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Poly(8-methyladenylic acid): A Single-Stranded Regular Structure with Alternating Syn–Anti Conformations†

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ABSTRACT: Poly(8-methyladenylic acid) has been prepared by chemical synthesis of 8-methyladenosine 5′-diphosphate and enzymatic polymerization with polynucleotide phosphorylase. The polymer exhibits a large hypochromism and cooperative melting in neutral solution. The transition temperature is independent of salt concentration at moderate ionic strength and decreases slightly at high salt. The adenine ring vibration at 1626 cm^{-1} is independent of temperature. A high-resolution nuclear magnetic resonance spectrum is observed near the bottom of the melting range. The chemical shift of the H2 proton exhibits a large upfield shift in the ordered form, and the temperature profile of H2 is cooperative and congruent with the UV melting curve. The CH_3 proton signal, in striking contrast to H2, is independent of temperature. These results support a regular, single-stranded helix in the ordered form, in contrast to both poly(adenylic acid) and poly(8-bromoadenylic acid). We suggest that the contrasting temperature dependence of the H2 and CH_3 proton signals can be accounted for by regularly alternating syn and anti conformations of the 8-methyladenylic acid residues.

One of the most extensively investigated aspects of polynucleotide conformation in recent years has been that of syn and anti isomers, which result from torsion about the glycosidic bond (Donohue & Trueblood, 1960; Sundaralingam, 1969; Lakschminarayanan & Sasisekharan, 1969; Haschemeyer & Rich, 1967). In the naturally occurring purine nucleotides, the rotational energy barrier is relatively low. Both syn and anti conformations are encountered in crystals, though with the latter clearly predominating. One fruitful approach to the study of the less common syn conformation has been the synthesis of purine monomers substituted at the 8-position with groups sufficiently bulky to restrict the anti range of conformation [see, for example, Ikehara et al. (1969), Michelson et al. (1970), Tavale & Sobell (1970), and Howard et al. (1974)]. This method has been extended to polymers, providing the first examples of syn polynucleotides. Thus, for example, in the homopolymer poly(8-bromoadenylic acid) [poly(8brA)] the Br substituent causes all residues to adopt a syn conformation and to have properties radically different from the parent poly(adenylic acid) [poly(A)] [cf. Howard et al. (1974, 1975) and Govil et al. (1981)]. Poly(8brA) forms a hydrogen-bonded double helix of high stability rather than the nonregular, partially stacked single-stranded structure possessed by poly(A) [cf. Leng & Felsenfeld (1966), Applequist & Damle (1966), Brahms et al. (1966), Holcomb & Tinoco (1965), and Eisenberg & Felsenfeld (1967)]. In this report, we are concerned with an electronically less perturbing 8-substituent of about the same size as Br, namely, the methyl group. A series of dinucleoside monophosphates investigated by Ikehara and co-workers (Uesugi et al., 1978; Ikehara et al., 1978) indicated that the 8-methyladenylic acid (8-meA) residues could adopt a syn conformation in some of the dimers and an anti structure in others, as well as a syn–anti structure in the same molecule. It is likely that the dimers in solution are present as mixtures of more than one conformation. We find in the present study that poly(8meA) forms a regular, single-stranded helix, the

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first such structure found to exist in aqueous solution. The glyosidic conformation appears to be a regularly alternating syn–anti structure. The new polymer is thus strikingly different from both poly(A) and poly(8brA). It is interesting that alternation of syn and anti conformations within a strand is also observed in the novel structure of Z DNA.

Materials and Methods

8-Methyladenosine 5′-Phosphate (8meAMP). To a stirred solution of 393 mg (1.22 mmol) of 2′,3′-*O*-isopropylidene-8-methyladenosine (Ikehara et al., 1977) in 4.5 mL of trimethyl phosphate was added 0.25 mL of phosphorus oxychloride at 0 °C. The reaction mixture was stirred in an ice bath for 3 h and then poured into 50 mL of ice water and stirred for 10 min.

The acidic solution was kept at 54 °C for 80 min to remove the isopropylidene group (slight depurination was observed). The reaction mixture was cooled in the ice bath, neutralized with 10% ammonium hydroxide to pH 8, and applied to a column of Dowex AG 1-X8 (formate form). After the water wash, the column was eluted with a linear gradient of formic acid: 1.5 L each of water and 0.12 M formic acid. The fractions corresponding to 8-methyladenosine phosphate were pooled and evaporated in vacuo. 8-Methyladenosine phosphate was obtained in 89% yield (1.09 mmol, determined by absorbance of 260 mm); paper electrophoresis yielded an $R_{f,AMP}$ of 0.74 in ammonium bicarbonate (0.05 M) at pH 7.5.

8-Methyladenosine 5′-Diphosphate. 8-Methyladenosine 5′-phosphate (380 mg) was dissolved in *tert*-butyl alcohol (10 mL) and water (10 mL). To this solution was added morpholine (0.33 mL). While the mixture was refluxed, dicyclohexylcarbodiimide (1.27 g) dissolved in *tert*-butyl alcohol (19 mL) was added dropwise. The reaction mixture was refluxed for 3 h, and the solution was then evaporated in vacuo. The resulting residue was dissolved in water (20 mL) and ether (20 mL). Dicyclohexylurea was removed by filtration. After the ether extraction to remove unreacted dicyclohexylcarbodiimide, the water layer was evaporated in vacuo. The residue was dried by repeated evaporation from dry pyridine.

To the residue was added tri-*n*-butylamine (1.26 mL) and inorganic phosphoric acid (0.35 mL), which had been previously dried by repeated evaporation with added dry pyridine. The mixture was further dried by addition and evaporation of pyridine and kept at room temperature for 60 h with the exclusion of moisture. The reaction was stopped by addition of water. Pyridine was evaporated, and the water solution was applied to a column of DEAE-Sephadex A-25 (2.4 cm × 30 cm). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer: 1 L each of water and 0.3 M buffer solution followed by an additional 1 L of 0.3 M buffer. The diphosphate was obtained in 79.8% yield (0.80 mmol). Paper electrophoresis yielded an $R_{f,ADP}$ of 1.19.

Poly(8-methyladenylic acid). Preparation of 8-methyladenosine 5′-diphosphate was polymerized with polynucleotide phosphorylase from *Micrococcus luteus*. The reaction mixture contained 0.0258 M substrate, 0.015 M $MgCl_2$, 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9.0), 2×10^{-4} M ethylenediaminetetraacetic acid (EDTA), 1×10^{-2} M dithiothreitol, and 55 units of polynucleotide phosphorylase in a total volume of 22 mL; 40.4% of the substrate was polymerized in 1 day at 38 °C as determined by release of inorganic phosphate. Protein was removed from the reaction mixture by repeated shaking with isoamyl alcohol–chloroform (1/3 v/v). The resulting water layer was dialyzed in turn against the following solutions: 4 L of 0.5 M NaCl–0.001 M EDTA, 3 L of 0.5 M NaCl, 4 L of 0.3 M NaCl, 4 L of 0.1 M NaCl, 3 L of distilled water, and 4 L of distilled water. The solution was lyophilized to yield 170 mg of polymer.

Ultraviolet experiments were performed with a Cary 15 or a Cary 118 spectrophotometer and infrared measurements with a Perkin-Elmer 580B spectrophotometer. NMR spectra were measured with a Varian 220-MHz spectrometer. Molar absorbance of the polymer was determined by phosphate analysis, as described previously (Howard et al., 1971; Muraoka et al., 1980).

Results

Acid Titration. Poly(A) (Rich et al., 1961) and some of its derivatives [cf. Ishikawa et al. (1973)] form regular helices in acid solution, protonated at N1 and hydrogen bonded at N1 and N7. Those polymers that form acid helices exhibit an elevation of about 1.5–2 p*K* units over that of the monomer [cf. Massoulié (1965) and Ishikawa et al. (1973)]. In the present case, the p*K* of 4.8 (cf. Figure 1) is not very different from that of the monomer. We also observe that, in contrast to poly(A), the ultraviolet absorbance increases rather than decreases on acidification, a result consistent with the destruction of an ordered neutral structure by protonation. These results suggest that a helical acid structure is not formed under these conditions.

Spectroscopic Properties of Neutral Poly(8meA). The ultraviolet spectrum of poly(8meA) has λ_{max} of 259 nm and ϵ_{max} of 12 300 (Na^+ 0.03 M; pH 6.3; 25 °C; Figure 1). These values may be compared to those for the nucleoside of 261 nm and 15 800. Temperature dependence of UV absorbance is presented in a later paragraph.

The infrared spectrum of poly(8meA) in D_2O has a strong band at 1626 cm^{-1} (ϵ = 1150 at 5 °C) and a weak one at 1578 cm^{-1}, both assigned to adenine ring vibrations. These are quite similar to corresponding bands of poly(A) but differ in showing essentially no frequency change with temperature. The intensities of both bands are independent of temperature. The two ring vibrations of poly(A) shift from 1630 and 1572 cm^{-1} at 10 °C to 1622 and 1576 cm^{-1} at 90 °C (Miles, 1971). The spectrum of helical poly(8brA) is totally different from those of both of the other polymers. The brA ring vibration, which is present at 1623 cm^{-1} in the single-stranded form, appears as a doublet (1633 and 1617 cm^{-1}) in the helix, possibly as a result of Fermi resonance of a combination mode with the fundamental at ~1625 cm^{-1} (Howard et al., 1974, 1975).

Bands observed in the range 1250–1550 cm^{-1} in D_2O show little temperature dependence of either frequency or intensity. The first value (cm^{-1}) of each pair of values was observed at 6 °C, the second (cm^{-1}) at 72 °C: 1530, 1532; 1494, 1497; 1442, 1438; 1389, 1391; ~820 (sh), 798 (sh). In the spectrum from 1300 to 900 cm^{-1}, some bands show significant temperature sensitivity (Figures 2 and 3). The band at 1237 cm^{-1} is assigned primarily to an antisymmetric PO_2^- stretch [cf. Shimanouchi et al. (1964)] and shows some change of intensity with temperature but not of frequency.

There is a discrete band at 1120 cm^{-1} at 5 and at 15 °C, which disappears by 45 °C. This presumably corresponds to the band observed at 1122 cm^{-1} in RNA by Tsuboi et al. (1963), who assigned it to a nearly degenerate stretching vibration of the C–C(O)–C structure at the 2′-position of ribose. We have observed the same band in poly(A)·poly(U) (Miles, 1980) and found that it is conformationally sensitive and disappears when the helix melts. The intense band (ϵ ~890) and 1079 cm^{-1} is assigned to the symmetric stretching vibration of $>PO_2^-$ [cf. Shimanouchi et al. (1966)]. A shoulder at 1095 cm^{-1} is presumably due to an A ring vibration. In poly(A) the relative intensities of these two bands are

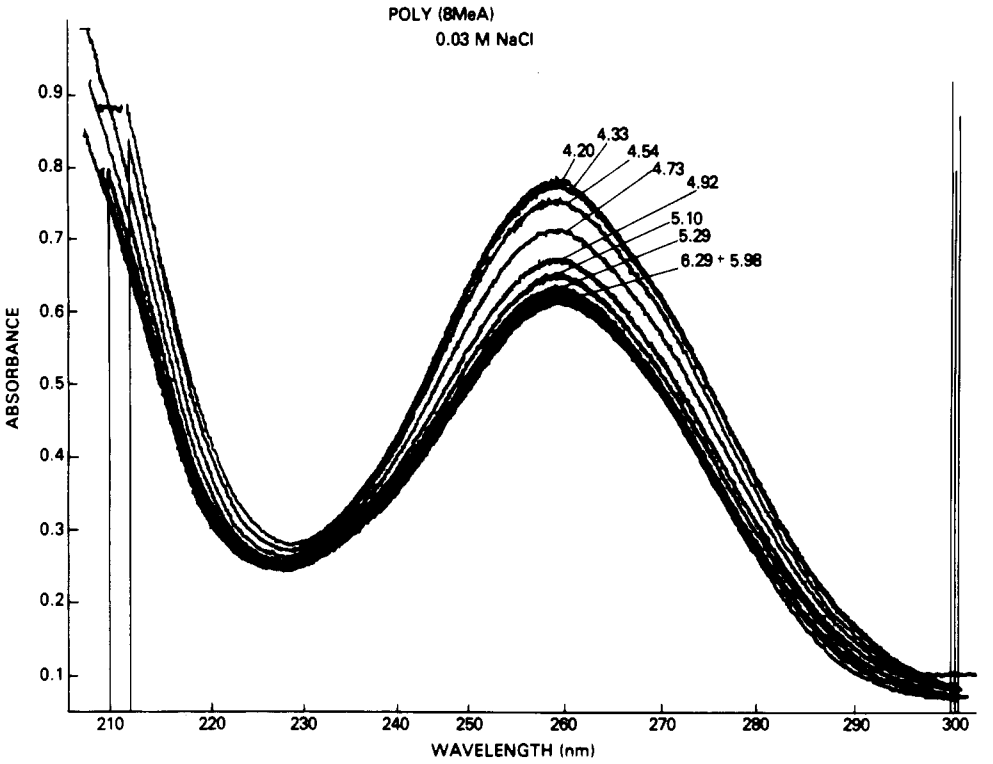

FIGURE 1: Ultraviolet spectra of poly(8meA) as a function of pH. The increase of absorbance as pH is reduced results from disruption of the neutral ordered structure when the base is protonated.

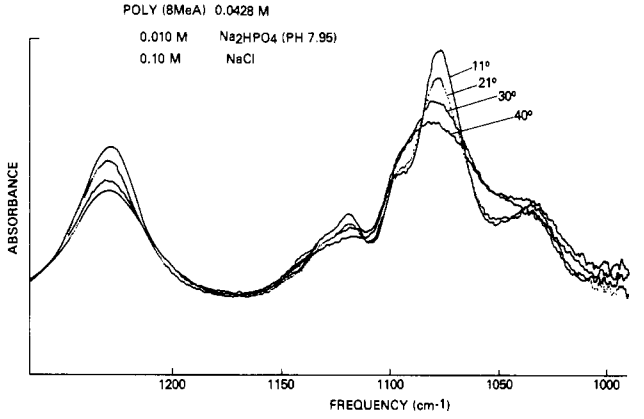

FIGURE 2: Infrared spectra in D_2O solution of poly(8meA) in the range 1000–1250 cm^{-1}. For assignments, see text.

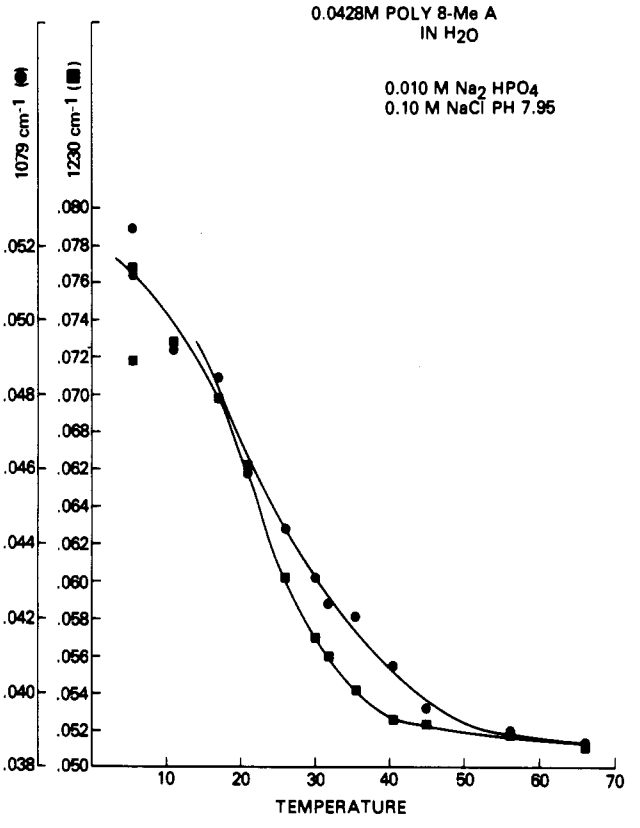

FIGURE 3: Infrared melting curves of bands assigned to symmetric (1079 cm^{-1}) and antisymmetric (1230 cm^{-1}) vibrations of the $>PO_2^-$ group.

reversed, with a resolved band at 1095 cm^{-1} and a shoulder at ~1080 cm^{-1}.

Heating causes a significant decrease in absorbance at 1079 cm^{-1} and a shift to 1085 cm^{-1} (Figure 2). This frequency is about the mean of the two observed at 5 °C and may represent a composite band. For comparison with the polymer bands in this region, we note that the monomer 2′,3′-*O*-isopropylidene-8-methyladenosine (KBr pellet) has bands at 1218 (m), 1108 (wide m), 1082 (ms), 1030 (wide m) cm^{-1}.

Infrared spectra were also measured in H_2O in 12-μm cells in order to observe the NH_2 deformation mode and ring vibration, which occur in a region usually obscured by the water deformation band at 1645 cm^{-1} (Miles & Frazier, 1978). δ NH_2 occurs at 1647 cm^{-1} and the ring vibration at 1613 cm^{-1} at 6 °C. By 34 °C, the frequencies have shifted slightly to 1650 and 1609 cm^{-1}. These values may be compared with 1659 and 1605 cm^{-1} (4 °C) for poly(A). As we have seen in D_2O solution, the meA ring vibration when unperturbed by coupling to the amino deformation mode occurs at 1626 cm^{-1}. The unperturbed δ NH_2 mode would probably occur near this frequency (in aniline, for example, it occurs at 1620 cm^{-1}), but the coupled modes are displaced to higher and lower frequencies at the observed values of 1647 and 1613 cm^{-1}. The fact that these displacements are considerably smaller than in unsubstituted poly(A) indicates that the coupling is weaker in poly(8meA). At lower frequencies, essentially the same

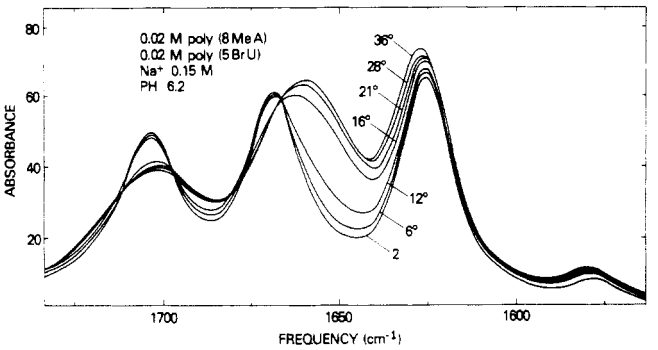

FIGURE 4: Infrared spectra of a 1/1 mixture of poly(8meA) and poly(brU) in D_2O solution, pD 6.2, [Na^+] 0.15 M. Changes in the brU carbonyl bands (the two higher frequency bands) are accounted for entirely by formation of the poly(brU) self-structure. The 8meA ring vibration at 1626 cm^{-1} is unaffected by the presence of poly(brU), indicating no A·U base pairing.

bands as those reported above are seen in H_2O with small (or occasionally moderate) frequency shifts in some cases: 1490, 1436, 1387, 1353, 1340, 1301, 1230, 1120, ~1096 (sh), 1079, and 1036 cm^{-1}.

Infrared spectroscopy was employed to determine whether base-pairing interaction occurs with potentially complementary polynucleotides. Poly(brU) was used to increase the stability of any A·U pair that might be formed. While there are spectral changes at low temperature (Figure 4), these are entirely accounted for by formation of the poly(brU) helical self-structure. No A·U interaction occurs at any temperature.

The NMR spectra of poly(8meA) and of 5′-meAMP are shown in Figure 5. At 19 °C the polymer is largely ordered, and the spectrum is considerably broadened, though the well-resolved signals of H2, H1′, and 8-CH_3 are readily identified. At 87 °C, the polymer is fully melted and has sharp resonances, comparable to the monomer at 19 °C. Upfield shifts of H2 and H1′ signals [(H2) 19 °C, 7.02 ppm; 87 °C, 7.91 ppm; (H1′) 19 C, 5.39 ppm; 87 °C, 5.66 ppm] arise from ring current shielding of these protons. Temperature insensitivity of the chemical shift of the 8-CH_3 signal (δ 2.70 ppm at both 19 and 87 °C) reflects lack of stacking of this group in the ordered form, as discussed below. The relationship of the proton vicinal spin–spin coupling constant with the dihedral angle between two CH bonds is well-known (Karplus, 1959) and has been used extensively in studies of nucleotides conformation [see, for example, Jardetsky (1962), Schleich et al. (1972), and Hruska et al. (1970)]. Schleich et al. (1972) have estimated a dihedral angle of 155° and $J_{1'-2'}$ of ~8.3 Hz for the C2′ endo conformation and 97° and ~0 Hz for C3′ endo. Vicinal coupling constants of both monomers and polymers have generally been interpreted in terms of rapid equilibrium between C2′ endo and C3′ endo conformers, with the value of J determined by a population-weighted time average of the values for these forms [cf. Davies & Danyluk (1974, 1975), Hruska et al. (1977), and Lee et al. (1976)]. In poly(A), $J_{1'-2'}$ is 4.5 Hz and in poly(8brA) 3.5 Hz, and the C3′-endo population of these polymers at 70 °C has been estimated as 49 ± 10% and 65 ± 10%, respectively (Govil et al., 1981). The coupling constant observed for poly(8meA) at 87 °C is 5.0 Hz, indicating roughly equal populations of C2′ endo and C3′ endo. The signals are too broad for reliable measurement of $J_{1'-2'}$ at low temperature, but an extrapolated value is discussed below.

Thermal Transition of Neutral Poly(8meA). The temperature profile of UV absorbance is quite cooperative (Figure 6), in complete contrast to that of poly(A). Poly(8brA) exhibits a similar cooperativity but a much higher T_m (56 compared to 27 °C in 0.1 M Na^+). Measurement of melting curves at different salt concentrations shows that T_m is independent of ionic strength at low and moderate ionic strength and decreases slightly in high salt (cf. Figure 6).

The temperature profile of the chemical shift of the H2 proton in Figure 7 is shown to correspond very closely to the

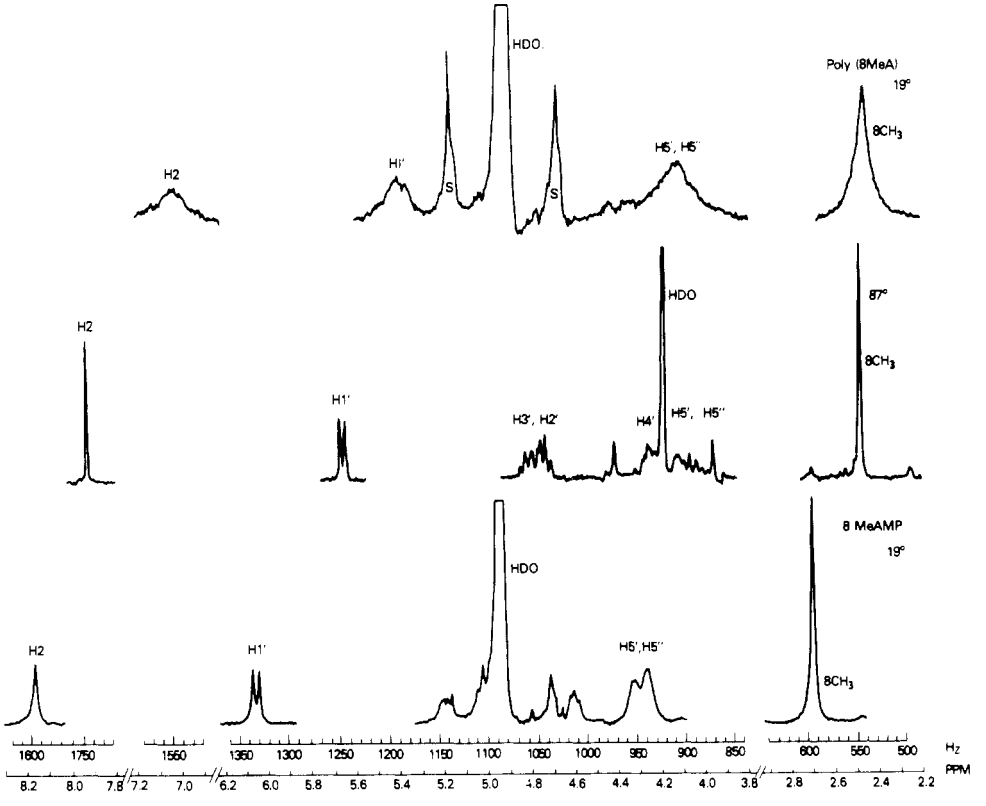

FIGURE 5: NMR spectra in D_2O solution of poly(8meA) at 19 and 87 °C and of 8meAMP at 19 °C. TSP was an internal standard.

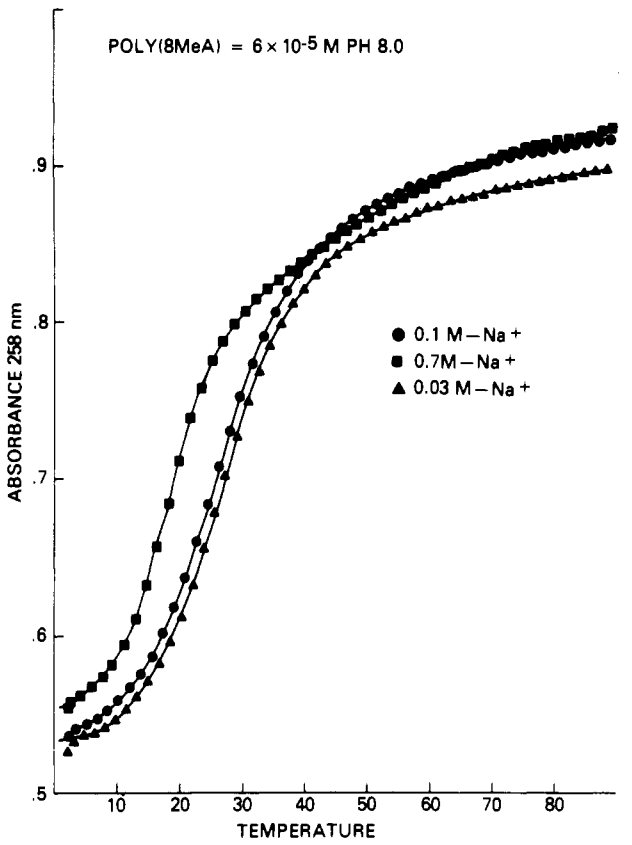

FIGURE 6: Ultraviolet temperature profiles of poly(8meA) at pH 8. The sigmoid curves reflect melting of a regular, ordered structure.

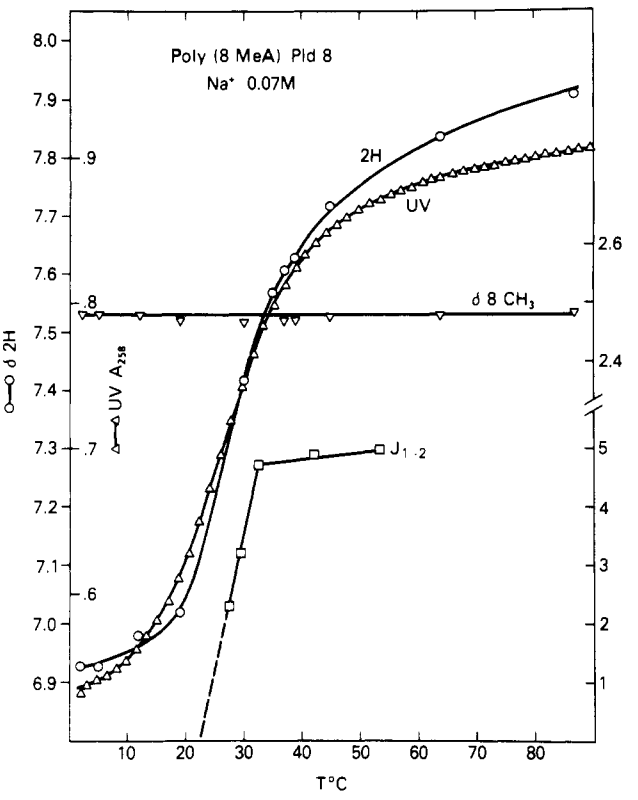

FIGURE 7: Temperature profiles of chemical shifts of the H2 and 8-CH_3 protons and of the $J_{H1'-H2'}$ coupling constant. The H2 melting curve is quite cooperative and is almost congruent with the ultraviolet curve (△). The 8-CH_3 chemical shift is independent of temperature. $J_{1'-2'}$ is given on the right ordinate in hertz. For discussion, see text.

UV absorbance profile. Evidently, both reflect in the same way the cooperative stacking of an ordered form.

Discussion

The foregoing results clearly suggest that poly(8meA) has a novel structure at low temperature. The large hypochromism (~74% increase in absorbance on melting) indicates extensive stacking, and the cooperative melting curve suggests the structure is regular. Poly(A), in contrast, exhibits little cooperativity and has less than half the hypochromism. The double helix of poly(8brA), however, has similar cooperativity and hypochromism (Howard et al., 1974). The large upfield shift of the H2 resonance and the cooperative temperature profile, parallel to the UV curve, further support the conclusion of a regular, stacked structure, presumably helical. The temperature profile of the 8-CH_3 protons, on the other hand, is dramatically different from that of H2: δ CH_3 is completely independent of temperature (Figure 7), though it is at higher field than the 8-CH_3 signal of the monomer. The 8-CH_3 is thus evidently not in close proximity to other bases in the polymer, while the 2-proton must be stacked over adjacent bases. The sharply contrasting temperature profiles of the H2 and 8-CH_3 protons also argue strongly for regularity of the ordered structure. Appreciable randomness in the structure would lead to intermediate responses of these signals: there should be some variation of the 8-CH_3 resonance with temperature and a smaller, less cooperative dependence of the H2 signal. A structure consistent with these observations is presented below.

We consider next the questions of base pairing and helix strandedness. In all known cases of base pairing in a polynucleotide helix, the A ring vibration at ~1625 cm^{-1} undergoes major changes of intensity or frequency or both [cf. Miles (1971) and Howard et al. (1974)]. The 1625-cm^{-1} A band is, however, relatively insensitive to stacking. Complete lack of the temperature sensitivity of this vibration in poly(8meA) clearly suggests that base pairing is not involved in the ordered structure.

The fact that a good high-resolution NMR spectrum can be observed near the bottom of the melting curve indicates that the structure has considerable flexibility. The rigid double helix of poly(8brA), in contrast, does not undergo motional averaging of nuclear dipole–dipole broadening and has no detectable high-resolution proton spectra under conditions comparable to those of Figure 5 [cf. Howard et al. (1975)].

As noted above, the coupling constant $J_{1'-2'}$ indicates about 50% of C2′ endo and C3′ endo sugar conformations at higher temperature (Figures 5 and 7). Upon entering the melting range, however, $J_{1'-2'}$ decreases abruptly. Though broadening prevents measurement of the coupling constant at low temperature, extrapolation indicates a value near 0 Hz at room temperature (Figure 7). This would correspond to essentially pure C3 endo conformation, a result in conformity with most fully ordered RNA structures.

All nonprotonated double helices exhibit a positive dependence of T_m upon salt concentration as a result of the need for counterion screening of interstrand phosphate repulsion [cf. Krakauer & Sturtevant (1968) and Schildkraut & Lifson (1965)]. The dependence is linear in log $[M^+]$ and the slope of dT_m/d log $[M^+]$ is usually in the range 15–20 °C. For poly(8brA), the slope is 15 °C (Howard et al., 1975). The T_m of poly(8meA), however, is independent of $[Na^+]$ in the intermediate range and shows a slight negative dependence at higher salt (cf. Figure 6). We therefore conclude that the ordered structure is single stranded because of the salt independence of T_m, the temperature independence of the 1625-cm^{-1} A ring vibration, and the high-resolution NMR spectrum.

The structure is then stacked, regular, and single-stranded, but how can the striking contrast in shielding behavior of the H2 and 8-CH_3 protons be explained? Ikehara and co-workers (Ikehara et al., 1978; Uesugi et al., 1978) have pointed out the possible conformational arrangements of the various dimers containing 8meA residues as anti–anti, syn–syn, and syn–anti. These studies have shown that 8-methyl substitution moves the glycosidic rotational equilibrium toward the syn conformation, but anti structures can exist in the same dimer molecule. We suggest that in the polymer, the bulky 8-methyl groups are expelled to the outside of the stacked array of bases and are not exposed to ring currents of the bases. The 2-protons, however, are overlapped on both sides by the stacked bases and experience an unusually large ring current deshielding effect. To achieve this favorable arrangement, the nucleoside residues could adopt regularly, alternating syn and anti conformations. This regular, single-stranded structure thus occupies an intermediate position between all-syn poly(8brA) double helix and the anti, stacked, nonregular structure of poly(A).

Registry No. Poly(8meA), 86456-35-1; 2′,3′-*O*-isopropylidene-8-methyladenosine, 65627-13-6; 5′-8meAMP, 68045-12-5; 5′-8meADP, 86456-34-0.

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