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Synthesis and Properties of Polyribonucleotides Containing N^2-Methyl- and N^2-Dimethylguanylic Acid in Polyguanylic Acid*

Morio Ikehara† and Masao Hattori

ABSTRACT: Polyribonucleotides containing m^2G and G or m_2^2G and G in various ratios were synthesized from the corresponding nucleoside diphosphates by polynucleotide phosphorylase. While the amount of m_2^2G incorporated into the polynucleotide was proportional to the ratio of m_2^2GDP in the reaction mixture, m^2GDP acted inhibitory to the GDP polymerization and the incorporation was less extensive. The T_m of poly(G,m_2^2G) was 30–50° when the amount of m_2^2G in the polynucleotides <74% and over 80° when m_2^2G >51%. Poly(G,m^2G) and poly(G,m_2^2G) containing a lower amount of methylated guanosines form 1:1 complexes with poly(C), but the polynucleotides containing higher amount of methylated nucleotides could not. These results were supported by mixing curves and CD spectra.

N^2-Methylguanosine (m^2G) and N^2-dimethylguanosine (m_2^2G) are constituents of some tRNAs (Holley *et al.*, 1965; Zachau *et al.*, 1966; Madison *et al.*, 1966; RajBhandary *et al.*, 1967; Takemura *et al.*, 1968; Staehelin *et al.*, 1968). The fact that these modified nucleosides occur at specific positions of tRNA chains suggested some roles of these nucleotides in the function of tRNAs. Pochon and Michelson (1969) studied the physical properties of homopolynucleotides of m^2G and m_2^2G and found that these polynucleotides could not form double-stranded complexes with poly(C).[1] Iwamura *et al.* (1970) have shown strong stacking tendency of a compound containing dimethylguanine. Since Yamazaki *et al.* (1967) developed a method suitable for large-scale synthesis of methylated guanosine, it became easier to obtain larger amounts of substrates needed for polynucleotide synthesis.

In this paper we describe the enzymatic synthesis of analogs of polyguanylic acid (poly(G)) containing m^2G and m_2^2G in various ratios using polynucleotide phosphorylase (Grunberg-Manago *et al.*, 1956). The thermal denaturation of these polynucleotides, their complex formation with poly(C), and their CD properties were studied in order to gain more information about the effect of methylguanosine or dimethylguanosine on the secondary structure of RNA.

Materials and Methods

Synthesis of m^2GDP and m_2^2GDP. m^2GDP and m_2^2GDP were prepared from m^2GMP and m_2^2GMP, respectively (Yamazaki *et al.*, 1968), by the method described by Moffatt and Khorana (1961). m^2GMP or m_2^2GMP (1 mmole) was dissolved in a mixture of water (10 ml) and *tert*-butyl alcohol (10 ml). To the solution was added morpholine (0.54 ml). The mixture was

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[1] The same results were obtained by us independently (reported at Annual Meeting of Pharmaceutical Society of Japan, 1968).

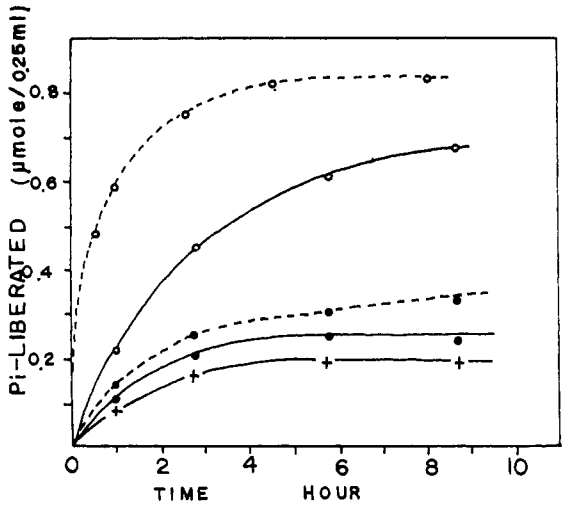

FIGURE 1: Copolymerization of GDP and m^2GDP. (O---O---O) Polymerization with GDP, (O—O—O) GDP:m^2GDP = 4:1, (●---●---●) 3:2, (●—●—●) 2:3, and (—+—+—) 1:4. Other conditions as described in the text.

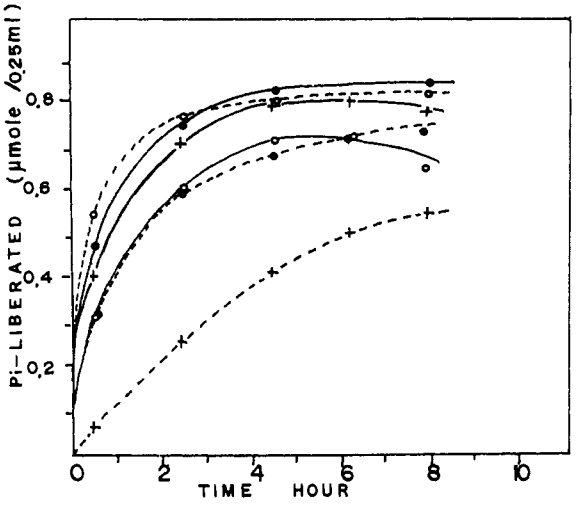

FIGURE 2: Copolymerization of GDP and m_2^2GDP. (●—●—●) Polymerization with GDP, (O---O---O) GDP:m_2^2GDP = 4:1, (—+—) 3:2, (●--●--●) 2:3, (O—O—O) 1:4, and (+---+---) m_2^2GDP.

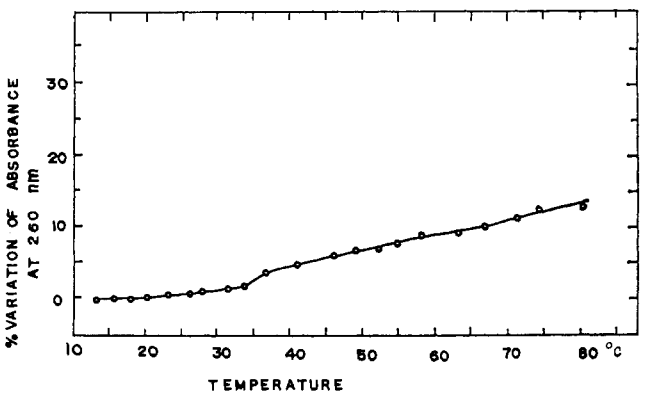

FIGURE 3: Thermal denaturation profile of poly(G_{52}, $m_2^2G_{48}$).

TABLE I: Paper Chromatography and Paper Electrophoresis.[a]

Compound	Solvent A R_F	Solvent B R_F	Solvent C R_F	Paper Electrophoresis R_{GMP}
m^2GMP	0.42	0.21	0.39	1.0[b]
m^2GMP	0.39	0.13	0.29	1.3
m_2^2GMP	0.49	0.24	0.42	1.0
m_2^2GMP	0.46	0.19	0.32	1.3

Phosphate Analysis of m^2GDP and m_2^2GDP

Compound	Base	Acid-Labile P	Total P
m^2GDP	1.0	1.05	2.01
m_2^2GDP	1.0	0.99	2.04

Uv Absorption (nm) Properties of Methylated Guanosine Derivatives and GDP

Compound	pH 1	pH 7	pH 13
m^2G	259.5	255	259
m^2GDP	260	255	259
m_2^2G	265	261	262.5
m_2^2GDP	265	261	262

[a] Solvents used: A, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v); B, ethanol–1 M ammonium acetate (7:3, v/v); C, *n*-butyl alcohol–acetic acid–water (5:2:3, v/v). [b] Relative migration distance to GMP in ammonium bicarbonate (0.05 M) at pH 7.5.

refluxed for 3.5 hr with dropwise addition of *N,N*-dicyclohexylcarbodiimide (830 mg) dissolved in *tert*-butyl alcohol (15 ml) in 1.5 hr. The solvent was evaporated *in vacuo* and the precipitated dicyclohexylurea was filtered off. The filtrate was washed with ether and the water phase evaporated to give morpholidate in almost quantitative yield. The morpholidate (1 mmole) was dried by evaporation with added pyridine and combined with a mixture of 80% phosphoric acid (0.3 ml) and tri-*n*-butylamine (0.95 ml), which were previously dried the same way. Reactants were dissolved in pyridine (2 ml) and kept at room temperature for 2 days under exclusion of moisture. Reaction was stopped by addition of water (2 ml), solvent

TABLE II: Yield and Properties of m^2G–G Copolynucleotides.

Polynucleotide	Ratio of GDP: m^2GDP	Yield[a] (%)	Base Ratio[b] G:m^2G	λ_{max} (nm)[c] (pH 7.0)	ϵ(P) × 10^{-3}
Poly(G)		15		253.5	10.8
Poly(G,m^2G)	4:1	26	75:25		
Poly(G,m^2G)	3:2	8	57:43	254.5	7.2
Poly(G,m^2G)	2:3	4	45:55	255	7.7
Poly(G,m^2G)	1:9		Only m^2G	254.5	
Poly(m^2G)		7		254.5	6.8

[a] Ignoring hypochromicity. [b] Digestion and measurement were done as described in text. [c] At 0.15 M ionic concentration.

TABLE III: Yield and Properties of m_2^2G–G Copolynucleotides.

Polynucleotides	Ratio of GDP:Analog	Yield (%)	Base Ratio $G_p:m_2^2G_p$	λ_{max} (nm) (pH 7.0)	$\epsilon(P) \times 10^{-3}$	T_m[a] (°C)
Poly(G)		15		253.5	10.8	>80
Poly(G,m_2^2G)	4:1	10	85:15	255.0	10.0	>80
Poly(G,m_2^2G)	3:2	22	70:30	255.5	10.0	>80
Poly(G,m_2^2G)	1:1	25	52:48	256.5	9.9	>80
Poly(G,m_2^2G)	2:3	13	49:51	256.5	9.9	>80
Poly(G,m_2^2G)	1:4	29	26:74	257.5	8.5	39.5
Poly(G,m_2^2G)	1:5	24	10:90	257.5	8.4	41
Poly(m_2^2G)		20		256.5	7.9	40

[a] At 0.15 M salt concentration.

was evaporated *in vacuo*, and the residue was extracted with ether. The aqueous phase was made slightly acidic with acetic acid and adsorbed on charcoal. Extracts of the charcoal with ethanol–water (1:1, v/v) containing 2% ammonia were evaporated *in vacuo*. The residue was dissolved in a small amount of water and applied to a column of DEAE-cellulose (4 × 50 cm). Appropriate fractions were evaporated to give m^2GDP or m_2^2GDP in 60–70% yields. The properties of these diphosphates are summarized in Table I.

Guanosine 5′-diphosphate (GDP) was purchased from Sigma Chemical Co.

Synthesis of Polynucleotides. Poly(G), poly(m^2G), and poly(m_2^2G) were synthesized by essentially the same procedure described by Thang *et al.* (1965) and Pochon and Michelson (1969).

Copolynucleotides, poly(G, m^2G) and poly (G,m_2^2G), were synthesized as follows. The incubation mixture contained in a total volume of 12.5 ml 0.5 mmole of nucleoside diphosphate, 6.25 mmoles of Tris·HCl (pH 8.5), 0.63 mmole of $MnCl_2$, and polynucleotide phosphorylase (Grunberg-Manago *et al.*, 1965) (50 units). Incubation was performed at 60° for 8 hr. After stopping the reaction by chilling the mixture in an ice bath, proteins were removed by $CHCl_3$–isoamyl alcohol (3:1, v/v) extraction. The resulting solution was lyophilized, dissolved in 0.5–1.0 ml of water, and applied to a column (1.5 × 80 cm) of Sephadex G-25. Elution with water gave polynucleotides as the first main peak. The yield was 10–29%. Hypochromicity of polymers is neglected. The properties of these polynucleotides are summarized in Tables II and III.

Analysis of the Base Ratios in the Polynucleotides. Polynucleotides (*ca.* 5 A_{260} units) were digested with 3.0 N KOH for 18 hr at 37°. After neutralization of the digest with Dowex 50 (H^+ form), the whole mixture was subjected to paper chromatography in the solvent *n*-propyl alcohol–ammonia–water

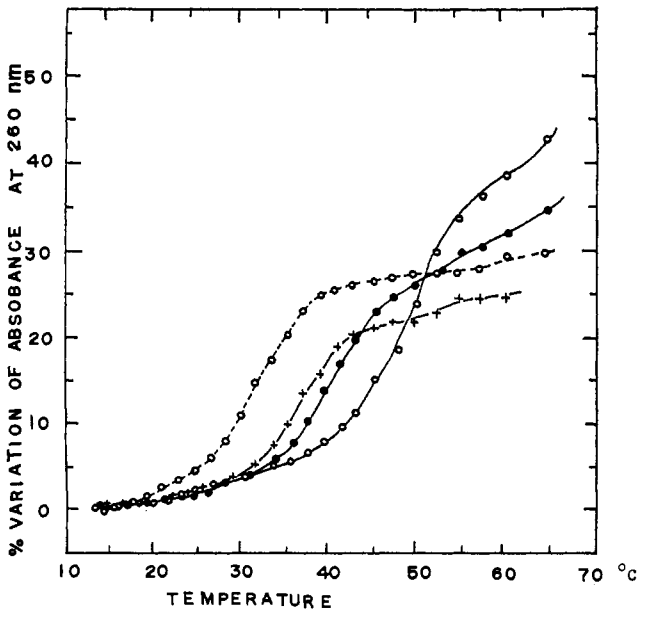

FIGURE 4: Thermal denaturation profile of poly(G_{26},$m_2^2G_{74}$). (O—O—O) With 1.5 M NaCl, (●—●—●) 0.15 M NaCl, (+—+—+) 0.075 M NaCl, and (O--O--O) 0.015 M NaCl.

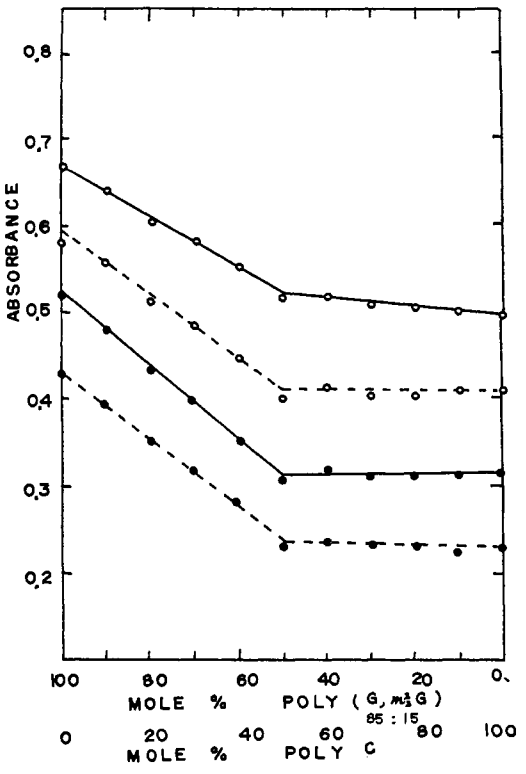

FIGURE 5: Mixing curves of poly(G_{85},$m_2^2G_{15}$) and poly(C). (O—O—O) At 272.5 nm, (O--O--O) 275.5 nm, (●—●—●) 282.5 nm, and (●--●--●) 287.5 nm.

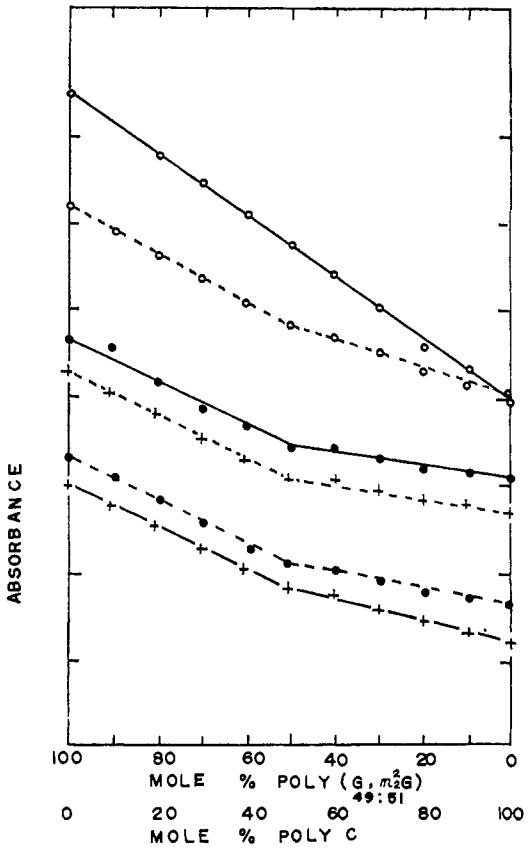

FIGURE 6: Mixing curves of poly(G_{49},$m_2^2G_{51}$) and poly(C). (O—O—O) 270 nm, (O--O--O) 265 nm, (●—●—●) 277.5 nm, (+--+--+) 280 nm (●--●--●), 287.5 nm, and (+—+—+) 290 nm.

(55:10:35, v/v). Spots corresponding to each mononucleotides were cut out and eluted with water. Amount of nucleotides were estimated by uv absorption at the absorption maximum. Since spots corresponding to nucleosides could not be detected, the chain length would be over 100 nucleotides.

Uv and CD Measurements. Uv absorption spectra were taken with a Hitachi 124 and EPS-3T spectrophotometer in 0.15 M NaCl or equivalent ionic strength. CD spectra were taken with a Jasco ORD/UV-5 spectropolarimeter equipped with a CD attachment. Temperature was 20–25° and calibration was made with *d*-10-camphorsulfonic acid.

Measurement of Melting Temperature. T_m's were measured with a Shimadzu AQV 50 spectrophotometer installed with a Komatsu thermostated cell. The temperature inside of the cell was measured by a thermister, Takara SPD-ID.

Results and Discussion

Polymerization of m²GDP and m_2^2GDP with GDP in Various Ratios. The time course of the polymerization of m²GDP incubated with various ratios of GDP was shown in Figure 1. The rate of the reaction was retarded and the yields of the copolymer decreased as the fraction of m²GDP increased in the incubation mixture. The extent of the reaction was about 20% in the case of GDP:m²GDP = 1:4. As summarized in Table II, the yield in the case of GDP:m²GDP = 4:1 was comparable to the polymerization of GDP and the ratio G:m²G in the polymer was 75:25. When the ratio reversed to 2:3, the yield of the polymer decreased to only 4% and the ratio was 45:55. This means that inhibitory effect of m²GDP

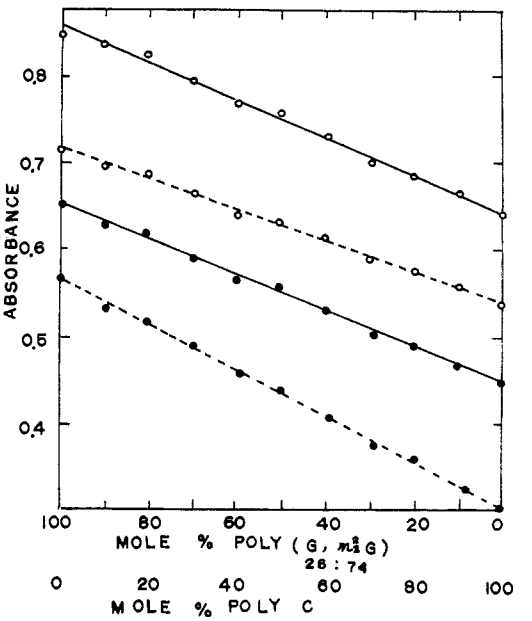

FIGURE 7: Mixing curves of poly(G_{26},$m_2^2G_{74}$) and poly(C). (O—O—O) 270 nm, (O--O--O) 275 nm, (●—●—●) 280 nm, and (●--●--●) 286 nm.

to GDP polymerization may be ascribed to a lower polymerization rate of m²GDP. In the case of poly(m²G) formation, the incubation condition (Thang *et al.*, 1965) was somewhat different from that used here. As judged from absorption properties in the neutral solution, the incorporation of m²G does not change absorption maxima of poly(G) significantly and decreased ϵ(P) about 30%.

For the copolymerization of m_2^2GDP and GDP, results are summarized in Table III. There is no inhibitory effect with m_2^2GDP on the polymerization as shown in Figure 2 and the yield of polymers was 10–29%. The amount of m_2^2G in polynucleotides increased almost proportional to the increase of the molar fraction of m_2^2G in the incubation mixture. It increased from 15% in the case of GDP:m_2^2GDP = 4:1 to the extreme of 90% in the case of 1:5 mixture. Uv absorption properties also showed a gradual change in ϵ(P) and in absorption maxima. The λ_{max} changed from 255 to 257 nm as the molar fraction of m_2^2G increased from 15 to 90% and ϵ(P) decreased from 10.0×10^3 to 8.4×10^3. These features suggested that introduction of two methyl groups on the 2-NH_2 group of guanosine did not interfere significantly with substrate–enzyme binding.

Thermal Denaturation Profiles of Poly(G,m_2^2G). In contrast to the uv absorption properties of poly(G,m_2^2G), thermal denaturation profiles (T_m curves) showed a significant difference between polymers with a low-m_2^2G and high-m_2^2G content. As shown in Figure 3, poly(G_{52},$m_2^2G_{48}$)[2] had no marked transition between 20 and 78°. Similarly polymers poly(G_{85},$m_2^2G_{15}$), poly(G_{70},$m_2^2G_{30}$), and poly(G_{49},$m_2^2G_{51}$) showed only gradual increase of absorption in 5–12%. However, as shown in Figure 4, in poly(G_{26},$m_2^2G_{74}$) a cooperative melting was observed. The T_m increased from 31 to 48.5° as the salt concentration increased. In some curves a second inflexion was recorded. Similarly T_m values of 30–50.5° were recorded for poly(G_{10},$m_2^2G_{90}$) in various salt concentrations. This is slightly lower than that of poly(m_2^2G). These results suggested an ordered structure different from that of poly(G) may exist in

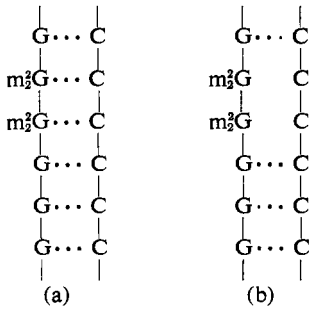

FIGURE 8: Schematic representation of the poly(G,m_2^2F)–poly(C) complex. The solid line denotes the polynucleotide backbone and dotted lines represent hydrogen bonds.

the polymer containing relatively high content of m_2^2G (>50%).

Mixing Curves of Poly(G,m²G) and Poly(G,m_2^2G) with Poly(C). Poly(G) is known to form a 1:1 complex with poly(C) in 0.1 M salt concentration at pH 7.0 (Pochon and Michelson, 1965). In contrast, poly(m²G) and poly(m_2^2G) do not form a complex with poly(C) (Pochon and Michelson, 1969). We therefore investigated the mixing of poly(G,m²G) and poly(G,m_2^2G), which contain methylated nucleotides in various ratios, with poly(C).

When poly(G_{85},$m_2^2G_{15}$) was mixed with poly(C) in a solution containing 0.1 M NaCl and 0.05 M sodium cacodylate at pH 7, these polymers formed a 1:1 complex (Figure 5). The same phenomenon was observed in the mixing curve of poly(G_{49},$m_2^2G_{51}$) with poly(C) (Figure 6). The same 1:1 complex was formed in the case of poly(G_{52},$m_2^2G_{48}$). However, as shown in Figure 7, when poly(G_{26},$m_2^2G_{74}$) was mixed with poly(C), no inflex point was detected at all wavelengths tested. In the case of poly(G_{10},$m_2^2G_{90}$) also no complex was formed with poly(C). These results suggest that poly(G,m_2^2G) mixed with poly(C) forms a 1:1 complex under appropriate conditions, if the former polynucleotide contained low amounts of methylated guanylic acids. Although this situation is similar in the case of poly(G)–poly(C) interaction, the question of hydrogen bonds between m_2^2G and C remained unsolved. For the 1:1 complex, two forms illustrated in Figure 8a,b may be drawn. Form a contained fully hydrogen-bonded nucleotide pairs, G·C or m_2^2G·C. In contrast to this, form b contained only G·C pairs and m_2^2G residues were left in the polynucleotide double strand unpaired. Although these two forms may be differentiated by infrared study (Tsuboi *et al.*, 1968), we may prefer the b form, because homopolymer of m_2^2G could not form double-stranded complex with poly(C) due presumably to the sterical distorsion of the $N(Me)_2$ group (Pochon and Michelson, 1969). Increase of the amount of m_2^2G in the polymer chain destroys the stable conformation of the double helix. m_2^2G residues in the b form may be retained in the double strand mainly by stacking with neighboring bases.

The same phenomenon was observed in the case of poly(G,m²G)–poly(C). As shown in Figure 9, poly(G_{57},m^2G_{43}) could form a complex with poly(C). This was true both for poly(G_{75},m^2G_{25}) and poly(G_{45},m^2G_{55}) again. In contrast, poly(G_{30},m^2G_{70}) could not form a complex with poly(C). These results suggested that a double helical 1:1 complex could be formed between poly(G,m²G) and poly(C), unless the former contained higher ratios of m²G.

[2] Poly(G_{52},$m_2^2G_{48}$) stands for polynucleotide composed of 52% guanylic acid and 48% dimethylguanylic acid.

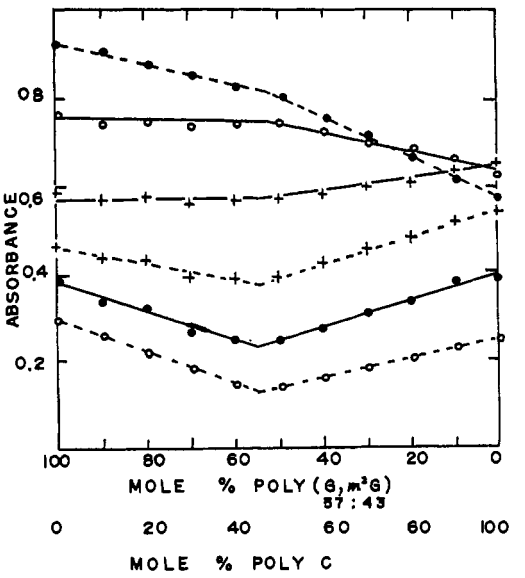

FIGURE 9: Mixing curves of poly(G_{57},m^2G_{43}) and poly(C). (●--●--●) 260 nm, (○—○—○) 265 nm, (+—+—+) 270 nm, (+--+--+) 275 nm, (●—●—●) 285 nm, and (○--○--○) 290 nm.

CD Curves of Poly(G,m²G)- and Poly(G,m_2^2G)–Poly(C) Complex. As studied by several investigators (Yang and Samejima, 1969), the formation of double-helical complex can be seen by ORD and CD spectra. We therefore investigated CD spectra of the polynucleotides containing m²G and m_2^2G together with those of complexes with poly(C).

As shown in Figure 10, poly(G_{52},$m_2^2G_{48}$) and poly(C) showed a positive Cotton band at 274 nm, a shoulder at 245 nm, and a trough at 236 nm. This curve is significantly different from a curve obtained by summation of curves of poly(G_{52},$m_2^2G_{48}$) and poly(C). Magnitudes of the Cotton effect decreased both at the peak and the trough and the crossing-

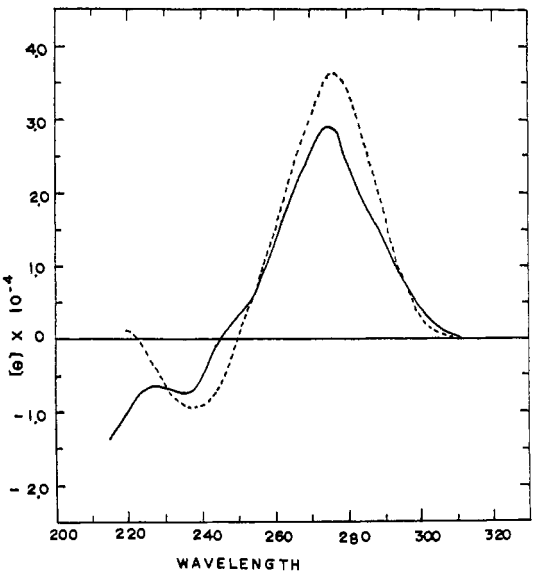

FIGURE 10: CD spectra of the poly(G_{52},$m_2^2G_{48}$)–poly(C) complex. The solid line represents the CD curve of the poly(G,m_2^2G)–poly(C) complex and the dotted line shows the calculated sum of CD of poly(Gm_2^2G) and poly(C).

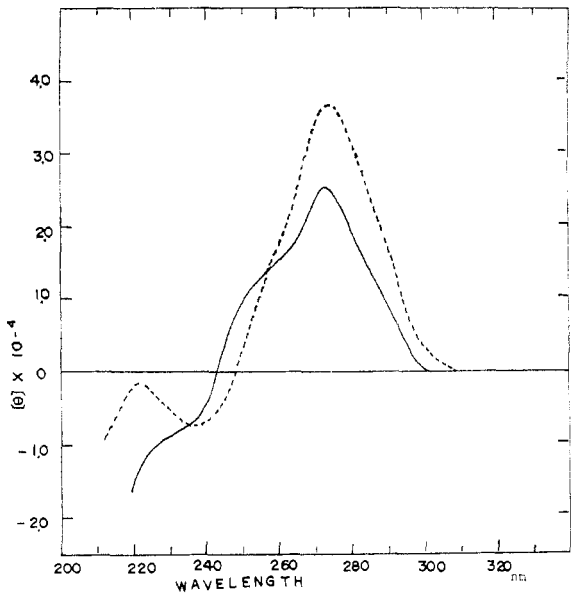

FIGURE 11: CD curve of the poly(Gm²G)–poly(C) complex. The solid line represents the CD curve of the complex, poly(G_{57},m^2G_{43})–poly(C). The dotted line shows the calculated sum of CD of poly-(G_{57},m^2G_{42}) and poly(C).

over point shifted toward shorter wavelengths. These features suggested the formation of double-helical structure for the mixed polynucleotides. When poly(m_2^2G) and poly(C) were mixed, no difference between the observed and calculated curve was found. Therefore, the complex formation of poly-(G_{52},$m_2^2G_{48}$) and poly(C) predicted by the mixing curve was supported. Similarly, when poly(G_{57},$m_2^2G_{43}$) was mixed with poly(C), a characteristic CD curve (Figure 11) was observed. In this case a positive Cotton band at 272 nm, a positive shoulder at about 248 nm, and a negative shoulder at about 238 nm were observed. In the calculated curve of poly-(G_{57},$m_2^2G_{43}$) + poly(C), it showed peaks at 273 and 221 nm, and a trough at 238 nm. The magnitude of the peak at 273 nm decreased about 35%. These features clearly showed that a complex was formed between these polynucleotides. The same type of differences of CD curves between mixed polynucleotides and the calculated were observed also in a poly(G_{75},m^2-G_{25})–poly(C) mixture. Therefore in the case of poly(G,m^2G) containing low amounts of m^2G, it interacts with poly(C) to form a double-helical complex as predicted by the mixing experiment.

The results obtained with the polynucleotides containing m^2G and m_2^2G showed that a single m^2G or m_2^2G residue in the polynucleotide chain could not prevent the formation of double-helical complexes with poly(C). The role of the methylated guanosines in the tRNA chain is still an open question.

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