Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates†

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ABSTRACT: Oligodeoxyribonucleoside methylphosphonates with base sequences complementary to the anticodon loop of $tRNA^{Lys}$ and to the –ACCA–OH amino acid accepting stem of tRNA were prepared by chemical synthesis. Oligodeoxyadenosine methylphosphonates form stable, triple-stranded complexes with both poly(U) and poly(dT). These analogues selectively inhibit cell-free aminoacylation of $tRNA^{Lys}_{E.coli}$ but have no effect on aminoacylation of $tRNA^{Lys}_{rabbit}$. The extent of inhibition is temperature dependent and parallels the ability of the oligomer to bind to poly(U), which suggests that inhibition occurs as a result of oligomer binding to the –UUUU– anticodon loop of $tRNA^{Lys}_{E.coli}$. The failure of the oligodeoxyadenosine methylphosphonates to inhibit $tRNA^{Lys}_{rabbit}$ aminoacylation suggests that there may be a difference between the structure of $tRNA^{Lys}$ or its interaction with aminoacyl synthetase in the *Escherichia coli* and rabbit systems. The oligodeoxyadenosine analogues also effectively inhibit polyphenylalanine synthesis in cell-free translation systems derived from both *E. coli* and rabbit reticulocytes. The extent of inhibition parallels the T_m values of the oligo(A) phosphonate–poly(U) complexes and suggests that the inhibition is a consequence of complex formation with the poly(U) message. Tritium-labeled oligodeoxyribonucleoside methylphosphonates with a chain length of up to nine nucleotidyl units are taken up intact by mammalian cells in culture. All the oligomer analogues tested inhibited, to various extents, colony formation by bacterial, hamster, and human tumor cells in culture.

Nonionic oligonucleotide analogues have been shown to be useful nucleic acid analogues for probing nucleic acid sequence–function relationships both in biochemical experiments and in living cells. Previous reports from this laboratory have described the interaction of nonionic, oligonucleotide ethyl phosphotriesters with transfer RNA (Miller et al., 1974) and the effects of these analogues on cell-free aminoacylation of tRNA (Barrett et al., 1974). A trinucleotide analogue, $G^m_p(Et)G^m_p(Et)U$[1] was shown to be taken up by mammalian cells in culture and to have specific inhibitory effects on cellular protein synthesis and cell growth (Miller et al., 1977).

Recently we described the syntheses of a series of novel nonionic oligonucleotide analogues, the dideoxyribonucleoside methylphosphonates (Miller et al., 1979). These analogues have an isosteric, 3′–5′-linked methylphosphonate group which replaces the normal phosphodiester linkage of nucleic acids. Extensive physical studies by ultraviolet, circular dichroism, and nuclear magnetic resonance spectroscopic techniques revealed that the conformation of these analogues is similar to those of the corresponding phosphodiesters and that the analogues form stable complexes with complementary polynucleotides (Miller et al., 1979; Kan et al., 1980). Since these phosphonate analogues can penetrate mammalian cells and the methylphosphonate linkage is resistant to cleavage by a variety of nucleases, it is of interest to determine if these analogues could be used as probes of the sequence–function relation of nucleic acids in living cells. In this paper we report the synthesis of a series of oligonucleoside methylphosphonates whose base sequences are complementary to the anticodon loops of $tRNA^{Lys}$ species and to the –ACCA–OH amino acid accepting stem of tRNA. The effects of these analogues on cell-free aminoacylation and cell-free protein synthesis were studied. The uptake of selected analogues by mammalian cells in culture and the effects of these compounds on bacterial and mammalian cell growth are reported.

Experimental Section

Materials. Nucleosides were purchased from P-L Biochemicals and were checked for purity by paper chromatography before use. *N*-Benzoyldeoxyadenosine, *N*-isobutyryldeoxyguanosine, their 5′-*O*-dimethoxytrityl derivatives, and 5′-*O*-(methoxytrityl)thymidine were prepared according to published procedures (Schaller et al., 1963; Büchi & Khorana, 1972). d-[$(MeO)_2$Tr]bzA*p*bzA*p*CNEt, d-[$(MeO)_2$Tr]-bzA*p*bzAOAc, d-[(MeO)Tr]T*p*T*p*CNEt, d-A*p*T, d-A*p*[^{3}H]T, d-T*p*T, and d-T*p*[^{3}H]T were synthesized by procedures previously described (Miller et al., 1979). Dimethyl methylphosphonate (K & K Laboratories) and benzenesulfonic acid (Eastman) were used without further purification. Hydracrylonitrile (Eastman) was dried over 4-Å molecular sieves. Methylphosphonic acid dipyridinium salt and mesitylenesulfonyl tetrazolide were prepared as previously described (Miller et al., 1979). Anhydrous pyridine was prepared by refluxing reagent-grade pyridine (3 L) with chlorosulfonic acid (40 mL) for 7 h, followed by distillation onto sodium hydroxide pellets (40 g). After being refluxed for 7 h, the pyridine was distilled onto 4-Å molecular sieves and stored in the dark.

Silica gel column chromatography was carried out by using Baker 3405 silica gel (60–200 mesh). Thin-layer silica gel chromatography (TLC) was performed on E. Merck silica gel 60 F_{254} plastic-backed TLC sheets (0.2 mm thick). High-pressure liquid chromatography (LC) was carried out by using a Laboratory Data Control instrument on columns (2.1 mm × 1 m) packed with HC Pellosil (Whatman, Inc.). The columns were eluted with a linear gradient (40 mL total) of chloroform to 20% (v/v) methanol in chloroform at a flow rate

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[1] Abbreviations used: Np(Et)N, an oligonucleotide ethyl phosphotriester; d-N*p*N*p*N, oligodeoxyribonucleotide analogues containing 3′–5′ internucleoside methylphosphonate linkages (in this abbreviation an italic p represents the methylphosphonate linkage); MST, mesitylenesulfonyl tetrazolide. The symbols used to represent protected nucleosides and oligonucleoside methylphosphonates follow the IUPAC–IUB Commission on Biochemical Nomenclature (1970) recommendations.

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of 1 mL/min. Ultraviolet spectra were recorded on a Cary 14 or a Varian 219 ultraviolet spectrophotometer with a temperature-controlled cell compartment. The following extinction coefficients (260 nm) were used: dT, 9100; d-[(MeO)Tr]T, 10 200; d-[$(MeO)_2$Tr]bzA, 12 500; d-bzA, 10 600; d-[$(MeO)_2$Tr]ibuG, 17 400; d-ibuG, 16 700. Paper chromatography was carried out on Whatman 3 MM paper using solvent A: 2-propanol–concentrated ammonium hydroxide–water (7:1:2 v/v).

Preparation of d-[$(MeO)_2$Tr]ibuGpCNEt. d-[$(MeO)_2$Tr]ibuG (12 g, 18.7 mmol) and the pyridinium salt of methylphosphonic acid (21 mmol) were dried by evaporation with anhydrous pyridine (4 × 20 mL) and the residue in 40 mL of pyridine was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (12.7 g, 42 mmol) for 8 h at room temperature. Hydracrylonitrile (4.5 g, 63 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (0.61 g, 2 mmol) were added and the reaction mixture was kept at room temperature. After 2 days the reaction mixture was poured into 500 mL of ice-cold 5% $NaHCO_3$ solution. The solution was extracted with ethyl acetate (2 × 250 mL) and the combined extracts were dried over anhydrous Na_2SO_4. Examination of the extract by TLC showed the presence of both d-[$(MeO)_2$Tr]ibuG*p*CNEt (R_f 0.31, silica gel TLC, 10% MeOH–$CHCl_3$) and d-ibuG*p*CNEt (R_f 0.14, silica gel TLC, 10% MeOH–$CHCl_3$). After concentration the ethyl acetate extract was chromatographed on silica gel (4 × 35 cm) by using ether (1 L) and a 0–20% linear gradient of methanol in chloroform (1.6 L total) as solvents. d-[$(MeO)_2$Tr]ibuG*p*CNEt (2.75 mmol) was obtained in 15% yield while d-ibuG*p*CNEt (2.46 mmol) was obtained in 13% yield. Additional d-[$(MeO)_2$Tr]ibuG*p* (3.69 mmol, 20%) was obtained from the aqueous bicarbonate solution after extraction with chloroform (2 × 200 mL).

Preparation of Protected Oligonucleoside Methylphosphonates. The same general procedures were used as previously described for the preparation of dinucleoside methylphosphonates (Miller et al., 1979). The specific conditions used in the condensation reactions and the yields obtained after silica gel column chromatography are given in Table I. The ultraviolet spectroscopic characteristics and the mobilities of the protected oligonucleotides on silica gel TLC and silica gel high-pressure LC are given in Table II.

Preparation of Oligonucleoside Methylphosphonates. The protecting groups were removed from the blocked oligonucleoside methylphosphonates by using conditions described previously (Miller et al., 1979). In the case of the dA-containing oligomers, the *N*-benzoyl groups were removed by treatment with hydrazine (Miller et al., 1979). The oligomers were purified by preparative paper chromatography using solvent A. For the ^{3}H-labeled oligothymidine methylphosphonates, d-$(Tp)_n$[^{3}H]T, the condensation reactions containing d-[(MeO)Tr]$(Tp)_n$ plus [^{3}H]TOAc were run on 0.01 (n = 1) and 0.005 (n = 4 and 8) mmol scales while d-G*p*G*p*[^{3}H]T was prepared on a 0.012-mmol scale. The protecting groups were removed without isolation of the protected ^{3}H-labeled oligomers and the entire reaction mixture was chromatographed on paper. The oligonucleoside methylphosphonates were eluted from the paper with 50% aqueous ethanol. The ethanol solutions were passed through DEAE-cellulose columns (0.5 × 1 cm) and stored at 0 °C. The following overall yields were obtained: d-$(Tp)_n$[^{3}H]T (n = 1, 41%; n = 4, 22%; n = 8, 17%) and d-G*p*G*p*[^{3}H]T (15%). The UV spectral properties and chromatographic mobilities of the oligonucleoside methylphosphonates are given in Table III. For use in the physical, biochemical, and biological experiments described below, aliquots containing the required amount of oligomer were evaporated to dryness, and the oligomer was dissolved in the buffer used in the particular experiment.

Interaction of Oligodeoxyadenylate Methylphosphonates with Polynucleotides. The continuous variation experiments and melting experiments were carried out as previously described (Miller et al., 1971). The extinction coefficients of the oligomers were determined by comparing the absorption of a solution of the oligomer in water at pH 7.0 to the absorption of the same solution at pH 1.0. The oligomer extinction coefficient was calculated from the observed hyperchromicity of the oligomer at pH 1.0 by using the following extinction coefficients: dA, pH 1.0, 14.1 × 10^3; dG, pH 1.0, 12.3 × 10^3. The molar extinction coefficient of poly(U) is 9.2 × 10^3 (265 nm) and of poly(dT) is 8.52 × 10^3 (264 nm).

Cell-Free Aminoacylation. (*1*) *E. coli System.* Unfractionated *Escherichia coli* tRNA was purchased from Schwarz/Mann and unfractionated *E. coli* aminoacyl synthetase was purchased from Miles Laboratories, Inc. Reactions were run in 60 μL of buffer containing 100 mM Tris-HCl, pH 7.4, 10 mM $Mg(OAc)_2$, 5 mM KCl, 2 mM ATP, 4 μM ^{3}H-labeled amino acid, 1.8 μM $tRNA_{E.coli}$, and 0–100 μM oligonucleotide, following the procedure of Barrett et al. (1974). Reactions were initiated by addition of 4 μg of aminoacyl synthetase. Aliquots (10 μL) were removed at various times and added to 1 mL cold 10% trichloroacetic acid and the resulting precipitate was filtered on Whatman G/F filters. After being washed with four (1 mL) portions of 2 N HCl and four (1 mL) portions of 95% ETOH, the filters were dried and counted in 7 mL of New England Nuclear 949 scintillation mixture.

(*2*) *Rabbit Reticulocyte System.* A rabbit reticulocyte cell-free translation system was purchased from New England Nuclear (lot no. J1157AW). Reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 79 mM potassium acetate, 0.6 mM magnesium acetate, 57 μM [^{3}H]lysine, and 50 μM oligomer. The reactions wre initiated by addition of 5 μL of reticulocyte lysate and were assayed as described for the *E. coli* system.

Cell-Free Protein Synthesis. (*1*) *E. coli System.* A cell-free protein synthesizing system was isolated from *E. coli* B cells (S-30) according to the procedure of Nirenberg (1963). The system incorporates 300 pmol of [^{3}H]phenylalanine/mg of S-30 protein after 15-min incubation at 37 °C when poly(U) is used as a message.

(*2*) *Rabbit Reticulocyte.* The reticulocyte translation system prepared by New England Nuclear was used. For the translation of globin mRNA, the reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 0.10 μg of globin mRNA (Miles Laboratories), 79 mM potassium acetate, 0.2 mM magnesium acetate, 0–50 μM oligomer, and 20.5 μM [^{3}H]leucine. For the translation of poly(U), the reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 120 mM potassium acetate, 0.8 mM magnesium acetate, 367 μM poly(U), 0–200 μM oligomer (base concentration), and 32 μM [^{3}H]phenylalanine. Reactions were initiated by addition of 5 μL of reticulocyte lysate. Aliquots (2 μL) were removed at various times and added to 1.0 mL of bovine serum albumin (100 μg) solution. The protein was precipitated by heating with 1 mL of 10% trichloroacetic acid at 70 °C, filtered on G/F filters, and counted in 7 mL of Betafluor.

Uptake of Oligodeoxyribonucleoside Methylphosphonates. The uptake of d-A*p*[^{3}H]T, d-G*p*G*p*[^{3}H]T, and d-$(Tp)_n$[^{3}H]T

Table I: Preparation of Protected Oligodeoxyribonucleoside Methylphosphonates

3'-methylphosphonate component (mmol)	5'-OH component (mmol)	MST (mmol)	product (mmol)	yield (%)
d-[$(MeO)_2$Tr]ibuG*p* (0.50)	d-ibuG*p*CNEt (0.50)	2.0	d-[$(MeO)_2$Tr]ibuG*p*ibuG*p*CNEt (0.82)	16
d-[$(MeO)_2$Tr]ibuG*p* (1.0)	d-bzAOAc (1.5)	4.0	d-[$(MeO)_2$Tr]ibuG*p*bzAOAc (0.42)	42
d-[(MeO)Tr]T*p*T*p* (0.33)	d-T*p*T*p*CNEt (0.50)	1.6	d-[(MeO)Tr]T*p*T*p*T*p*T*p*CNEt (0.168)	50
d-[(MeO)Tr]T*p*(T*p*)$_2$T*p*CNEt (0.0324)	d-T*p*(T*p*)$_2$T*p*CNEt (0.0524)	0.16	d-[(MeO)Tr]T*p*(T*p*)$_6$T*p*CNEt (0.0138)	43
d-[$(MeO)_2$Tr]ibuG*p*ibuG*p* (0.07)	d-TOAc (0.15)	0.28	d-[$(MeO)_2$Tr]ibuG*p*ibuG*p*TOAc (0.0153)	22
d-[$(MeO)_2$Tr]bzA*p*bzA*p* (0.065)	d-bzAOAc (0.043)	0.163	d-[$(MeO)_2$Tr]bzA*p*bzA*p*bzAOAc (0.023)	53
d-[$(MeO)_2$Tr]bzA*p*bzA*p* (0.13)	d-bzA*p*bzAOAc (0.20)	0.52	d-[$(MeO)_2$Tr]bzA*p*bzA*p*bzA*p*bzAOAc (0.031)	24
d-[$(MeO)_2$Tr]bzA*p*bzA*p* (0.0168)	d-ibuG*p*bzAOAc (0.0168)	0.0735	d-[$(MeO)_2$Tr]A*p*bzA*p*ibuG*p*bzAOAc (0.0029)	17

Table II: Ultraviolet Spectral Properties and Chromatographic Mobilities of Protected Oligodeoxyribonucleoside Methylphosphonates

	UV spectra[a]						silica gel TLC R_f[b] in MeOH–$CHCl_3$				silica gel HPLC[c]
			$\epsilon_{260}/\epsilon_{235}$		$\epsilon_{260}/\epsilon_{280}$						retention
oligomer	λ_{max} (nm)	λ_{min} (nm)	calcd	obsd	calcd	obsd	5%	10%	15%	20%	time (min)
d-[(MeO)Tr]T*p*T*p*T*p*T*p*CNEt	265 235 sh	243	1.34	1.31	1.55	1.64	–	–	0.08	0.29	–
d-[(MeO)Tr]T*p*(T*p*)$_6$T*p*CNEt	265	243	1.75	0.92	1.57	1.56	–	0.00	–	0.13	–
d-[$(MeO)_2$Tr]ibuG*p*ibuG*p*CNEt	238 253 260 280	225 245 256 270	1.19	1.05	1.33	1.32	–	0.16	–	–	19.2
d-[$(MeO)_2$Tr]ibuG*p*bzAOAc	235 278	256	0.82	0.75	0.88	0.87	–	0.29	–	–	12.3
d-ibuG*p*bzAOAc	260 280	239 267	1.63	1.27	0.90	0.90	–	0.18 0.14	–	–	15.5 17.6
d-[$(MeO)_2$Tr]ibuG*p*ibuG*p*TOAc	240 sh 260 275 sh	228	1.34	1.51	1.38	1.45	–	0.18	–	–	16.0
d-[$(MeO)_2$Tr]bzA*p*bzA*p*bzAOAc	234 280	227 255	0.66	0.61	0.59	0.59	–	0.41 0.38	0.55 0.53	–	13.4 14.3
d-[$(MeO)_2$Tr]bzA*p*bzA*p*bzA*p*bzAOAc	233 sh 280	253	0.71	0.60	0.59	0.60	–	–	0.31	–	19.3
d-[$(MeO)_2$Tr]bzA*p*bzA*p*ibuG*p*bzAOAc	235 sh 280	255	0.89	0.74	0.73	0.75	–	0.15	0.44	–	23.8

[a] Measured in 95% EtOH. [b] E. Merck silica gel 60 F_{254} sheets, 0.2 mm thick. [c] HC Pellosil (2.1 mm × 1 m); 0–20% methanol in chloroform; 1 mL/min; 40-mL total volume.

by transformed Syrian hamster fibroblasts was determined as previously described (Miller et al., 1977).

Effects of Oligodeoxyribonucleoside Methylphosphonates on Colony Formation. (1) E. coli. *E. coli* B was grown in M-9 medium (Bolle et al., 1968) supplemented with glucose (36 g/L) and 1% casamino acids. The cells were harvested in mid-log phase and resuspended in 50 μL of fresh medium containing 0–160 μM oligomer at a final cell density of 1×10^4 cells/mL. The cells were incubated for 1 h at 37 °C and then diluted with 0.9 mL of medium. A 0.8-mL aliquot was added to 2.5 mL of 0.8% Bactoagar at 45 °C. This solution was quickly poured onto a 100-mm plate containing solid 1.2% Bactoagar. After solidification, the plates were incubated overnight at 37 °C and the resulting colonies were counted.

(2) Transformed Syrian Hamster Embryonic Fibroblasts (BP-6) and Transformed Human Fibroblasts (HTB1080). Colony formation by the fibroblasts in the presence of the methylphosphonate analogues was carried out as previously described (Miller et al., 1977).

Results

Synthesis of Oligodeoxyribonucleoside Methylphosphonates. The synthetic scheme used for preparing the oligonucleoside methylphosphonates followed the basic approach used to synthesize dideoxyribonucleoside methylphosphonates (Miller et al., 1979). Suitably protected monomers or oligomer blocks carrying a 3'-terminal methylphosphonate group were condensed with protected mono- or oligonucleotides bearing a free 5'-hydroxyl group. Mesitylenesulfonyl tetrazolide (Stawinsky et al., 1977) was used as the condensing agent. The fully protected oligomers were purified by silica gel column chromatography. The reaction conditions used and the yields obtained are given in Table I. The oligomers were characterized by ultraviolet spectroscopy, thin-layer chromatography, and high-pressure liquid chromatography as indicated in Table II.

The protecting groups were removed as previously described (Miller et al., 1979). In the case of the deoxyadenosine-containing oligomers, the *N*-benzoyl groups were first removed by treatment with hydrazine hydrate (Letsinger et al., 1968). The remaining 3'-*O*-acetyl and 5'-*O*-dimethoxytrityl groups were removed by sequential treatment with ammonium hydroxide and 80% acetic acid. The oligomers were purified by preparative paper chromatography and were characterized by UV spectroscopy (Table III).

Interaction of Oligodeoxyribonucleoside Methylphosphonates with Complementary Polynucleotides. Table IV summarizes thhe melting temperatures of complexes formed between oligodeoxyadenosine methylphosphonates and poly(U) or poly(dT). For comparison, the melting temperatures of complexes formed by oligodeoxyribo- and oligoriboadenosines are included. Each oligomer forms a triple-stranded complex with a stoichiometry of 2U:1A or 2T:1A. The melting temperatures increase as the chain length of the oligonucleotide increases. For a given chain length, the complexes formed by the methylphosphonate analgoues melt at higher temperatures than those formed by the natural diester oligomers. With the exception of r-ApApApA, the complexes formed by the oli-

Table III: Spectral Properties and Chromatographic Mobilities of Oligodeoxyribonucleoside Methylphosphonates

oligomer	UV spectra[a] λ_{max} (nm)	λ_{min} (nm)	$\epsilon_{260}/\epsilon_{280}$	ϵ at λ_{max}	paper chromatography[b] R_f, solvent A
d-G*p*GpT[c]	257 270 sh	230	1.45	33.4×10^3	0.31
d-A*p*ApA	258	232	4.27	39.0×10^3	0.29
d-A*p*ApApA	258	230	3.77	50.4×10^3	0.11
d-A*p*ApGpA	258	227	3.03	50.3×10^3	0.11
d-T*p*[^{3}H]T	267	235	1.53		0.59
d-(T*p*)$_4$[^{3}H]T	266	235	1.49		0.21
d-(T*p*)$_8$[^{3}H]T	266	235	1.56		0.17

[a] Measured in water, pH 7.0. [b] R_f^A of pT = 0.11. [c] The UV spectrum is similar to that of d-GpGpT (Miller et al., 1974).

Table IV: Interaction of Oligonucleoside Methylphosphonates with Complementary Polynucleotides[a]

oligomer	T_m with poly(U) (2U:1A) (°C)	T_m with poly(dT) (2T:1A) (°C)
d-A*p*A: isomer 1	15.4	18.7
isomer 2	19.8	18.4
d-A*p*ApA	33.0	36.8
d-A*p*ApApA	43.0	44.5
d-ApA	7.0	9.2
d-ApApApA	32.0	35.5
r-ApApApA	36.2	2.4

[a] 5×10^{-5} M total [nucleotide], 10 mM Tris, and 10 mM $MgCl_2$, pH 7.5.

gomers with poly(dT) have slightly higher melting temperatures than the corresponding complexes formed with poly(U).

The interaction of d-G*p*Gp[^{3}H]T with unfractionated $tRNA_{E.coli}$ was measured by equilibrium dialysis (Miller et al., 1974). The apparent association constants at 0, 22, and 37 °C are 1100 M^{-1}, 200 M^{-1}, and 100 M^{-1}, respectively. These binding constants are much lower than those of the 2′-*O*-methylribooligonucleotide ethyl phosphotriester, G_p^m(Et)-G_p^m(Et)[^{3}H]U, which are 9300 M^{-1} (0 °C), 1900 M^{-1} (22 °C), and 2000 M^{-1} (37 °C) (Miller et al., 1977).

Effect of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Aminoacylation of tRNA. The effects of selected oligodeoxyribonucleoside methylphosphonates on aminoacylation of unfractionated $tRNA_{E.coli}$ are shown in Table V. Three amino acids were tested at various temperatures. The deoxyadenosine-containing analogues which are complementary to the –UUUU– sequence of the anticodon of $tRNA^{Lys}_{E.coli}$ have the largest inhibitory effect on aminoacylation of $tRNA^{Lys}_{E.coli}$. The percent inhibition increases with increasing chain length and decreases with increasing temperature. Inhibition by d-A*p*ApG*p*A and by the diesters d-ApApApA and r-ApApApA is less than that exhibited by d-A*p*A*p*ApA. In contrast to their behavior with $tRNa^{Lys}_{E.coli}$, neither the methylphosphonates, d-A*p*A*p*ApA and d-A*p*A*p*G*p*A, nor the phosphodiesters, d-ApApApA and r-ApApApA, had any inhibitory effect on $tRNA^{Lys}_{rabbit}$ in the rabbit reticulocyte cell-free system (data not shown).

Effects of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Protein Synthesis. The ability of deoxyadenosine-containing oligonucleoside methylphosphonates to inhibit polypeptide synthesis in cell-free systems directed by synthetic and natural messages was tested. The results of these experiments are given in Table VI. Poly(U)-directed phenylalanine incorporation and poly(A)-directed lysine incorporation are both inhibited by oligodeoxyadenosine methylphosphonates and diesters in the *E. coli* system at 22 °C. The percent inhibition increases with oligomer chain length and is greater for polyphenylalanine synthesis. The methylphosphonate analogues are more effective inhibitors than either

Table V: Effects of Oligonucleoside Methylphosphonates on Aminoacylation in an *E. coli* Cell-Free System

oligomer[a]	% inhibition[b] Phe, 0 °C	Leu, 0 °C	Lys 0 °C	Lys 22 °C	Lys 37 °C
d-A*p*A	6	0	7		
d-A*p*ApA	9	0	62	15	0
d-A*p*ApApA	9	12	88	40	16
d-A*p*ApG*p*A	12	12	35	0	
d-G*p*G*p*T	31	5	34	9	15
dGpGpT (400 μM)	23				
d-ApApApA	0	7	71[c]	15[c]	
r-ApApApA			78[d]	17[d]	

[a] [oligomer] = 50 μM. [b] [$tRNA_{E.coli}$] = 2 μM. [c] [oligomer] = 100 μM. [d] [oligomer] = 125 μM.

Table VI: Effects of Oligonucleoside Methylphosphonates on Bacterial and Mammalian Cell-Free Protein Synthesis at 22 °C

oligomer	% inhibition *E. coli* poly(U) directed[a]	*E. coli* poly(A) directed[b]	rabbit reticulocyte poly(U) directed[a]	rabbit reticulocyte globin mRNA directed[c]
d-A*p*A	20	10		
d-A*p*ApA	84	30	81	
d-A*p*ApApA	100	65	77	0
d-A*p*ApG*p*A	22			0
d-ApApApA	13	19	18	0
r-ApApApA	18	17	85	0

[a] [poly(U)] = 360 μM in U; [oligomer] = 175–200 μM in base. [b] [poly(A)] = 300 μM in A; [oligomer] = 175–200 μM in base. [c] [oligomer] = 200 μM in base.

d-ApApApA or r-ApApApA at the same concentration. Although both the oligodeoxyadenosine methylphosphonates and the phosphodiesters inhibit translation of poly(U) in the rabbit reticulocyte system, no effect on the translation of globin message was observed. As in the case of the *E. coli* system, inhibition of phenylalanine incorporation increased with oligomer chain length and was greater for the methylphosphonate analogues than for the diesters.

Uptake of Oligodeoxyribonucleoside Methylphosphonates by Mammalian Cells. Figure 1 shows the incorporation of radioactive 100 μM d-G*p*Gp[^{3}H]T with time into transformed Syrian hamster embryonic fibroblasts growing in monolayer. The incorporation is approximately linear for the first hour and begins to level off after 1.5 h. The concentration of radioactivity inside the cells is ~117 μM after 1.5 h assuming a cell volume of 1.5 μL/10^6 cells (Hempling, 1972).

Cells were incubated with 25 μM d-G*p*Gp[^{3}H]T for 18 h. The medium was removed, and the cells were washed with phosphate buffer and then lysed with $NaDodSO_4$. Approx-

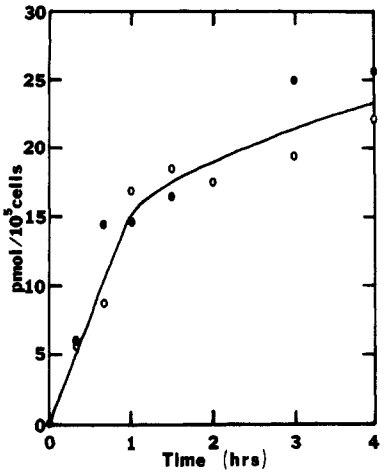

FIGURE 1: Transport of (○) 100 μM d-G*p*G*p*[^{3}H]T and (●) 100 μM d-(T*p*)$_8$[^{3}H]T into transformed Syrian hamster fibroblasts growing in monolayer at 37 °C.

imately 30% of the total radioactivity from the lysate was found in Cl_3AcOH-precipitable material. The DNA was precipitated from the lysate and digested with deoxyribonuclease and snake venom phosphodiesterase. The culture medium, the DNA-free lysate, and the DNA digest were each examined by paper chromatography. Only intact d-G*p*G*p*-[^{3}H]T was found in the medium. Radioactivity corresponding to [^{3}H]TTP (6%) and to d-G*p*G*p*[^{3}H]T (94%) was found in the lysate, while the DNA digest gave [^{3}H]dpT and [^{3}H]dT as products.

Similar uptake studies were carried out with d-A*p*[^{3}H]T and with a series of oligothymidylate analogues, d-(T*p*)$_n$[^{3}H]T (n = 1, 4, and 8). The rates and extents of uptake of these analogues were very similar to that of d-GpGp[^{3}H]T (Figure 1). Examination of the culture medium and cell lysate after overnight incubation with these oligonucleotides gave results similar to those found for d-G*p*G*p*[^{3}H]T.

Effects of Oligodeoxyribonucleoside Methylphosphonates on Colony Formation by Bacterial and Mammalian Cells. The effects of selected oligodeoxyribonucleoside methylphosphonates on colony formation by *E. coli* B, transformed Syrian hamster fibroblast (BP-6), and transformed human fibroblast (HTB 1080) cells are summarized in Table VII. The d-(A*p*)$_n$*A* analogues appear to inhibit *E. coli* colony formation at high concentrations (160 μM). However, no inhibitory effects on the incorporation of [^{3}H]leucine into cellular protein or [^{3}H]thymidine into cellular DNA could be detected in the presence of these compounds.

Colony formation of both transformed hamster and human cells are inhibited to various extents by the oligonucleoside methylphosphonates. Both the hamster and human cells appear to be affected to a similar extent by a given analogue. It appears in the case of d-A*p*A that each diastereoisomer exerts a different inhibitory effect on the growth of the hamster cells. As in the case of *E. coli*, no inhibition of cellular protein synthesis could be detected.

Discussion

Oligodeoxyribonucleoside methylphosphonates with sequences complementary to the anticodon loop of tRNA^{Lys} and to the –ACCA–OH amino acid accepting stem of tRNA were prepared in a manner similar to that used to prepare dideoxyribonucleoside methylphosphonates (Miller et al., 1979). The present studies demonstrate the ability to join blocks of protected methylphosphonates to give oligomers with chain lengths up to nine nucleotidyl units. The yields in these

Table VII: Effects of Oligonucleoside Methylphosphonates on Colony Formation by Bacterial and Mammalian Cells in Culture

	% inhibition[a]			
	E. coli B			
oligomer	50 μM	160 μM	BP-6, 50 μM	HTB 1080, 50 μM
d-A*p*T	4	5	5, 16[b]	12
d-A*p*A	8	58	6, <1[b]	5
d-A*p*A*p*A	3	44	29	31
d-A*p*A*p*A*p*A	19	78	36	19
d-G*p*G*p*T	7	11	7	9

[a] The results are the average of two or three experiments. Each experiment consisted of two plates (bacterial cells) or three plates (mammalian cells). The average variation is ±3% in percent inhibition. The cells were treated with and grown in the presence of the oligomer at 37 °C. [b] The percent inhibition of isomers 1 and 2, respectively.

condensation reactions are acceptable, although reactions involving deoxyguanosine residues appear to proceed in low yield. Similar difficulties have been encountered in the syntheses of oligonucleotide phosphotriesters. Unlike the dideoxyribonucleoside methylphosphonates previously reported, the oligodeoxyribonucleoside methylphosphonates prepared for this study were not resolved into their individual diastereoisomers.

The oligodeoxyadenosine analogues form triple-stranded complexes with both poly(U) and poly(dT). These complexes are more stable than similar complexes formed by either oligoribo- or oligodeoxyribonucleotides. As previously suggested for oligonucleotide ethyl phosphotriesters (Miller et al., 1971, 1974; Pless & Ts'o, 1977) and dideoxyribonucleoside methylphosphonates (Miller et al., 1979), this increased stability is attributed to the decreased charge repulsion between the nonionic backbone of the analogue and the negatively charged complementary polynucleotide backbone. With the exception of r-ApApApA (Table IV), the stability of the complexes formed with poly(dT) are slightly higher than those formed with poly(U), a situation which is also observed for the interaction of poly(dA) with poly(dT) and with poly(U) (Chamberlin, 1965). The lower stability of the (r-ApApApA)·2[poly(dT)] complex is also reflected at the polymer level. Thus, under the conditions of the experiments described in Table IV, we found that the T_m of poly(rA)·2[poly(rU)] is 83 °C while the T_m of poly(rA)·2[poly(dT)] is 59 °C. These results are consistent with those of Riley et al. (1966). They observed that formation of the poly(rA)·2[poly(dT)] complex occurs only at a sodium ion concentration of 2.5 M in the absence of magnesium, while poly(rA)·2[poly(rU)] forms in 0.1 M sodium phosphate buffer.

The oligodeoxyadenosine methylphosphonates and their parent diesters selectively inhibit cell-free aminoacylation of $tRNA^{Lys}_{E.coli}$. The extent of inhibition is temperature dependent and parallels the ability of the oligomers to bind to poly(U). These observations and the previously demonstrated interaction of r-ApApApA with $tRNA^{Lys}_{E.coli}$ (Möller et al., 1978) suggest that inhibition occurs as a result of oligomer binding to the –UUUU– anticodon loop of the tRNA. The reduced inhibition observed with d-A*p*A*p*G*p*A is consistent with this explanation, since interaction of this oligomer with the anticodon loop would involve formation of a less stable G·U base pair.

Recent studies by Ramberg et al. (1978) have shown that the rate of aminoacylation of $tRNA^{Lys}_{E.coli}$ substituted with 5-fluorouracil is considerably lower than that of nonsubstituted $tRNA^{Lys}_{E.coli}$. The increased K_m of the 5-fluorouracil-substituted tRNA suggested a decreased interaction with the lysyl aminoacyl synthetase. These results and those of Saneyoshi

& Nishimura (1971) suggest that the anticodon loop of $tRNA^{Lys}_{E.coli}$ is part of the synthetase recognition site. Thus, inhibition of aminoacylation by the oligodeoxyribonucleoside methylphosphonates could result from the reduction in the affinity of the synthetase for $tRNA^{Lys}$–oligonucleotide complexes. The greater inhibition observed with d-A*p*A*p*A*p*A vs. the diesters, d-ApApApA or r-ApApApA, may result from greater binding of the analogue to the anticodon loop or to the decreased ability of the synthetase to displace the nonionic oligonucleotide analogue vs. the phosphodiester oligomers from the anticodon loop. Alternatively, oligomer binding to the anticodon loop could induce a conformational change in the tRNA, leading to a lower rate and extent of aminoacylation. Such conformational changes have been detected when r-ApApApA binds to $tRNA^{Lys}_{E.coli}$ (Möller et al., 1979; Wagner & Garrett, 1979).

None of the oligomers have any effect on the aminoacylation of $tRNA^{Lys}_{rabbit}$ in a cell-free system. Since the anticodon regions of tRNAs from bacterial and mammalian sources probably are similar, the oligo(A) analogues are expected to interact with the anticodon region of both $tRNA^{Lys}$'s. The failure to observe inhibition of aminoacylation of $tRNA^{Lys}_{rabbit}$ in the presence of these oligo(d-A) analogues suggests that there may be a difference between the interaction of the lysine aminoacyl synthetase with $tRNA^{Lys}$ from *E. coli* and from rabbit systems or a difference between the structure of these two $tRNA^{Lys}$'s in response to the binding of oligo(d-A) analgoues.

The trimer, dG*p*G*p*T, inhibits both phenylalanine and lysine aminoacylation at 0 °C but has little effect on leucine aminoacylation. The aminoacyl stems of both $tRNA^{Lys}_{E.coli}$ and $tRNA^{Phe}_{E.coli}$ terminate in a G-C base pair between nucleotides 1 and 72, while a less stable G-U base pair is found at this position in $tRNA^{Leu}_{E.coli}$ (Sprinzl et al., 1978). Thus the observed differences in inhibition of aminoacylation by d-G*p*G*p*T may reflect differences in the ability of this oligomer to bind to the different –ACC– ends of the various tRNAs.

Inhibition of lysine aminoacylation by dG*p*G*p*T is very temperature sensitive and parallels the decrease in binding to tRNA with increasing temperature. This behavior of d-G*p*G*p*T contrasts that of $G^m_p(Et)G^m_p(Et)U$ (Miller et al., 1977). Although both oligomers can potentially interact with the same sequences in tRNA, the 2′-*O*-methylribotrinucleotide ethyl phosphotriester binds more strongly and more effectively inhibits aminoacylation. The differences in binding ability may be due to overall differences in the conformation of the deoxyribo vs. 2′-*O*-methylribo backbones of these oligomers.

The oligodeoxyribonucleoside methylphosphonates effectively inhibit polyphenylalanine synthesis in cell-free systems derived from both *E. coli* and rabbit reticulocytes. In the *E. coli* system, the extent of inhibition by the oligodeoxyadenosine analogues parallels the T_m values of the oligomers with poly(U). The tetramer, d-A*p*A*p*G*p*A, which would have to form a G·U base pair with poly(U), was 4.5-fold less effective than d-A*p*A*p*A*p*A. These results suggest that the oligomers inhibit polypeptide synthesis as a consequence of forming complexes with the poly(U) message. A similar inhibitory effect by poly(dA) on the translation of poly(U) was observed by Williamson et al. (1967). It is unlikely that inhibition results from nonspecific interaction of the methylphosphonates with protein components of the translation systems. In the *E. coli* system, poly(A) translation is inhibited to a lesser extent than is translation of poly(U), while in the reticulocyte system, no inhibition of globin mRNA translation is observed.

The data suggest that the magnitude of inhibition of poly(U)-directed polypeptide synthesis in the *E. coli* system does not reflect proportionally the ability of the oligomer to bind to poly(U). Although the oligomer pairs d-A*p*A*p*A/d-ApApApA and d-A*p*A*p*A*p*A/r-ApApApA form complexes with poly(U) which have very similar T_m values (see Table IV), in each case the methylphosphonate analogues inhibit 5.5–6.5 times better than do the diesters. This stronger inhibitory effect could result from a decreased ability of the ribosome to displace the nonionic oligodeoxyribonucleoside methylphosphonates from the poly(U) meassage, or, alternatively, there may be a degradation of the oligonucleotides (phosphodiesters) by nucleases in the cell-free translation systems but not the corresponding phosphonate analogues.

Experiments with radioactively labeled oligonucleotide methylphosphonates show that these analogues are taken up by mammalian cells growing in culture. The extent of uptake is consistent with passive diffusion of the oligomer across the cell membrane. Both d-T*p*[^{3}H]T and d-$(Tp)_8$[^{3}H]T are taken up to approximately the same extent, which suggests that there is no size restriction to uptake over this chain length range. This behavior is in contrast to results obtained with *E. coli* B cells (K. Jayaraman et al., unpublished results).

Examination of lysates of mammalian cells exposed to labeled oligomers for 18 h showed that ~70% of the labeled thymidine was associated with intact oligomer with the remainder found in thymidine triphosphate and in cellular DNA. These observations indicate that the oligodeoxyribonucleoside methylphosphonates, which are recovered intact from the culture medium, are slowly degraded within the cell. Failure to observe shorter oligonucleotides and the known resistance of the methylphosphonate linkage to nuclease hydrolysis suggests that degradation may result from cleavage of the 3′-terminal [^{3}H]thymidine *N*-glycosyl bond with subsequent reutilization of the thymine base.

The uptake process of the oligonucleoside methylphosphonates is quite different from that of previously studied oligonucleotide ethyl phosphotriesters (Miller et al., 1977; P. S. Miller et al., unpublished results). In the case of $G^m_p(Et)G^m_p(Et)$[^{3}H]U, the oligomer is rapidly taken up by the cells and is subsequently deethylated. Further degradation to smaller oligomers is then observed, presumably as a result of nuclease-catalyzed hydrolysis of the resulting phosphodiester linkages. Approximately 80% of the oligomer is metabolized within 24 h. Although the rate of uptake of d-Gp(Et)Gp(Et)[^{3}H]T is similar to that of d-G*p*G*p*[^{3}H]T, examination of the cell lysate showed extensive degradation of the phosphotriester analogue. The relatively long half-lives of the oligodeoxyribonucleoside methylphosphonates may be of value in potential pharmacological applications of these oligonucleotide analogues.

The effects of these analogues on cell colony formation confirmed that the methylphosphonates are taken up by both mammalian and bacterial cells. All the oligomers tested inhibited colony formation of both cell types to various extents. The mechanism(s) by which these compounds exert their inhibitory effects is (are) currently under investigation. No decrease in either overall short-term cellular protein synthesis or DNA synthesis was detected by the present procedure in the presence of these compounds. This does not rule out the possibility that the syntheses of certain critical proteins are perturbed by these oligomers. We are currently studying this possibility by examination of the cellular proteins using two-dimensional gel electrophoresis.

The experiments described in this paper extend our studies on the use on nonionic oligonucleotides as sequence–function probes of nucleic acids both in biochemical experiments and

in living cells. In a future publication we will describe the effects of an oligodeoxyribonucleoside methylphosphonate complementary to the 3′ terminus of 16S rRNA on bacterial protein synthesis and growth (K. Jayaraman et al., unpublished results). Our results suggest that sequence-specific oligonucleoside methylphosphonates may find important applications in probing and regulating nucleic acid function within living cells.

References

Barrett, J. C., Miller, P. S., & Ts'o, P. O. P. (1974) *Biochemistry 13*, 4898.

Bolle, A., Epstein, R. H., Salser, W., & Geiduschek, E. P. (1968) *J. Mol. Biol. 33*, 339.

Büchi, H., & Khorana, H. G. (1972) *J. Mol. Biol. 72*, 251.

Chamberlin, M. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 1446.

Hempling, H. G. (1972) in *Transport and Accumulation in Biological Systems* (Harris, E. J., Ed.) p 272, Baltimore University Park Press, Baltimore, MD.

IUPAC–IUB Commission on Biochemical Nomenclature (1970) *Biochemistry 9*, 4022.

Kan, L. S., Cheng, D. M., Miller, P. S., Yano, J., & Ts'o, P. O. P. (1980) *Biochemistry 19*, 2122.

Letsinger, R. L., Miller, P. S., & Grams, G. W. (1968) *Tetrahedron Lett.*, 2621.

Miller, P. S., Fang, K. M., Kondo, N. S., & Ts'o, P. O. P. (1971) *J. Am. Chem. Soc. 93*, 6657.

Miller, P. S., Barrett, J. C., & Ts'o, P. O. P. (1974) *Biochemistry 13*, 4887.

Miller, P. S., Braiterman, L. T., & Ts'o, P. O. P. (1977) *Biochemistry 16*, 1988.

Miller, P. S., Yano, J., Yano, E., Carroll, C., Jayaraman, K., & Ts'o, P. O. P. (1979) *Biochemistry 18*, 5134.

Möller, A., Schwarz, U., Lipecky, R., & Gassen, H. G. (1978) *FEBS Lett. 89*, 263.

Möller, A., Wild, U., Riesner, D., & Gassen, H. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3266.

Nirenberg, M. (1963) *Methods Enzymol. 6*, 17.

Pless, R. C., & Ts'o, P. O. P. (1977) *Biochemistry 16*, 1239.

Ramberg, E. S., Ishag, M., Rulf, S., Moeller, B., & Horowitz, J. (1978) *Biochemistry 17*, 3978.

Riley, M., Maling, B., & Chamberlin, M. J. (1966) *J. Mol. Biol. 20*, 359.

Saneyoshi, M., & Nishimura, S. (1971) *Biochim. Biophys. Acta 246*, 123.

Schaller, H., Weiman, G., Lerch, B., & Khorana, H. G. (1963) *J. Am. Chem. Soc. 85*, 3821.

Sprinzl, M., Grüter, F., & Gauss, D. H. (1978) *Nucleic Acids Res. 5*, r15.

Stawinsky, J., Hozumi, T., Narang, S. A., Bahl, C. P., & Wu, R. (1977) *Nucleic Acids Res. 4*, 353.

Wagner, R., & Garrett, R. A. (1979) *Eur. J. Biochem. 97*, 615.

Williamson, A. R., Hausmann, E., Heintz, R., & Schweet, R. (1967) *J. Mol. Biol. 26*, 267.