R_P diastereomer (Bartlett & Eckstein, 1982; Romaniuk & Eckstein, 1982). This same observation has been reported by Nelson

et al. (1984) for the (R_P)- and (S_P)-2′,5′-phosphorothioate dimers. The difference of δ for S_PR_P trimer core compared to R_PS_P trimer core may be characteristic for 2′,5′-phosphorothioates where there are alternating R_P and S_P configurations.

Finally, our earlier report showed that the enzymatically synthesized $p_3A_3\alpha S$ and $p_3A_4\alpha S$ inhibited protein synthesis in intact L929 cells (Lee & Suhadolnik, 1985), which suggested that the inhibition of protein synthesis might be proceeding via activation of RNase L. Direct evidence is presented here by the core–cellulose and rRNA cleavage assays to establish that indeed the enzymatically synthesized 2′,5′-phosphorothioates ($p_3A_2\alpha S$ and $p_3A_3\alpha S$) act via the activation of RNase L. In addition, the trimer 5′-monophosphorothioate ($pA_3\alpha S$) can activate RNase L better than authentic pA_3 (Figure 5B). The enhanced activation of RNase L by $pA_3\alpha S$ prompted us to convert the four phosphorothioate trimer cores to their respective 5′-monophosphates with T_4 polynucleotide kinase to determine the effect of the 5′-monophosphate moiety on RNase L activation in the presence of R_P or S_P chirality in the internucleotide linkages [see second of three papers in this issue (Karikó et al., 1987)].

Our finding that the 2′,5′-phosphorothioate dimer with only one 2′,5′-internucleotide linkage can activate RNase L is important theoretically in the evaluation of the role of 3′,5′/2′,5′-branched polyadenylated mRNA in the mRNA splicing process. Baglioni and co-workers suggested that activation of RNase L is involved in the cleavage of heterogeneous nuclear RNA (Nilsen et al., 1982). More recently, Wallace and Edmonds (1983) characterized the nuclear intermediate of mRNA as a polyadenylated RNA containing 2′,5′- and 3′,5′-phosphodiester bonds that form a branch at the 2′,3′-hydroxyl groups. Because no consecutive 2′,5′-internucleotide linkages have been demonstrated in branched RNA (one branched nucleotide occurs per every 40 000 nucleotides) (Wallace & Edmonds, 1983), it was of great importance to determine whether a single 2′,5′-linkage is sufficient for activation of RNase L. With cytoplasmic RNase L from L929 cell extracts, we have demonstrated that chemically synthesized 3′,5′/2′,5′-branched trimer (A_C^G and A_U^G) (Vial et al., 1987) and tetramer (GA_C^G) (Kierzek et al., 1986) nucleotides were not able to bind to or activate RNase L as determined by radiobinding and rRNA cleavage assays (unpublished results). If the nuclear RNase L is involved in the splicing process, it may be that a branched polynucleotide is required for activation (and not trimer or tetramer 2-5A). We are currently synthesizing oligo- and polynucleotides covalently linked to the 5′-hydroxyl and/or 3′-hydroxyl of 2′,5′/3′,5′ branches to determine whether the splicing process involves the nuclear RNase L.

In summary, we have demonstrated that phosphodiester bond modification results in a dramatic alteration of the 2-5A binding/activation site on RNase L. The implication of this finding is more fully discussed in the second of three papers in this issue (Karikó et al., 1987). The results described here add new dimensions to the introduction of chirality into the 2-5A molecule via phosphorothioate substitution and have revealed heretofore unknown aspects of the stereodynamics of the 2-5A synthetase/RNase L system. Introduction of chirality into 2-5A by replacement of the nonbridging oxygens with sulfur will permit us to evaluate the effects of the P–S bond vs the P–O bond related to changes in bond length, pK_a, charge modulation, degree of hydration, etc. (see also the following two papers in this issue). Finally, studies are now under way in this laboratory to prepare (R_P)- and (S_P)-[α,-α,α-^{16}O,^{17}O,^{18}O]ATP and (R_P)- and (S_P)-[β,β,β-^{16}O,^{17}O,^{18}O]ATP to begin the assessment of the effects of chirality exclusive of sulfur vs oxygen on the binding and activation of RNase L.

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SUPPLEMENTARY MATERIAL AVAILABLE

Chemical synthesis, UV spectra, and ^1H NMR spectra of the phosphorothioate dimer and trimer cores (13 pages). Ordering information is given on any current masthead page.

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