Towbin, H., Staehelin, T., & Godon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Tsu, T.-T., & Herzenberg, L. A. (1980) in *Selected Methods in Cellular Immunology* (Mishell, B. B., & Shiigi, S. M., Eds.) pp 373–397, W. H. Freeman, San Francisco, CA.
Van Duin, J., & Wijnands, R. (1981) *Eur. J. Biochem. 118*, 615–619.
Van Duin, J., Van Dieijen, G., Zipori, P., & Van Prooijen, W. (1979) *Methods Enzymol. 60*, 436–446.
Zinker, S. (1980) *Biochim. Biophys. Acta 606*, 76–82.

Synthesis and Biological Activity of Tubercidin Analogues of ppp5′A2′p(5′A2′p)$_n$5′A†

Jean-Claude Jamoulle, Jiro Imai, Krystyna Lesiak, and Paul F. Torrence*

ABSTRACT: A series of tubercidin (7-deazaadenosine) analogues of 2–5A of the general formula p5′(c^{7}A)2′p[5′(c^{7}A)-2′p]$_n$5′(c^{7}A) (n = 0–5) were prepared by lead ion catalyzed polymerization of the 5′-phosphoroimidazolidate of tubercidin. Through the corresponding imidazolidates, these oligonucleotide 5′-monophosphates were converted to the 5′-triphosphates. All reported structures were corroborated by enzyme digestion and ^{1}H or ^{31}P nuclear magnetic resonance. When evaluated for its ability to bind to the 2–5A-dependent endonuclease of mouse L cells, the tubercidin analogue of trimeric 2–5A, namely, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), and the corresponding tetramer were bound as effectively as 2–5A itself; nonetheless, it and the corresponding tetramer, ppp5′-(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), failed to stimulate the 2–5A-dependent endonuclease as judged by its inability to inhibit translation in extracts of mouse L cells programmed with encephalomyocarditis virus RNA and to give rise to ribosomal RNA cleavage in the same cell system under conditions where 2–5A showed activity at 10^{-9} M. The trimer, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), was an antagonist of 2–5A action in the L cell extract. In the lysed rabbit reticulocyte system, both the trimeric and tetrameric tubercidin 2–5A analogues were bound to the 2–5A-dependent endonuclease as well as 2–5A, but in this case, the tetramer triphosphate, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), was just as potent an inhibitor of translation as 2–5A tetramer triphosphate. Moreover, this inhibition was prevented by the established 2–5A antagonist p5′A2′p5′A2′p5′A. The tubercidin analogues of 2–5A also were bound to the endonuclease of Daudi lymphoblastoid cells albeit slightly less effectively than 2–5A. Thus, it appears that the purine N(7) moieties of 2–5A are not involved in binding to the 2–5A-dependent endonuclease but one or more are required for activation of the 2–5A-dependent endonuclease of mouse L cells. These results also serve to underscore the inherent differences between the mouse L cell enzyme and the rabbit reticulocyte RNase L.

The probable role of the unique oligoribonucleotide 2–5A[1] (Kerr & Brown, 1978) and its associated enzymes, 2–5A synthetase,[1] RNase L, and a 2′,5′-phosphodiesterase, in the antiviral action of interferon [reviewed by Revel (1979), Lengyel (1982), Sen (1982) and Torrence (1982)] and the possible role of the above elements in the antiproliferative effects of interferon (Kimchi et al., 1981), as well as in cellular regulation of growth or differentiation (Stark et al., 1979; Etienne-Smekens et al., 1983), have stimulated considerable interest in the synthesis and biological activities of 2–5A analogues [reviewed by Imai & Torrence (1983) and Johnston & Torrence, 1984)]. Although a number of modifications have been executed at the 5′- and/or 2′-terminus (Imai et al., 1982; Silverman et al., 1981; Haugh et al., 1983; Torrence et al., 1982, 1984; Imai & Torrence, 1984) or in the sugar–phosphate backbone (Baglioni et al., 1981; Lesiak et al., 1983; Sawai et al., 1983; Eppstein et al., 1982), relatively little is understood regarding the influence of base modifications on binding to and activation of RNase L.

One base modification of interest is the substitution of a CH moiety for the adenine N(7), resulting in the replacement of adenosine with tubercidin (7-deazaadenosine, 4-amino-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidine). In some instances, the adenine N(7) may be a recognition element in enzymic transformation; for example, tubercidin 5′-triphosphate was not a substrate of adenosine deaminase (Ikehara & Fukui, 1974) or nucleoside phosphorylase (Bloch, 1975). Incorporation of tubercidin or its inosine counterpart, 7-deazainosine, into polynucleotides results in significant changes in interferon-inducing ability (DeClercq et al., 1974; Torrence et al., 1974) and nucleic acid conformation (Ikehara & Fukui, 1968; Bobst et al., 1976; Miles et al., 1979). Finally, Wreschner et al. (1981b) and Floyd-Smith et al. (1981) have noted that the preferred cleavage sites of RNase L all contain uridylate residues, which are complementary to the adenosines of 2–5A. Replacement of adenosine with tubercidin allows a test of the possibility that Hoogsteen hydrogen bonding could be involved in activation of RNase L.

Materials and Methods

Chromatography. Thin-layer chromatography (TLC) was carried out on PEI–cellulose F in 0.25 M NH_4HCO_3 (system a) or on cellulose F in isobutyric acid–concentrated NH_4OH–0.2 M EDTA (100:60:0.8) (system b) or on silica gel Merck 60F254 in 2-propanol–concentrated NH_4OH–H_2O (7:1:2) (system c) or PEI–cellulose F in 0.1 M ammonium bicarbonate (system d). High-performance chromatography (HPLC) was

† From the Laboratory of Chemistry, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Building 4, Room 126, Bethesda, Maryland 20205. *Received August 1, 1983.* J.-C.J. thanks FNRS and NATO for support for a portion of his stay at NIH.

[1] Abbreviations: 2–5A, ppp5′A2′p(5′A2′p)$_n$5′A, where n = 1 to about 10; RNase L, 2–5A-dependent endoribonuclease; 2–5A synthetase, enzyme that, after activation by double-stranded RNA, effects the conversion nATP → pppA(pA)$_{n-1}$ + (n–1)PP$_i$; PEI, poly(ethylenimine); EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Table I: Characteristic Proton NMR Signals of 2',5'-Oligodeazaadenylate 5'-Monophosphates[a]

compd	chemical shifts (ppm)[b] aromatic ring protons C-2 and C-7	protons C-8 and anomeric protons C-1
pc^7A	7.90 (1 H, s), 7.38 (1 H, d, 5 Hz)	6.46 (1 H, d, 5 Hz), 6.03 (1 H, d, 7 Hz)
$Impc^7A$	7.97 (1 H, s), 7.31 (1 H, d, 4 Hz), 7.80 (1 H, s),[d] 7.18 (1 H, s),[d] 6.92 (1 H, s)[d]	6.54 (1 H, d, 4 Hz), 6.12 (1 H, d, 6 Hz)
$(pc^7A)_2$	7.80 (1 H, s), 7.56 (1 H, s), 7.04 (1 H, d, 4 Hz), 6.95 (1 H, d, 4 Hz)	6.08 (1 H, d, 5 Hz), 6.02 (1 H, d, 4 Hz), 5.98 (1 H, d, 4 Hz), 5.75 (1 H, d, 5 Hz)
$(pc^7A)_3$[c]	7.87 (1 H, s), 7.66 (1 H, s), 7.58 (1 H, s), 6.94 (1 H, d, 2.5 Hz), 6.85 (1 H, d, 2.5 Hz), 6.66 (1 H, d, 2.5 Hz)	6.01 (3 H, m), 5.85 (1 H, d, 2.5 Hz), 5.72 (2 H, m)
$(pc^7A)_4$	7.86 (1 H, s), 7.63 (3 H, br m), 7.00 (1 H, d), 6.87 (1 H, d), (1 H, d), 6.69 (1 H, d), 6.62 (1 H, d)	6.04 (1 H, d), 5.97 (2 H, m), 5.76 (4 H, m), 5.24 (1 H, d)

[a] ^1H NMR spectra were determined at 220 MHz in D_2O with acetone (2.05 ppm) as the internal standard. [b] s, singlet; d, doublet; m, multiplet; br, broad. Coupling constants are expressed in hertz and not recorded when less than 2 Hz. [c] Sodium salt. [d] Imidazole ring protons.

performed with a Beckman instrument with Model 110A pumps with columns and solvents indicated in the text. Alternatively, HPLC was with a Waters Associates 440.

Materials. Tubercidin 5'-monophosphoric acid was purchased from Calbiochem. DEAE-Sephadex A-25 was from Pharmacia. Enzymes such as bacterial alkaline phosphatase (BAP) or snake venom phosphodiesterase were from Worthington.

NMR Spectra. Proton NMR spectra (220 MHz) were obtained on a Varian HR220 instrument at 25 °C. The concentration of the samples was 75–250 OD_{270} units in 0.8 mL of D_2O. ^{31}P NMR were recorded with a Jeol FX instrument at 100 MHz.

Biological Activity Studies. The preparation of mouse L cell and human Daudi cell extracts, reticulocytes lysates, and encephalomyocarditis virus RNA, as well as the techniques and conditions for cell-free protein synthesis assays, has been described elsewhere (Torrence & Friedman, 1979; Johnston et al., 1980; Sawai et al., 1983). Radiobinding assays were performed according to Knight et al. (1980) with ppp5'A2'p5'A2'p5'A2'p5'A3'[^{32}P]p5'pC3'p of specific activity 3000 Ci/mmol (Amersham, Chicago, IL). Ribosomal RNA cleavage assays were carried out according to Wreschner et al.(1981a).

Methodology for Determination of Degradation of Oligonucleotides. Stability of the 5'-monophosphates of 2–5A and of 2–5A tubercidin analogues was determined at 30 °C under protein synthesis conditions [10% cell extract, 200 mM HEPES (pH 7.5), 110 mM KCl, 2.0 mM $Mg(OAc)_2$, 0.05 μL/mL β-mercaptoethanol, and water added instead of the other usual components]. The concentration of added oligonucleotides was 2×10^{-5} M. After the indicated incubation time, further degradation of sample was stopped by 2-min heating at 100 °C. After centrifugation, the mixtures were analyzed by HPLC on an Altex ultrasphere column (4.6 mm × 15 cm) with a linear gradient of 0–70% solvent B in solvent A (solvent A, 0.05 M ammonium phosphate, pH 7.0; solvent B, CH_3OH–H_2O, 1:1). The relative concentrations of oligonucleotides were determined from corresponding peak area integrations.

Tubercidin 5'-Phosphoroimidazolidate. Tubercidin 5'-monophosphate (255 mg, 7141 OD_{270} units, 0.7 mmol) was dissolved in dry Me_2SO (5 mL); imidazole (255 mg, 3.75 mmol), triphenylphosphine (392 mg, 1.5 mmol), triethylamine (125 μL), and dipyridyl disulfide (330 mg, 1.5 mmol) were mixed together. The yellow solution was held at ambient temperature for 40 min, and the completion of the reaction was checked by TLC on silica gel with system c. The solution was transferred dropwise to a stirred solution of sodium iodide (565 mg, 3.75 mmol) in dry acetone (25 mL), and the precipitate was collected by centrifugation and washed 3 times with acetone (3 times 4 mL). The resulting sodium salt of tubercidin 5'-phosphoroimidazolidate was dried 3 h in vacuo to give 6090 OD_{270} units (85% on the basis of starting monophosphate) of product. The material was homogeneous as judged by TLC in system c. Its proton NMR characteristics are detailed in Table I.

Preparation of 2',5'-Linked Oligomers of Tubercidin 5'-Monophosphate of the General Formula $(pc^7A)_n$. The sodium salt of tubercidin 5'-phosphoroimidazolidate (6090 OD_{270} units, 261 mg, 0.6 mmol) was dissolved in 0.2 M imidazolium buffer (pH 8.0, 14.4 mL) containing lead nitrate (0.25 M, 0.75 mL). The mixture was stirred for 12 days at 4 °C. The suspension then was treated with Chelex (100–200 mesh, NH_4^+ form, 10 mL) at room temperature for 1 h. After removal and washing (10 mL of H_2O) of the Chelex by filtration, the pH of the filtrate was adjusted to 7.2 with dilute acetic acid (10%). The clear solution was evaporated to dryness in vacuo, the residue was taken up with alcohol (20 mL), and the resulting suspension was filtered. The insoluble precipitate was washed with alcohol (1 mL) and then dried for 3 h in vacuo. The residue was dissolved in a buffer (7 mL) containing 4-morpholinethanesulfonic acid (50 mM, pH 6.0), EDTA (1.0 mM), and ribonuclease P_1 (0.25 mg), and the entire mixture was incubated overnight at 37 °C. The incubation mixture then was heated at 100 °C for 4 min, and the denatured insoluble protein was removed by centrifugation at 10000*g*. Oligodeazaadenylates then were purified from this mixture by HPLC on a reverse-phase column (Zorbax ODS, 9.4 mm × 25 cm). This required 18 injections of approximately 350 OD_{270} units of crude reaction mixture and elution of the column with a linear gradient of 0–35% buffer B in 35 min (buffer A, 50 mM ammonium phosphate, pH 7.0; buffer B; methanol–H_2O, 1:1; flow rate 2.7 mL/min). Oligomers up to octamer were eluted from the column in less than 25 min. Appropriate fractions containing each oligomer were pooled and evaporated in vacuo, and the residues were applied to a DEAE-Sephadex A-25 (HCO_3^-) column to remove phosphate from the HPLC buffer. Elution was with a linear gradient of 0.2–0.5 M (dimer) 0.7 M (trimer or tetramer) triethylammonium bicarbonate (pH 7.5). Fractions containing the oligomer in question were pooled, evaporated to dryness in vacuo at 40 °C, and then taken up in H_2O and reevaporated. This latter process was repeated twice in order to remove all triethylammonium bicarbonate. Finally, the residue was dissolved in anhydrous methanol, and the sodium salt was collected as described for tubercidin 5'-phosphoroimidazolidate (vide supra). The yields (based on OD_{270} units) were as follows: 2',5'-$(pc^7A)_2$, 31.5%; 2',5'-$(pc^7A)_3$, 7.0%; 2',5'-$(pc^7A)_4$, 2.4%. Also obtained from the HPLC column but not further

Table II: Comparison of ^{31}P NMR Chemical Shifts of 2-5A and the Corresponding Tubercidin Analogues[a]

compounds	internucleotide phosphate	5'-phosphate
ppp5'A2'p5'A2'p5'A	−1.06, −1.38 (2 P)	−11.38 (d) (1 P), −22.52 (1 P), −6.84 (1 P)
ppp5'(c^7A)2'p5'(c^7A)	−1.01 (1 P)	−11.26 (1 P), −22.60 (1 P), −9.48 (1 P)
ppp5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)	−0.96, −0.96 (2 P)	−11.08 (1 P), −22.10 (1 P), −8.71 (1 P)

[a] NMR values were determined in D_2O with 0.85% H_3PO_4 as external standard.

processed were putative 2',5'-(pc^7A)$_5$ (1.5%), 2',5'-(pc^7A)$_6$ (1%), and 2',5'-(c^7A)$_7$ (<1%). Each oligomer (dimer, trimer, and tetramer) was homogeneous by TLC (systems a and b) and by HPLC.

In addition, each oligomer was completely resistant to the action of RNase P_1 but completely degraded by snake venom phosphodiesterase to give tubercidin 5'-monophosphate as the only detectable product. Proton and phosphorus NMR data for each oligomer are given in Tables I and II.

Synthesis of 5'-Triphosphates and 5'-Diphosphates of the Dimer, Trimer, and Tetramer, 2',5'-(pc^7A)$_n$ (n = 2–4). For example, tubercidin trimer monophosphate [2',5'-(pc^7A)$_3$, 210 OD_{270} units, 8.4 μmol) was dissolved in a dry Me_2SO (600 μL) solution containing imidazole (28.5 mg, 42 μmol), triphenylphosphine (44 mg, 16.8 μmol), triethylamine (1.25 μL), and dipyridyl disulfide (37 mg, 16.8 μmol). The solution was stirred at room temperature for 40 min, and then the sodium salt of the trinucleotide imidazolidate was isolated as described above for the sodium salt of tubercidin 5'-phosphoroimidazolidate. The imidazolidate was dried by repeated evaporations with dry pyridine and finally with dry benzene to remove traces of pyridine. The dried residue was dissolved in DMF (200 μL) and tri-*n*-butylammonium pyrophosphate in DMF (0.5 M, 108 μL, 54 μmol). The reaction was kept under a dry atmosphere at ambient temperature for 24 h.

The DMF then was evaporated in vacuo, and the residue was diluted with triethylammonium bicarbonate buffer (0.25 M, 500 μL). The pH of the solution was adjusted to 7.5, and then the entire solution was applied to a DEAE-Sephadex (HCO_3^-) A-25 column (1 × 20 cm). The column was eluted with a linear gradient of 0.2 (250 mL)–0.5 M (250 mL) triethylammonium bicarbonate (pH 7.6). Fractions containing the triphosphate were pooled and repeatedly evaporated with water to remove triethylammonium bicarbonate. The product, ppp5'(c^7A)2'p5'(c^7A)2'p5'(c^7A) was obtained as the triethylammonium salt in 42% yield (88 OD_{270} units, 3.5 μmol) on the basis of the starting monophosphate. The corresponding 5'-diphosphate was obtained as a byproduct in 19% yield. Both the dimer triphosphate, ppp5'(c^7A)2'p5'(c^7A), and the tetramer triphosphate, ppp5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A), were prepared in the identical manner to give 108 OD_{270} units (45%) of dimer triphosphate and 44 OD_{270} units (52%) of tetramer triphosphate.

Table III: Thin-Layer Chromatographic Mobilities of Synthetic Oligonucleotides

compounds	R_f system a[a]	R_f system b[b]
pA	0.66	0.70
$(pA)_2$	0.63	0.69
$(pA)_3$	0.61	0.65
$(pA)_4$	0.56	0.64
p(c^7A)	0.56	0.75
(pc^7A)$_2$	0.50	0.69
(pc^7A)$_3$	0.32	0.69
(pc^7A)$_4$	0.26	0.64
p(pc^7A)$_2$	0.29	0.62
pp(pc^7A)$_2$	0.14	0.47
p(pc^7A)$_3$	0.22	0.52
pp(pc^7A)$_3$	0.10	0.45
p(pA)$_2$	0.45	0.61
pp(pA)$_2$	0.28	0.39
p(pA)$_3$	0.56	0.50
pp(pA)$_3$	0.30	0.40

[a] PEI-cellulose, 0.25 M NH_4HCO_3. [b] Cellulose F, isobutyric acid–concentrated NH_4OH–EDTA (0.2 M) (100:60:0.8).

Table IV: Column Chromatography and HPLC Characteristics of 2',5'-Oligo-7-deazaadenylates[a]

compounds	TEABC[b] gradient (M/L)	TEABC[b] concn of elution (M/L)	HPLC retention time (min)
pc^7A	0.0–0.3	0.23	6.23
(pc^7A)$_2$	0.2–0.5	0.28	10.28
(pc^7A)$_3$	0.2–0.7	0.39	12.75
(pc^7A)$_4$	0.2–0.7	0.52	15.26
p(pc^7A)$_2$	0.2–0.5	0.32	7.57
pp(pc^7A)$_2$	0.2–0.5	0.38	7.1
p(pc^7A)$_3$	0.2–0.7	0.46	10.68
pp(pc^7A)$_3$	0.2–0.7	0.53	9.6
p(pc^7A)$_4$	0.2–0.8	0.59	13.14
pp(pc^7A)$_4$	0.2–0.8	0.67	12.38
(c^7A)2'p5'(c^7A)2'p5'(c^7A)	0.05–0.25	0.15	24.60

[a] Analytical (HPLC) high-performance liquid chromatography was performed with a Beckman apparatus with a Model 110A pump. The column (Bondapak, 30 × 0.39 cm, C-18) was eluted with a linear gradient of 0–50% buffer B in 25 min (buffer A, 50 mM ammonium phosphate, pH 7.0; buffer B, methanol–H_2O, 1:1; flow rate 1 mL/min). [b] TEABC, triethylammonium bicarbonate.

Results

Synthesis and Characterization of 2',5'-Oligo-7-deazaadenylates. Tubercidin 5'-phosphoroimidazolidate, prepared by a modification of the general method developed by Mukayama & Hashimoto (1971), could be polymerized by lead ion catalysis according to the procedures published by Sawai and collaborators (Sawai & Ohno, 1981 a,b; Sawai et al., 1979, 1981). After digestion with nuclease P_1 to remove 3',5'-phosphodiester linkage isomers and purification by HPLC, the dimer, p5'(c^7A)2'p5'(c^7A), was obtained in 31.5% yield, the trimer, p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A), in 7% yield, and the tetramer, p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A), in 2.4% yield, as well as pentamer, hexamer, and heptamer (all about 1% yield). As expected, each oligomer was resistant to the action of nuclease P_1 but was degraded by snake venom phosphodiesterase to give tubercidin 5'-monophosphate as the only detectable product. In addition, the behavior of the oligo-7-deazaadenylate 5'-monophosphates on TLC (Table III), DEAE column chromatography (Table IV), or HPLC (Table IV) was that to be expected on the basis of earlier results with the parent oligoadenylates (Sawai et al., 1981; P. F. Torrence et al., unpublished results). For each oligomer, the requisite number of C-2, C-7, and C-8 protons as well as anomeric protons further confirmed the assigned structures (Table I). The synthetic triphosphates also showed the requisite number of internucleotide phosphates and the characteristic multiplicity and chemical shifts for the α-, β, and γ-phosphorus atoms of a 5'-triphosphate (Table II). Table V gives the extinction

Table V: Extinction Coefficients of 2',5'-Oligo-7-deazaadenylates[a]

compounds	E_{270}
c^7A	12 200
pc^7A	10 200
$(pc^7A)_2$	8 820
$(pc^7A)_3$	8 330
$(pc^7A)_4$	7 930

[a] Extinction coefficients were determined as $E = [(A_D - A_I)/A_D]E_{pc^7A}$. Hypochromicity was calculated for each oligonucleotide by measurement of the absorbance at 270 nM before (A_I) and after (A_D) digestion with snake venom phosphodiesterase, carried out for 2.5 h at 37 °C in a mixture (50 μL) containing the substrate (pH 8.8), 0.01 M $MgCl_2$, 0.01 M Tris-acetate (pH 8.8), and the enzyme solution (0.1 unit).

coefficients for the compounds.

Biological Activity of the Oligo-7-deazaadenylates. The 5'-triphosphates of the oligo-7-deazaadenylate trimer and tetramer first were evaluated for their ability to bind to the 2–5A-dependent endonuclease (RNase L) of mouse L cells, rabbit reticulocyte lysates, and Daudi lymphoblastoid cells. This binding assay was 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 as first detailed by Knight et al. (1980). These results are summarized in Table VI in which the relative binding ability of each oligomer is presented in terms of the concentration necessary to prevent binding of 50% of the radiolabeled probe to the endonuclease. With enzyme from mouse L cells or rabbit reticulocytes, it was evident that the two tubercidin 2–5A analogues ppp5'-(c^7A)2'p5'(c^7A)2'p5'(c^7A) and ppp5'(c^7A)2'p5'(c^7A)2'p5'-(c^7A)2'p5'(c^7A) were equally effective, within experimental error, at displacing probe and that both tubercidin 2–5A analogues were, again within experimental error, as effective as 2–5A trimer or tetramer 5'-triphosphates or 2–5A trimer 5'-monophosphate in displacing 2–5A[^{32}P]pCp from the endonuclease–nitrocellulose complex. Similarly to the parent oligoadenylate dimer, trimer, and tetramer 5'-monophosphates, p5'A2'p5'A, p5'A2'p5'A2'p5'A, and p5'A2'p5'A2'p5'A2'p5'A, the tubercidin analogues, p5'(c^7A)2'p5'(c^7A), p5'(c^7A)-2'p5'(c^7A)2'p5'(c^7A), and p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)-2'p5'(c^7A), were bound to the 2–5A-dependent endonuclease with the dimer showing an affinity about 4 orders of magnitude less than the trimer and tetramer, which were bound to the endonuclease as well as 2–5A itself.

A slightly different result was revealed when Daudi lymphoblastoid cell extract was used as a source of human enzyme (Table VI). In this case, the tubercidin 2–5A analogues apparently were bound to the endonuclease about 2–4 times less effectively than 2–5A itself.

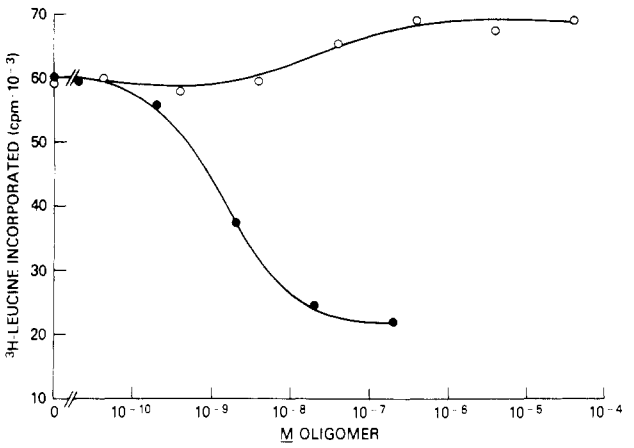

FIGURE 1: Effect of varying concentrations of ppp5'A2'p5'A2'p5'A (●) or ppp5'(c^7A)2'p5'(c^7A)2'p5'(C^7A) (○) on translation in extracts of mouse L cells programmed with encephalomyocarditis virus RNA.

Two different experimental approaches were used to ascertain the ability of the tubercidin analogues to activate the 2–5A-dependent endonuclease. First, the ability of the analogues to inhibit translation in an encephalomyocarditis virus RNA-programmed L cell free system was determined. As is apparent from Figure 1, under conditions where ppp5'A2'p5'A2'p5'A inhibited protein synthesis by 50% at a concentration of 10^{-9} M, the corresponding tubercidin analogue, ppp5'(c^7A)2'p5'(c^7A)2'p5'(c^7A), had no inhibitory effect at any concentration tested. To the contrary, at higher concentrations, a slight stimulatory effect was noted. In this same system, the tubercidin 2–5A tetramer triphosphate, ppp5'-(c^7A)2'p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A), also failed to inhibit translation (data not illustrated). In a second separate approach to determine analogue ability to activate the endonuclease, ribosomal RNA cleavage was examined in the same manner as originally worked out by Wreschner et al. (1981). In this case, cell extracts with or without 2–5A or analogue were incubated under protein synthesis conditions, and the ribosomal RNA was extracted, denatured, and analyzed by electrophoresis on 1.8% agarose gels. RNA was located by ethidium bromide staining. As is apparent from Figure 2, although 2–5A gave significant rRNA cleavage even at 10^{-9} M, the tubercidin analogues, ppp5'(c^7A)2'p5'(c^7A)2'p5'(c^7A) and ppp5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A), caused no rRNA cleavage even at 10^{-5} M.

Two separate lines of evidence suggested that the ability of the tubercidin analogues of 2–5A to inhibit protein synthesis was not due to premature destruction in the protein synthesis incubation conditions. First, the trimer monophosphate, p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A), was degraded at the same rate as the oligoadenylate p5'A2'p5'A2'p5'A itself (Figure 3); in

Table VI: Ability of Oligo-7-deazaadenylates To Bind to the 2–5A-Dependent Endonuclease of Mouse L Cells, Rabbit Reticulocytes, or Daudi Lymphoblastoid Cells As Determined by Radiobinding Assay

oligomer	IC_{50} (M)[a]		
	mouse	rabbit	human (Daudi)
p5'(c^7A)2'p5'(c^7A)	7×10^{-6}		
p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)	8×10^{-10}		
p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)	8×10^{10}		
ppp5'(c^7A)2'p5'(c^7A)2'p5'(c^7A)	6×10^{-10}	1.4×10^{-10}	1×10^{-9}
ppp5'(c^7A)2'p5'(c^7A)2p5'(c^7A)2'p5'(c^7A)	8×10^{-10}	1.5×10^{-10}	$(1.5\text{–}2) \times 10^{-9}$
ppp5'A2'p5'A2'p5'A	5×10^{10}		
ppp5'A2'p5'A2'p5'A2'p5'A		2.5×10^{-10}	6×10^{-10}
p5'A2'p5'A2'p5'A	8×10^{-10}		

[a] IC_{50} is defined as the concentration of oligonucleotide required to displace 50% of the 2–5A[^{32}P]pCp probe from the endonuclease–nitrocellulose complex.

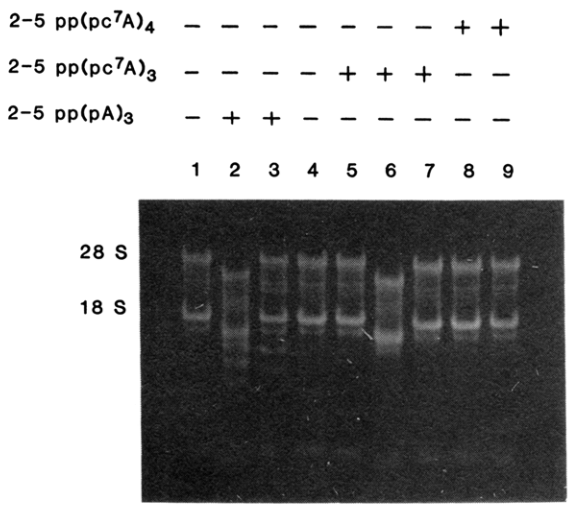

FIGURE 2: Activation of the 2–5A-dependent nuclease of mouse L cells as monitored by rRNA cleavage. Incubation of the extract from interferon-treated cells was for 2 h at 30 °C in the absence of any added oligomers (tracks 1 and 4), in the presence of ppp5′A2′p5′A2′p5′A at 2×10^{-7} M (track 2) or 2×10^{-9} M (track 3), in the presence of ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) at 2×10^{-5} M (track 5), 2×10^{-7} M (track 6), or 2×10^{-9} M (track 7), or in the presence of ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) at 2×10^{-5} M (track 8) or 2×10^{-7} M (track 9). Due to the presence of some salts in the samples of tracks 2 and 6, and rRNA bands moved faster than usual; however, the ladder-like cleavage bands due to 2–5A are clearly present in track 2 but absent from track 6.

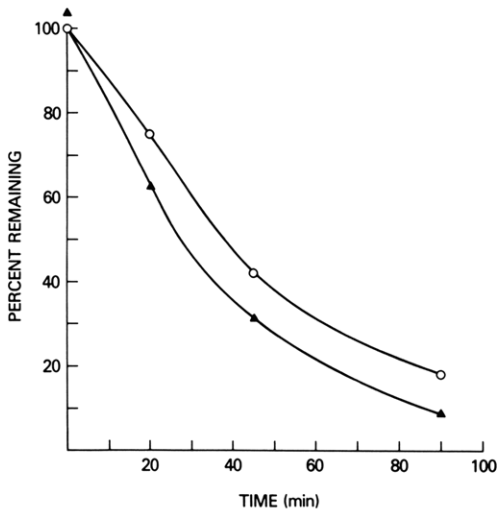

FIGURE 3: Comparison of the rate of degradation of p5′A2′p5′A2′p5′A (○) and p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) (▲) in extracts of mouse L cells under conditions of protein sythesis. Concentrations of oligonucleotides were determined by HPLC as described under Materials and Methods.

addition, under the same protein synthesis conditions, the trimer triphosphates, ppp5′A2′p5′A2′p5′A and ppp5′(c^{7}A)-2′p5′(c^{7}A)2′p5′(c^{7}A), disappeared at the same rate (data not shown). Second, the tetramer, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′-(c^{7}A)2′p5′(c^{7}A), was able to prevent the translational inhibitory effects of ppp5′A2′p5′A2′p5′A itself, thereby functioning as a 2–5A antagonist (data not illustrated) and establishing its persistence under assay conditions.

The ability of the tubercidin analogues to activate the 2–5A-dependent endonuclease also was examined in rabbit reticulocyte lysates by determining their ability to inhibit translation of an endogenous (globin) message. 2–5A trimer triphosphate, ppp5′A2′p5′A2′p5′A, has been found previously to be relatively inactive in reticulocyte lysates (Williams et al., 1979), and in accord with this, the trimeric tubercidin analogue ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) was without activity

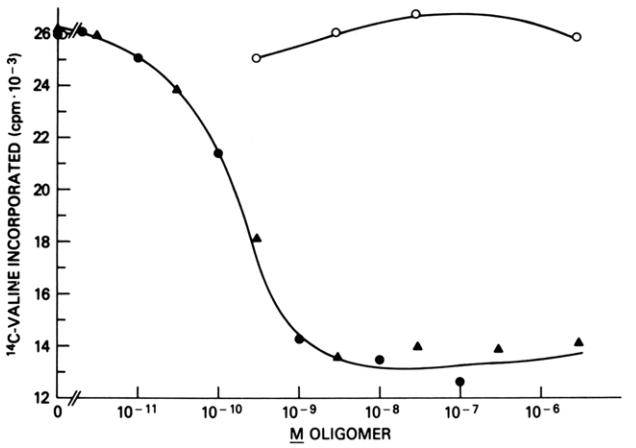

FIGURE 4: Comparison of the ability of ppp5′A2′p5′A2′p5′A2′p5′A (●), ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) (○), and ppp5′(c^{7}A)2′p5′-(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) (▲) to inhibit protein synthesis in rabbit reticulocyte lysates engaged in endogenous globin synthesis. Lysates were incubated in the presence of the indicated concentration of the oligonucleotide, and then the necessary salts, creatine phosphate, creatine phosphokinase, and radioactive and nonradioactive amino acids were added to start translation, which was terminated after 1-h incubation.

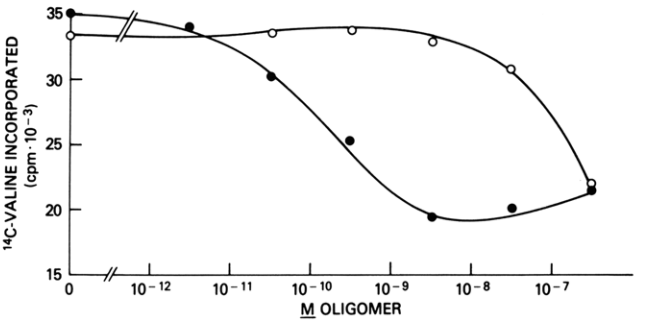

FIGURE 5: Prevention of the protein synthesis inhibitory effects of the tetramer ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) by p5′A2′p5′A2′p5′A in rabbit reticulocyte lysates. The experiment was performed exactly as described in the legend to Figure 4. Lysates were preincubated with either varying concentrations of ppp5′-(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) alone (●) or with ppp5′(c^{7}A)-2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) and p5′A2′p5′A2′p5′A, with the latter at a final concentration of 10^{-6} M (○).

(Figure 4). However, in stark contrast to the results of the mouse L cell experiments (Figures 1 and 2), the tubercidin tetramer 5′-triphosphate, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)-2′p5′(c^{7}A), was just as potent as 2–5A tetramer triphosphate as an inhibitor of protein synthesis (Figure 4). That this inhibition was mediated by the 2–5A-dependent endonuclease was indicated by the ability of an established antagonist of 2–5A action, p5′A2′p5′A2′p5′A (Torrence et al., 1981), to prevent the translational inhibiory effects of ppp5′(c^{7}A)-2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A) (Figure 5) and also by the fact that the tubercidin 2–5A tetramer analogue gave the same characteristic ribosomal RNA cleavage pattern as did 2–5A tetramer triphosphate itself (Figure 6). Finally, neither tubercidin itself nor any other derivative, including tubercidin 5′-monophosphate, tubercidin trimer core, (c^{7}A)2′p5′(c^{7}A)-2′p5′(c^{7}A), or the corresponding dimer, trimer, or tetramer 5′-monophosphates, showed any inhibition of translation, even at concentrations as high as 10^{-4} M.

Discussion

In this paper, we have synthesized, by lead ion catalyzed polymerization of tubercidin 5′-phosphoroimidazolidate, a series of oligo-7-deazaadenylates and their corresponding

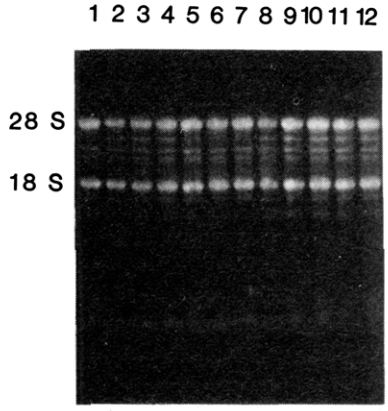

FIGURE 6: Activation of the 2–5A-dependent endonuclease of rabbit reticulocytes as monitored by rRNA cleavage. Conditions were similar to those in Figure 2. The extract was incubated in the presence of the following: (lane 1) no additions, control; (lanes 2–4) 2′,5′-$pppA_4$ at 4×10^{-8}, 4×10^{-9}, and 4×10^{-10} M, respectively; (lane 5) 2′,5′-$pppA_3$ at 4×10^{-7} M; (lane 6) 2′,5′-$ppp(c^7A)_3$ at 4×10^{-5} M; (lane 7) control; (lanes 8–11) 2′,5′-$ppp(c^7A)_4$ at 4×10^{-7}, 4×10^{-8}, 4×10^{-9}, and 4×10^{-10} M, respectively; (lane 12) 2′,5′-$pppA_4$ at 4×10^{-7} M. In lanes 2–5 and 8–12, the 2–5A-specific cleavage band is visible just below the 28S rRNA band [see also Wreschner et al. (1981a)].

5′-triphosphates. All of these products have been characterized by thin-layer chromatography and HPLC, and the assigned structures have been corroborated by proton and/or phosphorus NMR, UV spectral data, and enzymic degradation.

The tubercidin analogues of 2–5A and its derivatives, with the expected exception of the dimer p5′(c^7A)2′p5′(c^7A), all were able to bind to the 2–5A-dependent endonuclease as well as 2–5A itself. This was true for the enzyme of both mouse L cells and rabbit reticulocytes: the tubercidin analogues had a slightly lower affinity for the endonuclease present in Daudi cells. In spite of their high affinities for the mouse enzyme, neither ppp5′(c^7A)2′p5′(c^7A)2′p5′(c^7A) nor ppp5′(c^7A)-2′p5′(c^7A)2′p5′(c^7A)2′p5′(c^7A) was capable of activating the mouse enzyme, although the tetramer was as effective as ppp5′A2′p5′A2′p5′A2′p5′A itself as an inhibitor of protein synthesis in rabbit reticulocyte lysates. Thus, these data imply that none of the purine N(7) moieties of 2–5A are necessary for optimal binding to mouse L cell or rabbit reticulocyte RNase L and have very little influence on binding to the enzyme of Daudi cells. This conclusion is not completely unexpected since it has been shown earlier (Torrence et al., 1984) that the 2–5A trimer monophosphate analogues, p5′U2′p5′U2′p5′U, p5′C2′p5′C2′p5′C, and p5′I2′p5′I2′p5′I, all had low and nearly equal affinities for RNase L, indirectly implying that the purine N(7)'s made no substantial contribution to binding. Second, and perhaps most important, these data demonstrate that one or more of the purine N(7)'s of 2–5A are necessary for activation of the 2–5A-dependent endonuclease of mouse L cells, but that none of the purine N(7) moieties are involved in activation of the enzyme of rabbit reticulocyte lysate.

The divergent behavior of the rabbit reticulocyte RNase L and RNase L of other sources has been observed previously (Williams et al., 1979; Knight et al., 1981; Sawai et al., 1983). Whereas 2–5A trimer triphosphate activates the 2–5A-dependent endonuclease of mouse and human cells, it does not efficiently activate the enzyme of rabbit reticulocytes although it binds just as well as the tetramer, ppp5′A2′p5′A2′p5′A2′p5′A, which is an efficient activator of rabbit reticulocyte RNase L. In addition, Sawai et al. (1983) found that while the cordycepin analogue of 2–5A trimer or tetramer bound to but could not activate the RNase L of mouse L cells, the cordycepin tetramer analogue ppp5′-(3′dA)2′p5′(3′dA)2′p5′(3′dA)2′p5′(3′dA) was only 20–100 times less efficient than 2–5A tetramer triphosphate as an activator of the reticulocyte RNase L. Haugh et al. (1983) also found that the cordycepin 2–5A trimer analogue could not activate the 2–5A-dependent endonuclease; however, Doetsch et al. (1981) claimed that the cordycepin 2–5A trimer was at least as effective or more effective than 2–5A tetramer as an inhibitor of protein synthesis.

Two previous papers have reported on the synthesis of a tubercidin analogue of 2–5A (Hughes et al., 1983; Hughes & Robins, 1983). An enzymatic method involving polymerization of tubercidin 5′-triphosphate by the double-stranded RNA-dependent 2–5A synthetase was used to prepare the putative ppp5′(c^7A)2′p5′(c^7A)2′p5′(c^7A); however, no proof of structure of any kind has been reported. From the data presented, it is difficult to evaluate, qualitatively or quantitatively, the biological activity of the reported tubercidin analogue of 2–5A. First, in calcium phosphate treated L cells, the putative tubercidin 2–5A analogue inhibited translation, albeit not as effectively as 2–5A itself. In this case, no control was run to check for the established cytotoxicity of tubercidin and its nucleotides (Suhadolnik, 1979). In rabbit reticulocyte lysate, it was reported that the tubercidin analogue trimer triphosphate was comparable to 2–5A trimer triphosphate as an inhibitor of translation (Hughes et al., 1983); however, other studies have found that the 2–5A trimer triphosphate was not an effective inhibitor of protein synthesis in the reticulocyte system (Williams et al., 1979; Knight et al., 1981; Sawai et al., 1983). Finally, two assays designed to determine activation of the 2–5A-dependent endonuclease through its degradation of RNA were at odds according to the published results: the tubercidin 2–5A trimer triphosphate did accelerate cleavage of cytoplasmic RNA but did not give ribosomal RNA cleavage.

There are several possible explanations for the dependence of RNase L activation on one or more of the purine N(7) moieties of 2–5A. One is that one or more of these N(7) residues are involved in a protein–oligonucleotide binding interaction that must be completed before enzyme activation is possible. A second possibility is that alteration of the N(7) residues may change the oligonucleotide's conformation, still permitting binding to RNase L but not its activation. A third possibility is that the purine N(7) is involved in Hoogsteen-type hydrogen bonding with the RNA substrate. Floyd-Smith et al. (1981) previously have pointed out that 2–5A-dependent endonuclease cleaves after UpNp residues and that the penultimate nucleotide is complementary (in a Watson–Crick hydrogen binding scheme) to the adenine bases of 2–5A; however, UA matching also can occur via Hoogsteen base pairing. Another possibility may be related to the considerable change in pK_a for the protonated nucleoside when effecting the alteration of adenosine (pK_a = 3.55) (Sober, 1970) to tubercidin (pK_a = 5.2) (Sober, 1970).

Registry No. Tubercidin 5′-monophosphate, 16719-46-3; tubercidin 5′-phosphoroimidazolidate sodium salt, 89656-09-7; endonuclease, 9055-11-2; $(pc^7A)_2$, 84876-74-4; $(pc^7A)_3$, 84876-77-7; $(pc^7A)_4$, 84876-78-8; ppp5′(c^7A)2′p5′(c^7A), 84876-76-6; ppp5′(c^7A)2′p5′-(c^7A)2′p5′(c^7A), 84824-03-3; $p(pc^7A)_2$, 84876-75-5; $p(pc^7A)_3$, 84824-04-4; $p(pc^7A)_4$, 84895-04-5; $pp(pc^7A)_4$, 84876-79-9; (c^7A)-2′p5′(c^7A)2′p5′(c^7A), 89656-10-0.

References

Baglioni, C., D'Alessandro, S. R., Nilsen, T. W., den Hartog, J. A. J., Crea, R., & van Boom, J. H. (1981) *J. Biol. Chem. 256*, 3253–3257.

Bloch, A. (1975) *Ann. N.Y. Acad. Sci. 255*, 576–572.
Bobst, A. M., Torrence, P. F., Kouidou, S., & Witkop, B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3788–3792.
DeClercq, E., Torrence, P. F., & Witkop, B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 182–186.
Doetsch, P., Wu, J. M., Sawada, Y., & Suhadolnik, R. J. (1981) *Nature (London) 291*, 355–358.
Eppstein, D. A., Marsh, Y. V., Schryver, B. B., Larsen, M. A., Barnett, J. W., Verheyden, J. P. H., & Prisbe, E. J. (1982) *J. Biol. Chem. 257*, 13390–13397.
Etienne-Smekens, M., Vandenbussche, P., Content, J., & Dumont, J. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4609–4613.
Floyd-Smith, G., Slattery, E., & Lengyel, P. (1981) *Science (Washington, D.C.) 212*, 1030–1032.
Haugh, M. C., Cayley, P. J., Serafinowska, H. T., Norman, D. G., Reese, C. B., & Kerr, I. M. (1983) *Eur. J. Biochem. 132*, 77–84.
Hughes, B. G., & Robins, R. K. (1983) *Biochemistry 22*, 2127–2135.
Hughes, B. G., Srivastava, P. C., Muse, D. D., & Robins, R. K. (1983) *Biochemistry 22*, 2116–2126.
Ikehara, M., & Fukui, T. (1974) *Biochim. Biophys. Acta 338*, 512–520.
Imai, J., & Torrence, P. F. (1983) *Protein, Nucleic Acid Enzymes 28*, 801–814.
Imai, J., & Torrence, P. F. (1984) *Biochemistry 23*, 766–774.
Imai, J., Johnston, M. I., & Torrence, P. F. (1982) *J. Biol. Chem. 257*, 12739–12745.
Johnston, M. I., & Torrence, P. F. (1984) in *Interferon: Mechanisms of Production and Action* (Friedman, R. M., Ed.) Elsevier, Amsterdam (in press).
Johnston, M. I., Friedman, R. M., & Torrence, P. F. (1980) *Biochemistry 19*, 5580–5585.
Kerr, I. M., & Brown, R. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 256–280.
Kimchi, A., Shure, H., & Revel, M. (1979) *Nature (London) 282*, 849–851.
Knight, M., Cayley, P. J., Silverman, R. H., Wreschner, D. H., Gilbert, C. S., Brown, R. E., & Kerr, I. M. (1980) *Nature (London) 288*, 189–192.
Knight, M., Wreschner, D. H., Silverman, R. H., & Kerr, I. M. (1981) *Methods Enzymol. 79*, 216–227.
Lengyel, P. (1982) *Annu. Rev. Biochem. 51*, 251–282.
Lesiak, K., Imai, J., Floyd-Smith, G., & Torrence, P. F. (1983) *J. Biol. Chem. 258*, 13082–13088.
Miles, D. L., Miles, D. W., & Eyring, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1018–1021.
Mukaiyama, T., & Hashimoto, M. (1971) *Bull. Chem. Soc. Jpn. 44*, 2284.
Revel, M. (1979) *Interferon (London) 1*, 101–163.
Sawai, H., & Ohno, M. (1981a) *Bull. Chem. Soc. Jpn. 54*, 2752–2759.
Sawai, H., & Ohno, M. (1981b) *Chem. Pharm. Bull. 29*, 2237–2245.
Sawai, H., Shibata, T., & Ohno, M. (1979) *Tetrahedron Lett. 47*, 4573–4576.
Sawai, H., Shibata, T., & Ohno, M. (1981) *Tetrahedron 37*, 481–485.
Sawai, H., Imai, J., Lesiak, K., Johnston, M. I., & Torrence, P. F. (1983) *J. Biol. Chem. 258*, 1671–1677.
Sen, G. C. (1982) *Prog. Nucleic Acid Res. Mol. Biol. 27*, 105–156.
Silverman, R. H., Wreschner, D. H., Gilbert, C. S., & Kerr, I. M. (1981) *Eur. J. Biochem. 115*, 79–85.
Sober, H. (1970) *Handbook of Biochemistry*, 2nd ed., pp J-65, GH-204, CRS Press, Boca Raton, FL.
Stark, G. R., Dower, W. J., Schimke, R. T., Brown, R. E., & Kerr, I. M. (1979) *Nature (London) 278*, 471–473.
Suhadolnik, R. J. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 193–291.
Torrence, P. F. (1982) *Mol. Aspects Med. 5*, 129–171.
Torrence, P. F., & Friedman, R. M. (1979) *J. Biol. Chem. 254*, 1259–1267.
Torrence, P. F., DeClercq, E., Waters, J. A., & Witkop, B. (1974) *Biochemistry 13*, 4400–4408.
Torrence, P. F., Imai, J., & Johnston, M. I. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5993–5997.
Torrence, P. F., Imai, J., Lesiak, K., Johnston, M. I., Jacobsen, H., Friedman, R. M., Sawai, H., & Safer, B. (1982) in *Interferons* (Merrgan, T., Friedman, R., & Fox, C. F., Eds.) pp 123–142, Academic Press, New York.
Torrence, P. F., Imai, J., Lesiak, K., Jamoulle, J.-C., & Sawai, H. (1984) *J. Med. Chem.* (in press).
Williams, B. R. G., Golgher, R. R., Brown, R. E., Gilbert, C. S., & Kerr, I. M. (1979) *Nature (London) 282*, 582–586.
Wreschner, D. H., James, T. C., Silverman, R. H., & Kerr, I. M. (1981a) *Nucleic Acids Res. 9*, 1571–1581.
Wreschner, P. H., McCauley, J. W., Skehel, J. J., & Kerr, I. M. (1981b) *Nature (London) 289*, 414–417.