Poly(2-methyl-N^6-methyladenylic acid): Synthesis, Properties, and Interaction with Poly(uridylic acid)†

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ABSTRACT: A modified poly(adenylic acid) containing methyl groups at the 2 and N(6) positions has been prepared by enzymic polymerization of the chemically synthesized 5′-diphosphate. Poly(2Me6NMeA) is strongly stacked in neutral solution by ultraviolet and circular dichroic criteria. The polymer appears to be fully stacked at 30° and less than half melted by 80° in 0.1 M Na^+. The new polymer does not form a regular helix in acid solution even at low ionic strength. Failure to form an acid helix is probably due to blocking of interchain NH···OP hydrogen bonds and the relatively less favorable geometry which would be required to accommodate the N–Me groups in the interior of the helix. Poly(2Me-6NMeA) forms a 1:1 helical complex with poly(U). Since Watson–Crick pairing is prevented by steric interference of the 2-Me group with C_2=O of the uracil residues, the complex must be hydrogen bonded at N(7) rather than N(1) of the purine residues (Hoogsteen or reverse Hoogsteen pairing). In marked contrast to Watson–Crick A·U helices the new complex is not significantly destabilized by the 6NMe group. We suggest that this difference is primarily due to restricted rotation about the exocyclic C–N bond of the methyladenine residues, the form with Me syn to N(1) being more stable than its geometrical isomer. T_m of the new A·U complex is 17° in 0.15 M Na^+. All of the Hoogsteen or reverse Hoogsteen A·U helices so far described have T_m in the range 35–50° below those of corresponding Watson–Crick helices, indicating a markedly lower stability. This difference can account for exclusive formation of Watson–Crick helices with polynucleotides which, in principle, would be capable of either kind of pairing.

All two-stranded A·U helices appear to be formed with Watson–Crick hydrogen bonding to the exclusion of other plausible bonding arrangements, though it had not been clear until recently whether this conclusion indicated that other structures were not capable of independent existence. Recent studies have shown that non-Watson–Crick A·U helices can be formed by introducing a Me (Ikehara *et al.*, 1972) or a NMe_2 (Ishikawa *et al.*, 1972) substituent into the 2 position of poly(A).[1] The objective, in these papers and in the present one, of substitution in the 2 position of adenine is to introduce a group sufficiently bulky to prevent base pair formation by steric interference with approach of uracil residues, while leaving both hydrogen bond donor and acceptor positions of the adenine intact. Though less bulky than a NMe_2 group, a Me group meets the steric requirement stated above. If we represent a Watson–Crick A·U pair with the usual hydrogen bond distances (Figure 1A), we see that a substituent M in the 2 position is about 2.8–2.9 Å from the C(2) carbonyl oxygen of uracil. When M is NH_2, a third hydrogen bond is formed, increasing the stability of the helical complex (Howard *et al.*, 1966; Ikeda *et al.*, 1970). When M is a methyl group, however, no such bond can be formed, and the Me···O distance is too close to be allowed. Pauling (1960) estimates 2.0 Å as the van der Waals radius of a methyl or methylene group and about 3.4 Å for an intermolecular CH_2···O contact. The shaded area in Figure 1 has a radius of 3.4 Å about the methyl group M and would represent a nonallowed region for the oxygen. Since the oxygen cannot be removed from this region while maintaining an approximately coplanar base pair with acceptable A·U hydrogen bond distances, we conclude that Watson–Crick base pairing is not possible with this polymer. We also cite the failure of poly(2MeI) to interact with poly(C) (Ikehara and Hattori, 1972) as further evidence that the carbonyl to methyl distance is too close to permit base pair formation.

Previous studies have also shown that poly(A) with a single Me residue on the amino group could still form a 1:1 complex with poly(U) (Griffin *et al.*, 1964; Ikeda *et al.*, 1970). The helices had Watson–Crick bonding (Ikeda *et al.*, 1970; Figure 1D), though they were markedly destabilized by the presence of a 6-NMe group.

We describe in this paper a new substituted poly(A) containing two of the modifications (2-Me and 6-NMe) which had previously been studied separately. The properties of the new polymer are strongly affected by these modifications, which greatly aid the interpretation of previous studies.

The neutral polymer appears to be more strongly stacked than poly(A) or most of its derivatives, but no helical acid self-structure could be detected at lower pH.

The new polymer forms a 1:1 complex with poly(U), which must have non-Watson–Crick bonding as a result of steric interference by the 2-methyl group (Figure 1B or C).

Both of the methyl substitutions considered in this paper are known to occur naturally in the nucleic acids. 2-Methyladenine has been found in $tRNA^{Glu}$ of *Escherichia coli* (Ohasi *et al.*, 1970) and N^6-methyladenine in $tRNA^{Val}$ of the same organism (Yaniv and Barrell, 1969; Kimura *et al.*, 1971). N^6-Methyladenine is also found in some bacterial DNA, where it appears to be the chemical modification responsible for species or strain specificity of restriction enzymes (Smith *et al.*, 1972; Meselson *et al.*, 1972; Arber and Linn, 1969). Introduction of one such methyl group in approximately 10^4 bases of double-stranded DNA appears to protect host DNA of certain bacterial strains from cleavage by their own restriction enzymes. While the basis for recognition by the enzyme of this chemical modification is at present unknown,

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[1] Abbreviations used are: poly(A), poly(adenylic acid); poly(2Me-6NMeA), poly(2-methyl-N^6-methyladenylic acid); poly(2Me6NMeA)·poly(U) and r(2Me6NMeA)·r(U), 1:1 complex of poly(2Me6NMeA) with poly(U).

studies with model systems containing the 6-NMe group may contribute to an understanding of the mechanism.

Materials and Methods

2-Methyl-N^6-methyladenosine 5'-Monophosphate. To a stirred solution of 110 mg (0.37 mmol) of 2-methyl-N^6-methyladenosine (nucleoside previously reported by Yamazaki *et al.*, 1968) in 1.0 ml of trimethyl phosphate was added 0.05 ml of phosphorus oxychloride at 0°. The reaction mixture was stirred in an ice bath for 3 hr. The reaction mixture was then poured into 100 ml of ice–water, neutralized with 1 N KOH, and applied to a column of Dowex 1-X8 (formate form; 1.5 cm × 17 cm). After the water wash, the column was eluted with 0.1 N formic acid. The fractions corresponding to 2-methyl-N^6-methyladenosine monophosphate were pooled and applied to a column of active charcoal (1.0 cm × 2 cm). After the water wash nucleotidic material was eluted with 50% ethanol containing 2% ammonia; the solvent was evaporated to dryness *in vacuo*. 2-Methyl-N^6-methyladenosine monophosphate was obtained in 92% yield (0.34 mmol, determined by absorbance at 269 nm); paper electrophoresis: R_{AMP} 0.87 in ammonium bicarbonate (0.05 M) at pH 7.5.

To establish the identity of the product, a sample of the material was dephosphorylated with crude venom 5'-phosphatase. The incubation mixture (150 μl) contained 100 μl of 2-methyl-N^6-methyladenosine monophosphate (10 μmol/ml), 10 μl of $MgCl_2$ (100 mM), 20 μl of Tris-HCl (pH 8.7) (250 mM), and 20 μl of crude venom 5'-phosphatase (20 mg/ml). Incubation was carried out at 37° for 3 hr. Under these conditions 2-methyl-N^6-methyladenosine monophosphate was completely converted to 2-methyl-N^6-methyladenosine, indicating that direct phosphorylation of unblocked nucleoside 2-methyl-N^6-methyladenosine with phosphorus oxychloride in trimethyl phosphate produced only 5'-monophosphate, as previously observed in the case of other nucleosides (Yoshikawa and Kato, 1967).

2-Methyl-N^6-methyladenosine 5'-Diphosphate. 2-Methyl-N^6-methyladenosine 5'-monophosphate (0.33 mmol), thus obtained, was converted to the morpholidate and allowed to react with inorganic phosphate according to the method of Moffatt and Khorana (1961).

2-Methyl-N^6-methyladenosine 5'-monophosphate (morpholinium salt) was dissolved in *tert*-butyl alcohol (5 ml) and water (5 ml). To this solution was added morpholine (0.17 ml) and water (5 ml). To this solution was added morpholine (0.17 ml). While the mixture was refluxed, dicyclohexylcarbodiimide (0.55 g) dissolved in *tert*-butyl alcohol (10 ml) was added dropwise. The reaction mixture was refluxed for 2 hr and kept overnight at room temperature. Dicyclohexylurea was removed by filtration. *tert*-Butyl alcohol was evaporated *in vacuo* and the water solution was extracted with ether to remove unreacted dicyclohexylcarbodiimide. The water layer was evaporated *in vacuo*. The residue was dissolved in a small amount of dry pyridine and evaporated again. This procedure was repeated three times for complete drying.

To the residue was added tri-*n*-butylamine (0.5 ml) and inorganic phosphoric acid (0.075 ml), which had been previously dried by repeated evaporation with added dry pyridine. The mixture was further dried by repeated addition and evaporation of pyridine and kept at 30° for 2 days with the exclusion of moisture.

The reaction was stopped by the addition of water. Pyridine was evaporated and the water solution was applied to a column of active charcoal (1.0 cm × 5 cm). After the column was washed with water, nucleotidic material was eluted with 50% ethanol containing 2% ammonia. Eluents were concentrated by evaporation *in vacuo* and applied to a column of DEAE-Sephadex (1.5 cm × 15 cm) (bicarbonate form). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer: 1 l. each of 0.05 M solution and 0.25 M solution. 5'-Diphosphate was obtained in 72% yield (0.24 mmol). Acidic-labile phosphate was determined by measuring release of inorganic phosphate after the 5'-diphosphate in 1 N HCl was incubated at 100° for 10 min. Total phosphate was determined after digestion under the conditions described by Howard *et al.* (1971). Inorganic phosphate was determined by the procedure of Fiske and Subba Row (1925). Nucleoside–acid labile P_i–total P_i ratio was 1.0:1.2:2.1: λ_{max} (pH 7.4) 269 nm, λ_{max} (0.1 N HCl) 262.5 nm, λ_{max} (0.1 N NaOH) 269 nm. Paper electrophoresis (Toyo filter paper No. 51A) was carried out at pH 7.5 in 0.05 M triethylammonium bicarbonate for 1 hr at 20 or 60 V/cm; R_{AMP} 1.13.

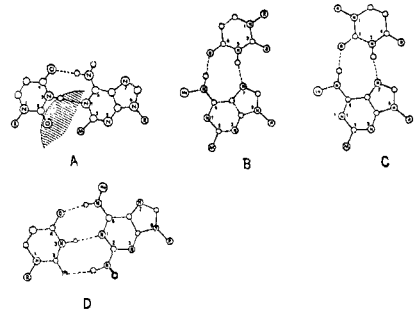

FIGURE 1: Hydrogen bonding arrangements between poly(U) and substituted poly(A) molecules which have been chemically modified to control the site of interaction. In A the substituent M in the adenine 2 position would exclude the uracil residue from the shaded region when M = methyl and from a larger region when M = NMe_2. For this reason the Watson–Crick pair shown cannot be formed (*cf.* text). The pairs B and C can be formed even when the 2 position of adenine is blocked (M = Me or NMe_2), but the 6-NMe group must be syn to N(1). In D the stability of a Watson–Crick pair is enhanced by a third hydrogen bond. Here the 6-NMe group of the modified adenines must be anti to N(1).

Polymerization of 2-Methyl-N^6-methyladenosine 5'-Diphosphate. The incubation mixture (12.5 ml) contained 2-methyl-N^6-methyladenosine diphosphate, 50 μmol (4 mM), $MgCl_2$ (2 mM), Tris-HCl (50 mM), and *E. coli* polynucleotide phosphorylase (Grunberg-Manago *et al.*, 1956) (1.4 units/ml). After incubation at 37° for 9 hr, proteins were removed with a chloroform–isoamyl alcohol (3:1) mixture and the water layer was lyophilized. The residue was dissolved in water (2.0 ml) containing 0.01 M EDTA and 0.05 M Tris-HCl (pH 7.5). The solution was applied to a column (2.0 cm × 100 cm) of Sephadex G-50. The column was eluted with water and 6.2 ml fractions were collected. Product polynucleotide was obtained in the first peak, and its isolated yield was 2.35 μmol (47%). For the purpose of studying physical properties, this polynucleotide was further dialyzed against 0.5 M sodium chloride containing 0.01 M Tris-HCl (pH 7.3), and 0.001 M EDTA containing 0.01 M Tris-HCl (pH 7.3) and water.

Ultraviolet spectra were measured with a Cary Model 15 spectrophotometer or a Zeiss PMQ II spectrophotometer.

Circular dichroic spectra were measured with a Cary Model 60 spectrophotometer with a Model 6001 circular dichroism (CD) attachment. Data were transmitted on-line to a Honeywell DDP-516 computer. Communication between the laboratory and the computer system was accomplished with a remote operator's console designed and built at NIH (Shapiro and

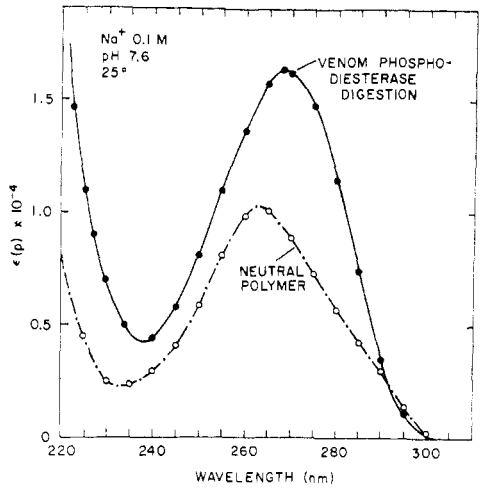

FIGURE 2: Ultraviolet spectrum of poly(2Me6NMeA) (8.3×10^{-5} M; Na^+, 0.1 M; pH 7.6; 25°) (○). Spectrum of the same solution after treatment with venom phosphodiesterase (●).

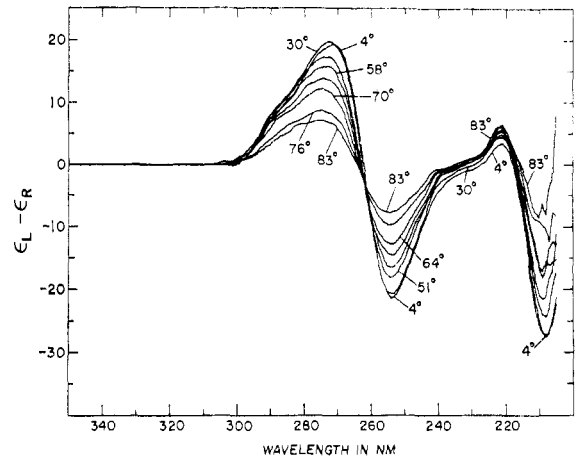

FIGURE 3: Circular dichroic spectra of poly(2Me6NMeA) (8.3×10^{-5} M; Na^+, 0.1 M; phosphate buffer, 0.002 M, pH 7.5) as a function of temperature. Data were reduced and plotted by computer as described in *Materials and Methods*.

Schultz, 1971). Data reduction, scaling to a molar basis, and summation of spectra were carried out by the computer. The spectra were drawn by a peripheral Calcomp plotter. We are indebted to Marie Chang for writing many of the necessary programs.

Temperature measurements were made with a Digitec digital thermometer and a calibrated Yellow Springs Instrument thermistor probe.

Results and Discussion

Poly(2-methyl-N^6-methyladenylic acid). (a) Neutral Solution. The neutral polymer has λ_{max} 264 nm and ϵ_{max} 10,300 in 0.1 M Na^+ at 25°. Digestion with venom phosphodiesterase results in an increase of ϵ_{max} to 16,200 and of λ_{max} to 268 nm (Figure 2). The nucleoside has λ_{max} 269 nm and ϵ_{max} 16,300 (Table I).

The circular dichroic spectrum of the neutral polymer at 10° has λ_{max} 271 nm ($\Delta\epsilon$ 20.1) and 220 nm ($\Delta\epsilon$ 4.1) and λ_{min} at 253 nm ($\Delta\epsilon$ −21.3) and 208 nm ($\Delta\epsilon$ −30.4). There are also clearly defined shoulders at ~290 and ~240 nm (Figure 3). The pair of positive and negative extrema at 271 and 253 nm are equal in magnitude and equidistant from the crossover point ($\Delta\epsilon$ 0 at 263 nm), which occurs at λ_{max} of the absorption spectrum. These spectral features arise from exciton splitting of a transition in the adenine residues and satisfy very well the

TABLE I: Spectroscopic Data.

Material	Ultraviolet λ_{max}	$\epsilon_{max} \times 10^3$	λ_{min}
2-Methyl-N^6-methyladenosine[a]	269	16.3	231
2-Methyladenosine[b,c]	263.5	15	
Poly(2Me6NMeA)[a]	264	10.3	233
Poly(2MeA)[c]	259.5	9.58	
Poly(2Me6NMeA)·poly(U)[d]	260	7.8	232

	Circular Dichroism[e]							
	λ_{max}	$\epsilon_L - \epsilon_R$	λ_{min}	$\epsilon_L - \epsilon_R$	λ_{max}	$\epsilon_L - \epsilon_R$	λ_{min}	$\epsilon_L - \epsilon_R$
Poly(2Me6NMeA)[a]								
4°	271	+19.3	253	−21.5	222	+3.5	208	−28.1
70°	273	+12.4	253	−12.9	222	+7.1	209	−19.7
83°	276	+7.1	255	−7.8	220	+5.5	207	−15
Poly(2Me6NMeA)[f]								
pH 7.5	272	+17.7	255	−20.4	222	+3.4	209	−27.5
pH 5.1	276	+5.5	253	−8.4	222	+0.6	210	−9.8
pH 4.4	275	+0.76	254	−3.0	228	−0.1	210	−6.8
Poly(2Me6NMeA)·poly(U)[g]								
3.7° (helix)	265	+9.3	248	−6.7	221	+5.3	208	−9.1
15.6° (half melted)	271	+12.2	251	−9.5	221	+2.2	209	−12.0
26° (dissociated polymers)	271	+12.4	254	−10.3	221	+2.2	208	−12.9

[a] Na^+, 0.1 M; phosphate, 0.002 M, pH 7.5; 25°. [b] Na^+, 0.1 M cacodylate, 0.05 M, pH 7.0. [c] Data of Ikehara *et al.*, 1972, *loc. cit.* [d] Na^+, 0.1 M; phosphate, 0.002 M, pH 7.5; 5.3°. [e] Duplicate measurements of all CD spectra were averaged and normalized to a molar basis by computer. [f] Na^+, 0.001 M; 25°. Phosphate buffer for pH 7.5, acetate buffer for pH 5.1 and pH 4.4. There appears to be a slight dependence of CD spectrum of the neutral polymer on sodium ion concentration.

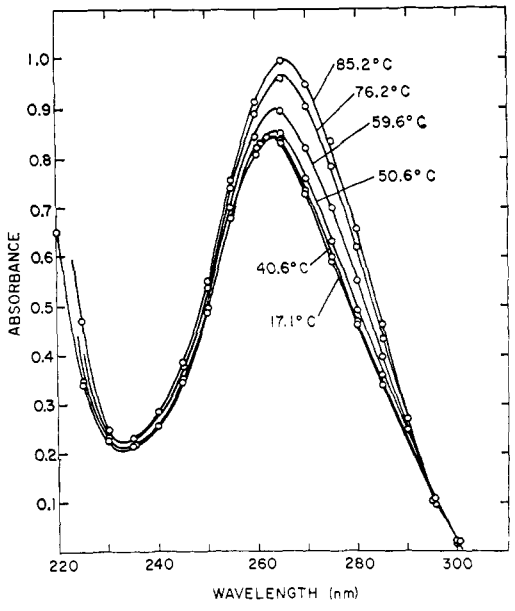

FIGURE 4: Temperature dependence of ultraviolet spectrum of poly(2Me6NMeA) (8.3×10^{-5} M; Na^+, 0.10 M; phosphate buffer, 0.002 M, pH 7.5).

theoretical criteria for coupling of a single transition. The CD spectra are quite similar to those of poly(2MeA) and poly(A) (Ikehara *et al.*, 1972).

(*b*) *Temperature Dependence of Optical Properties.* The ultraviolet spectrum of poly(2Me6NMeA) does not change significantly over the temperature range 0–40°, but above 50° exhibits an increase of absorbance and small red shift of λ_{max} (Figure 4). The temperature profile of absorbance is shown in Figure 5. The hypochromism $(\epsilon_{max}^{monomer} - \epsilon_{max}^{polymer})/\epsilon_{max}^{polymer} \cong 58.2\%$ at 25°) reflects stacking of the bases and is similar in magnitude to poly(A) and several of its derivatives.

The stacked configuration of the polymer appears to be considerably more stable than those of most other single-stranded polynucleotides. Both the uv and CD spectra are essentially constant between 0 and ~30° (Figures 4–6), indicating that the structure is fully stacked at room temperature (or at least that the configuration is saturated with respect to these optical properties). The temperature profile of absorbance appears to be noncooperative and indicates a high thermal stability of uv sensitive configurations. At 85° poly(2Me6NMeA) still retains about 70% of its hypochromism, compared to ~59% for poly(2MeA) and ~39% for poly(A) under comparable conditions (Ikehara *et al.*, 1972). While we have no accurate measure from an incomplete melting curve (Figure 5), we would estimate T_m to be somewhat above 80°. It appears that the 2-methyl group favors base stacking and that the 6-N-Me group further enhances this tendency. Poly(2$NH_2$6NMeA), which has the 6-N-Me substitution in common with the presently described polymer, is, by the ultraviolet criterion, almost fully stacked at room temperature (Ikeda *et al.*, 1970). It loses virtually all of its hypochromism by 85°, however, and exhibits a marked cooperativity of melting.

The CD spectra have an approximate isosbestic point at 259 nm (Figure 3), suggesting that exciton splitting of a single transition near 260 nm and the existence of only two configurational states can account for the pair of extrema between 240 and 280 nm.

(*b*) *Acid Solution.* Poly(A) forms a regular, helical self structure in acid solution, with hydrogen bonding of the amino

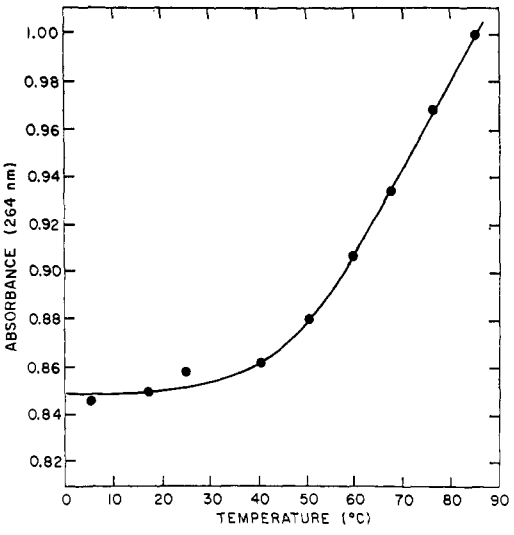

FIGURE 5: Temperature profile of absorbance of poly(2Me6NMeA) (conditions of Figure 3).

group to N_7 and protonation of N_1 (Fresco and Doty, 1957; Beers and Steiner, 1957; Rich *et al.*, 1961; Massoulié, 1965). Recent reports of poly(2MeA) (Ikehara *et al.*, 1972) and poly(2NMe_2A) (Ishikawa *et al.*, 1972) show that both Me and NMe_2 substituents in the 2 position of poly(A) greatly enhance the stability of the acid helix. The effect of a 6-NMe group, on the other hand, is strongly destabilizing. Ikeda *et al.* (1970) found that poly(2$NH_2$6NMeA) forms an acid helix, though with the $T_m \sim 30°$ below that of poly(A) under comparable conditions. Griffin *et al.* (1964) stated that poly(6NMeA) did not form an acid helix but reported no experiments at low ionic strength, necessary to detect an acid structure of low stability.

We were especially interested to see whether an acid helix would be formed by poly(2Me6NMeA). In the model of acid poly(A) proposed by Rich *et al.* (1961) a hydrogen bond between the second proton of the 6-amino group and a phos-

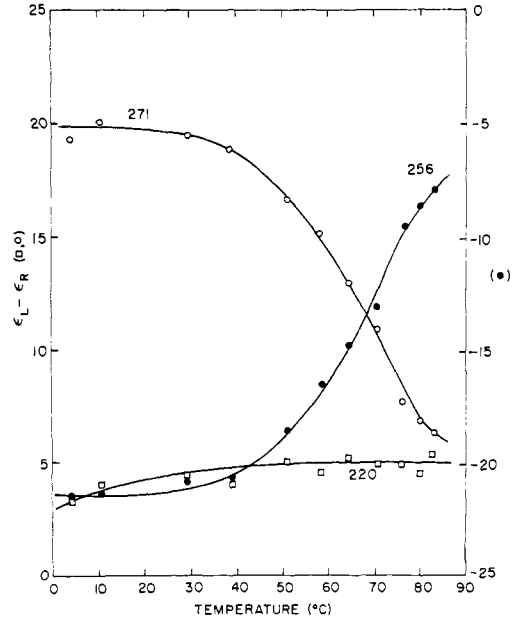

FIGURE 6: Temperature dependence of circular dichroism of poly(2Me6NMeA) (conditions of Figure 3) at wavelengths of the conservative pair of extrema and of the temperature-insensitive peak at 220 nm. Like the ultraviolet profiles (Figure 5) these curves suggest that the polymer is less than half melted at 85°.

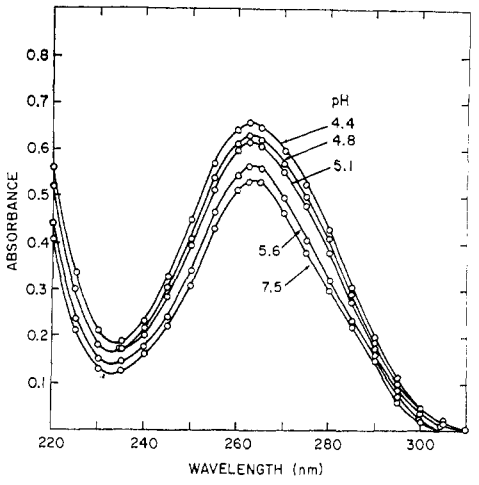

FIGURE 7: pH dependence of ultraviolet spectrum of poly(2Me6NMeA) at 25°. The solution was 0.001 M in sodium acetate buffer, adjusted with 1 N HCl to give the indicated pH values.

phate oxygen in the opposite chains plays an important role in producing an unusually compact structure. A 6-NMe group provides a valuable experimental probe of this structural feature since the relevant hydrogen bond is precluded, and the molecule would have to expand considerably to accommodate the NMe groups.

The ultraviolet spectrum of poly(2Me6NMeA) exhibits with decreasing pH a general increase in absorbance throughout the observed range, with no significant change in wavelength (Figure 7). Poly(A), in contrast, shows a marked decrease of absorbance on acidification (Steiner and Beers, 1957). The circular dichroic spectrum shows a progressive reduction in magnitude of all extrema upon acidification (Figure 8), finally reaching at pH 4.4 very low ellipticities, more suggestive of a monomer than of an ordered polynucleotide. The measurements were made under conditions potentially favorable to formation of an acid helix (Na^+, 0.001 M; 5° as well as 25°). Again in marked contrast to the present polymer, with poly(A) the longest wavelength extremum is significantly intensified upon acidification (Brahms, 1965; Ikehara *et al.*, 1972). We conclude that poly(2Me6NMeA) very probably does not form an acid helix even under these favorable conditions. The polymer does undergo ring protonation of the purine residues, however, as indicated by the spectroscopic changes upon

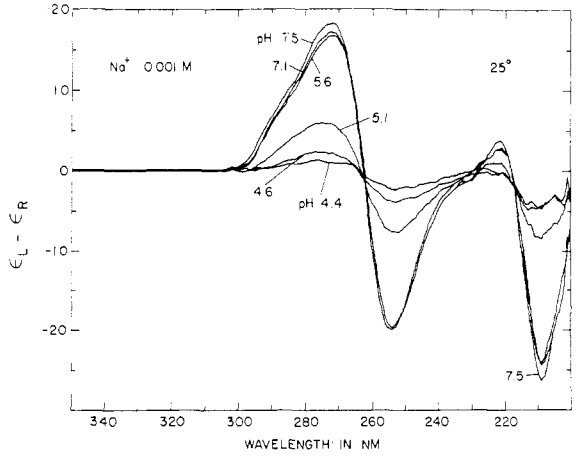

FIGURE 8: pH dependence of CD spectrum of poly(2Me6NMeA) (conditions of Figure 7). The uniform decrease of $\epsilon_L - \epsilon_R$ to very low values strongly suggests protonation of the bases without formation of any regular, helical structure.

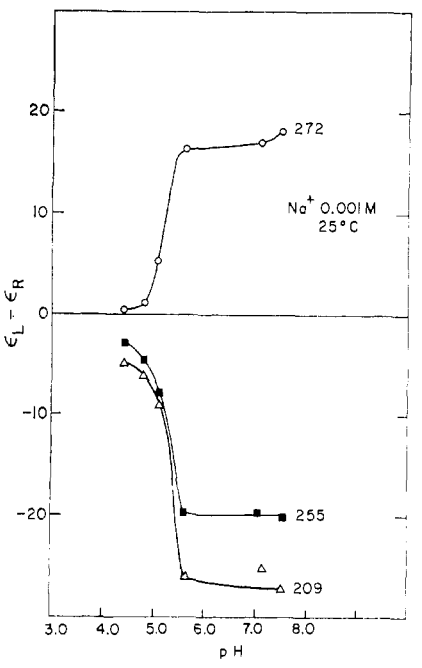

FIGURE 9: CD titration of poly(2Me6NMeA) (conditions of Figures 7 and 8).

acidification. An ultraviolet titration curve in 0.001 M Na^+ shows a transition with $pK = 5.0 \pm 0.1$, a plateau with midpoint at pH ~4 and a second increase in absorbance at pH ~3 (the pK of the nucleoside is 4.0). In 0.01 M Na^+ the apparent pK is ~4.8 and in 0.2 M Na^+, ~4.3. The CD titration in Figure 9 shows a pK of ~5.2 but was not carried to lower pH. The spectroscopic change of $pK \sim 5$ represents protonation of some or all of the bases. The absorbance change at lower pH might represent, for example, the response of protonated bases to protonation of phosphates (at an unusually high pH) or of a second class of bases (the two pK values being displaced both up and down from the monomer value by electrostatic interactions in the polymer chain). At higher [Na^+] (*e.g.*, >0.5 M) and at lower pH the polymer precipitates, but the data discussed above were obtained on homogeneous solutions. We do not know, and did not pursue further, the structure of the protonated polymer, but we conclude that it is not a regular structure, either single or double stranded.

Failure of poly(2Me6NMeA) to form an acid helix is presumably due to the factors discussed above: blocking of an interchain NH···OP hydrogen bond and of a favorable, compact structure. The prohibition of an acid structure by 6-NMe is evidently not absolute, however, but rather results from an unfavorable contribution which may in some polymers be compensated by favorable ones. Poly(2$NH_2$6NMeA) was shown by infrared and ultraviolet spectroscopy to form a regular acid self-structure, though one of rather low stability (Ikeda *et al.*, 1970). It is possible that the effect of the 2-NH_2 group in increasing the basicity of the latter polymer is responsible for stabilizing an otherwise unfavorable structure.

Interaction with Poly(U). The ultraviolet spectrum of 1:1 mixture of poly(2Me6NMeA) and poly(U) (Figure 10) is hypochromic over the range 237–290 nm, indicating complex formation. Ultraviolet mixing curves at four wavelengths exhibit sharp discontinuities at a mole fraction of poly(U) of 0.5, confirming the interaction and clearly establishing the stoichiometry to be 1:1 (Figure 11).

Temperature profiles of absorbance are sharp, sigmoid curves, the entire transition occurring over a range of ~6°

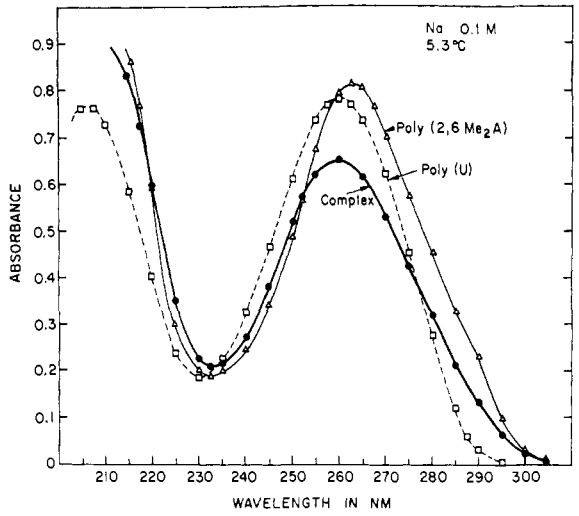

FIGURE 10: Ultraviolet spectrum of 1:1 mixture of poly(2Me6NMeA) and poly(U) (8.3×10^{-5} M in polymer phosphate for each homopolymer and for the complex; Na^+, 0.1 M; phosphate buffer, 0.002 M, pH 7.5; 5.3°) and of the component polymers. The complex has a lower absorbance than a summation of the components ($[A_\lambda^{MeA} + A_\lambda^U]/2$) between 237 and 290 nm, but is hyperchromic above 290 nm. Poly(U) under these conditions is partially helical and hypochromic with respect to the spectrum at 25°.

(Figure 12). T_m is 14° in 0.1 M Na^+, 17° in 0.15 M Na^+, 21.4° in 0.25 M Na^+, and 27.3° in 0.5 M Na^+, all in 0.001 M phosphate buffer at pH 7.5. The transition temperature of the complex is a linear function of sodium ion concentration and has a slope, $dT_m/d \log [Na^+] = 20°$. Poly(U)·poly(2Me6NMeA) must differ in the coordinates of its phosphate residues from Watson–Crick helices and hence in the detailed geometry of its charge distribution. This is the first such helix, structurally isomeric with Watson–Crick helices, for which the salt dependence is available. It is therefore significant that the magnitude of $dT_m/d \log [Na^+]$ is essentially the same as that observed for most two-stranded polynucleotide helices having Watson–Crick structures (for a theoretical treatment

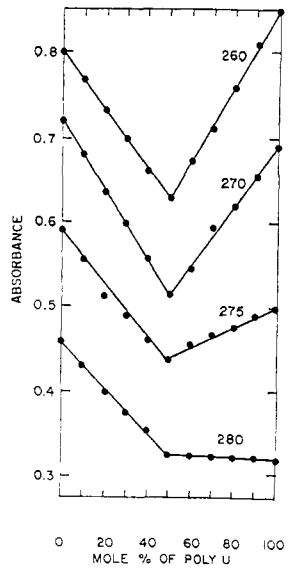

FIGURE 11: Ultraviolet mixing curves of poly(2Me6NMeA) and poly(U) in 0.1 M Na^+ and 0.002 M phosphate, pH 7.5, at 5.7 ± 0.2°. Sharp discontinuities at 50% poly(U) at all wavelengths demonstrate 1:1 stoichiometry.

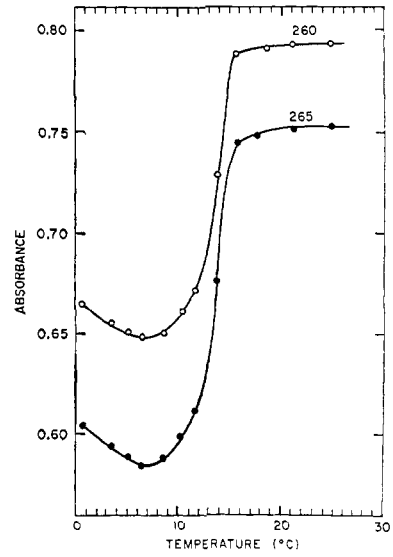

FIGURE 12: Ultraviolet melting curves of poly(2Me6NMeA)·poly(U) (conditions of Figure 11). $T_m = 14°$ in 0.1 M Na^+.

and relevant earlier references on electrostatic effects, see Record, 1967).

The circular dichroic spectrum of a 1:1 mixture of poly(2Me6NMeA) and poly(U) has positive extrema at 266 nm ($\Delta\epsilon$ 9.13) and 220 nm ($\Delta\epsilon$ 5.00) and negative extrema at 248 nm ($\Delta\epsilon$ −6.64) and 208 nm ($\Delta\epsilon$ −7.76) (Figure 13). The conservative pair of extrema is uniformly blue shifted by about 4 nm and diminished in magnitude in comparison with the summation curve. The 220-nm maximum increases in magnitude on helix formation and the 208-nm minimum decreases, both without change of wavelength. It is interesting to observe that the changes in circular dichroism upon helix formation as well as the final spectrum are similar for r(2Me6NMeA)· r(U), r(2MeA)·r(U), and r(A)·r(U) in sign, wavelength, and magnitude of the first pair of extrema (Table I, Figure 13, and Ikehara *et al.*, 1972). The similarity of the first two is not surprising since they should have the same bonding scheme and similar structures, either Hoogsteen (with parallel strands and H bonding to $C_4{=}O$ of U) or reverse Hoogsteen (with antiparallel strands and bonding to $C_2{=}O$ of U). r(A)·r(U), however, has a Watson–Crick structure, quite different from either of the others. The CD spectra of these complexes are evidently relatively insensitive to certain large differences in molecular structure, though

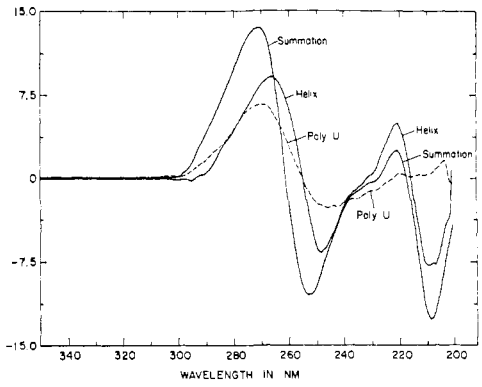

FIGURE 13: CD spectrum of poly(2Me6NMeA)·poly(U) and of a summation ($[\Delta\epsilon_\lambda^{MeA} + \Delta\epsilon_\lambda^U]/2$) of the components (solid curves, conditions of Figure 11). Ordinate, $\epsilon_L - \epsilon_R$, molarity expressed in terms of polymer phosphate.

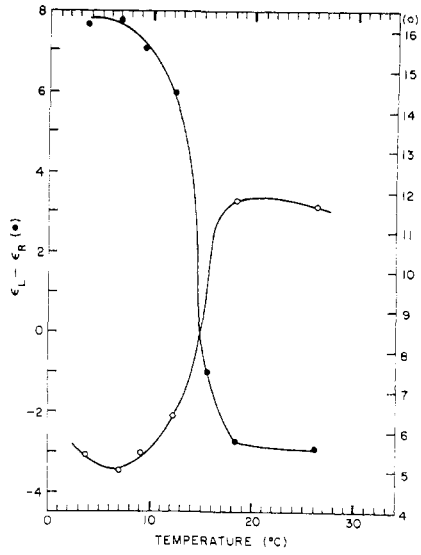

FIGURE 14: CD melting curves of poly(2Me6NMeA)·poly(U) measured at 260 nm (●, left ordinate) and 275 nm (○, right ordinate).

they are obviously quite sensitive to other structural differences.

The CD temperature profiles (Figure 14) reflect cooperative melting of the helix and are essentially congruent with ultraviolet curves (Figure 12).

We may now combine the present results with those of our previous studies to reach several conclusions of general significance to the polynucleotide field. We first observe that all of the four Hoogsteen or reverse Hoogsteen helices which have so far been described [r(2NMe$_2$A)·r(U); r(2NMe$_2$A)·r(BrU) (Ishikawa, Frazier, Howard and Miles, 1972); r(2MeA)·r(U) (Ikehara *et al.*, 1972); and r(2Me6NMeA)·r(U) (present work)] have transition temperatures 35–50° below those of comparable Watson–Crick helices, indicating a markedly lower stability. This difference can account for the fact that polynucleotides which could, in principle, form either bonding scheme appear to form Watson–Crick helices exclusively. Since this preference is not exhibited in monomer–monomer interactions (Hoogsteen, 1963; Katz, 1969; Nagel and Hanlon, 1972), we conclude that the Watson–Crick structure permits the ribose–phosphate backbone to assume an intrinsicially more favorable geometry.

We next consider the strongly destabilizing effect of a 6-NMe group in Watson–Crick helices ($\Delta T_m \cong -43°$, Ikeda *et al.*, 1970) and contrast that result with the negligible effect ($\Delta T_m = -4°$) observed in the present study. This difference could arise from a restricted rotation of the methylamino group of the adenine residues analogous to that demonstrated for the exocyclic methylamino group in cytosine monomers (Shoup *et al.*, 1972). The latter molecules were found to exist in two geometrically isomeric forms, with the methyl group syn and anti to the N(3) ring nitrogen. The equilibrium was strongly in favor of the syn form (96% at −20° in dimethylformamide; free energy difference between rotamers, −1.6 kcal) which would be sterically blocked from participating in Watson–Crick hydrogen bonding. For that reason the unfavorable equilibrium of the rotamers was suggested (Shoup *et al.*, 1972) as an explanation of the reported failure of poly(7NMeC) to interact with poly(I) (Brimacombe and Reese, 1966). In the case of methyladenines, however, both rotamers are capable of base pairing: Me syn to N(1), Hoogsteen (Figure 1B) and Me anti to N(1), Watson–Crick (Figure 1D). Crystallographic evidence (Sternglanz and Bugg, 1973a,b) shows that several different methylaminoadenines have Me syn to N(1) in the solid state and suggests that this form would be favored in other environments. The polynucleotide results of Ikeda *et al.* (1970) show that a 6-NMe group is capable of existing in a helix with a structure anti to N(1) (Figure 1D). It therefore appears very likely that rotational isomerism exists in methyladenines analogous to that in methylcytosines, with the syn (to N(1)) form being favored. Though we do not know the rotational equilibrium constant, we can calculate the free energy difference between rotamers for different assumed equilibrium compositions of the monomer. Thus, for 1% of the less common rotamer at equilibrium $\Delta(\Delta F°)$ would be 2.7 kcal/mol (at 25°), for 5%, 1.8 kcal/mol, and for 10%, 1.3 kcal/mol. The observed result that helices incorporating either of the possible rotamers (Figure 1B or C and D) are capable of existence may permit us to estimate a lower limit for the equilibrium constant. If the less stable rotamer were present to less than 1% at equilibrium, for example, the free energy difference would probably be too great to permit it to exist in a helix. That is, there are unlikely to be sufficiently large stabilizing contributions from other sources to overcome a positive rotational free energy contribution of the methyl group of 3 kcal or more.

If we use the estimate of Crothers and Zimm (1964) that a 50° difference in T_m corresponds to 1 kcal difference in free energy, we can obtain a very rough estimate of 860 cal as the free energy increase caused by introduction of a 6-NMe group into a Watson–Crick helix, assuming that the entire observed difference of ~43° (Ikeda *et al.*, 1970) is due to the methyl group. These calculations indicate that an unfavorable rotational equilibrium is capable of accounting both for the observed destabilization of 6-NMe groups in Watson–Crick helices and for the lack of an effect in Hoogsteen helices, though the actual numerical values for the free energies are not known. While other factors are presumably involved,[2] we suggest that this one may be the most important in accounting for the differences discussed above.

From these model studies we can draw certain conclusions about the base-pairing status of methyladenine residues in naturally occurring nucleic acids. We start with the finding that a 6-NMe group in adenine residues of a homopolymer does not prevent Watson–Crick base-pair formation in a two-stranded helix, though it is clearly destabilizing (Ikeda *et al.*, 1970). In a region of otherwise stable DNA helix we would expect an isolated N^6-methyladenine residue to be included in the helix and paired to thymine in the Watson–Crick manner. While a 2-methyl or a N^6-dimethyl substitution would prevent Watson–Crick pairing under all circumstances, substitution of a single methyl group on the exocyclic nitrogen would not have this exclusive role.

When the 6-NMe group occurs in shorter or less stable regions of a helix, however, either in tRNA or in DNA, the destabilizing effect of the methyl group could easily suffice to disrupt pair formation and cause looping out. The stacking free energy and *N*-methyl destabilization free energy may in some cases be approximately equal and of opposite sign, leading to a delicate balance between paired and unpaired structures for the methyladenine residues. In such cases the equilibrium might be shifted in one direction or the other by changes in temperature or in detailed sequence of the helix on either side of the methyladenine residues.

[2] We would note, for example, that a 6-NMe group in *monomer*–polymer Watson–Crick helices depressed T_m by only one-sixth the amount observed in polymer–polymer helices (Ikeda *et al.*, 1970).

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