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Synthesis and Biological Activity of 5′-Capped Derivatives of 5′-Triphosphoadenylyl(2′→5′)adenylyl(2′→5′)adenosine†

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ABSTRACT: The oligonucleotides A5′pp5′A2′p5′A2′p5′A and A5′ppp5′A2′p5′A2′p5′A were prepared by reaction of AMP or ADP, respectively, with the 5′-(phosphoimidazolidate) of A2′p5′A2′p5′A. A5′pppp5′A2′(p5′A)$_n$ (n = 1-3) were synthesized by reaction of p5′A2′(p5′A)$_n$ (n = 1-3) with adenosine 5′-trimetaphosphate. All structures were confirmed by enzyme digestion and ^{1}H and ^{31}P nuclear magnetic resonance (NMR). The products A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A were found to be identical with two of the products of the 2-5A synthetase catalyzed reaction of Ap_4A with ATP, thus confirming the structural assignments made by earlier investigators. In extracts of mouse L cells programmed with encephalomyocarditis virus RNA, A5′pppp5′A2′p5′A2′p5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A were equipotent with 2-5A itself as inhibitors of translation. The oligomers A5′ppp5′A2′p5′A2′p5′A and A2′pppp5′A2′p5′A were about 100 times less active than 2-5A, and A5′pp5′A2′p5′A2′p5′A was without translational inhibitory activity. When affinity for the 2-5A-dependent endonuclease was determined (by displacement of 2-5A[^{32}P]pCp from endonuclease), all of the analogues, as well as 2-5A itself, had similar affinities for the endonuclease except for A5′pppp5′A2′p5′A, which was bound ~100 times less effectively. Under conditions of the radiobinding assay, A5′pppp5′A2′p5′A2′p5′A was degraded ($t_{1/2}$ = 2 h) to ATP, ADP, AMP, ppp5′A2′p5′A2′p5′A, and p5′A2′p5′A2′p5′A. The same products were obtained when degradation was carried out under protein synthesis conditions at 30 °C except that the half-life of A5′pppp5′A2′p5′A2′p5′A was reduced to 3 min. The other unsymmetrical di- and triphosphates A5′pp5′A2′p5′A2′p5′A and A5′ppp5′A2′p5′A2′p5′A were degraded much more slowly than the tetraphosphate, and they did not give rise to 2-5A as a degradation product. When the degradation of A5′pppp5′A2′p5′A2′p5′A was examined in incubation mixtures containing human serum or Nalmalwa cell extract, it was found that the tetraphosphate was quite stable to the action of human serum but was readily degraded to 2-5A and p5′A2′p5′A2′p5′A by extracts of the human lymphoblastoid cells. Thus, "capping" (with adenosine) of the β- or γ-phosphates of the established translational inhibitors pp5′A2′p5′A2′p5′A or ppp5′A2′p5′A2′p5′A led to a loss of ability to activate the 2-5A-dependent endonuclease even though such oligomers still were bound to the endonuclease as well as 2-5A itself. However, capping with an adenosine tetraphosphate moiety gave a 2-5A derivative that was stable in the external milieu of the cell but that was rapidly cleaved by the enzyme(s) of the cytosol to give 2-5A itself.

The established role of 2-5A[1] (Kerr & Brown, 1978) in the antiviral action of interferon [for reviews see Torrence (1982), Lengyel (1982), and Sen (1982)], its possible role (Kimchi et al., 1981a,b) in interferon's antiproliferative action (Gresser & Tovey, 1978; Taylor-Papadimitriou, 1980), and its possible involvement in regulation of cell growth, differentiation, and/or development (Stark et al., 1979; Etienne-Smekens et al., 1983a,b; Krishnan & Baglioni, 1980; Oikarinen, 1982; Besancon et al., 1981; Kimchi, 1981) have resulted in interest in the 2-5A system as a new approach to antiviral and/or antitumor agents. Two problems associated with envisaged application of the 2-5A system have been defined (Torrence et al., 1982) to include the relative lability of 2-5A toward degrading enzymes and the cellular impermeability of the highly charged 2-5A molecule. While a solution of the first problem has been provided by chemical modification of the 2-5A molecule to yield analogues resistant to degradation

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[1] Abbreviations: 2-5A, pppA2′p(A2′p)$_n$A where n = 1 to about 10; TEAB, triethylammonium bicarbonate; Im(pA)$_3$, 5′-imidazolidate of p5′A2′p5′A2′p5′A; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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(Imai et al., 1982; Baglioni et al., 1981; Silverman et al., 1981) but still able to activate the 2–5A-dependent endonuclease, no approach to the second problem of cell permeability has been forthcoming. Therefore, relatively drastic methodology has been required to introduce 2–5A into the intact cell including microinjection (Higashi & Sokawa, 1982), hypertonic salt (Williams & Kerr, 1978) or lysolecithin (Fanet et al., 1981) treatments, or calcium phosphate coprecipitation (Hovanessian et al., 1979; Hovanessian & Wood, 1980). The use of 2–5A trimer core (5′-dephosphorylated 2–5A trimer or A2′p5′A2′p5′A) has been advanced as a means to circumvent the inability of 2–5A itself to penetrate the intact cell and to obtain an active antimitogenic agent (Kimchi et al., 1978, 1981a,b), but recently, it was found that optimal antimitogenic activity was obtained with the more highly charged core 5′-monophosphates such as p5′A2′p5′A2′p5′A and p5′A2′p5′A2′p5′A2′p5′A (Torrence et al., 1983). Moreover, it has been reported that treatment of Swiss 3T3 cells with 2–5A trimer core, under conditions that give an antimitogenic response, does not give rise to ribosomal RNA cleavage patterns that are known to be characteristic of 2–5A treatment (Eppstein et al., 1983).

The preponderance of the negative charge of the 2–5A trimer molecule is born at the 5′-triphosphate moiety. In addition, the state of phosphorylation of the 5′-terminus of the 2–5A core has a dramatic effect on the biological properties of the resistant molecule. The 5′-unphosphorylated molecule, e.g., A2′p5′A2′p5′A, binds to the 2–5A-dependent endonuclease about 1000 times less strongly than 2–5A itself (Knight et al., 1980; Torrence et al., 1982); the 5′-monophosphorylated molecule, p5′A2′p5′A2′p5′A, binds to the 2–5A-dependent nuclease approximately as well as does 2–5A, but rather than activating the nuclease, p5′A2′p5′A2′p5′A is an antagonist of 2–5A action (Torrence et al., 1981). Finally, the 5′-di- and 5′-triphosphates, viz., pp5′A2′p5′A2′p5′A and ppp5′A2′p5′A2′p5′A, are equally potent activators of the 2–5A-dependent endonuclease (Martin et al., 1979; Haugh et al., 1983; J. Imai and P. F. Torrence, unpublished observations). For these reasons, it was of interest to chemically modify the 5′-terminus of 2–5A core to determine if any of the negative charges of 2–5A could be dispensed with and still retain ability to activate the 2–5A-dependent endonuclease. To this end, we prepared and evaluated the biological activity of a series of adenosine "capped" 2–5A derivatives of the general formula A5′pp$_x$5′A2′(p5′A)$_n$ where x is from 1 to 3 and n is from 1 to 3. Additional interest in such capped 2–5A derivatives arises from the observations that the enzyme 2–5A synthetase can add 5′AMP in 2′–5′ linkage to the important metabolites ADP-Rib, nicotinamide adenine dinucleotide (NAD^+), and adenosine tetraphosphate (Ap_4A) (Ball & White, 1980; Ball, 1980; Ferbus et al., 1981; Cayley & Kerr, 1982) although none of these adducts have been found to occur naturally (Cayley & Kerr, 1982). Support for the assigned structures of these adducts has been obtained by enzymic analysis and NMR spectral data. No structure confirmation has been established by chemical synthesis, however. In this paper, we show that chemically synthesized A5′p$_4$5′A2′p5′A and A5′p$_4$5′A2′p5′A2′p5′A are identical with two products of the 2–5A synthetase catalyzed reaction of Ap_4A with ATP.

Materials and Methods

^{1}H NMR spectra were recorded with a Varian HR220 instrument operating at 220 MHz. The solvent was D_2O with acetone (2.05 ppm from Me_4Si) as an internal standard. Chemical shifts are reported in ppm. Multiplicity is abbreviated as s (singlet), d (doublet), t (triplet), or m (multiplet). ^{31}P NMR spectra were obtained with a Varian instrument at 109 MHz with D_2O as solvent and 0.85% H_3PO_4 as external reference.

Thin-layer chromatography (TLC) was on either E. Merck precoated PEI–cellulose F plates with solvent system A (0.1 M $NH_4HCO)_3$ or B (0.25 M NH_4HCO_3) or cellulose F plates with solvent system C (2-propanol–NH_4OH–H_2O, 55:10:35) or D (isobutyric acid–1 M NH_4OH–0.2 M EDTA, 100:60:0.8).

HPLC was carried out with a Beckman instrument with Model 110A pump in a Zorbax ODS column (9.4 mm × 25 cm) at a flow rate of 2.5 mL/min or a Accupac C_{18} column (4.6 mm × 10 cm) at a flow rate of 1.0 mL/min. Solvent A was 50 mM ammonium phosphate (pH 7.0). Solvent B was methanol–H_2O (1:1). Elution was with a linear gradient from 0 to 50% of solvent B in 20 min (program 1) or from 0 to 70% in 10 min (program 2). Detection was at either 258 or 280 nm. Digests with bacterial alkaline phosphatase or snake venom phosphodiesterase were carried out under conditions specified by Brownless (1972).

Preparation of 2′–5′-Oligoadenylate Monophosphates [$(pA)_n$ (n = 2–4)]. 2′–5′-Oligoadenylate monophosphates [$(pA)_n$] were prepared by phosphorylation of properly protected A2′p5′A or A2′p5′A2′p5′A (Imai & Torrence, 1981a,b) or by lead ion catalyzed polymerization of adenosine 5′-(phosphoimidazolidate) (ImpA) (Sawai et al., 1981). In the latter case, the polymerized reaction mixture was first treated with nuclease P_1 to remove undesired 3′–5′-phosphate linkage isomers and then separated on a DEAE-Sephadex (HCO_3^-) A-25 column eluted with a linear gradient of 0.1–0.7 M triethylammonium bicarbonate buffer (TEAB) (pH 7.6). The separated oligomers were further purified by preparative HPLC with a Zorbax ODS column. Elution was performed under program 1.

Preparation of Imidazolidates of p5′A2′p5′A2′p5′A [*Im*-$(pA)_3$]. (*1*) *Method A.* 2′–5′(pA)$_3$ (triethylammonium salt, 518 OD_{260} units, 15.0 μmol) was dissolved in dry pyridine and dried by two consecutive additions and evaporations of dry pyridine, followed by two consecutive additions and evaporations of dry benzene. The residue was dissolved in a mixture of dry dimethyl sulfoxide (200 μL), triethylamine (10 μL, 67 μmol), and tri-*n*-octylamine (5 μL, 11.4 μmol). Carbonyldiimidazole (DCI, 12.2 mg, 75 μmol) was added, and the reaction was stirred at room temperature for 40 min. The reaction mixture was poured into a 0.1 M solution of sodium iodide in dry acetone (5 mL) on a vortex mixer. After 1 min of being mixed, the resultant white precipitate was pelleted by centrifugation at 2500 rpm for 5 min. The precipitate was washed twice with dry acetone and then dissolved in dry methanol for absorbance measurements. The yield of Im(pA)$_3$ was 98% (506 OD_{260} units, 14.7 μmol). The methanol solution was evaporated to dryness, and the residue was dried over P_2O_5 for a few hours before further reaction.

(*2*) *Method B.* 2′–5′(pA)$_3$ (triethylammonium salt, 518 OD_{260} units, 15.0 μmol) dried in the same manner described above was suspended in dry dimethylformamide (DMF) (500 μL). Triphenylphosphine (19.7 mg, 75.0 μmol), dipyridyl disulfide (16.5 mg, 75.0 μmol), imidazole (10.2 mg, 150 μmol), and triethylamine (2 μL) were added to this suspension with stirring. After being stirred at room temperature for 60 min, the reaction mixture became homogeneous, and its color turned to yellow. Completion of the reaction was assured by PEI–cellulose TLC (solvent system A). The whole mixture was poured into a 0.1 M solution of sodium iodide in dry acetone (5 mL) on a vortex mixer. The white precipitate of Im(pA)$_3$

thus formed was collected by centrifugation and washed further with dry acetone 3 times. The yield of $Im(pA)_3$ was 93% (482 OD_{260} units).

Preparation of A5′pp5′A2′p5′A2′p5′A. $Im(pA)_3$ (sodium salt, 506 A_{260} units, 14.7 μmol) was dissolved in dry dimethylformamide (300 μL), and adenosine 5′-monophosphate (AMP, free acid, 30.0 mg, 75 μmol) dissolved in dry DMF (750 μL) was added to it. The reaction mixture was allowed to stand at room temperature for 3 days. The solution was diluted with H_2O (1.0 mL) and applied to a DEAE-Sephadex A-25 column (HCO_3^-, 1.0 × 20 cm) that was eluted with a linear gradient of 0.15–0.40 M TEAB (pH 7.6, total volume 500 mL, 136 fractions). Proper fractions (70–100) were pooled, concentrated under vacuum, and coevaporated with water several times until TEAB was completely removed. The resulting residue was further purified in preparative HPLC on a Zorbax ODS column. Elution was done under program 1. A peak that appeared at 17.3 min was collected and concentrated. The residue was reapplied to a DEAE-Sephadex A-25 column to remove inorganic phosphate contained in buffer solution A. The column was eluted with a linear gradient of 0.2–0.4 M TEAB (pH 7.6, total volume 500 mL, 136 fractions. After a pooling and evaporation of proper fractions (50–78), A5′pp5′A2′p5′A2′p5′A was isolated as a sodium salt (312 A_{260} units, 7.5 μmol, yield 50.0%). The product was homogeneous on a PEI–cellulose plate developed in solvent B (R_f 0.59).

Preparation of A5′ppp5′A2′p5′A2′p5′A. Adenosine 5′-diphosphate (sodium salt, 152 mg, 0.30 mmol) was dissolved in H_2O (1.5 mL) and applied to a Dowex 50W-8X column (H^+ form, 0.9 cm × 6 cm). The column was eluted with H_2O (12 mL), and the eluate was concentrated under vacuum to 3 mL. Tri-*n*-butylamine (0.3 mL) dissolved in acetone (3 mL) was added to this solution, and the mixture was evaporated to dryness. The residue was coevaporated twice with dry pyrridine (3 mL each) and twice with dry benzene (3 mL each) to remove trace amounts of water. The resultant white powder was dissolved in dry DMF (3 mL) to make a 0.1 M solution of the tri-*n*-butylammonium salt of ADP. $Im(pA)_3$ (406 A_{260} units, 116 μmol) was then dissolved in this 0.1 M ADP solution (464 μL). After this was mixed on a vortex mixer, the reaction mixture was allowed to stand at room temperature for 3 days. The solution was diluted with H_2O (1.5 mL) and then applied to a DEAE-Sephadex A-25 column (HCO_3^-, 1.0 cm × 20 cm), which was eluted with a linear gradient of 0.15–0.50 M TEAB (pH 7.6, total volume 500 mL, 137 fractions). Proper fractions (86–110) were pooled, concentrated under vacuum, and coevaporated with water to remove TEAB. The crude product was further purified on HPLC in a Zorbax ODS column. Elution was performed under program 1. The main peak that appeared at 15.2 min was pooled and evaporated. The residue was rechromatographed on a DEAE-Sephadex A-25 column (HCO_3^-, 1.0 × 20 cm) that was eluted with a linear gradient of 0.28–0.50 M TEAB (pH 7.6, total volume 500 mL, 136 fractions). After a pooling and evaporation of proper fractions (60–85), A5′ppp5′A2′p5′A2′p5′A was isolated as a sodium salt (130 A_{260} units, 3.12 μmol, yield 26.9%). The product was homogeneous on a PEI–cellulose plate developed in solvent B (R_f 0.43).

Preparation of Adenosine 5′-Trimetaphosphate (ATMP). ATMP was synthesized by a slightly modified method originally described by Knorre et al. (1976). Briefly, ATP (dry tributylammonium salt, 60 mol) dissolved in dry Me_2SO (600 μL) was treated with dicyclohexylcarbodiimide (DCC) (79.5 mg, 390 μmol) under a dry argon atmosphere at room temperature for 1 h. After filtration of dicyclohexylurea deposited from the solution, the reaction mixture was extracted 3 times with dry ether (7 mL each) to remove Me_2SO. The resulting gummy residue was subsequently used for the next reaction.

Preparation of A5′pppp5′A2′p5′A2′p5′A. 2′–5′$(pA)_3$ (triethylammonium salt, 518 A_{260} units, 15 μmol) was dissolved in a mixture of tributylamine (10 μL, 40 μmol) and dry DMF (500 μL), and to the solution was added the ATMP (60 μmol) prepared above. After being mixed in a vortex mixer, the reaction mixture was allowed to stand at room temperature for 7 days. The solution was diluted with H_2O (1.5 mL) and applied to a DEAE-Sephadex A-25 column (HCO_3^-, 1.0 × 20 cm), which was eluted with a linear gradient of 0.24–0.60 M TEAB (pH 7.6, total volume 500 mL, 136 fractions). Fractions 105–130 were combined together and concentrated. The crude product thus obtained was further purified on preparative HPLC equipped with a Zorbax ODS column. Elution was performed under program 1. A peak that appeared at 14.38 was collected and concentrated. The residue was rechromatographed on a DEAE-Sephadex A-25 column (HCO_3^-, 1.0 × 20 cm) to remove inorganic phosphate. The column was eluted with a linear gradient of 0.40–0.60 M TEAB (pH 7.6, total volume 500 mL, 136 fractions). After a pooling and evaporation of proper fractions (58–87), A5′pppp5′A2′p5′A2′p5′A was isolated as a sodium salt (296 A_{260} units, 7.1 μmol, yield 47.4%). The product was homogeneous on a PEI–cellulose plate developed in solvent B (R_f 0.27).

A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A2′p5′A were prepared essentially in the same manner as described here. Briefly, 2′–5′$(pA)_2$ (10 μmol) was reacted with ATMP (40 μmol) at room temperature for 2 days. The yield of A5′pppp5′A2′p5′A was 51.0% (5.07 μmol), and its R_f on PEI–cellulose TLC was 0.27 (solvent B). A5′pppp5′A2′p5′A2′p5′A2′p5′A was synthesized by the reaction of 2′–5′$(pA)_4$ (6 μmol) with ATMP (30 μmol) at room temperature for 7 days. The yield was 43.3% (2.6 μmol), and its R_f on PEI–cellulose TLC was 0.24 (solvent B).

Biological Activity Studies. The preparations of mouse L cell and human Nalmalwa cell extracts and encephalomyocarditis virus RNA, as well as the techniques and conditions for cell-free protein synthesis assays, have been described elsewhere (Torrence & Friedman, 1979; Johnston et al., 1980). Radiobinding assays were performed according to Knight et al. (1980) with ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′Cp of specific activity ~3000 Ci/mmol (Amersham, Chicago, IL), and the source of the 2–5A-dependent endoribonuclease was unfractionated mouse L cell 510 extracts.

Results

Synthesis of Capped 2–5A Analogues. Two different synthetic approaches were used to synthesize the capped oligoadenylates. The first method utilized a displacement reaction on the 5′-(phosphoimidazolidate) of A2′p5′A2′p5′A by either 5′AMP or 5′ADP after the methodology developed by Schaller et al. (1961) for the synthesis of unsymmetrical pyrophosphates or that of Bornemann & Schlimme (1981) for the preparation of unsymmetrical or symmetrical α,γ-disubstituted triphosphates. Thus, reaction of Imp5′A2′p5′A2′p5′A with 5′AMP gave A5′pp5′A2′p5′A2′p5′A in 50% yield whereas reaction of Imp5′A2′p5′A2′p5′A with 5′ADP gave A5′ppp5′A2′p5′A2′p5′A in only fair yield (26.9%). In a separate approach, unsymmetrical P^1,P^4-substituted tetraphosphates were obtained by the reaction of an oligoadenylate 5′-monophosphate with adenosine trimetaphosphate prepared by treatment of ATP with dicyclohexylcarbodiimide in dry

Table I: Characteristic Proton NMR Signals of Capped Oligoadenylates

oligoadenylate	chemical shift (ppm)[a] anomeric protons (C-1′ H)	aromatic ring protons (C-2 H and C-8 H)
A5′pp5′A2′p5′A2′p5′A	5.67 (3 H, m), 5.59 (1 H, d, J = 2)	7.92 (1 H, s), 7.81 (1 H, s), 7.80 (1 H, s), 7.73 (1 H, s), 7.70 (1 H, s), 7.68 (1 H, s), 7.60 (1 H, s), 7.49 (1 H, s)
A5′ppp5′A2′p5′A2′p5′A	5.75 (3 H, m), 5.61 (1 H, d, J = 2)	7.98 (1 H, s), 7.91 (1 H, s), 7.79 (1 H, s), 7.78 (1 H, s), 7.75 (1 H, s), 7.74 (1 H, s), 7.62 (1 H, s), 7.54 (1 H, s)
A5′pppp5′A2′p5′A	5.92 (1 H, d, J = 3), 5.77 (1 H, d, J = 6), 5.65 (1 H, d, J = 3)	8.14 (1 H, s), 8.01 (1 H, s), 7.94 (1 H, s), 7.92 (1 H, s), 7.82 (1 H, s), 7.70 (1 H, s)
A5′pppp5′A2′p5′A2′p5′A	5.75 (2 H, d, J = 8), 5.72 (1 H, d, J = 6), 5.61 (1 H, d, J = 6)	8.13 (1 H, s), 7.92 (1 H, s), 7.88 (1 H, s), 7.83 (1 H, s), 7.74 (1 H, s), 7.71 (1 H, s), 7.61 (1 H, s), 7.58 (1 H, s)
A5′pppp5′A2′p5′A2′p5′A2′p5′A	5.80 (2 H, m), 5.73 (1 H, d, J = 6), 5.62 (2 H, m)	8.17 (1 H, s), 7.95 (1 H, s), 7.89 (1 H, s), 7.71 (2 H, s), 7.63 (3 H, s), 7.60 (1 H, s)

[a] s, singlet; d, doublet; m, multiplet. Coupling constants are expressed in hertz.

Table II: ^{31}P NMR Chemical Shifts of Capped Oligoadenylates

oligoadenylate	chemical shifts (ppm)[a] internucleotide phosphate	5′-terminal phosphates
A5′pp5′A2′p5′A2′p5′A	−0.26 (1 P, s), −0.50 (1 P, s)	−10.63 (2 P, br s, P_α and P_β)
A5′ppp5′A2′p5′A2′p5′A	−0.27 (2 P, s), −0.59 (1 P, s)	−10.90 (2 P, d, J = 16 Hz, P_α and P_γ), −22.14 (1 P, t, J = 17 Hz, P_β)
A5′pppp5′A2′p5′A	−0.86 (1 P, s)	−11.00 (2 P, d, J = 6 Hz, P_α and P_δ), −22.41 (2 P, m, P_β and P_γ)
A5′pppp5′A2′p5′A2′p5′A	−0.69 (2 P, s), −1.01 (1 P, s)	−10.95 (1 P, m, P_α or P_δ), −11.03 (1 P, m, P_α or P_δ), −22.47 (2 P, m, P_β and P_γ)
A5′pppp5′A2′p5′A2′p5′A2′p5′A	−0.71 (1 P, s), −0.86 (1 P, s), −1.05 (1 P, s)	−11.02 (2 P, m, P_α and P_δ), −22.39 (2 P, m, P_β and P_γ)

[a] s, singlet; d, doublet; t, triplet; m, multiplet.

Me_2SO (Glonek et al., 1974). This method had been exploited previously for the synthesis of P^1,P^5-bis(5′-adenosyl)pentaphosphate by Hampton et al. (1982), who extended the finding (Knorre et al., 1976) that adenosine trimetaphosphate, in reactions with amines or alcohols, produced P^3-substituted ATP derivatives. By this method, the tetraphosphates A5′pppp5′A2′p5′A, A5′pppp5′A2′p5′A2′p5′A, and A5′ppppA2′p5′A2′p5′A2′p5′A were obtained in 51, 47, and 43% yield, respectively.

As could be expected, all of the unsymmetrical polyphosphates synthesized by the above procedures were completely resistant to the action of alkaline phosphatase but were completely degraded to 5′AMP by venom phosphodiesterase. Further confirmation of the assigned structures was obtained from the proton and phosphorus NMR's of these capped 2–5A derivatives. Table I shows that for each synthetic polyphosphate, the requisite number and multiplicity of anomeric and purine aromatic protons could be observed. The same result was obtained from ^{31}P NMR (Table II); for each analogue, the number and multiplicity of internucleotide phosphate agreed with the assigned structure. Most characteristic, however, were the phosphorus resonances associated with the polyphosphate functionality. For the diphosphate A5′pp5′A2′p5′A2′p5′A, ^{31}P NMR showed two broad singlets; for the triphosphate A5′ppp5′A2′p5′A2′p5′A, two doublets and one triplet were revealed; for the tetraphosphate A5′pppp5′A2′p5′A, two doublets and two multiplets were obtained. In addition, the chemical shifts of each phosphorus atom was characteristic of its position in the polyphosphate residue.

Identity of Chemically Synthesized A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A with the Product of the Reaction of ATP with Ap_4A As Catalyzed by 2–5A Synthetase. Adducts of ATP with Ap_4A were prepared according to Cayley & Kerr (1982) with 2 mM each of ATP and Ap_4A in the synthetase reaction; however, instead of extracts of interferon-treated HeLa cells, extracts of interferon-treated mouse L cells were used as a synthetase source, and the synthetase was bound to poly(I)·poly(C)–Sepharose rather than to poly(I)·poly(C)–cellulose. HPLC analysis of the reaction was carried out in a μBondapak C_{18} column as shown in Figure 1a. The result was very similar to that of Cayley & Kerr (1982), who also used a μBondapak column for HPLC analyses. The enzymatically prepared putative A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A appeared approximately 1 and 2 min, respectively, after the peak of unreacted Ap_4A. The retention times of the enzymatically prepared polyphosphates matched the retention times of the chemically synthesized materials (Table III). However,

Table III: HPLC Retention Times of Synthetic Capped 2–5A Analogues As Compared to Those of Some Standard Compounds[a]

compound	retention time (min)
p5′A (AMP)	7.89
pp5′A (ADP)	6.96
ppp5′A (ATP)	6.36
p5′A2′p5′A2′p5′A	14.79
pp5′A2′p5′A2′p5′A	13.50
ppp5′A2′p5′A2′p5′A	12.93
A5′pppp5′A	14.18
A5′pppp5′A2′p5′A	15.23
A5′pppp5′A2′p5′A2′p5′A	16.36
A5′pppp5′A2′p5′A2′p5′A2′p5′A	17.44
A5′ppp5′A2′p5′A2′p5′A	16.63
A5′pp5′A2′p5′A2′p5′A	18.02

[a] Conditions: μBondapak C_{18} column (4.6 mm × 25 cm); (solvent A) 50 mM ammonium phosphate (pH 7.0); (solvent B) methanol–H_2O (1:1); gradient 0–50% B (in 25 min); flow rate 1.0 mL/min.

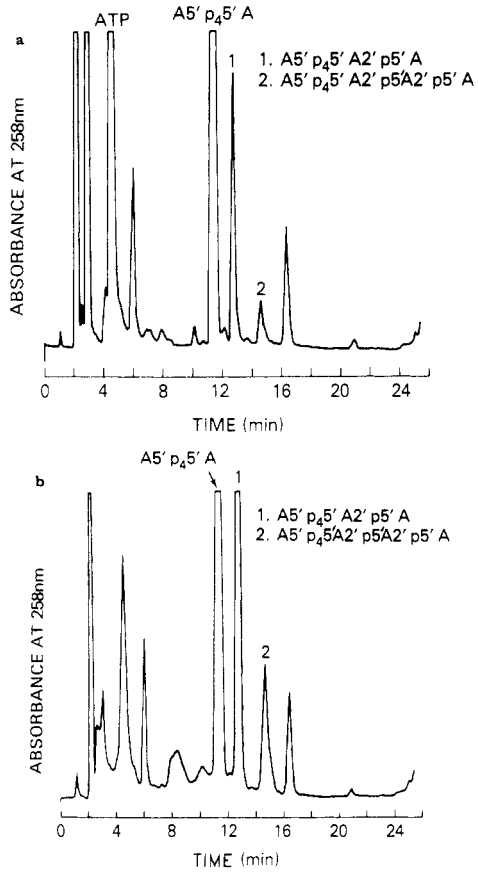

FIGURE 1: (a) Enzymatic synthesis of A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A from Ap_4A and ATP. The 2–5A synthetase of mouse L cell extracts was adsorbed to poly(I)·poly(C)–Sepharose, and synthesis of Ap_4A adducts was according to the conditions described by Cayley & Kerr (1982). After reaction, the Sepharose was centrifuged down, and the supernatant was boiled for 5 min and then centrifuged. The supernatant was analyzed by HPLC on a μBondapak C_{18} column (4.6 mm × 25 cm) with a 0–35% B 25-min linear gradient where solvent A was 50 mM ammonium phosphate (pH 7.0) and solvent B was methanol–water (1:1). (b) Identity of chemically synthesized A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A with enzymatically synthesized material. Authentic synthetic tetraphosphates were coinjected with the enzymatic reaction mixture and the components separated according to the program of (a). The positions of elution of ATP, Ap_4A, and authentic A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A are indicated on the chromatogram.

definitive proof of their identity was provided when one or the other chemically synthesized tetraphosphate was coinjected with the enzymic reaction mixture. Figure 1b shows such an experiment wherein both chemically prepared A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A were injected with the enzyme reaction mixture. The two peaks (~15.2 and 16.3 min) assigned by Cayley & Kerr (1982) to A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2p5′A, respectively, were specifically enhanced just as they were in experiments wherein only one or the other chemically prepared adduct was coinjected (not illustrated). Table III provides characteristic μBondapak HPLC retention times of the various synthetic polyphosphates as compared to those of some standard reference materials.

Biological Activities of the Capped 2–5A Analogues. As one measure of their ability to interact with the 2–5A-dependent endonuclease, the potential of each of the synthetic capped 2–5A analogues to inhibit translation in an encephalomyocarditis virus RNA programmed mouse L cell free system was examined. For comparison purposes, diadenosine tetraphosphate and the 2–5A dimer and trimer triphosphates, ppp5′A2′p5′A and ppp5′A2′p5′A2′p5′A, were included as

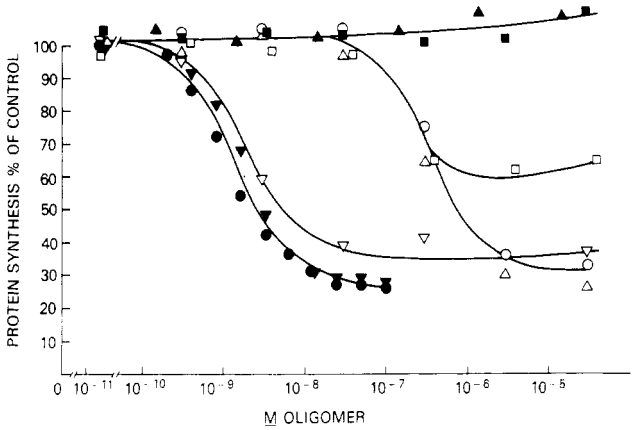

FIGURE 2: Inhibition of translation in extracts of mouse L cells programmed with encephalomyocarditis virus RNA: ppp5′A2′p5′A2′p5′A (●); ppp5′A2′p5′A (○); AppppA (▲); A5′pppp5′A2′p5′A (△); A5′pppp5′A2′p5′A2′p5′A (▼); A5′pppp5′A2′p5′A2′p5′A2′p5′A (▽); A5′pp5′A2′p5′A2′p5′A (■); A5′ppp5′A2′p5′A2′p5′A (□).

controls. The results as presented in Figure 2 can be compared in terms of IC_{50}'s, the concentration of analogue needed to effect a half-maximal inhibition of protein synthesis. In accord with many previous studies, ppp5′A2′p5′A2′p5′A was a potent inhibitor of translation causing a half-maximal inhibition at about 10^{-9} M. Two analogues were virtually as active as 2–5A trimer triphosphate itself: these included A5′pppp5′A2′p5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A2′-p5′A. Also in agreement with earlier work, ppp5′A2′p5′A was much less effective as a protein synthesis inhibitor than 2–5A trimer triphosphate. The 2–5A analogue A5′ppp5′A2′p5′A2′p5′A showed about the same activity as the 2–5A dimer triphosphate but differed in the extent to which it was able to inhibit translation. The tetraphosphate A5′pppp5′A2′p5′A on the other hand behaved virtually identically with the unmodified dimer triphosphate ppp5′A2′p5′A. Finally, neither A5′pp5′A2′p5′A2′p5′A nor Ap_4A itself showed any inhibition of translation at any concentration. In summary, the oligoadenylates cold be listed in the following (decreasing) order of translational inhibitory activity with their IC_{50} values listed in parentheses: ppp5′A2′p5′A2′p5′A (1.3×10^{-9} M) ≥ A5′pppp5′A2′p5′A2′p5′A (1.8×10^{-9} M) ≥ A5′pppp5′A2′p5′A2′p5′A2′p5′A (2×10^{-9} M) ≫ A5′ppp5′A2′p5′A2′p5′A (2×10^{-7} M) ≥ A5′pppp5′A2′p5′A (3×10^{-7} M) ≥ ppp5′A2′p5′A (4×10^{-7} M) ≫ A5′pp5′A2′p5′A2′p5′A (≫3×10^{-5} M) ~ A5′pppp5′A (≫3×10^{-5} M).

While the protein synthesis inhibition assay described above represents a combination of an oligoadenylate's ability to bind to and to activate the 2–5A-dependent endonuclease, the radiobinding assay developed by Knight et al. (1980) provides an estimate of binding ability only since it is based on the displacement of the radiolabeled probe ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′C3′p from an endonuclease–nitrocellulose complex. This assay was carried out with the endonuclease of mouse L cell extracts, and the results are presented in Figure 3. With only one exception (A5′pppp5′A2′p5′A), all of the capped 2–5A analogues were bound as well or nearly as well as 2–5A trimer triphosphate to the endonuclease. An IC_{50} could be defined in this assay as the concentration of oligomer required to displace 50% of the radiolabeled probe. The compounds evaluated could be listed in the following (descending) order of activity with the respective IC_{50} values listed in parentheses:

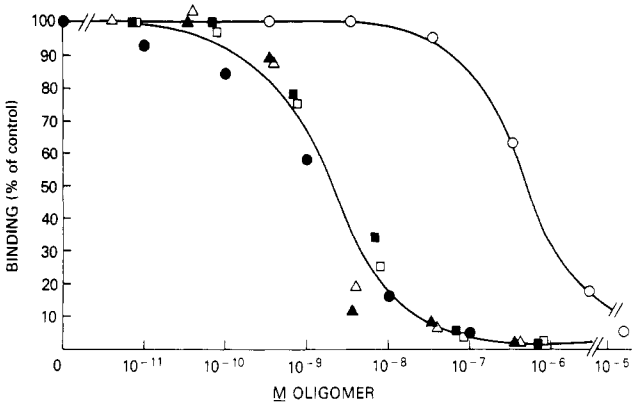

FIGURE 3: Radiobinding assay. Ability of various 5'-capped 2–5A derivatives to displace ppp5'A2'p5'A2'p5'A2'p5'A3[^{32}P]p5'Cp from the endonuclease of mouse L cell extracts according to the procedure of Knight et al. (1980): ppp5'A2'p5'A2'p5'A (●); A5'pppp5'A2'p5'A (○); A5'pppp5'A2'p5'A2'p5'A2'p5'A (▲); A5'pppp5'A2'p5'A2'p5'A (△); A5'pp5'A2'p5'A2'p5'A (■); A5'ppp5'A2'p5'A2'p5'A (□).

ppp5'A2'p5'A2'p5'A (1.5×10^{-9} M) ~ A5'pppp5'A2'p5'A2'p5'A (1.5×10^{-9} M) ~ A5'pppp5'A2'p5'A2'p5'A2'p5'A (1.6×10^{-9} M) ≥ A5'ppp5'A2'p5'A2'p5'A (2.5×10^{-9} M) ≥ A5'pp5'A2'p5'A2'p5'A (2.5×10^{-9} M) ≫ A5'pppp5'A2'p5'A (5×10^{-7} M).

Degradation of Capped 2–5A Analogues in Extracts of Mouse and Human Cells. The degradation of the various capped 2–5A analogues was studied under conditions of the radiobinding assay as well as under protein synthesis conditions. First, the nature of the degradation products of A5'pppp5'A2'p5'A2'p5'A was established by carrying out the reaction under radiobinding assay conditions (2-h incubation at 0 °C) and determining the product spectrum by HPLC in a μBondapak C_{18} column. Figure 4a presents such an HPLC run. Components were identified from their retention times and were confirmed by coinjection of authentic material with the degradation mixture to obtain enhancement of the assigned peak. When the degradation of A5'pppp5'A2'p5'A2'p5'A was studied under the above conditions at an initial starting concentraion of 10^{-4} M capped analogue and 20% mouse L cell extract in the reaction mixture, the components listed in Figure 4a could be identified as degradation products; i.e., p5'A2'p5'A2'p5'A, ppp5'A2'p5'A2'p5'A, 5'ATP, 5'ADP, and 5'AMP. The latter three nucleotides also were present in cell extracts but were increased by 23, 50, and 81%, respectively, when A5'pppp5'A2'p5'A2'p5'A was incubated at 10^{-4} M with 20% L cell extract under radiobinding assay conditions (data not illustrated). Under radiobinding assay conditions, the half-life of A5'pppp5'A2'p5'A2'p5'A was approximately 2 h; however, when protein synthesis conditions were used at a temperature of 30 °C, degradation was greatly accelerated (Figure 4b). Under these conditions, the half-life of A5'pppp5'A2'p5'A2'p5'A was reduced to approximately 3 min. The disappearance of the capped 2–5A analogue was paralleled by the appearance of the two degradation products ppp5'A2'p5'A2'p5'A and p5'A2'p5'A2'p5'A. When compared to the unsymmetrical di- or triphosphates, A5'pp5'A2'p5'A2'p5'A or A5'ppp5'A2'p5'A2'p5'A, under protein synthesis conditions, it was clear (Figure 4c) that the tetraphosphate A5'pppp5'A2'p5'A2'p5'A ($t_{1/2}$ = 3 min) was much more rapidly degraded than either A5'pp5'A2'p5'A2'p5'A ($t_{1/2}$ = 27 min) or A5'ppp5'A2'p5'A2'p5'A ($t_{1/2}$ = 50 min). Degradation products of the unsymmetrical triphosphate A5'ppp5'A2'p5'A2'p5'A included p5'A2'p5'A2'p5'A and

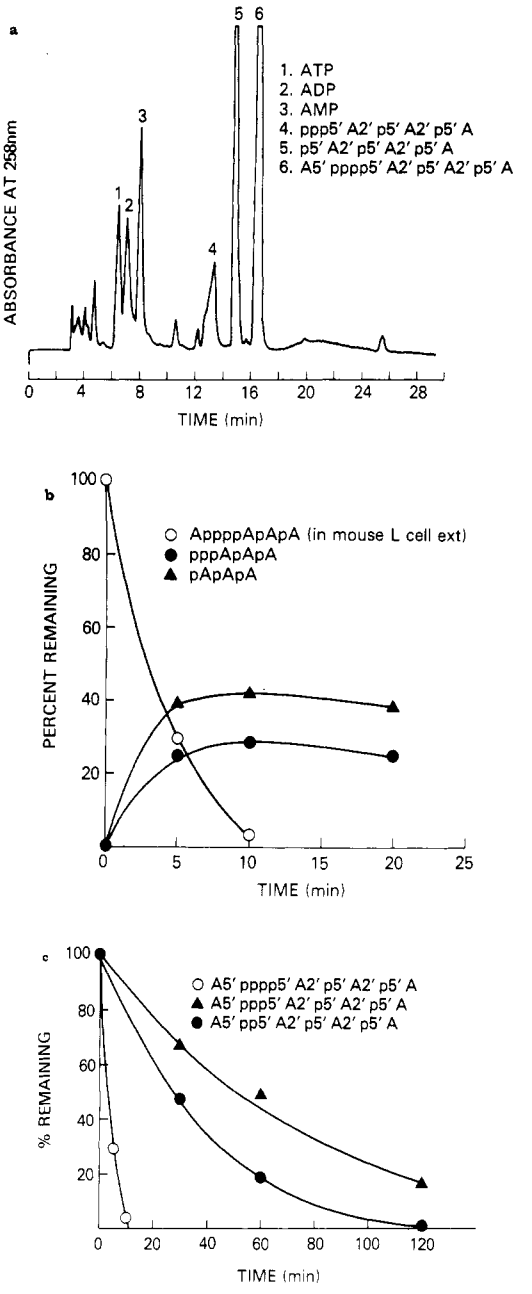

FIGURE 4: (a) Degradation of A5'pppp5'A2'p5'A2'p5'A by the enzymes of mouse L cell extract under radiobinding assay conditions except that no ATP was present: i.e., 0 °C, 2 h, 10 μL of extract, 10 μL of 2–5A analogue in H_2O, 30 μL of 85 mM KCl, 20 mM Tris-HCl (pH 7.6), 5 mM magnesium acetate, and 5% glycerol. After incubation, the mixture was boiled for several minutes and centrifuged to remove denatured protein. Analysis was by HPLC with a μBondapak C_{18} column (4.6 mm × 25 cm) and a 25-min linear gradient of 0–50% B where B was methanol–water (1:1) and A was 50 mM ammonium phosphate (pH 7.0). Assigned peaks are so indicated on the chromatogram. These were determined from the retention times of authentic samples and by coinjection of authentic material to obtain peak enhancement. (b) Degradation of A5'pppp5'A2'p5'A2'p5'A in extracts of mouse L cells under conditions of protein synthesis except that no encephalomyocarditis RNA, creatine kinase, or added energy mix (ATP, CTP, GTP, creatine phosphate) was added. Conditions, including 30 °C incubation temperature, were otherwise the same as those described by Torrence & Friedman (1979). Aliquots were removed at the indicated times and, after being boiled and centrifuged, were analyzed by HPLC according to the program of (a): A5'pppp5'A2'p5'A2'p5'A (○); ppp5'A2'p5'A2'p5'A (●); p5'A2'p5'A2'p5'A (▲). (c) Comparison of degradation of A5'pppp5'A2'p5'A2'p5'A (○), A5'ppp5'A2'p5'A2'p5'A (▲), and A5'pp5'A2'p5'A2'p5'A (●) under protein synthesis conditions with extracts of mouse L cells. Aliquots was removed at the indicated times and assayed as described in the legend to (b).

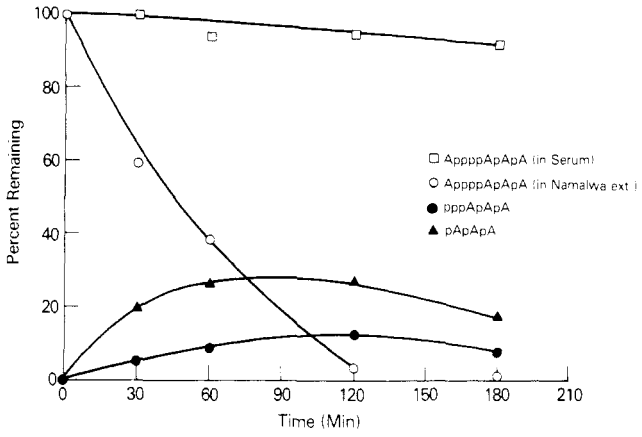

FIGURE 5: Comparison of degradation of A5′pppp5′A2′p5′A2′p5′A by the enzymes of human serum (□) or by the enzymes of Namalwa cell extracts (○). Degradation was at 30 °C under conditions of protein synthesis as described in Figure 4. Since the human serum extract contained nearly twice the amount of protein as the Namalwe extract as judged by OD_{280} readings, it was diluted with an appropriate amount of saline to normalize protein concentration before assay. Samples were removed at the indicated times and analyzed according to the legend of Figure 4. The concentrations of the degradation products were also determined in this way: ppp5′A2′p5′A2′p5′A (●); p5′A2′p5′A2′p5′A (▲).

5′AMP, but no ppp5′A2′p5′A2′p5′A was detected. In the case of A5′pp5′A2′p5′A2′p5′A, no tri- or monophosphate of 2–5A core was detected; rather, 5′AMP was a major product of degradation along with an unidentified product appearing at an approximately 17.5-min retention time (data not shown). Under radiobinding assay conditions (0 °C), both A5′pp5′A2′p5′A2′p5′A and A5′ppp5′A2′p5′A2′p5′A did not undergo detectable degradation even after 2-h incubation.

The degradation of the tetraphosphate A5′pppp5′A2′p5′A2′p5′A also was studied under protein synthesis conditions with extracts of a human Nalmalwa cell line as compared to human serum (Figure 5). Degradation of the tetraphosphate by Nalmalwa cell extracts was slower than that seen in the case of mouse L cell extracts, but this could have been in part related to the lowered protein content of the Nalmalwa extracts. When, however, incubation mixtures containing human serum or Nalmalwa cell extract were normalized for protein content, it was clear that A5′pppp5′A2′p5′A2′p5′A was dramatically more stable in the presence of serum than in the presence of Nalmalwa cell extract (Figure 5). The products of degradation with Nalmalwa cell extract were the same as those in the case of mouse L cell extract (data not shown and Figure 5).

Discussion

Adenosine capped analogues of 2–5A can be obtained by reaction of 5′AMP or 5′ADP with the 5′-(phosphoimidazolidate) of p5′A2′p5′A2′p5′A or by reaction of the trimetaphosphate of 5′ATP with an oligoadenylate 5′-monophosphate. With these procedures, A5′pp5′A2′p5′A2′p5′A, A5′ppp5′A2′p5′A2′p5′A, A5′pppp5′A2′p5′A, A5′pppp5′A2′p5′A2′p5′A, and A5′pppp5′A2′p5′A2′p5′A were prepared. The tetraphosphates A5′pppp5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A were identical with two of the products of the reaction of diadenosine tetraphosphate with 5′ATP as catalyzed by 2–5A synthetase, thus proving the structures originally asisgned by others (Cayley & Kerr, 1982; Ball & White, 1980; Ball, 1980; Ferbus et al., 1981; Figure 1).

Of the five capped 2–5A derivatives synthesized, only two, A5′pppp5′A2′p5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A2′-p5′A, possessed substantial activity as inhibitors of translation (Figure 2). Cayley & Kerr (1982) reported previously that both of these tetraphosphates, obtained as products of 2–5A synthetase, could inhibit protein synthesis as well as 2–5A itself in extracts of Ehrlich ascites tumor cells and could activate the endonuclease of rabbit reticulocyte lysates. In concert with these data, both A5′pppp5′A2′p5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A2′p5′A were comparable to 2–5A in their ability to bind to the 2–5A-dependent endonuclease of mouse L cells (Figure 3). Since, however, these tetraphosphate analogues of 2–5A were degraded to 2–5A itself, both under conditions of the protein synthesis assay and under conditions of the radiobinding assay (Figures 4 and 5), it was not possible to say if the capping is truly consistent with endonuclease binding and activation.

The unsymmetrical triphosphate A5′ppp5′A2′p5′A2′p5′A was bound to the 2–5-dependent endonuclease about as well as 2–5A itself (Figure 3); however, it was nearly 200 times less active than 2–5A as an inhibitor of translation. No evidence for degradation of A5′ppp5′A2′p5′A2′p5′A to ppp5′A2′p5′A2′p5′A could be obtained by HPLC analysis (see results); only AMP and p5′A2′p′5A2′p5′A could be detected. The evidence is therefore strong that this analogue remains intact under conditions of protein synthesis or of the radiobinding assay. In this case, the adenosine cap on the 2–5A triphosphate structure did not impede binding to the 2–5A-dependent endonuclease but did prevent its subsequent activation to give rise to inhibition of translation.

The unsymmetrical diphosphate A5′pp5′A2′p5′A2′p5′A also was bound to the 2–5A-dependent endonuclease as well as 2–5A (Figure 3). This molecule was completely devoid of protein synthesis inhibitory activity (Figure 2). A5′pp5′A2′p5′A2′p5′A acted rather similarly to another unsymmetrical diphosphate, P^1-(D-mannopyranos-6-yl) P^2-[adenylyl(5′→2′)adenylyl(5′→2′)adenos-5′-yl diphosphate, which was an effective antagonist of 2–5A action (Torrence et al., 1982; K. Lesiak and P. F. Torrence, unpublished results). The remaining analogue, A5′pppp5′A2′p5′A, was bound to the 2–5A-dependent endonuclease 500 times less strongly than 2–5A (Figure 3) and, in addition, could not activate the enzyme (Figure 2). Thus, for all the capped analogues examined, optimal binding to the 2–5A-dependent endonuclease occurred regardless of the nature of the cap so long as the P^2-substituent was a 2′,5′-oligoadenylate trimer or tetramer. The behavior of these capped 2–5A derivatives therefore was similar to that of the unmodified 2′,5′-oligoadenylate in that 2–5A dimer bound to but did not activate the endonuclease (Kerr & Brown, 1978; Hoavanesian et al., 1979; Williams & Kerr, 1978; Haugh et al., 1983; P. F. Torrence, I. Imai, and K. Lesiak, unpublished observations). In addition, these results show that blocking of the β- and γ-phosphates of the translational inhibitors pp5′A2′p5′A2′p5′A or ppp5′A2′p5′A2′p5′A leads to a loss of ability to activate the 2–5A-dependent endonuclease even though the oligomers bind to the endonuclease as well as 2–5A itself.

An interesting possibility is raised by the data of Figure 5, which showed that while A5′pppp5′A2′p5′A2′p′A was virtually undegraded by the enzymes of human serum, it was quickly cleaved to the monophosphate p5′A2′p5′A2′p5′A and 2–5A itself, ppp5′A2′p5′A2′p5′A, by the enzyme(s) of extracts of human Nalmalwa cells. Thus, capping the 2–5A molecule may provide a means of modifying the 2–5A structure with a 5′-terminus that would be resistant to degradation while in serum or the external milieu of the cell but which quickly would give rise to free 2–5A once the molecule had penetrated

the cell. Since 2–5A is quickly degraded by a phosphodiesterase (Williams et al., 1978; Schmidt et al., 1978), even higher concentrations of protein synthesis inhibitory 2–5A-like molecules may be achieved by chemical modification [e.g., Imai et al. (1982)] prior to or after the capping reaction.

Since mouse L cell S10 extracts were used as a source of the 2–5A-dependent endonuclease, the degradation of the various capped 2–5A analogues was a factor to consider in any attempt to relate oligonucleotide structure to binding to and activation of the 2–5A-dependent endonuclease. Since both A5′pppp5′A2′p5′A2′p5′A and A5′pppp5′A2′p5′A2′p5′A2′p5′A were degraded to free 2–5A molecules [ppp5′A2′-(p5′A2′)$_n$p5′A] under conditions of the radiobinding assay or of the protein synthesis assay, it was not possible to ascertain if these compounds could bind to or activate the 2–5A-dependent endonuclease; however, the lability of these tetraphosphates has suggested a possible approach to latentiation of the 2–5A structure (vide supra). On the other hand, the capped diphosphate, A5′pp5′A2′p5′A2′p5′A, or the capped triphosphate, A5′ppp5′A2′p5′A2′p5′A, was not detectably degraded under conditions of the radiobindiing assay. It was, therefore, possible to conclude that the capping modification did not adversely affect the binding of either pp5′A2′p5′A2′p5′A or ppp5′A2′p5′A2′p5′A to the 2–5A-dependent endonuclease. In addition, both A5′pp5′A2′p5′A2′p5′A and A5′ppp5′A2′p5′A2′p5′A were relatively stable under conditions of the protein synthesis assay ($t_{1/2}$'s of 27 and 50 min, respectively). Thus, it could be concluded that the capping modification was not consistent with effective activation of the 2–5A-dependent endonuclease. These results will have to be extended when sufficient purified enzyme is available; however, at this point, it is of interest to note that activation of the 2–5A-dependent endonuclease by a capped analogue could be achieved only when that analogue underwent degradation to the free 2–5A structure.

Registry No. p5′A2′p5′A2′p5′A, 61172-40-5; 2′–5′(pA)$_3$, 65954-93-0; CDI, 530-62-1; imidazole, 288-32-4; A5′pp5′A2′p5′A2′p5′A, 87841-74-5; AMP, 61-19-8; A5′ppp5′A2′p5′A2′p5′A, 87841-73-4; ADP, 58-64-0; ATMP, 53355-60-5; ATP, 56-65-5; DCC, 538-75-0; A5′pppp5′A2′p5′A2′p5′A, 77063-72-0; A5′pppp5′A2′p5′A, 81674-97-7; A5′pppp5′A2′p5′A2′p5′A2′p5′A, 81689-40-9; 2′–5′(pA)$_2$, 65954-94-1; 2′–5′(pA)$_4$, 65954-95-2; Ap$_4$A, 5542-28-9; pp5′A2′p5′A2′p5′A, 76991-64-5; (2′→5′)-oligoadenylate synthetase, 69106-44-1; ribonuclease L, 76774-39-5.

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Modulation of the Actin-Activated Adenosinetriphosphatase Activity of Myosin by Tropomyosin from Vascular and Gizzard Smooth Muscles†

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ABSTRACT: Tropomyosins from bovine aorta and pulmonary artery exhibit identical electrophoretic patterns in sodium dodecyl sulfate but differ from tropomyosins of either chicken gizzard or rabbit skeletal muscle. Each of the four tropomyosins binds readily to skeletal muscle F-actin as indicated by their sedimentation with actin and by their ability to maximally stimulate or inhibit actin-activated ATPase activity at a molar ratio of one tropomyosin per seven actin monomers. Smooth and skeletal muscle tropomyosins differ in their effects on activity of skeletal myosin or heavy meromyosin (HMM); the former can enhance activity under conditions in which the latter inhibits. Gizzard and arterial tropomyosins are usually equally effective in stimulating ATPase activity of skeletal acto-HMM, but at high concentrations of Mg^{2+} gizzard tropomyosin is more effective, a result that cannot be attributed to differences in the binding of the two tropomyosins to F-actin. The effects of tropomyosin also depend on the type of myosin; tropomyosin enhances activity of gizzard myosin under conditions in which it inhibits that of skeletal myosin. Increasing the pH or the Mg^{2+} concentration can reverse the effect of tropomyosin on actin-stimulated ATPase activity of skeletal HMM from activation to inhibition, but this reversal is not found with gizzard myosin. Activity in the absence of tropomyosin is independent of pH, and the loss of activation with increasing pH is not accompanied by loss of binding of tropomyosin to actin.

In skeletal muscle, tropomyosin plays a direct role in Ca^{2+}-dependent regulation of the ATPase[1] activity of actomyosin, acting together with troponin to inhibit activity in the absence but not in the presence of Ca^{2+} (Ebashi et al., 1969). Many of the structural studies on Ca^{2+}-dependent regulation (Haselgrove, 1972; Huxley, 1972; Taylor & Amos, 1981) indicate that in the absence of Ca^{2+}, tropomyosin moves to a position on the actin filament where it sterically blocks the binding of the myosin head. Recent biochemical findings show that at very low ionic strengths removal of Ca^{2+} produces inhibition of acto-S-1 catalyzed hydrolysis of ATP but does not inhibit the binding of S-1 to regulated actin (Chalovich et al., 1981; cf. Wagner & Giniger, 1981), a finding not readily explained by a simple steric blocking model.

In smooth muscle the role of tropomyosin is less clear. Smooth muscle tropomyosin stimulates rather than inhibits activity (Chacko et al., 1977; Hartshorne et al., 1977; Ebashi et al., 1977; Sobieszek & Small, 1977) and according to most reports plays no direct role in Ca^{2+}-dependent activation of actomyosin ATPase activity, which requires enzymatic phosphorylation of the myosin light chain (Chacko et al., 1977; Gorecka et al., 1976; Sobieszek & Small, 1976). A possible link to Ca^{2+}-dependent regulation in smooth muscle is suggested by the requirement of both Ca^{2+} and tropomyosin for optimal actin-activated ATPase activity with phosphorylated smooth muscle myosin (Chacko et al., 1977; Chacko & Rosenfeld, 1982; Nag & Seidel, 1983), while a report of a similar requirement with unphosphorylated myosin (Ebashi et al., 1977) has not yet been confirmed.

In addition to stimulating the actomyosin ATPase of smooth muscle, tropomyosin activates or potentiates the activity of several other actomyosin systems including those of rabbit skeletal muscle (Bremel et al., 1972; Shigekawa & Tonomura, 1972) and Limulus (Lehman & Szent-Gyorgyi, 1972). The resemblence of the activation by tropomyosin in skeletal and smooth muscle (Bremel et al., 1972; Chacko et al., 1977;

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[1] Abbreviations: ATPase, adenosinetriphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HMM, heavy meromyosin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; S-1, subfragment 1; TM, tropomyosin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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