Poly(2-dimethylaminoadenylic acid). Synthesis and Characterization of the Homopolymer†

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ABSTRACT: We have prepared poly(2-dimethylaminoadenylic acid) by chemical synthesis and enzymatic polymerization of the nucleoside pyrophosphate. The new polymer forms an unusually stable protonated helix even at neutral pH. Introduction of the 2-NMe_2 residue results in an elevation of T_m of the protonated helix approximately 50° above that of poly(A) measured under comparable conditions. The neutral polymer forms a nonregular stacked structure, apparently similar in many respects to that of poly(A). The dependencies of different optical properties (uv, CD, ir) of poly(2-dimethylaminoadenylic acid) upon temperature, however, are quite different from each other. We suggest that these variations in temperature dependence may arise from differing sensitivities of the different optical properties to base stacking or to the melting of more than one ordered structure over the temperature range 0–90°.

One of the most fruitful approaches to understanding the chemical and physical properties of nucleic acids is the synthesis and investigation of chemically modified polynucleotides. Such modification may permit a large measure of control over the properties of the polymer, thus facilitating the experimental testing of current hypotheses and the development of new ones.

We describe in this paper the chemical synthesis and enzymatic polymerization of 2-dimethylaminoadenosine 5′-pyrophosphate. The resulting poly(2-dimethylaminoadenylic acid) was examined by the chemical and spectroscopic methods reported below.

The new polymer forms an acid helix of extraordinary stability, having a pK of 6.9 (Na^+ 0.1 M) and a T_m approximately 50° higher than that of poly(A) under comparable conditions.

The neutral polymer forms a stacked single stranded structure, presumably similar in many respects to that of poly(A). The temperature profiles of the observed optical properties (uv, CD, ir) of the new polymer, however, are quite different from each other. These thermal differences apparently arise from different sensitivities of each spectroscopic property to the geometrical arrangement of the bases. In contrast these three spectroscopic methods give essentially identical temperature profiles when they are used to monitor melting of the complexes formed by poly(2NMe_2A)[1] with poly(U) and with poly(BrU) (Ishikawa *et al.*, 1972).

Materials and Methods

Polynucleotide phosphorylase was obtained as a nuclease-free preparation (type 15) from P-L Biochemicals.

2-Dimethylaminoadenine was purchased from Cyclo Chemical Corp.

Ultraviolet spectra were measured with a Cary 15 spectrophotometer.

Circular dichroic spectra were measured with a Cary 60 spectrophtometer with circular dichroism attachment.

Infrared spectra were measured with a Beckman IR-7 spectrophotometer as described in previous reports (Miles and Frazier, 1964; Howard *et al.*, 1966; Miles, 1968, 1971).

Temperature measurements were made with a Digitec digital thermometer and Yellow Springs Instrument thermistor probe, which was calibrated with an accurate mercury thermometer with 0.1° graduations.

Synthesis of 2-Dimethylaminoadenine Monoacetate. A mixture of 3.56 g (20 mM) of 2-dimethylaminoadenine (Cyclo lot no. R-6629) and 40 ml of acetic anhydride was heated to reflux. One drop of 85% phosphoric acid was added, and the solution was refluxed for 5 hr. The acetic anhydride was removed *in vacuo* at 60–70°. The residual, yellow solid was stirred with 40 ml of ice–water for 10 min, collected on a filter, washed successively with water, and dried to obtain 5.3 g of pale yellow solid, mp 178–210°. This material was not characterized in detail but appeared to be a mixture of mono-, di-, and triacetates of the purine. The higher acetates were converted to the desired monoacetate by the following treatment of the reaction product.

The acetylated material was dissolved in 400 ml of hot methanol. After cooling to 30°, 5 ml of aqueous ammonia (28%) was added to the solution, which was then allowed to stand for 15 min at room temperature. The crystals which separated from the solution were filtered, washed with methanol, and dried to get 2.91 g of monoacetate, mp 274–275°. The filtrate and washings were concentrated to dryness *in vacuo*, and 10 ml of 50% aqueous methanol was added to the residue. The crystals were filtered, washed with methanol, and dried to get a second crop (0.87 g) of monoacetate, mp 273–274°. Total yield of monoacetate was 3.78 g (86%).

Ultraviolet spectra had the following λ_{max} (nm) and extinction coefficients: pH 1, 334 (ϵ 5300), 239 (ϵ 20,000); pH 7, 336 (ϵ 4,600), 237 (ϵ 25,200), 222 (ϵ 24,300); pH 13, 324 (ϵ 5200), 232 (ϵ 29,100). Infrared maxima (Nujol mull) occurred at the following frequencies (cm^{-1}): 3580, 1693, 1678, 1635, 1585. Thin-layer chromatography (tlc): silica gel 6060; solvent benzene–ethyl acetate–methanol (6:3:2); single spot, R_F

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[1] Abbreviations used are: poly(2NMe_2A), poly(2-dimethylaminoadenylic acid); poly(BrU), poly(bromouridylic acid); poly(2$NH_2$6MeA), poly(2-amino-6-methyladenylic acid); poly(2$NH_2$6NMeA), poly(2-amino-6-methylaminoadenylic acid.

0.32. *Anal.* Calcd for $C_9H_{12}N_6O$: C, 49.08; H, 5.45; N, 38.16. Found: C, 49.13; H, 5.22; N, 38.47.

Synthesis of 2-Dimethylaminoadenosine. To a mixture of 2.64 g (12 mM) of 2-dimethylaminoadenine monoacetate, 3.1 g (12 mM) of mercuric cyanide and 6.0 g of anhydrous calcium sulfate in 120 ml of dried nitromethane was added 2′,3′,5′-tri-*O*-benzoylribofuranosyl chloride (Yamaoka *et al.*, 1965). The latter compound was obtained by treating 6.38 g of 1-*O*-acetyl-2′,3′,5′-tri-*O*-benzoyl-D-ribofuranose with AcCl and HCl in ether solution (Yung and Fox, 1963). The ether was evaporated, and the compound was dissolved in 20 ml of nitromethane. The reaction mixture was refluxed and stirred for 4 hr.

The hot reaction mixture was filtered and the precipitate was washed with 20 ml of hot nitromethane. The filtrate was combined with washings and the combined solution was evaporated to dryness *in vacuo*. The residue was dissolved in 50 ml of chloroform and the solution was washed three times with 20 ml of 5% potassium iodide and then with water. The chloroform solution was dried over magnesium sulfate and evaporated to dryness *in vacuo* to get 8.9 g of amorphous solid. This solid was then dissolved in 100 ml of warm methanol containing 1.30 g (24 mM) of sodium methoxide. The solution was refluxed for 2 hr.

Water (100 ml) was added to the cooled solution, and the mixture was applied to a column of 40 ml of Amberlite CG-50 resin, H^+ form. The column was washed with 200 ml of methanol until the eluate did not show ultraviolet absorption. The effluent was concentrated to about 100 ml *in vacuo*. The aqueous solution was extracted three times with 30 ml of chloroform and evaporated to dryness *in vacuo*. The residue was crystallized from methanol to get 2.45 g of product (66%, from 2-dimethylaminoadenine acetate), mp 220–221°. This nucleoside has been synthesized previously by an alternative method (Schaeffer and Thomas, 1958) and reported to have a melting point of 213° dec.

Ultraviolet λ_{max} (nm) and ϵ_{max} were as follows: pH 1, 218 (ϵ 21,400), 261 (ϵ 16,300), 305 (ϵ 8800); pH 7, 228 (ϵ 21,400), 262 (ϵ 13,000), 295 (ϵ 8340); pH 13, 227 (ϵ 26,507), 262 (ϵ 12,600), 295 (ϵ 8,340). Tlc: silica gel 6060; in solvent used above for the acetylated purine, R_F 0.20. *Anal.* Calcd for $C_{12}H_{18}N_6O_4$: C, 46.49, H, 5.85; N, 27.11. Found: C, 46.32; H, 5.67; N, 26.93.

Synthesis of 2-Dimethylaminoadenosine 5′-Phosphate. A mixture of 0.62 g (2 mM) of 3-dimethylaminoadenosine, 4.0 ml, of methyl orthoformate and 25 ml of dimethylformamide containing 0.15 g of hydrogen chloride gas was allowed to stand at room temperature for 20 min (Jarman and Reese, 1964; Zemlicka, 1964; Jarman *et al.*, 1967). Tlc examination of aliquots at increasing time intervals showed that the reaction was essentially complete in 5 min. The reaction mixture was neutralized with triethylamine and examined by tlc. There were two spots with R_F values of 0.56 and 0.48 (solvent system benzene–ethyl acetate–methanol, 6:3:2, v/v; Eastman chromogram sheet 6060, silica gel). The desired product, R_F 0.48, is the 2′,3′-orthoformate of starting material (R_F 0.20) and constitutes about 70% of the reaction mixture. The by-product (probably the bis(2′,3′–5′ orthoester) present to about 30%) was converted to the desired 2′,3′-orthoester by the following procedure. Trimethylamine (2 ml) was added to the reaction mixture, and the mixture was evaporated to dryness *in vacuo* below 45°. The residue was dissolved in 8 ml of methanol and 2 ml of water. The solution was allowed to stand at room temperature overnight. There was then one spot (R_F 0.48) on tlc. The reaction is complete after about 7 hr. No conversion of the bis orthoester to the desired product occurs unless water is added; 20 ml of 5% sodium bicarbonate solution was added to the mixture, which was then evaporated to about 20 ml *in vacuo*. The precipitated oily material was dissolved in 30 ml of chloroform. The chloroform layer was separated from aqueous phase, washed with 10 ml of water, dried over magnesium sulfate, and evaporated to dryness *in vacuo* to get 0.61 g (87%) of colorless crystalline material. This material was phosphorylated by the method of Tener (1961).

A solution of 0.75 g (2.0 mM) of 2′,3′-methoxymethylidene-2-dimethylaminoadenosine and 6 mM of β-cyanoethyl phosphate in 20 ml of pyridine was concentrated to dryness *in vacuo* below 25°. The residue was dissolved in 20 ml of pyridine and evaporated to dryness. This procedure was repeated three times. The residue was then dissolved in 20 ml of pyridine, and 6.0 g of dicyclohexylcarbodiimide was added. The mixture was allowed to stand at room temperature for ~18 hr.

Water (4 ml) was added to the reaction mixture. After 1 hr, the mixture was evaporated to dryness, and residual pyridine was removed by addition and evaporation of water; 80 ml of 0.4 N lithium hydroxide was added to the residue, and the mixture was heated under reflux and stirred for 1 hr. After cooling, the mixture was filtered and the precipitate was washed with 20 ml of 0.01 N lithium hydroxide. The filtrate and washings were neutralized to pH 7.0 with AG50WX2 ion-exchange resin, H^+ form, which was then removed by filtration. The resin was washed with water and the filtrate and washings were concentrated to dryness *in vacuo*. The residue was dissolved in 60 ml of 10% aqueous acetic acid, and the solution was then adjusted to pH 2.7 with 1 N hydrochloric acid. The solution was heated under reflux for 40 min. After cooling, the mixture was filtered and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml of water and the solution was applied to a column (2.2 × 10 cm) of AG-50WX2, H^+ form, which was washed with water until the eluate did not show uv absorption. The column was then eluted with 250 ml of 2 N ammonium hydroxide and the eluate was concentrated to about 15 ml *in vacuo*. Two milliliters of 1.0 M barium acetate was added to the solution, and the mixture was concentrated to about 3 ml *in vacuo*. Ethanol (6 ml) was added to the mixture and the precipitate was collected by centrifugation, washed with ethanol and ether, and dried *in vacuo* to get 0.960 g of the barium salt of the nucleoside 5′-monophosphate.

This product was chromatographically homogeneous and had the following properties: R_{AMP} 1.67 by tlc (propanol–concentrated ammonia–water, 6:3:1, Eastman chromagram sheet 6065 cellulose); R_{AMP} 0.86 by paper electrophoresis (0.1 M borate buffer, pH 9.2); uv absorption at pH 7 λ_{max} (nm) 295, 262, 227. The product contained 1.54 mM (77.2% yield) of nucleotide. The diphosphate was prepared by the method of Moffatf and Khorana (1961).

Synthesis of 2-Dimethylaminoadenosine 5′-Diphosphate. Barium 2-dimethylaminoadenosine 5′-phosphate (0.80 mM) was dissolved in 20 ml of water containing AG-50WX2 ion-exchange resin, H^+ form. The solution was passed through a column (3 × 2.5 cm) of AG-50WX2 ion-exchange resin, morpholinium form, which was washed with water until the eluate did not show ultraviolet absorption. The eluate was concentrated to dryness *in vacuo* and the residue was dissolved in 5 ml of water.

A solution of 0.66 g (3.2 mM) of dicyclocarbodiimide in 10 ml of *tert*-butyl alcohol was added dropwise to a refluxing solution of 0.8 mM of 2-dimethylaminoadenosine 5′-phosphate and 0.280 ml (3.2 mM) of morpholine in 20 ml of 50% aqueous

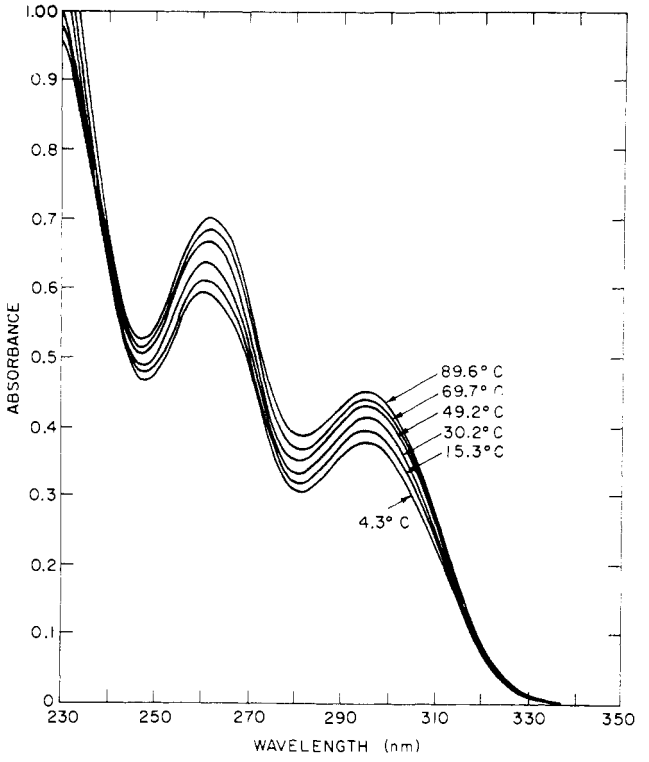

FIGURE 1: Ultraviolet spectra of neutral poly(2NMe$_2$A) at different temperatures in 0.05 M sodium cacodylate buffer (pH 7.8). Polymer concentration, 6.76×10^{-5} M; Na^+, 0.10 M.

tert-butyl alcohol. After the addition was completed (2.0 hr), the mixture was refluxed for 3 hr.

The mixture was cooled and concentrated to about 5 ml *in vacuo*. The residual mixture was filtered and washed with water. The filtrate and washings were extracted three times with 10 ml of ether, and the aqueous phase was concentrated to dryness *in vacuo*.

The residue contained 0.70 mM (yield 86%) of nucleoside 5′-phosphomorpholidate as determined by absorbance at 295 nm. The solution of nucleoside 5′-phosphomorpholidate (0.70 mM) was concentrated *in vacuo* to dryness and the residue was dried by addition and evaporation of dry pyridine.

Separately, 85% orthophosphoric acid (0.205 ml, 3 mM) was dissolved in 10 ml of pyridine containing 0.71 ml (3 mM) of tri-*n*-butylamine, and the solution was dried by four evaporations of pyridine.

Separate pyridine solutions of the nucleoside 5′-phosphomorpholidate and of tri-*n*-butylammonium orthophosphate were prepared, mixed, and evaporated two additional times. The final residue was dissolved in 10 ml of dry pyridine and allowed to stand at 25°. After 5 days, the reaction mixture was evaporated to dryness *in vacuo*.

Residual pyridine was removed by addition and evaporation of water. The residue was dissolved in 10 ml of water containing lithium acetate (410 mg, 4 mM), and the solution was adjusted to pH 12.0 with lithium hydroxide. The mixture was stored at 0° for 30 min. The precipitate was removed by filtration and washed with 0.01 M lithium hydroxide. The filtrate and washings were combined and adjusted to pH 8.0 with AG-50WX2, H^+ form, which was washed with water. The combined filtrate and washings were applied to a column of DEAE-cellulose (2.2 × 40 cm, bicarbonate form), and the column was washed with water until the eluate did not show uv absorption. Then the column was eluted by linear gradient of 1.5 l. of 0.4 M triethylammonium bicarbonate (pH 7.5) and 1.5 l. of water.

The fraction of nucleoside 5′-diphosphate was combined and concentrated to dryness *in vacuo*. Residual triethylammonium bicarbonate was removed by addition and evaporation of methanol. The final residue was dissolved in 20 ml of water, passed through ion-exchange resin AG-50WX2, sodium form, which was washed with water. The eluate was concentrated to dryness *in vacuo*. The residue was dissolved in 5 ml of water. This solution contained 0.42 mM of 2-dimethylaminoadenosine 5′-diphosphate as determined by absorbance at 295 nm. The yield was 61% from nucleoside 5′-phosphomorpholidate.

Preparation of Poly(2-dimethylaminoadenylic acid). 2-Dimethylaminoadenosine 5′-diphosphate was polymerized with polynucleotide phosphorylase from *Micrococcus luteus*. The reaction mixture contained 0.04 M substrate, 0.011 M $MgCl_2$, 0.1 M Tris buffer (pH 9.0), 2×10^{-4} M EDTA, 5×10^{-3} M dithiothreitol, and 38 units of polynucleotide phosphorylase (Singer and Guss, 1962) in a total volume of 5.00 ml; 62% of the substrate was polymerized in 1 day as determined by release of inorganic phosphate. To the reaction mixture was added 1.75 ml of mixture of isoamyl alcohol–chloroform (2:5, v/v).[2] The mixture was vigorously shaken for 5 min and separated by centrifugation. The water layer was removed and extracted 12 more times in the same way until no more precipitate was present at the interface. The combined water layer from the extraction was dialyzed[3] in turn against the following solutions: 3 l. of 0.5 M NaCl–0.001 M EDTA–0.001 M Tris buffer (pH 8.0), 3 l. of 0.6 M NaCl–0.001 M Tris buffer (pH 8.0), 4 l. of 0.1 M NaCl, 4 l. of distilled water, and 4 l. of distilled water. The solution was lyophilized to yield 53.1 mg of polymer. This material showed ϵ 9350 at 260 nm in 0.01 M pyrophosphate buffer (pH 7.8), containing 0.10 M sodium ion at room temperature. Alkaline hydrolysis of this polymer with 1.5 M NaOH in 70 hr at room temperature was found to be 99% complete by ultraviolet measurement of the hydrolysate. The combined organic layer was extracted three times with 0.1 M Tris buffer (2.5 ml), pH 8.0.

The weight-average molecular weight of the polymer was estimated by short-column equilibrium sedimentation using uv optics (Inners and Felsenfeld, 1970) to be about 190,000 in 1.0 M sodium chloride at 15°. When the rotor speed was increased all of the material was seen to migrate, indicating that the preparation was essentially free of oligomers (<5%). We are indebted to Dr. Eugene Achter for measuring and interpreting the ultracentrifugal data.

Results and Discussion

Spectroscopic Properties of Neutral Poly(2NMe$_2$A). The ultraviolet spectrum of the polymer (Figure 1 and Table I) has absorption maxima at 261 nm (ϵ 9580) and 295 nm (ϵ 6230)

[2] In a previous preparation we had employed phenol extraction of the reaction mixture to remove protein. We found that the polynucleotide dissolved in the phenol layer and experienced major losses in attempting to recover it. Professor Morio Ikehara (personal communication, 1971) informed us of his finding that poly(2NMe$_2$G) is also soluble in phenol. It appears that the Sevag procedure is preferable to phenol extraction for deproteinizing polynucleotides containing more than one alkyl residue.

[3] The customary ethanol precipitation of the polymer was omitted, without apparent disadvantage to the preparation. The unreacted substrate was accounted for in the first dialysate, and none of the subsequent dialysates had significant ultraviolet absorption.

TABLE I: Spectroscopic Data.

a. Ultraviolet

Material	λ_{max} (nm)	ϵ_{max}	λ_{min} (nm)	ϵ_{min}
2-Dimethylaminoadeno-	228	21,400	247	8900
sine (pH 7.0)	262	13,000	279	6640
	295	8,340		
Poly(2NMe$_2$A) (pH 7.9)[a]	261	9,580	248	7250
	295	6,230	281	4940
Poly(2NMe$_2$A) (pH 6.3)[a]	263	10,900	242	5240
	303	5,610	281	3850

b. Circular Dichroism

Material	λ_{max} (nm)	$\epsilon_l - \epsilon_r$	λ_{min} (nm)	$\epsilon_l - \epsilon_r$
Poly(2NMe$_2$A)	270	+1.97	255	+0.67
(pH 7.9; −0.5°)[b]	302	+0.41	287	+0.03
			320	−0.35
(pH 7.9; 25.3°)[b]	272	+1.56	326	−0.11
	297	+1.34	283	+1.17
			~252	+0.65
(pH 7.9; 60.2°)[b]	251	+0.53	267	+0.35
	295	+1.91		
Poly(2NMe$_2$A)	227	+20.8	247	+1.21
(pH 6.5)[c]	272	+9.02	292	−0.67
	312	+11.4		

c. Infrared

Material	ν_{max} (cm^{-1})	ϵ_{max}
Poly(2NMe$_2$A) (pD 8.0)[d]	1611.5	1260
	1550.5	(430)
	1505	(210)
Poly(2NMe$_2$A) (pD 6.6)[e]	1655.5	890
	1601.5	1350
	~1589 (shoulder)	770

[a] Sodium cacodylate buffer, 0.05 M; Na^+, 0.10 M; 25°. [b] Sodium pyrophosphate buffer, 0.01 M, pH 6.5; Na^+, 0.12 M, pH 7.9. [c] Sodium pyrophosphate buffer, 0.01 M, pH 6.5; Na^+, 0.12 M; 20°. [d] Sodium pyrophosphate buffer, 0.0048 M, pD 8; Na^+, 0.087 M; 29°. A rising base line below ~1520 caused by water in the sample led us to discard the value of ϵ^{1505} from this run and select instead the value of 212 obtained in two other runs at somewhat higher [Na^+]. [e] Sodium cacodylate buffer, 0.061 M, pD 6.6; Na^+, 0.167 M; 25°.

at 25°. The corresponding nucleoside, 2-dimethylaminoadenosine, has absorption maxima at 228 nm (ϵ 21,400), 262 nm (ϵ 13,000), and 295 nm (ϵ 8340) (Figure 2 and Table I). Polymerization of these purine nucleotide residues thus results in significant reduction of molar absorbance of both longer wavelength peaks but little change in wavelengths of the maxima.

The circular dichroic spectrum (Figure 3) of the polymer at −0.5° has a negative first extremum at 320 nm, a crossover point at 310 nm, positive peaks at 302 and 269 nm and minima at 287 and 255 nm. The first two extrema (320 and 302 nm) are approximately equal in area and equidistant from the crossover point, suggesting that they arise from exciton splitting (Tinoco, 1964). Though we do not detect a band in the absorption spectrum at ~310 nm, a weak transition could

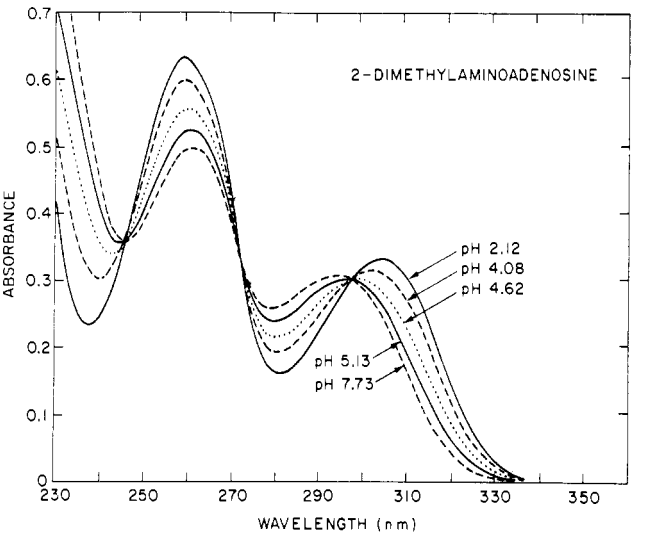

FIGURE 2: Ultraviolet spectra of 2-dimethylaminoadenosine as a function of pH. The three isosbestic points indicate that only two species, the uncharged molecule and a single protonated form, are present in this pH range. A pK value of 4.55 was calculated from the spectra.

give rise to the observed CD peaks without being evident in the absorption spectrum. At temperatures high enough to abolish the interactions responsible for the 302–320-nm pair of extrema (*e.g.*, 47°, Figure 3; 60°, Table I) the wavelength of the lowest energy peak (295 nm) corresponds to λ_{max} of the absorption spectrum and presumably arises from a transition of this wavelength.

The infrared spectrum of the neutral polymer in the region of double bond stretching vibrations is shown in Figure 4. The strongest band is a ring vibration (predominantly C=N stretch) at 1614 cm^{-1} (5°), which decreases in frequency in heating but shows no significant change in intensity. Similar behavior is exhibited by poly(A) (Miles, 1971) and poly-

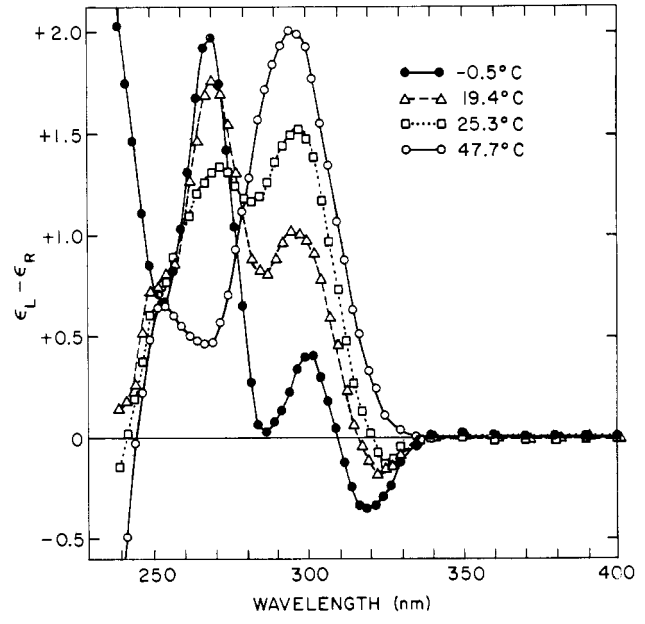

FIGURE 3: Circular dichroic spectra of poly(2NMe$_2$A), 2.0×10^{-4} M, pH 7.8, 0.01 M pyrophosphate buffer, Na^+, 0.12 M. The first negative and positive extrema may arise from exciton splitting of a transition near 310 nm, though a band is not observed at this wavelength in the absorption spectrum (*cf.* Figure 1 and text).

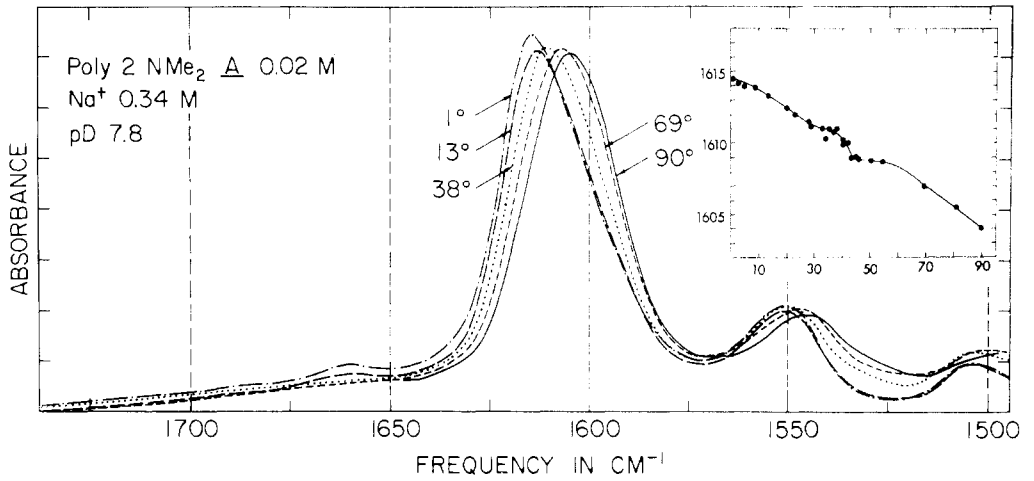

FIGURE 4: Infrared spectra of poly(2NMe$_2$A), 0.02 M, 0.018 M phosphate buffer, pD 7.8, Na^+, 0.34 M. Other spectra observed at pD 8.0, 0.08 M Na^+, were essentially the same but lacked the very slight protonation band (1656 cm^{-1}) seen here at low temperature. In both runs ν_{max} has the temperature dependence shown (inset), but ϵ_{max} changes little with temperature. Path length, 49.7 μ; scale expansion 5.85-fold. The ordinate index marks are 0.1 absorbance unit apart here and in Figure 9.

(2NH$_2$6MeA) (Ikeda *et al.*, 1970). Less intense bands appear at 1550 (predominantly C=C stretch), 1505, 1467, 1402, 1335, and 1312 cm^{-1}. The very weak band at 1660 cm^{-1} indicates a slight amount of protonation at 1° (<5%) and disappears by room temperature.

Acid Form of Poly(2NMe$_2$A). The pH dependence of the ultraviolet spectrum of the nucleoside 2-dimethylaminoadenosine is shown in Figure 2. The peak at 262 nm in the neutral molecule shifts to 258 nm in acid with an increase of intensity, and that at 295–305 nm with a slight increase of ϵ_{max}. There are isosbestic points at 246, 272, and 298 nm. The pK of the nucleoside determined from these spectra is 4.55.

Poly(2NMe$_2$A) exhibits a pH dependence of ultraviolet absorption (Figures 5 and 6) which is significantly different from that of the monomer. The peak at 261 nm increases slightly in wavelength at lower pH to 263 nm, with an increase in intensity smaller than that of the nucleoside. The higher wavelength peak at 296 nm shifts to 303 nm on protonation of the base and undergoes a decrease rather than an increase in ϵ_{max}. There are isosbestic points at 252 and 279 nm but none near 300 nm. The spectrophotometric titration (Figure 6) shows a strongly cooperative dependence on pH and a large shift in apparent pK to 6.95 (0.1 N Na^+) from the monomer value at 4.45. These observations and those discussed below indicate that the polynucleotide forms a stable acid helix.

When the acid helix is heated the ultraviolet spectrum (Figure 7) undergoes a change of λ_{max} from 303 to 296 mn (88°), indicating that deprotonation of the base residues accompanies thermal dissociation. We note for later reference the approximate constancy of absorption at ~305 and ~277 nm, though neither of these is an isosbestic point. The peak at 261 nm undergoes little change in intensity upon melting, probably because the gain of intensity resulting from helix

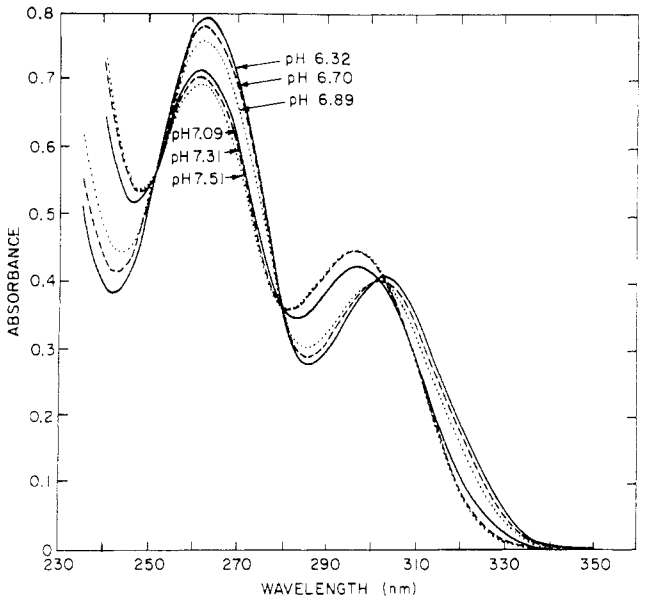

FIGURE 5: Ultraviolet spectra of poly(2NMe$_2$A) as a function of pH in 0.1 M Na^+ at 25°. The peak at 295 nm shifts to 303 nm on protonation of the polymer, and the 261-nm peak increases in intensity and shifts to 263 nm.

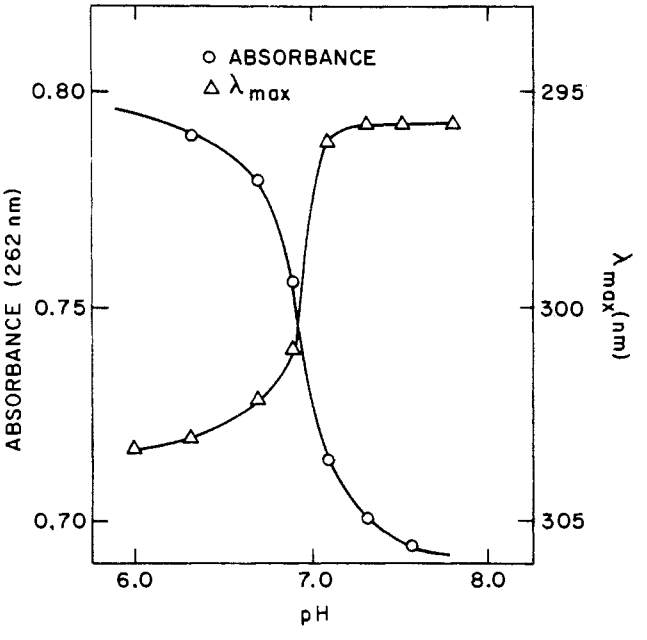

FIGURE 6: Ultraviolet titration of poly(2NMe$_2$A) in 0.1 M Na^+ at 25°. The strongly cooperative dependence of absorbance on pH and the high pK value of 6.95 indicate the formation of a stable acid helix.

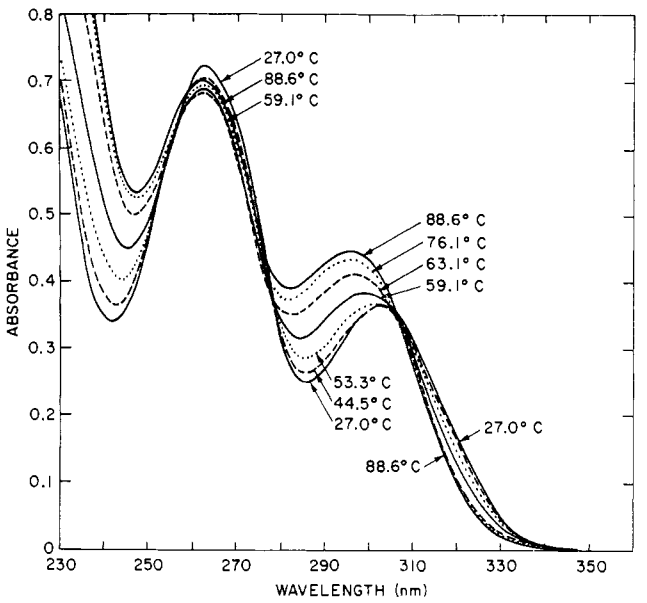

FIGURE 7: Ultraviolet spectra of poly(2NMe$_2$A) in 0.05 M cacodylate buffer, pH 6.2, Na^+, 0.1 M. The shift to a spectrum characteristic of the unprotonated polymer (*cf.* Figure 1) when the solution is heated indicates that the protons at N_1 of the purines are dissociated when the helix melts.

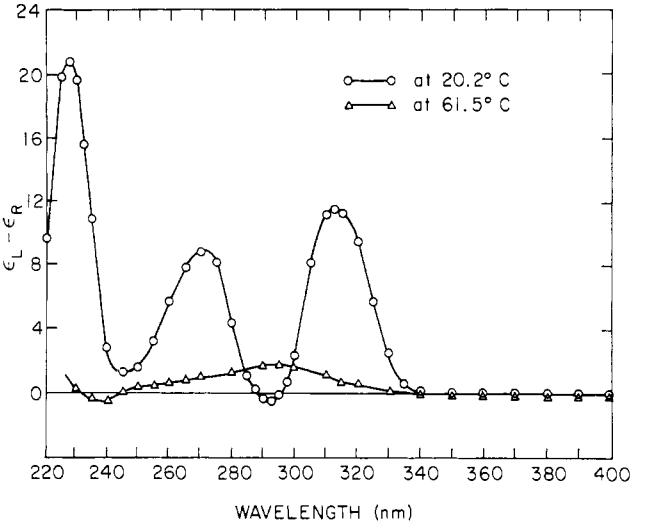

FIGURE 8: Circular dichroic spectrum of acid helix formed by poly(2NMe$_2$A), 1.08×10^{-4} M, in 0.01 M pyrophosphate buffer, pH 6.5, Na^+, 0.12 M, 20.2°.

dissociation is compensated by the loss resulting from deprotonation (*cf.* Figures 5 and 7).

The circular dichroic spectrum of the acid helix (Figure 8 and Table I) has maxima at 312, 272, and 227 nm, and minima at 292 and 247 nm. The magnitudes of the peaks of the acid helix are much larger than those of the neutral polymer, and the longest wavelength peak is positive rather than negative. A practical consequence of this difference in magnitude and sign is that even a small proportion of protonated polymer (*e.g.*, less than 10%) would be detectable in the presence of a much larger amount of neutral polymer. The first two bands in the acid helix (at 312 and 292 nm) have quite different areas, though the mean value of λ_{max} and λ_{min} corresponds to λ_{max} of the absorption spectrum (303 nm) (Figure 7, 27°). There are strong maxima at 272 and 230 nm in both acid and neutral structures, presumably with similar origins in the two cases.

The infrared spectrum of the acid helix has intense bands at 1655.5 cm^{-1}, 1602 cm^{-1}, ~1590 (shoulder) and weak bands at 1465 cm^{-1}, 1435 cm^{-1}, ~1416 cm^{-1} (shoulder), 1490 cm^{-1}, ~1394 cm^{-1} (shoulder), and 1356 cm^{-1} (Figure 9 and Table I). The band at 1655.5 cm^{-1} is characteristic of the N_1 protonated adenine ring (*cf.* Ikeda *et al.*, 1970), presumably with a major contribution from vibrations of the $[HN_1{=}C_6{=}N\text{-}H]^+$ system. The band with ν_{max} at 1602 cm^{-1} may contain contributions from both neutral and protonated purine ring vibrations. The shoulder at ~1590 cm^{-1} is visible only in acid solution, though the asymmetric low frequency side of the most intense band at pD 7.8 may well conceal a similar band (*cf.* Figure 4). We note a similarity to the spectrum of poly(2NH$_2$6NMeA) which has a band of moderate intensity at ~1600 cm^{-1}, in both acid and neutral solution, in addition to a strong ring vibration at ~1620 cm^{-1} (Ikeda *et al.*, 1970). There thus appear to be two vibrations in the region between ~1590 and 1615 cm^{-1}, their frequencies and intensities varying somewhat with protonation and with secondary structure of the polymer. In order to monitor separately the protonated bases (exclusively) and the neutral bases (predominantly) we can observe the spectra at 1655.5 and 1610 cm^{-1}, respectively. The latter frequency is that of the neutral polymer at a temperature at which the acid helix has dissociated (*cf.* Figures 4 and 9), and it provides a reasonably large increase in intensity on going from the protonated to the unprotonated polymer. Plotting these frequencies from spectra of the heated solution gives sigmoid melting curves with $T_m = 52°$ (inset, Figure 9; ultraviolet melting curves also have $T_m = 52°$ in 0.15 M Na^+; *cf.* Figure 10).

The increase in apparent pK (2.4 pK units) of the 2-dimethylaminoadenosine residues in the polymer as compared to the monomer is due to helix formation when the bases are protonated. Thermal dissociation of the helix in a solution buffered 2 pH units above the pK of the dissociated bases, therefore, results in simultaneous deprotonation of the bases. The essentially parallel temperature dependence of the protonation band (decrease at 1655 cm^{-1}) and the neutral ring vibration (increase at 1610 cm^{-1}) (Figure 9) provide experimental evidence that base deprotonation occurs simultaneously with helix dissociation. In more acid buffers the bases in the single-strand polymers resulting from helix dissociation would remain protonated but on further heating would undergo a more gradual dissociation of acid protons, depending on the enthalpy of ionization of the purine residues in the single-strand polymer.

Dependence of T_m of the Acid Helix upon pH and Ionic Strength. Temperature profiles of ultraviolet absorbance are sharp, sigmoid curves (Figure 10) and reflect the melting of a regular helical self-structure. There is a marked negative dependence of T_m on ionic strength (Figures 10 and 11), indicating a net stabilization of the helix by electrostatic interaction The dependence of T_m on log [Na^+] is linear and the slope, $dT_m/d \log [Na^+]$, is $-15°$, invariant with pH in the accessible range. Below pH 6 precipitation of the polymer interferes with accurate measurement.

The dependence of T_m on pH is ~+34° per unit decrease in pH over the range 0.03–0.30 M Na^+.

Stability and Structure of the Acid Helix. From the above data it is clear that the acid helix formed by poly(2NMe$_2$A) has a significantly higher stability than that formed by poly(rA). At pH 6 in 0.1 M Na^+ the T_m of the former is ~48° higher than that of poly(rA). The increase in apparent pK of the polymer as compared with its constituent nucleoside is 2.4 for

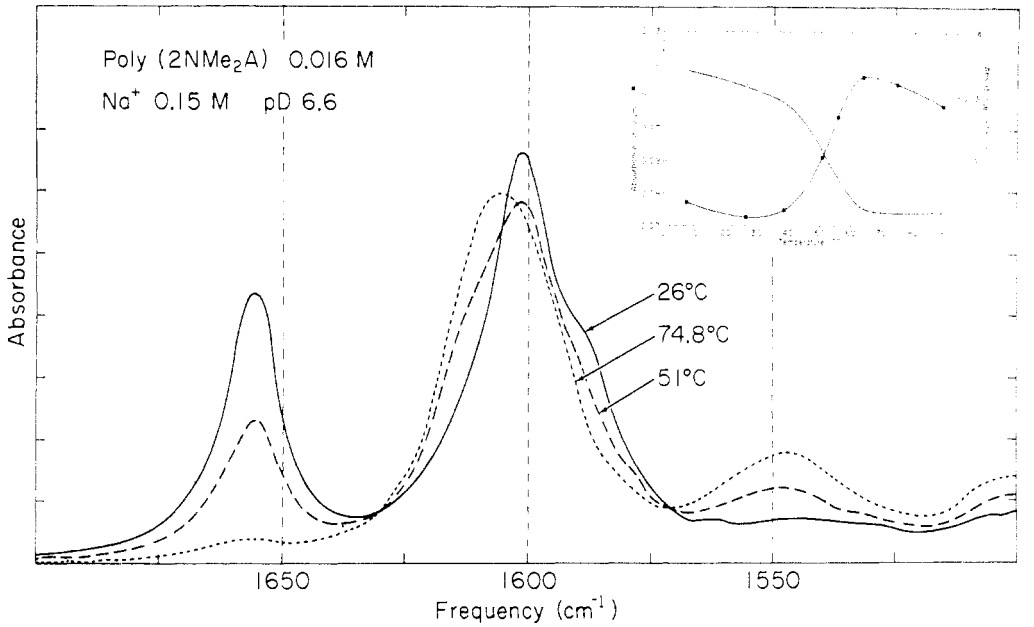

FIGURE 9: Infrared spectra of poly(2NMe$_2$A) in acid solution. Polymer 0.0167 M; Na^+, 0.168 M; cacodylate buffer, 0.061 M, pD 6.6; path length, 58.3 μ; scale expansion, 5.05-fold. The band at 1655.5 cm^{-1} is entirely due to protonated bases and that at 1550 cm^{-1} to the neutral polymer. In the acid helix (26°) there are ring vibrations at 1602 cm^{-1} and ~1589 (shoulder). The neutral polymer (75°) has a ring vibration at 1609 cm^{-1} (*cf.* Figure 4) and may also have a weaker, unresolved band under the envelope of the more intense one. This spectrum demonstrates that deprotonation of the purine bases occurs when the helix undergoes thermal dissociation. Inset, infrared melting curves of the acid helix.

poly(2NMe$_2$6NA) in 0.1 M Na^+ and about 2.0 for poly(rA) (Massoulié, 1965; Steiner and Beers, 1957).

The bonding scheme of the acid helix is presumably like that proposed for poly(rA) (Rich *et al.*, 1961) and for poly(2NH$_2$6NMeA) (Ikeda *et al.*, 1970). Protonation occurs at N_1 and mutual hydrogen bonding of the bases occurs between N_7 and the 6-amino group. If this proposed structure is essentially correct, the methyl residues would extend in a regular manner along the exterior of the helix, not appreciably overlapped by the adjacent bases. It appears that they might be in contact with adjacent methyl residues, if that is energetically favorable. If the contacts were so close as to be destabilizing, they could be relieved by slight rotation about the C_2–N bond, though at some cost in assuming a configuration not coplanar with the ring. It is not clear at present how or whether this regular arrangement of the methyl residues would contribute to the stability of the helix. One consequence of the arrangement might be a reduction of local dielectric constant in the vicinity of the positively charged ring with a resulting increase in electrostatic attraction between this charge and the negative charge of the phosphate in the opposite strand. Such an explanation would be consistent with increased stability and may be related to the somewhat lower ionic strength dependence of T_m as compared with poly(rA) (*i.e.*, a slope $dT_m/d \log [Na^+] = -15°$, compared to $\sim -22°$ for poly(rA).

Temperature Dependence of Optical Properties of the Neutral Polymer. Ultraviolet melting curves exhibit a slight but definite sigmoid character below 30° and more gradual increase with a smaller slope above this temperature in 0.1 M Na^+, pH 7.95 (Figure 12). Though we have not studied the salt dependence of melting in detail, the sigmoid character at lower temperature becomes somewhat more pronounced in lower salt [Na^+], 0.03 and 0.06 M), and disappears in high salt ([Na^+], 1.0 M). The T_m, to the extent that it can be estimated for these broad curves, appears to increase with salt concentration.

It might be supposed, in view of the high pK of poly(2NMe$_2$A), that changes in optical properties of the neutral polymer with temperature at pH 7.9–8.0 could be attributed to the presence of a small amount of acid helix. We shall present evidence from each spectroscopic method that this is not the origin of the changes. In the case of temperature dependence of the ultraviolet spectrum (Figure 1) the absorbance increases regularly with temperature at ~278 and 305 nm instead of remaining approximately constant as it does during melting of the acid helix (Figure 7). Similarly the absorbance at ~260 nm increases with temperature (Figure 1) instead of decreasing as it does with the protonated self-structure (Figure 7). Melting curves measured at pH values of 7.5, 7.9, and 8.3 (0.1 M Na^+, 0.002 M pyrophosphate buffer) are essentially superimposable.

The circular dichroic melting curves (Figure 13), in contrast to the ultraviolet curves, exhibit marked sigmoid character, as well as upper and lower plateaus at which the magnitude is nearly independent of temperature. The curves, moreover, show significant differences with wavelength as well as differences from the ultraviolet curves. The peak at 270 nm maintains constant magnitude from 0 to +15° and after a sigmoid increase does not become constant by 50°. At 295 nm the curve begins to change sooner than that at 270 nm and becomes constant by ~35°. As noted above, the much larger magnitude of peaks of the acid helix (Figure 8) would cause even a small amount of the latter structure to dominate the circular dichroic spectrum of the neutral polymer. In particular the weak first negative peak at 320 nm (Figure 3) would be entirely obscured by the very strong positive peak at this wavelength in the acid helix (Figure 8), and the increase observed on melting at 320 nm (Figure 13) would instead be a decrease.

The infrared spectrum shows little change in intensity of the ring vibration at ~1615 cm^{-1}, though the frequency decreases regularly with temperature over the range ~0–25° (Figure 4). The vibrational spectra show a quite different

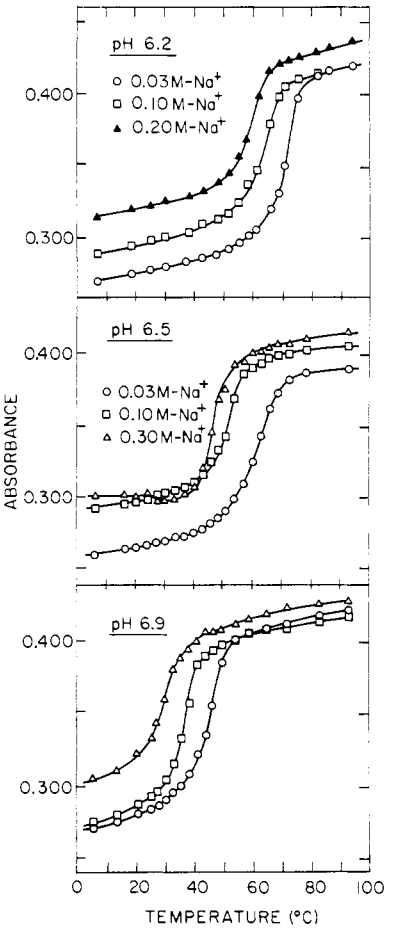

FIGURE 10: Ultraviolet melting curves of acid helix of poly(2NMe$_2$A) in sodium cacodylate buffer of the indicated pH values (0.05 M buffer for the 0.1 and 0.3 M Na^+ solutions, and 0.025 M for the 0.03 M Na^+ solutions; sodium chloride was added to give indicated values of total [Na^+]. The absorbance is plotted at 285 nm because of the large change at this wavelength (*cf.* Figure 7).

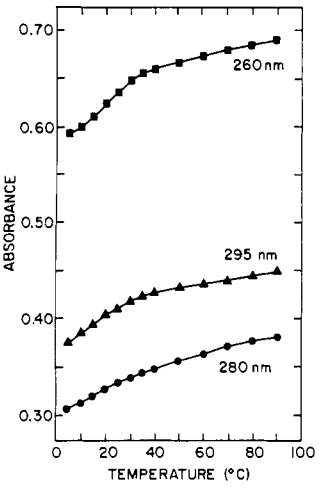

FIGURE 12: Temperature profiles of absorbance of neutral poly(2NMe$_2$A), 0.8×10^{-5} M, cacodylate buffer, 0.05 M, pH 7.8, Na^+, 0.1 M. Curves plotted at wavelengths of the strong peaks (260 and 295 nm, *cf.* Figure 1) show a slight but definite sigmoid character, which is enhanced in lower salt and abolished in higher salt. Melting curves in pyrophosphate buffer, 0.002 M, at pH values 7.5, 7.9, and 8.3 in 0.1 M Na^+ essentially duplicated the results shown here.

sensitivity from the electronic spectra for structural changes occurring over the same temperature range. The infrared spectra possess a specific protonation band at 1656 cm^{-1} which is well resolved from all others (*cf.* Figure 9). The extent of protonation can be estimated on this basis to be less than 5% (Figure 4). This point was further checked by another melting run of the neutral polymer under slightly different conditions (pD 8.0, Na^+, 0.06 M). The spectra and temperature dependence were essentially identical with those shown in Figure 4 below 1650 cm^{-1}. Absence of the 1656-cm^{-1} band showed that no acid helix is present under these conditions.

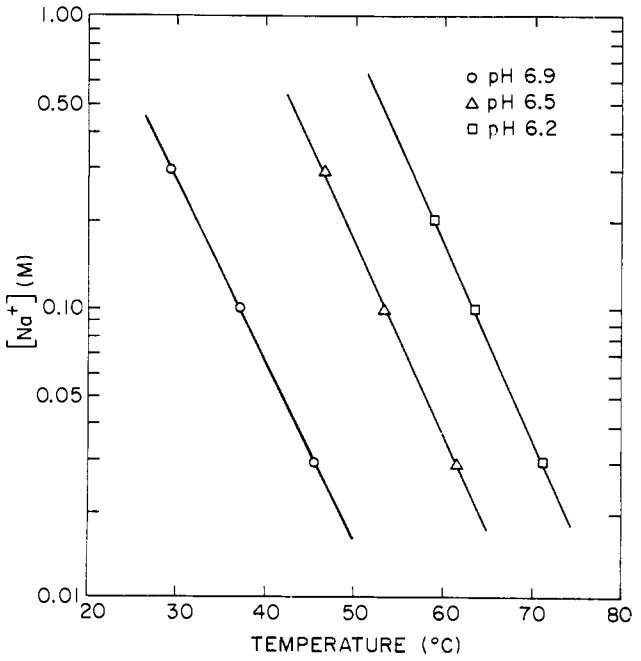

FIGURE 11: T_m of the acid helix has a linear dependence on log [Na^+] with a slope, $dT_m/d \log [Na^+] = -15°$. The cation dependence does not vary over the observed pH range.

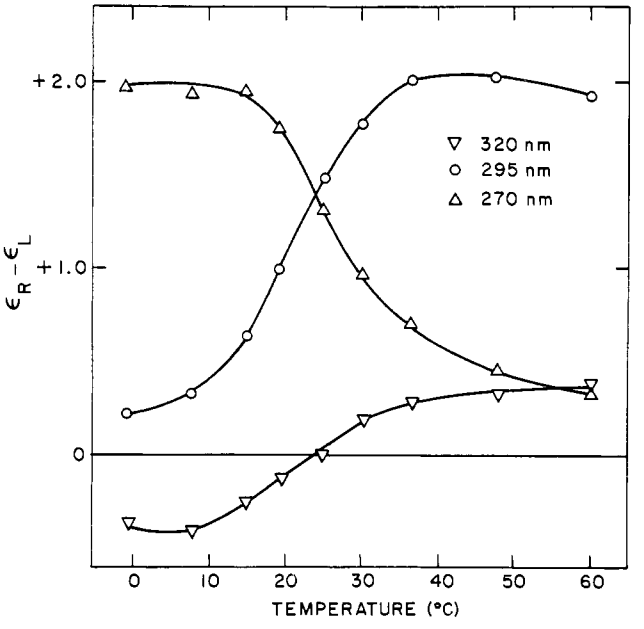

FIGURE 13: Circular dichroic melting curves of neutral poly(2NMe$_2$A), conditions of Figure 3. The curves are plotted at wavelengths of prominent extrema (Figure 3 and Table I) and show significant differences among themselves as well as differences from the ultraviolet curves. Possible explanations of the sigmoid temperature dependencies are discussed in the text.

Though the changes of the optical properties of neutral poly(2NMe$_2$A) with temperature are undoubtedly related to structural changes of the polymer, the detailed nature of the relationship is at present not clear. It is clear, however, that the common assumption that the fraction of bases stacked is directly proportional to change with temperature of an optical property is not valid in the present case. The difficulty here is not that upper and lower limits of the optical melting curves cannot be attained experimentally but that the temperature profiles vary considerably with the optical property and with the wavelength being observed. In the absence of independent and definitive structural evidence the selection of a particular optical property as a linear monitor of stacking is essentially arbitrary.

We shall consider two possible structural interpretations of the temperature dependence of optical properties observed in Figures 1, 3, 12, and 13. In the first of these two models two qualitatively different processes occur in the temperature range 0–90°. The portion of the melting curves above about ~35° would correspond to essentially noncooperative stacking of the kind proposed for neutral poly(A) (*cf.* Leng and Felsenfeld, 1966; Applequist and Damle, 1966; Brahms *et al.*, 1966; Holcomb and Tinoco, 1965; Poland *et al.*, 1966; Eisenberg and Felsenfeld, 1967; and other references cited in these papers). The curves from 0 to ~35° would reflect either (a) a distinct kind of single-strand stacking, differing in geometrical detail and probably corresponding to a narrower range of positions of the bases than the stacking occurring above ~35°, or (b) a two-stranded, hydrogen-bonded structure. Two centrosymmetric hydrogen-bonding schemes could be envisaged, the first involving mutual bonding of the two rings with an amino NH to ring N_1, and the second of amino NH to N_7, as in the acid helix (*cf.* Ikeda *et al.*, 1970, Figure 8a,b).

A second structural model would invoke a single kind of stacking, relatively noncooperative, over the entire temperature range of 0–90°. The circular dichroic bands observed in the ordered form at low temperature (Figure 3) may arise from interactions which require several consecutive stacked bases rather than depending primarily upon nearest-neighbor interactions. A dependence of the interactions on continuous sequences would lead to CD curves highly sensitive to the occurrence of runs of stacked bases rather than to overall extent of stacking. If the melting is relatively noncooperative, as the second model proposes, the fraction of longer runs of stacked bases will decrease much more rapidly with temperature than the total fraction of stacked bases. The CD melting curves under these circumstances would thus appear to be quite cooperative even though the physical process of stacking is itself not very cooperative. Ultraviolet hypochromism of poly(2NMe$_2$A) could have a different structural sensitivity, depending less on long runs of stacked bases, and so would show a more gradual increase with temperature according to this interpretation.

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