Copolymers of Adenylic and 2-Aminoadenylic Acids. Effect of Progressive Changes in Hydrogen Bonding and Stacking on Interaction with Poly(uridylic acid)[†]

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Appendix: Calculation of Copolymer Probabilities

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ABSTRACT: Random copolymers of adenylic acid and 2-aminoadenylic acid form both double and triple helices with poly(uridylic acid) [poly(U)]. Transition temperatures of two-stranded helices increase with $2NH_2A$ content, exhibiting a slight positive departure from linearity and indicating that the contribution to helix stability arising from introduction of the 2-amino group does not significantly depend upon base sequence. We have shown previously that poly($2NH_2A$)·poly(U) does not undergo a disproportionation reaction (2 → 3 transition). Extrapolation from melting curves of 1:1 complexes between A,$2NH_2A$ copolymers and poly(U) indicates a T_m for the 2 → 3 transition of poly($2NH_2A$)·poly(U) which is too high to be observable under normal conditions. Addition of an organic solvent (50% ethylene glycol), however, lowers T_m by promoting unstacking of single-stranded poly($2NH_2A$) sufficiently to permit observation of the disproportionation of poly($2NH_2A$)·poly(U) for the first time. Transition breadths of 1:1 complexes of A,$2NH_2A$ copolymers with poly(U) are greater than those of either of the homopolymer complexes in the middle range of composition (67 and 48% A) but not at 25% A. These results are consistent with previous calculations on the effect of heterogeneity in base-pair stability on DNA transition breadths. In the poly(A), poly(U) system, Et_4N^+ counterion reduces the T_m of the double and triple helices by 26 and 41 °C, respectively. The larger depression in the latter case arises from the higher charge density of the triple helix and less effective counterion screening by Et_4N^+. In the poly($2NH_2A$), poly(U) system $T_{m,2\rightarrow1}$ is reduced by 24 °C, but extrapolation of the copolymer results indicates a reduction of ~100 °C for $T_{m,3\rightarrow2}$, accounting for previous failure to observe a triple helix in this system. CD spectra of A,$2NH_2A$ copolymers suggest that much of the spectral region can be regarded as a combination of the CD spectra of the parent polymers poly(A) and poly($2NH_2A$) but that the region from 255 to 275 nm requires that contributions made by longer range interactions be taken into account.

The use of simple chemical substituents as probes to clarify structural and energetic relationships in polynucleotides has proved to be one of the best methods available for investigating these complex macromolecules. We have previously examined the consequences of modifying one of the better studied polynucleotides, poly(A),[1] by introducing an amino group into the 2 position of the purine ring (Howard et al., 1966, 1976). This modification results in marked changes in some of the properties of the polymer and in the way that the polymer reacts with the complementary polynucleotide, poly(U). In this report we attempt to provide a clearer understanding of the manner by which the 2-amino group causes these changes by studying the properties and reactions of random copolymers containing A and $2NH_2A$ residues in varying proportions. Observation of the manner in which a property varies with copolymer composition then may permit the cause of the change in property to be determined or suggest which experimental conditions should be altered to allow detection of a phenomenon not previously observed.

Thermal stability of nucleic acid helices varies with base composition, a well-known example being the linear dependence in T_m with G-C content of DNA (Marmur & Doty, 1962). Although the observed linearity in the relationship has been theoretically justified (Crothers & Kallenbach, 1966), the result depends on the assumption that the free energy of a sequence of bonded pairs depends only on the number of A-T and G-C pairs and not on the order in which they are arranged (Crothers & Kallenbach, 1966). Theoretical calculations of stacking energies (Pullman, 1968), however, suggest a rather large dependence upon sequence, raising the question of why this dependence is not observed experimentally. Interaction between A,$2NH_2A$ copolymers and poly(U) resembles that in DNA in the occurrence of both two and three hydrogen-bond base pairs. The amino substituent of $2NH_2A$ serves the function in A-U interactions that the amino group serves in G·C pairs in causing an increase in helix stability. A determination of the relationship between helix stability and base sequence in helices formed between A,$2NH_2A$ copolymers and poly(U) is thus relevant to an understanding of base sequence in natural nucleic acids.

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly($2NH_2A$), poly(2-aminoadenylic acid); poly(A,$2NH_2A$)(X, 100 − X), random copolymer containing X mole percent adenylic acid and 100 − X mole percent 2-aminoadenylic acid residues; poly(U), poly(uridylic acid); poly(A,$2NH_2A$)(X, 100 − X)·poly(U), 1:1 complex of these components; poly(A,$2NH_2A$)(X, 100 − X)·2poly(U), 1:2 complex of these components; poly(I), poly(inosinic acid); poly(C,BrC), random copolymer of cytidylic and 5-bromocytidylic acid residues; poly(U,5MeU), random copolymer of uridylic and 5-methyluridylic acid residues; Et_4N^+, tetraethylammonium ion; UV, ultraviolet; CD, circular dichroism.

The $2NH_2$ group not only changes helix stability when it forms an additional hydrogen bond but also alters the external appearance of the helix by its presence in the minor groove. This change can result in important biological effects when 2-aminoadenine replaces adenine in DNA. Stahl & Chamberlin (1976, 1978) found that substitution of 2-aminoadenine for adenine in one T7 DNA promoter site prevents utilization of the promoter by T7 RNA polymerase. The analogue did not affect transcription which starts outside the modified region. The NH_2 group in the minor groove thus appears to be involved in the initial recognition of the promoter site by T7 RNA polymerase, presumably causing the $2NH_2A{\cdot}U$ pair to be interpreted as G·C by the promoter-recognition site of the polymerase. Once transcription has started outside the modified promoter, however, Watson–Crick pairing specificity will cause $2NH_2A$ to be recognized as A and to specify U in the transcript.

Experimental Section

Mixing curves were determined by the technique described previously (Howard et al., 1971, 1976), a separate solution being prepared for each point. Spectra were measured with a Cary Model 118 spectrophotometer connected on line to a Honeywell Model DDP-516 computer (Shapiro & Schultz, 1971). Use of the computer to calculate wavelength dispersion of the angle of intersection of mixing curves has been described (Howard et al., 1976).

Ultraviolet melting data were measured automatically, the spectrophotometer, sampling accessories, and temperature programmer operating in a closed-loop mode with the computer (Howard et al., 1977). A Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroism accessory was used to measure circular dichroism spectra. Digital data were collected on line and processed with the Honeywell Model DDP-516 computer.

Poly(U) was purchased from Schwarz Bio-Research, Inc. (Lot No. 6701), and 2-aminoadenosine was purchased from Cyclo Chemical Corp. Polynucleotide phosphorylase from *Micrococcus luteus* was purchased from P-L Biochemicals, Inc. (Type 15). Poly(A) was purchased from P-L Biochemicals, Inc. (No. 179-14). $s_{w,20}$ for this polymer was reported by the manufacturer to be 7.73 ± 0.15 S. $s_{w,20}$ values of the polynucleotides synthesized for this study were 9.6, 9.1, and 10.2 S for A,$2NH_2A$ copolymers containing 67, 48, and 25% A, respectively, and 8.2 S for poly($2NH_2A$). We thank Dr. Gary Felsenfeld of this Institute for making these measurements. The similarity of these values provides assurance that the variation in transition breadth with copolymer composition of the 1:1 complexes formed with poly(U) (see below) cannot be attributed to differences in the degree of polymerization.

Synthesis of 2-Aminoadenosine-5′-phosphoric Acid. 2-Aminoadenosine was phosphorylated with phosphorus oxychloride in redistilled trimethyl phosphate at 0 °C (Yoshikawa et al., 1969). To an ice-cold, stirred suspension of 1.0 g (3.54 mmol) of dry (heated in vacuo at 110 °C for 16 h) 2-aminoadenosine in 10 mL of redistilled trimethyl phosphate was added 0.5 mL (5.5 mmol) of phosphorus oxychloride. Progress of the reaction was monitored by thin-layer chromatography. Samples (5 μL) were neutralized with 20 μL of 0.1 M ammonium bicarbonate and 15 μL was applied to a cellulose sheet (Eastman No. 6065). 2-Aminoadenosine 5′-phosphate had an R_f of 0.18 [1-propanol–ammonium hydroxide–water (60:30:10)] and was completely resolved from 2-aminoadenosine (R_f 0.50). After being stirred for 4 h in an ice bath, the reaction mixture was poured into 50 mL of a 1:1 mixture of ice and water. The pH of the solution was raised with several additions of 1 M ammonium hydroxide until it remained at 8.5. The solution of impure 2-aminoadenosine 5′-phosphate was applied to a 2.4 × 23 cm column of anion-exchange resin (Bio-Rad AG1X8; 100–200 mesh; formate form). The column was developed at a flow rate of 2.6 mL/min (18.2 mL/tube) with a linear gradient consisting of 1 L of 0.01 M formic acid in the mixing bottle and 1 L of 0.1 M formic acid in the reservoir. Elution was completed with an additional 3 L of 0.1 M formic acid. 2-Aminoadenosine 5′-phosphate appeared in tubes 79–200 (2.2 L). Pooled material was concentrated to dryness in vacuo (bath <30 °C), and the monophosphate was recovered in 20 mL of water and neutralized from pH 4.2 to 7.1 with 1 M morpholine. The yield was 3.35 mmol (95.2%), based on UV measurements.

2-Aminoadenosine 5′-phosphate (0.3 μmol) in 90 μL of 0.11 M Tris buffer, pH 8.0, was dephosphorylated with 100 μL of crude venom of *Crotalus adamanteus* (10 mg/mL) at room temperature for 20 h. Thin-layer chromatography of the digest (same solvent as above) revealed a single, UV-absorbing substance with the same R_f (0.50) as authentic 2-aminoadenosine.

2-Aminoadenosine 5′-phosphomorpholidate and 2-aminoadenosine 5′-diphosphate were synthesized by the methods described previously (Howard et al., 1976).

Synthesis of Copolymers Containing Adenylic and 2-Aminoadenylic Acids. To a solution (18.0-mL total volume) containing 0.05 M nucleoside diphosphate, 1.5×10^{-3} M magnesium chloride, 0.1 M Tris buffer, pH 9.0, 2×10^{-4} M ethylenediaminetetraacetate, and 0.01 M 1,4-dithiothreitol was added 125 units [as defined by Singer & Guss (1962)] of polynucleotide phosphorylase, and the mixture was incubated at 37 °C. Progress of the reaction was followed by measurement of the release of inorganic phosphate. Three copolymers were prepared, of input composition 70, 50, and 30% adenosine 5′-diphosphate, respectively. The extent of polymerization was 53.5% in 5 h, 56% in 8 h, and 56% in 8 h for the three copolymers. Copolymers were purified by phenol extraction and dialyzed as described previously (Howard et al., 1971). Yields of freeze-dried copolymers were 221, 195, and 201 mg, in the order listed above.

Molar extinction coefficients of copolymers were determined by analysis for phosphorus, in the manner described previously (Howard et al., 1971). Aliquots containing 0.4, 0.6, 0.8, and 1.0 μmol of phosphorus were digested for 24 h at 120–130 °C, and inorganic phosphate was determined. Parallel aliquots (0.2, 0.4, and 0.6 μmol of phosphorus) of stock solutions were diluted into 5 mL of 0.002 M sodium pyrophosphate, pH 8.0, for measurement of UV spectra. Data were fitted by computer to least-squares linear regression lines, which were used to obtain estimates of polymer concentration and absorbance and of standard deviation. Extinction coefficients were 9170 ± 60 (257 nm), 8620 ± 90 (257 nm), and 8110 ± 70 (258 nm) for the three copolymers, in the order given above. Extinction coefficients (257 nm) of copolymers were found to have a linear dependence upon composition (Figure 1S; see paragraph at end of paper regarding supplementary material). The least-squares line $\hat{\epsilon} = 10040 - 26.25Z$ ($\hat{\epsilon}$ = estimated molar extinction coefficient at 257 nm; Z = mole percent $2NH_2A$ in copolymer) was found to describe the data with a correlation coefficient of 0.9995.

Copolymer composition was determined from UV spectra of polymer samples hydrolyzed to a mixture of adenosine 3′(2′)-phosphate and 2-aminoadenosine 3′(2′)-phosphate with 1 N sodium hydroxide at 25 °C for 18 h. A least-squares best fit to each normalized experimental spectrum was computed

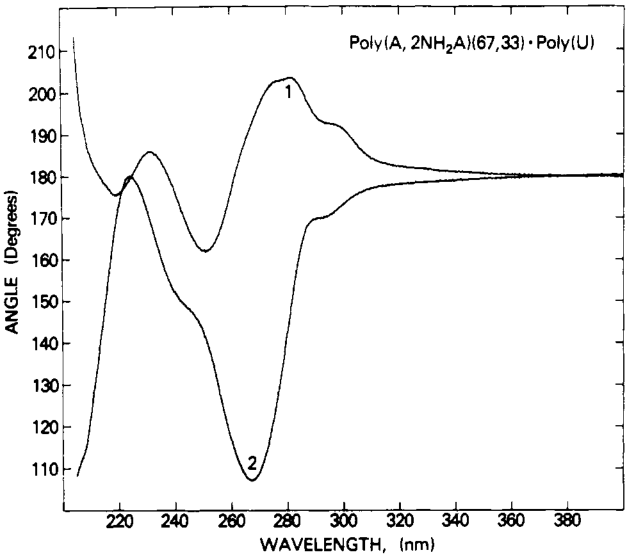

FIGURE 1: Dependence on wavelength of angles of intersection of poly(A,$2NH_2A$)(67, 33), poly(U) mixing curves at 50% poly(U) (θ_1, curve 1) and at 66.7% poly(U) (θ_2, curve 2). The most favorable wavelengths for detecting the 1:1 complex occur at the minimum at 251 nm (curve 1) and the maximum at 282 nm (curve 1). That for the 1:2 complex is at the minimum at 267 nm (curve 2).

in terms of catalog spectra of adenosine 3′(2′)-phosphate and 2-aminoadenosine 3′(2′)-phosphate (Miles, 1971). Calculated and experimental spectra were displayed on an oscilloscope to confirm closeness of fit over the spectral range 220–400 nm. The means and standard deviations of three determinations of composition for each copolymer were 66.7 ± 1.0, 48.0 ± 0.2, and 25.3 ± 1.0% A for input values of 70, 50, and 30% adenosine 5′-diphosphate, respectively. Computer analysis of synthetic mixtures known to contain 70 and 50% A resulted in values of 69.7 and 51.9% A, respectively.

Results

Stoichiometry. Recently we have described an improved, computer-assisted technique for analyzing ultraviolet spectra which permits extraction of all the information contained in the data with regard to the combining ratio of interacting polynucleotides (Howard et al., 1976, 1977). The method reduces or eliminates the uncertainties frequently associated with determinations of stoichiometry from UV-mixing curves. A computer is used to calculate the angles between intersecting limbs of mixing curves as an explicit function of wavelength. The computer calculates the angles (defined as dependent variables, θ_1 and θ_2) at 0.2-nm intervals over the range 200–400 nm, and, using these values, creates dispersion curves of θ_i as a function of wavelength. Since detectability of breaks in mixing curves increases with $|180° - \theta_i|$, the most favorable wavelengths for plotting mixing curves occur at maxima and minima in the dispersion curves, as seen in Figure 1.

Since poly(A) and poly($2NH_2A$) both interact with poly(U) to form two- and three-stranded complexes, we would expect to detect both two- and three-stranded helices as products of interaction of poly(U) with A,$2NH_2$ copolymers of any composition. The dispersion curve (θ_1) for detection of poly(A,$2NH_2A$)(67, 33)·poly(U) (Figure 1, curve 1) has a maximum at 282 nm and a minimum at 251 nm. Accordingly, these are the optimum wavelengths for detecting the 1:1 complex, and mixing curves (Figure 2) plotted at these wavelengths have clear breaks at the 1:1 ratio. A minimum occurs at 267 nm in the dispersion curve (θ_2) for detecting the 1:2 complex, and mixing curves at this and other wavelengths (Figure 2) demonstrate the formation of poly(A,$2NH_2A$)(67, 33)·2poly(U).

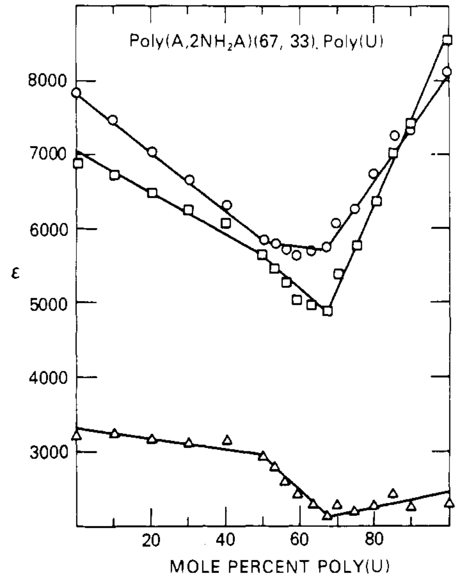

FIGURE 2: Mixing curves plotted at 251 (○) and 282 nm (△) and at 267 nm (□) demonstrate formation of poly(A,$2NH_2A$)(67, 33)·poly(U) and poly(A,$2NH_2A$)(67, 33)·2poly(U). The curves are derived from UV spectra measured on solutions containing 8 × 10^{-5} M total polymer. Conditions: 0.002 M sodium pyrophosphate, pH 8.0, 0.1 M Na^+, and 25 °C in this and all succeeding figures unless noted otherwise.

Table I: Most Favorable Wavelengths for Detection of Complexes from Mixing Curves

	wavelength (nm)			
	1:1 complex		1:2 complex	
reactants	max	min	max	min
poly(A,$2NH_2A$)(67, 33), poly(U)				
UV spectra	282	251		267
derivative UV spectra	260	242	282	255
CD spectra	250	264	260	
poly(A,$2NH_2A$)(48, 52), poly(U)				
UV spectra	283	225		269
		251		
derivative UV spectra	226	242	284	230
	261			258
CD spectra	248	270	263	
poly(A,$2NH_2A$)(25, 73), poly(U)				
UV spectra	282	252		270
derivative UV spectra	226	242	284	230
	262			258
CD spectra	250	280	264	248

Mixing curves demonstrating formation of both two- and three-stranded helices between two other A,$2NH_2A$ copolymers and poly(U) were measured, and optimum wavelengths for detecting these complexes (Figures 2S–5S, supplementary material) are listed in Table I.

Evidence confirming our assignment of combining ratios was obtained by differentiating the ultraviolet spectra and applying the same method of analysis to the derivative spectra, as described by Howard et al. (1976). Use of derivative spectra offers the advantage that in some cases mixing curves providing definitive evidence of stoichiometry may be plotted at wavelengths at which ultraviolet spectra are unable to show interaction (Howard et al., 1976, 1977). The derivative dispersion curve for poly(A,$2NH_2A$)(67, 33)·poly(U) (θ_1; curve 1, Figure 3) has a maximum at 260 nm and a minimum at 242 nm. Derivative mixing curves at these and other wave-

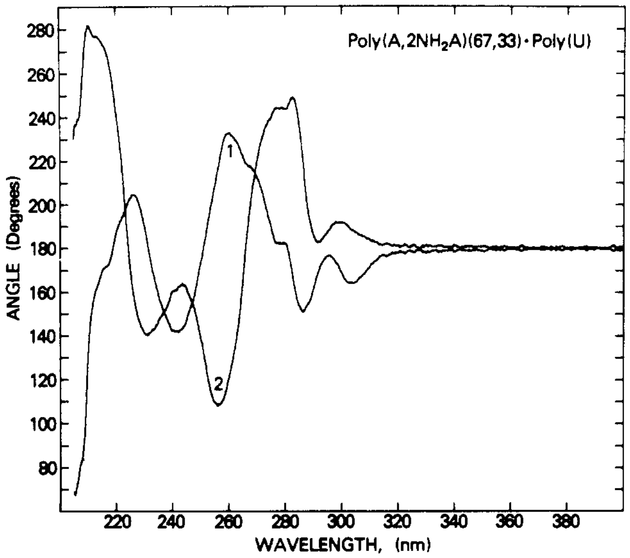

FIGURE 3: Wavelength dependence of the angle of intersection of derivative mixing curves [i.e., curves plotted from derivative UV spectra for the interaction of poly(A,2NH$_2$A)(67, 33) and poly(U)]. Curve 1 (θ_1) refers to the angle of intersection at 50% poly(U); curve 2 (θ_2) refers to that at 66.7% poly(U). Most favorable wavelengths for detecting the 1:1 complex occur at 242 and 260 nm (curve 1) and for detecting the 1:2 complex occur at 255 and 282 nm (curve 2).

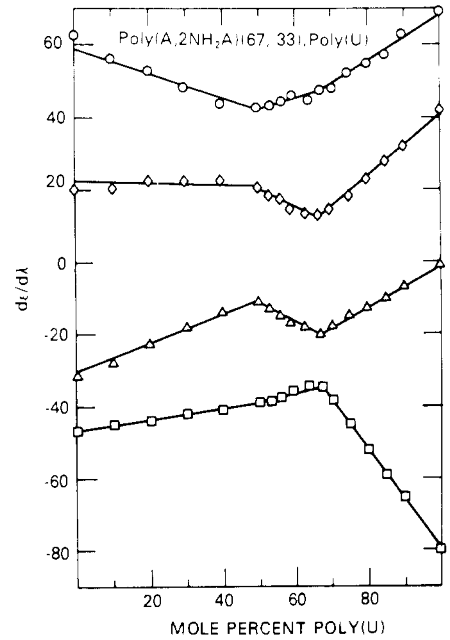

FIGURE 4: Derivative mixing curves for the interaction of poly(A,2NH$_2$A)(67, 33) with poly(U). Curves were plotted from derivatives of ultraviolet spectra (dϵ/dλ vs. λ) at 242 (○), 255 (◇), 260 (△), and 282 nm (□) and confirm formation of both double- and triple-stranded complexes.

lengths (Figure 4) break at the 1:1 ratio, demonstrating existence of the 1:1 complex. A maximum at 282 nm and a minimum at 255 nm in the derivative dispersion curve (θ_2) (Figure 3) represent the optimum wavelengths for detecting the 1:2 complex, and mixing curves at these wavelengths and at 242 and 260 nm have clear breaks, indicating formation of the triple helix.

Similar derivative mixing curves resulting in clear evidence for formation of both double and triple helices between poly(U) and the other A,2NH$_2$A copolymers are included in the supplementary material (Figures 6S–9S). The most favorable wavelengths for plotting these mixing curves are listed in Table I.

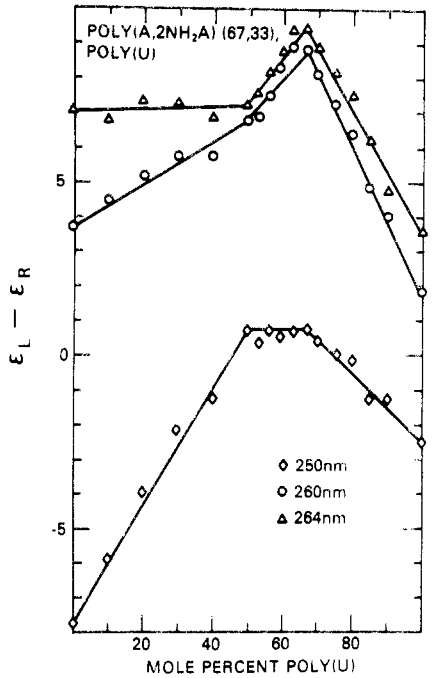

FIGURE 5: CD mixing curves of poly(A,2NH$_2$A)(67, 33) and poly(U). Large changes in CD spectra provide convincing evidence for formation of both 1:1 and 1:2 complexes by a method independent of the ultraviolet spectra.

We show in Figures 5 and 10S and 11S (supplementary material) that the same analytical procedures can be applied to CD data to provide a clear demonstration that A,2NH$_2$A copolymers form 1:1 and 1:2 complexes with poly(U) by a method which is independent of the ultraviolet spectra.

Circular Dichroism. CD spectra of nucleic acids are known to be quite sensitive to molecular structure and conformation (Yang & Samejima, 1969; Bush & Brahms, 1973; Bush, 1974; Bloomfield et al., 1974), and CD spectroscopy has proved to be a valuable technique in studies of polynucleotide interactions. Although there has been progress in the assignment of CD bands of nucleic acid monomers and some polymers to specific electronic transitions, our ability to relate the CD spectra resulting from interactions between such transitions to molecular geometry of polynucleotides is still far from adequate. Attempts have been made to calculate the CD spectra of oligo- and polynucleotides by use of a first-neighbor approximation (Gray & Tinoco, 1970; Tinoco & Cantor, 1970; Gray et al., 1972, 1973, 1978) with varying degrees of success, depending upon the nature and sequence of bases in the oligomer or polymer. The assumption is made in this approximation that the CD spectrum results from a summation of the spectra of nearest-neighbor dimers and that possible contributions from second or more distant neighbors or from the overall structure of the molecule may be neglected. Although locations of CD bands have sometimes been calculated with a reasonable degree of accuracy, large discrepancies are frequently encountered when attempts are made to reproduce magnitudes, and even signs of extrema, particularly when the sequence UpX (where X is a purine) is present. Since calculated and measured spectra in many cases agree rather poorly, it seems likely that different approaches will need to be taken if closer agreement is to be achieved. For the random copolymers reported in this study, we have applied the approximation that CD spectra have a linear dependence on the base composition (Gray & Tinoco, 1970). CD spectra of A,2NH$_2$A copolymers (Table II) ranging in composition from 100% A to 100% 2NH$_2$A are shown in Figure 6. These

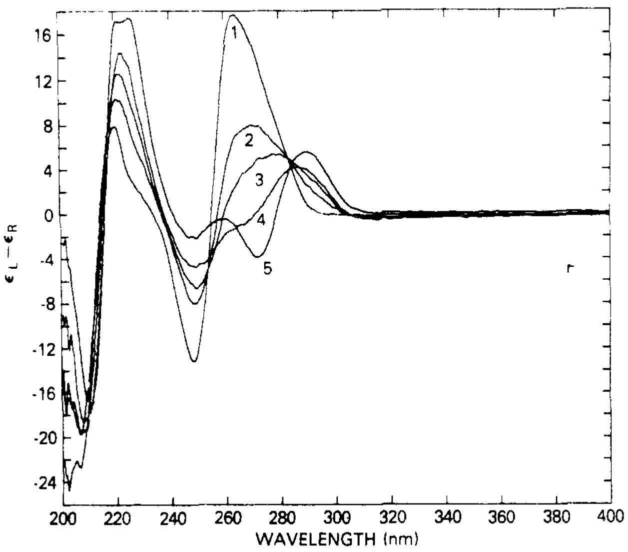

FIGURE 6: CD spectra of poly(A,2NH$_2$A)(X, 100 − X) where X = 100 (curve 1), 67 (curve 2), 48 (curve 3), 25 (curve 4), and 0 (curve 5). Each curve is the average of three spectra. Data recorded here and in Figures 12S and 13S (supplementary material) were collected, processed, smoothed, and plotted by computer.

spectra change progressively with composition, and there is an approximate isosbestic point at 285 nm. Use of the approximation that copolymer spectra may be accounted for in terms of a weighted sum of the spectra of poly(A) and poly(2NH$_2$A) produces excellent agreement between calculated and measured spectra over the ranges 200–255 nm and 275–400 nm, but poor agreement between 255 and 275 nm. Within this latter region, poly(A) has a first positive band at 263 nm and a negative band at 248 nm, which were assigned by Tinoco (1964) to exciton splitting of a strong transition at ~260 nm. It is now recognized that there are two π–π^* transitions under the 260-nm band of A [cf. Sprecher & Johnson (1977) for references], but the wavelengths and relative magnitudes of the B_{1u} and B_{2u} transitions have not been unambiguously established. The B_{1u} and B_{2u} transitions of poly(2NH$_2$A) are clearly resolved at 256.5 and 278 nm, respectively, and the four CD extrema in this region (291, 273, 260, and 249 nm) have been assigned to exciton splitting of these transitions (Howard et al., 1976).

The CD band of poly(A) at 263 nm has a large value of $\epsilon_L - \epsilon_R$ (+17.7) at a point where $\epsilon_L - \epsilon_R$ (−0.90) of poly(2NH$_2$A) is small. Introduction of a minor fraction of 2NH$_2$A residues results in a rapid, nonlinear decrease in $\epsilon_L - \epsilon_R$, but as the mole fraction of 2NH$_2$A in the copolymer increases, $\epsilon_L - \epsilon_R$ becomes less sensitive to further increases in 2NH$_2$A content.

We interpret this behavior as indicating that the rotational strength of the 263 band depends upon A residues being adjacent to each other. As 2NH$_2$A residues are introduced into the copolymer, the fraction of copolymer containing long runs of A residues will decrease rapidly at first and then less rapidly as the 2NH$_2$A content becomes predominant. Differences between the B_{1u} and B_{2u} transitions of A and 2NH$_2$A are evidently greater than between the other transitions, and it is possible that these differences are responsible for less extensive interaction between A and 2NH$_2$A transitions in the 260-nm region than at lower wavelengths. The region above 280 nm is dominated by the B_{2u} transition of 2NH$_2$A, and linear dependence of $\epsilon_L - \epsilon_R$ on composition above ~275 nm presumably reflects this fact. Madison & Schellman (1972) have concluded on the basis of their calculations on the α helix of short peptides that the π–π^* exciton system is very sensitive to the size of the system because of the long-range character of exciton interactions. It is possible to estimate the minimum number of adjacent A residues necessary for effective long-range interactions by calculating the fraction of copolymer present in runs of N or more A residues (N = 1, 2, ..., j) and then observing the value of N which results in a linear relationship between the calculated fraction and $\epsilon_L - \epsilon_R$ at 263 nm. Since the bases in A,2NH$_2$ copolymers are incorporated into the polymer chain in a random fashion (Heppel et al., 1957), the probability that a particular site in the chain is occupied by an A residue depends only upon the mole fraction of A in the copolymer. The random location of bases in the copolymer thus allows an expression to be derived (see the Appendix) which permits the fraction (E) of copolymer with sequences of chain length N or larger to be calculated:

$$E = p[1 - (1 - p)^2 \sum_{i=0}^{i=N-2} (i + 1)p^i]$$

where p = the mole fraction of base and i = the chain length of a particular sequence. Figure 7 shows that a linear relationship is observed when $\epsilon_L - \epsilon_R$ (263 nm) is plotted as a function of the fraction of copolymer with runs of A of four or more (but not of less than four). We conclude that the rotational strength of the A band at 263 nm arises largely from interactions between chromophores within units which include at least four A residues. Thus, while several of the CD bands of A,2NH$_2$A copolymers may be simply accounted for in terms of a linear combination of the corresponding bands of poly(A) and poly(2NH$_2$A), a more complex explanation is needed to account for the A band at 263 nm.

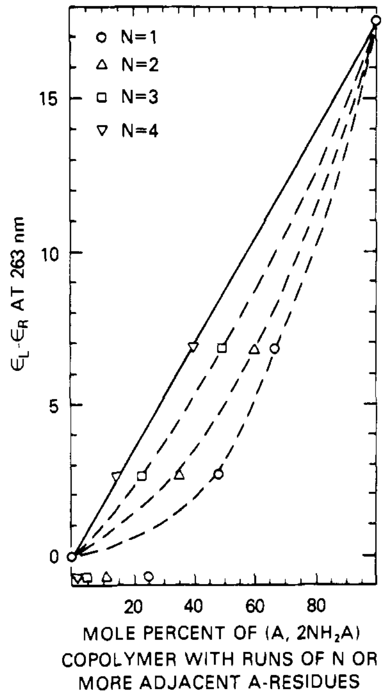

FIGURE 7: Molar ellipticity of the A band (263 nm) of A,2NH$_2$A copolymers has a linear dependence upon the mole percent of the copolymer containing runs of four (▽) or more adjacent A residues but a nonlinear dependence on runs of one (○), two (△), and three (□) or more adjacent A residues. $\epsilon_L - \epsilon_R$ has been corrected for the minor contribution of 2NH$_2$A residues.

CD spectra of 1:1 and 1:2 complexes of A,2NH$_2$A copolymers with poly(U) are shown in Figures 12S and 13S (supplementary material). Spectra of the 1:1 complexes change progressively as the copolymer composition varies from one extreme to the other, and there is an approximate isosbestic point at 250 nm. Attempts to calculate copolymer spectra as

Table II: Spectroscopic Data[a]

Ultraviolet

substance	λ_{max} (nm)	ϵ_{max}	λ_{min} (nm)	ϵ_{min}
poly(A)	255.6	10040	229.0	2860
poly(A,2NH$_2$A)(67, 33)	256.8	9170	232.6	3610
poly(A,2NH$_2$A)(48, 52)	257.2	8620	234.8	3940
poly(A,2NH$_2$A)(25, 75)	257.8	8110	236.2	4430
poly(2NH$_2$A)	257.4	7410	237.4	4600
	277.0	6980	267.2	6410
poly(A)·poly(U)	257.6	6940	231.8	2440
	~280 (sh)	3050		
poly(A,2NH$_2$A)(67, 33)·poly(U)	258.2	6830	234.3	2740
	~280 (sh)	3420		
poly(A,2NH$_2$A)(48, 52)·poly(U)	259.0	6680	235.4	2890
	~280 (sh)	3710		
poly(A,2NH$_2$A)(25, 75)·poly(U)	259.0	6470	236.4	2930
	~280 (sh)	3890		
poly(2NH$_2$A)·poly(U)	259.0	6130	236.6	2890
	~282 (sh)	4080		
poly(A)·2poly(U)	255.8	6040	233.4	2380
poly(A,2NH$_2$A)(67, 33)·2poly(U)	257.0	6400	234.0	2380
	~295 (sh)	380		
poly(A,2NH$_2$A)(48, 52)·2poly(U)	257.2	6360	234.6	2530
	~295 (sh)	660		
poly(A,2NH$_2$A)(25, 75)·2poly(U)	257.4	6630	235.4	2780
	~295 (sh)	880		
poly(2NH$_2$A)·2poly(U)	258.2	6620	235.0	2690
	~283 (sh)	2630		
	~295 (sh)	1210		

Circular Dichroism

	λ_{max} (nm)	$\epsilon_L - \epsilon_R$	λ_{min} (nm)	$\epsilon_L - \epsilon_R$
poly(A)	220	7.88	202	−25
	~235 (sh)	−0.6	248	−13.20
	263	17.68		
poly(A,2NH$_2$A)(67, 33)	221	10.34	207	−19.7
	~235 (sh)	1.3	248	−8.00
	270	8.04		
poly(A,2NH$_2$A)(48, 52)	221	12.54	208	−19.5
	~235 (sh)	1.9	249	−6.65
	280	5.44		
poly(A,2NH$_2$A)(25, 75)	223	14.38	208	−18.7
	285	4.29	249	−4.75
poly(2NH$_2$A)	226	17.42	210	−17.1
	260	−0.37	248	−2.14
	290	5.63	271	−3.87
poly(A)·poly(U)	223	2.41	208	−7.8
	~233 (sh)	0.4	244	−4.16
	~258 (sh)	10.4		
	267	11.29		
poly(A,2NH$_2$A)(67, 33)·poly(U)	224	3.82	207	−6.9
	~233 (sh)	1.7	245	−1.36
	264	7.27		
poly(A,2NH$_2$A)(48, 52)·poly(U)	225	5.13	209	−7.7
	~233 (sh)	2.4	244	−1.18
	261	6.41	297	−0.37
poly(A,2NH$_2$A)(25, 75)·poly(U)	225	5.98	208	−7.47
	262	6.66	243	−1.98
			288	−3.16
poly(2NH$_2$A)·poly(U)	223	10.42	209	−6.7
	262	10.31	245	−1.30
			285	−7.29
poly(A)·2poly(U)	223	7.81	210	−5.3
	264	10.89	245	−3.88
poly(A,2NH$_2$A)(67, 33)·2poly(U)	224	3.92	206	−4.8
	264	9.49	243	−1.82
			297	−0.42
poly(A,2NH$_2$A)(48, 52)·2poly(U)	224	4.38	204	−7.7
	263	9.72	245	−3.75
			294	−1.34
poly(A,2NH$_2$A)(25, 75)·2poly(U)	225	5.39	206	−5.2
	264	10.62	247	−4.16
			293	−2.15
poly(2NH$_2$A)·2poly(U)	223	7.81	210	−5.3
	264	10.89	245	−3.88
			290	−3.97

[a] Spectra were measured of solutions containing 8 × 10^{-5} M total polymer P, 0.002 M sodium pyrophosphate, pH 8.0, and 0.1 M Na$^+$, at 25.0 °C.

linearly weighted sums of the spectra of poly(A)·poly(U) and poly(2NH$_2$A)·poly(U) were unsuccessful. Although locations of bands were reproduced with reasonable accuracy, large deviations were observed when calculated values of $\epsilon_L - \epsilon_R$ were compared to experimentally determined values.

CD spectra of 1:2 complexes of A,2NH$_2$A copolymers and poly(U) of composition varying from 100% A to 100% 2NH$_2$A (Figure 13S, supplementary material) also have an approximate isosbestic point at 259 nm. Spectra calculated on the basis of a weighted summation of the spectra of poly(A)·2poly(U) and poly(2NH$_2$A)·2poly(U), in this example, are in good agreement between calculated and measured spectra. The markedly improved agreement between calculated and measured spectra for the 1:2 complexes when compared to those for the 1:1 complexes, however, may be largely due to the relatively large contribution made by U residues in the triple helices.

Ultraviolet Melting Curves: Salt Dependence. Ultraviolet melting curves of poly(A,2NH$_2$A)(X, 100 − X)·poly(U) (X = 100, 67, 48, 25, and 0) exhibit a single transition (Figure 14S, supplementary material) over the range of sodium ion concentration from 0.03 to 0.2 M. T_m increases with increasing 2NH$_2$A content at constant [Na$^+$]. The slope (dT_m/d log [Na$^+$]) of salt-dependence curves (Figure 8) decreases with decreasing A content, the mean of three determinations each [two for poly(2NH$_2$A)·poly(U)] being 17.1, 14.4, 13.6, 12.6, and 12.2 °C (Table III).

There is a progressive decrease in salt dependence with increase in 2NH$_2$A content. For poly(2NH$_2$A)·poly(U) the slope is 5 °C or ~30% lower than that of poly(A)·poly(U). While we do not have sufficient information to account unambiguously for this change of slope, we may note two factors which are probably related to it. The electrostatic contribution (presumably comparable in the two helices) to the free energy is relatively smaller in the more stable helix, tending to reduce dependence on counterion screening of interstrand repulsion. The enthalpy of interaction of poly(2NH$_2$A) with poly(U), moreover, is probably considerably higher than that of poly(A). Though no calorimetric measurements have been made on the polymer–polymer system, Scruggs & Ross (1970) found that 2-aminoadenosine had a heat of reaction with poly(U) ~3 kcal larger than that of adenosine.

Dependence of T_m of Two-Stranded Helices on 2NH$_2$A Content. The presence of an amino group at the 2 position of A permits formation of a third hydrogen bond with poly(U) and results in an increase in T_m of 33 °C (0.1 M Na$^+$; Howard et al., 1966, 1976). In assessing the contribution an energetic factor makes to helix stability, we can make a distinction

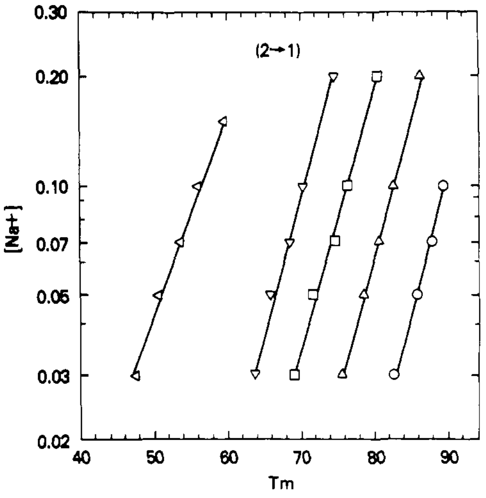

FIGURE 8: Dependence of T_m of the 2 → 1 transition of poly(A,2NH$_2$A)(X, 100 − X)·poly(U) [X = 100 (△), 67 (▽), 48 (□), 25 (◬), and 0 (○) on [Na$^+$]]. T_m values are from the melting curves of Figure 14S (supplementary material) and similar melting curves measured at other [Na$^+$]. Slopes of the 2 → 1 transitions are given in Table III. Conditions: 8×10^{-5} M total polymer P; 0.002 M sodium pyrophosphate, pH 8.0.

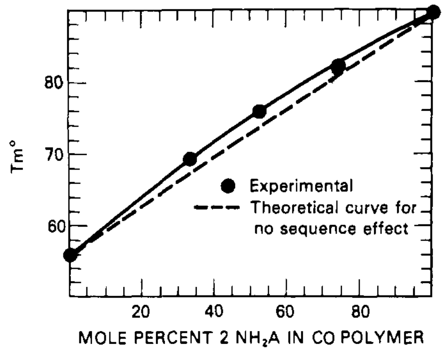

FIGURE 9: Dependence of T_m of 2 → 1 transition of poly(A,2NH$_2$A)(X, 100 − X)·poly(U) (X = 100, 67, 48, 25, and 0) upon mole percent of 2NH$_2$A residues in the A,2NH$_2$A copolymer. The slight deviation from linearity suggests that sequence effects play only a minor role in the thermal stability of these double helices.

Table III: Thermal Data

Transition Temperature substance	$T_m{}^a$ (°C)
poly(A)·poly(U)	56.0
	55.5
	55.7
poly(A,2NH$_2$A)(67, 33)·poly(U)	69.0
	69.0
	69.5
poly(A,2NH$_2$A)(48, 52)·poly(U)	75.6
	76.0
	75.9
poly(A,2NH$_2$A)(25, 75)·poly(U)	82.2
	81.9
	82.4
poly(2NH$_2$A)·poly(U)	89.6
	89.4
	89.8
	89.1

Dependence of T_m on Cation Concentration

substance	transition	dT_m/d log [Na$^+$]^b	dT_m/d log [Et$_4$N$^+$]^c
poly(A)·poly(U)	2 → 1	17.1 ± 0.8	17.5 ± 1.6
poly(A,2NH$_2$A)(67, 33)·poly(U)	2 → 1	14.4 ± 0.5	11.8 ± 1.7
poly(A,2NH$_2$A)(48, 52)·poly(U)	2 → 1	13.6 ± 0.7	10.5 ± 0.8
poly(A,2NH$_2$A)(25, 75)·poly(U)	2 → 1	12.6 ± 1.0	8.7 ± 1.4
poly(2NH$_2$A)·poly(U)	2 → 1	12.6 ± 0.9	9.9 ± 1.1
poly(A)·2poly(U)	3 → 2	27.7 ± 0.5	27.5 ± 1.0
poly(A,2NH$_2$A)(67, 33)·2poly(U)	3 → 2	31.0 ± 1.6	36.8 ± 0.8
poly(A,2NH$_2$A)(48, 52)·2poly(U)	3 → 2	33.3 ± 0.5	36.3 ± 1.5
poly(A,2NH$_2$A)(25, 75)·2poly(U)	3 → 2	29.3 ± 0.7	
poly(2NH$_2$A)·2poly(U)	3 → 2	26.0 ± 1.6	

[a] For the 2 → 1 transition measured at [Na$^+$] = 0.1 M and 0.002 M sodium pyrophosphate, pH 8.0. Although the statistical correlation coefficient for the linear regression equation $T_m = 33.28X + 51.7$ (X = mole fraction of 2NH$_2$A in copolymer) was 0.9956, an F test for lack of fit to the linear model was significant [F = 79.8; F (0.95;3,11) = 3.59]. F is the ratio of two variance estimates (Armitage, 1977). The parabolic equation $T_m = 55.79 + 42.98X - 9.028X^2$ was found to give a considerably improved, although still not perfect, fit to the data [F = 12.8; F (0.95;2,11) = 3.98]. [b] 0.002 M sodium pyrophosphate, pH 8.0. [c] 0.002 M tetraethylammonium pyrophosphate, pH 7.5.

between those factors operating within the plane of paired bases and those operating out of the plane. Since hydrogen bonds operate in the plane, stability should be independent of the position the 2-amino group occupies in the polymer chain and of the nature of the neighboring bases. In considering the dependence of T_m on composition of copolymers, we find there have been very few studies of chemically well-defined copolymer systems. In the well-known example of natural DNA (Marmur & Doty, 1962), T_m was found to be essentially linear in GC content. The dependence of T_m of the poly(I) complex with poly(C,BrC) copolymers was also shown to be linear in BrC content (Howard et al., 1969). In contrast, Swierkowski et al. (1965) reported that the relation between T_m and 5MeU content in both poly(A)·poly(U,5MeU) and the self-interacted poly(U,5MeU) helix was definitely nonlinear (T_m increases with 5MeU content in both cases). We have reinvestigated this system, however, and find that the dependence of T_m on 5MeU content is linear, as in the cases of poly(C,BrC) and DNA (F. B. Howard and H. T. Miles, unpublished experiments). Our data relating thermal stability to copolymer composition of poly(A,2NH$_2$A)(X, 100 − X)·poly(U) (X = 25, 48, and 57) helices is given in Figure 9. T_m of the double helix was measured three times (0.1 M Na$^+$) at each composition [four times in the case of poly(2NH$_2$A)·poly(U)]. A slight positive departure from linearity was observed (Figure 9), and replicate measurements were made to permit an analysis of the statistical significance of the departure (Table III). The maximum deviation from linearity is only 2.6 °C (Figure 9). These results suggest that helix stability depends upon sequence to only a minor degree and that while A–2NH$_2$A neighbors may offer a slightly more stable configuration than do either A–A or 2NH$_2$A–2NH$_2$A neighbors, the preference for such configuration is small.

Disproportionation Reaction. We have shown previously that the homopolymer helix poly(2NH$_2$A)·poly(U) does not undergo a 2 → 3 transition at any salt concentration (Howard et al., 1976). In the present copolymer systems we find that double helices in which the purine polymer has 67 or 48% A do undergo disproportionation under a limited range of conditions (Figure 10), but a complex containing only 25% A does not. Addition of ethylene glycol, however, lowers T_m of the 2 → 3 transition and permits this reaction to be observed for all copolymer systems as well as for the homopolymer helix

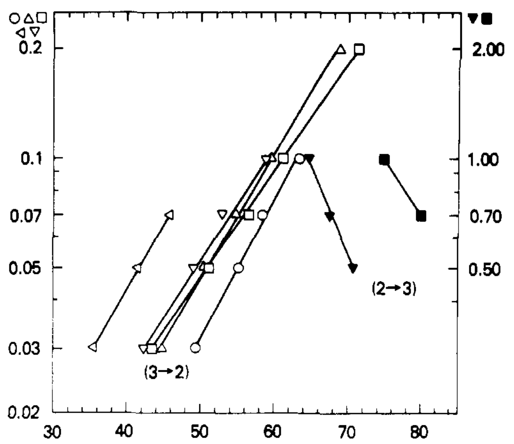

FIGURE 10: Dependence of the T_m of the 3 → 2 transition of poly(A,2NH$_2$A)(X, 100 − X)·2poly(U) [X = 100 (△), 67 (▽), 48 (□), 25 (△) and 0 (○)] and of the 2 → 3 transition of poly(A,2NH$_2$A)(X, 100 − X)·poly(U) [X = 67 (▼) and 48 (■)] upon [Na$^+$]. Slopes of the 3 → 2 transitions are listed in Table III. Conditions: see Figure 8.

poly(2NH$_2$A)·poly(U). These results are discussed in a later section.

Breadth of Melting Curves. The effect of heterogeneity of base pairs on transition breadth has been investigated theoretically (Crothers et al., 1965; Crothers & Kallenbach, 1966; Kallenbach, 1968; Lazurkin et al., 1970), but there have been few experimental studies of cases in which all bases in the helix form standard pairs (as distinct from mispairing or looping out). Data on transition breadths observed in the present study are given in Table IV. As expected, the differences with composition are relatively small and require statistical analysis for proper assessment. In order to utilize the larger data set, we treated each value of the transition breadth in Table IV as an independent observation, regardless of the ionic strength at which it was made. This treatment is justified by the finding (Table IV) that transition breadth is not affected by ionic strength in the present systems and by previous data on polydeoxynucleotides showing no effect of ionic strength on transition breadth (Inman & Baldwin, 1964; Riley et al., 1966). Salt effects are observed with strictly alternating copolymers such as poly[d(A-T)]·poly[d(A-T)], but these are presumably related to the ability of these polymers to form hairpin structures (Inman & Baldwin, 1962).

A major factor determining transition breadth of heterogeneous polymers is the relative stability of the two kinds of base pairs (Crothers et al., 1965). Crothers et al. (1965) have predicted that the transition breadth for DNA of low molecular weight is proportional to $T_G - T_A$ if other factors are held constant. Their calculations indicate that for DNA molecules 100 base pairs long there is little change in transition breadth for compositions ranging from 30 to 70% GC content but that there should be a slight progressive sharpening of the transition for GC contents of less than 30% or more than 70% (few natural DNAs are available in this range). Similar calculations have been made by Lazurkin et al. (1970), who report a satisfactory agreement between experimental and calculated values for transition breadths. Because of the relatively large difference (33 °C, 0.1 M Na$^+$; Howard et al., 1976) in T_m between poly(A)·poly(U) and poly(2NH$_2$A)·poly(U), an experimental test of theory can be made by use of the data listed in Table IV. Statistical tests of these data indicate that transition breadths of the complexes having mole percent A of X = 67 and X = 48 are greater than those of either of the homopolymer complexes but that of the helix with X = 25 is not statistically different from the breadths of the homopolymer pairs [Table IV, footnote *c*; Table 1S (supplementary material)]. Within the resolution of the measurements, our results are thus in agreement with the calculations of Crothers et al. (1965).

Table IV: Transition Breadths of Poly(A,2NH$_2$A) (X, 100 − X)·Poly(U)

[Na$^+$]^a (M)	transition breadth^b				
	$X = 0$	$X = 25$	$X = 48$	$X = 67$	$X = 100$
0.03	4.4	4.5	6.0	7.1	4.8
	4.0	3.7	4.7	4.2	3.0
	2.4	4.0	4.8	5.2	2.0
0.05	5.6	4.8	6.5	8.3	6.7
	3.9	5.0	4.8	6.0	4.6
	4.6	3.5	3.5	4.0	3.3
0.07	1.6	3.3	6.5	6.4	4.0
	3.6	3.2	4.0	5.6	3.0
	4.2	4.4	5.9	4.7	2.5
	1.7				
0.10	4.2	5.2	4.1	6.5	4.5
	2.8	5.3	5.2	5.5	3.5
	2.0	3.9	4.7	4.3	4.3
0.15					4.9
					3.0
					3.0
0.20		3.9	6.9	6.5	
		3.9	5.6	4.9	
			4.4	5.4	
av:^{c}	3.54 ± 1.23	4.19 ± 0.69	5.17 ± 1.02	5.64 ± 1.18	3.81 ± 1.19

^{a} The absence of dependence of transition breadth upon sodium ion concentration was determined by fitting each set of transition breadth values (X = 0, 25, 48, 67, and 100) with a linear least-squares regression line: transition breadth = A log [Na$^+$] + B, where A and B are the parameters of the linear model. In every case an F test for regression was not significant at $\alpha = 0.05$. ^{b} Transition breadth is the temperature interval between the intercepts of the line of maximum slope of the melting curve with straight lines drawn through the upper and lower limbs of the transition curve. ^{c} A one-way analysis of variance shows that the transition breadths are not all equal (see Table 1S, supplementary material). The Dunn-Bonferroni and Tukey statistical tests (Kirk, 1968) were used to identify significant differences between transition breadths of each duplex.

Stepwise Dissociation of Three-Stranded Helices. Melting curves of the triple helices formed by the copolymers with poly(U) have two transitions (Figure 11) over the range of sodium ion concentration from 0.03 to 0.2 M (Figure 10). The first transition reflects the conversion of the triple-stranded helix to double-stranded helix plus single-stranded poly(U) (3 → 2). The second transition occurs at the same T_m value observed for melting of the corresponding double helices (see above) and is identified as dissociation of these helices to random coils (2 → 1). T_m of the 3 → 2 transition increases nonlinearly with increasing 2NH$_2$A content, and poly(2NH$_2$A)·2poly(U) dissociates (3 → 2) at a temperature ~14 °C higher over the range of [Na$^+$] studied than does poly(A)·2poly(U), a somewhat larger difference than that reported previously (Howard et al., 1976).

Destabilizing Effect of Low Concentrations of Tetraethylammonium Counterion on Thermal Stability of Double and Triple Helices. T_m values of 1:1 complexes of A,2NH$_2$A copolymers and poly(U) are lowered when Et$_4$N$^+$ is substituted for Na$^+$ although T_m still increases with increasing 2NH$_2$A content. Cation-dependence curves are linearly dependent upon log [Et$_4$N$^+$] from 0.03 to 0.1 M Et$_4$N$^+$ (Figure 12). At [Et$_4$N$^+$] > 0.1 M, cation-dependence curves depart from

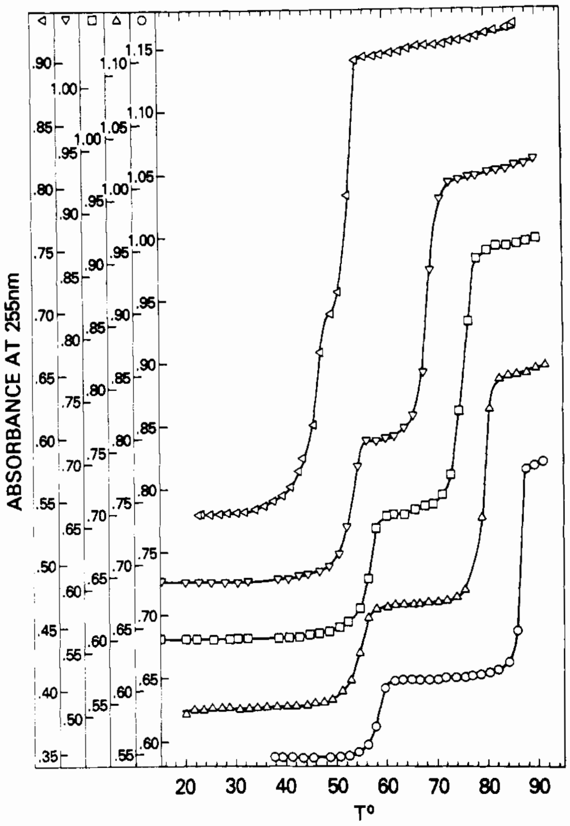

FIGURE 11: UV melting curves of poly(A,2NH$_2$A)(X, 100 − X)·2poly(U) [X = 100 (△), 67 (▽), 48 (□), 25 (△), and 0 (○)]. The first transition of each curve arises from dissociation of the triple helix to single-stranded poly(U) and the double helix (3 → 2 transition). The second transition occurs at the same T_m as was observed above (Figure 8) and is assigned to the dissociation of the double-stranded complex to single strands. Conditions: see Figure 8.

linearity (Figure 12), and T_m becomes nearly independent of cation concentration. This nonspecific destabilizing effect is presumably due to disruption of stacking interactions [cf. Howard et al. (1976)].

In the linear regions of these curves (Figure 12), dT_m/d log [Et$_4$N$^+$] decreases with increasing 2NH$_2$A content (Table III) but is nearly the same as dT_m/d log [Na$^+$] throughout the range of copolymer composition.

We have shown previously (Howard et al., 1971) that when Et$_4$N$^+$ is present as counterion both poly(A)·poly(T) and poly(A)·2poly(T) are destabilized compared to when sodium is present. Stability of the triple helix, however, is lowered much more than that of the double helix. Similarly, while T_m of poly(A)·poly(U) is decreased by 26 °C on substitution of Et$_4$N$^+$ for Na$^+$, T_m of poly(A)·2poly(U) is lowered by 41.5 °C at 0.07 M cation (Howard et al., 1976). We have attributed this selective destabilization of the three-stranded complex to its higher charge density and to less effective screening by Et$_4$N$^+$ than by sodium ion.

The electrostatic effect discussed above arises from the need for counterion screening and is general for multistranded polynucleotide structures. We find in addition, however, a surprising effect on triple helices which appears to be specific to the 2NH$_2$ substitution in A. The homopolymer does not form a triple helix with poly(U) at all in the presence of Et$_4$N$^+$, nor does the A,2NH$_2$A copolymer containing 75% of 2NH$_2$A. At lower 2NH$_2$A content, triple helices are formed with poly(U), but the 3 → 2 transition (which is the appropriate measure of triple helix stability under these conditions) is much lower than with Na$^+$ counterion. This depression, ΔT_m, is 41.5, 61.5, and 68.2 °C (0.07 M cation for copolymers containing 0, 33, and 52% 2NH$_2$A residues). The relation is essentially linear and can be extrapolated to give a value of $\Delta T_m \sim 98$ °C or $T_m \sim -49$ °C for the 3 → 2 transition of the hypothetical complex poly(2NH$_2$A)·2poly(U). In marked contrast is the behavior of the double helices: ΔT_m of poly(2NH$_2$A)·poly(U) (24 °C) is approximately the same as that of poly(A)·poly(U) (26 °C) on substitution of Et$_4$N$^+$ for Na$^+$ (Howard et al., 1976). It is not clear why Et$_4$N$^+$ has such a large destabilizing effect upon the triple helix when 2NH$_2$A is present. We may speculate that the anomalous destabilization of poly(2NH$_2$A)·2poly(U) may result from a different geometry of the helix backbone than that of poly(A)·2poly(U) or possibly that the hydrated 2NH$_2$ group in the minor groove may contribute to a steric exclusion of the bulky cation from a position suitable for efficient counterion screening. A major problem with such hypotheses is that ΔT_m for the double helix is the same as for poly(A)·poly(U).

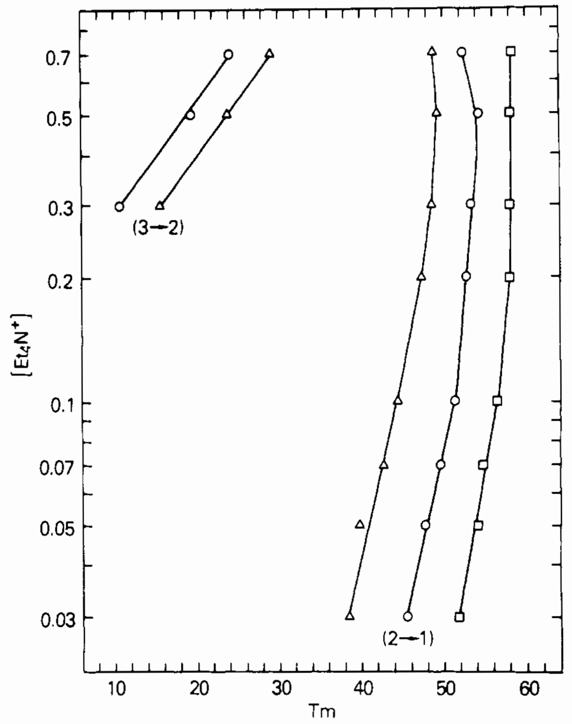

FIGURE 12: The two curves on the left show the dependence of the T_m of poly(A,2NH$_2$A)(X, 100 − X)·2poly(U) [X = 67 (△) and 48 (○)] upon [Et$_4$N$^+$] for the 3 → 2 transition. The three curves on the right are for the 2 → 1 transition of poly(A,2NH$_2$A)(X, 100 − X)·poly(U) [X = 67 (△), 48 (○), and 25 (□)]. Poly(2NH$_2$A)·2poly(U) was sufficiently unstable with Et$_4$N$^+$ present as cation so that the 3 → 2 transition could not be observed.

Discussion

We have previously shown that the third hydrogen bond of poly(2NH$_2$A) causes a differential elevation of the transition temperature of the double helix formed with poly(U) but relatively little change in T_m of the 3 → 2 transition. There results a wide separation of the curves defining dependence on cation concentration of the 2 → 1 and 3 → 2 transitions, which contrasts with the much greater proximity of these curves in the poly(A), poly(U) system [Figures 8 and 10; cf. Howard et al. (1976)]. In the copolymer systems described above, the salt-dependence curves for the 3 → 2 transition are almost independent of composition and ~8–10 °C above that for poly(A)·poly(U) (Figure 10). Curves for the 2 → 1 transition, however, respond in an almost linear fashion to changes in composition, and as 2NH$_2$A content decreases they approach the 3 → 2 curves (Figures 8 and 10). This alteration of the phase diagrams is consistent with the view that it is the third hydrogen bond which elevates T_m of the 2 → 1 transition and that a second poly(U) strand, lacking such a direct interaction, should be relatively unaffected by the $2NH_2$ substitution.

If an anticipated helix–coil transition is not observed in a new polynucleotide system, it is frequently not clear why the reaction did not occur or in what part of the phase diagram one should look for it. Should the ionic strength be increased or decreased? Does the hypothetical T_m lie above 100 °C or below 0 °C? Investigation of copolymers of the standard and modified bases can provide answers to these questions by extrapolation of T_m values of copolymer systems having a higher proportion of the standard base. We have seen in the preceding section, for example, that our previous failure to observe the triple helix poly($2NH_2A$)·2poly(U) with Et_4N^+ counterion was due to the low temperature (extrapolated $T_m \sim -50$ °C) of the 3 → 2 transition. A more interesting "missing" transition of the poly($2NH_2A$), poly(U) system is the disproportionation or 2 → 3 transition (Howard et al., 1976). Observation of this reaction at high temperatures in two of the copolymer systems (Figure 10) permitted an extrapolation which indicated a T_m of 109 °C for the 2 → 3 transition in 0.7 M Na^+ and 104 °C in 1 M Na^+. Two factors leading to this unusually high T_m may be identified. The net loss of one hydrogen bond in the reaction

$$2(U{\equiv}2NH_2A) \rightleftarrows (U{\equiv}2NH_2A{=}U) + 2NH_2A$$

would tend to prevent the 2 → 3 transition from occurring. The second factor depends not on the structure of the helix but on the stacking of single-stranded poly($2NH_2A$) produced by the above reaction. In the poly(A), poly(U) system Ross & Scruggs (1965) found that the enthalpy change was unfavorable for the 2 → 3 transition and concluded that the reaction must therefore be entropically driven. This entropic effect can presumably be attributed to decreased ordering of single-stranded poly(A). A more highly ordered single-stranded polymer such as poly($2NH_2A$) would contribute a smaller entropy increase to a 2 → 3 transition and so tend to prevent the reaction. Our results suggest that disproportionation of poly($2NH_2A$)·poly(U) could be observed, provided conditions were found to lower the transition temperature into the accessible range of measurement. Addition of an organic solvent such as ethylene glycol to aqueous solutions will reduce the T_m of double helices and lead to unstacking of partially ordered single strands. To the extent that single-strand stacking controls the 2 → 3 transition, therefore, we would expect ethylene glycol or other organic solvents to have an important influence on this reaction. The effect is in fact quite striking, especially for the polymers containing $2NH_2A$. Fifty percent ethylene glycol in water (0.25 M Na^+) reduces T_m of 2 → 1 and 3 → 1 transitions of poly(I)·poly(C), poly(I)·poly(BrC), poly(A)·poly(U), and poly($2NH_2A$)·poly(U) by only ~10–12 °C (Howard et al., 1969, 1976; Miles & Frazier, 1978; Figure 13). In contrast, T_m depression for the 2 → 3 transition is ~27 °C for poly(A)·poly(U) (Miles & Frazier, 1978) and ~54 °C for poly($2NH_2A$)·poly(U) (the value for the latter system in the absence of glycol is based upon extrapolation to 0.25 M Na^+ of results at 1.0 and 0.7 M Na^+; it is consistent with the copolymer results, which were measured directly). The two copolymer systems which undergo disproportionation in the absence of glycol exhibited T_m depressions under the same conditions of ~48 °C (48% A) and ~42 °C (67% A) (Figures 10 and 13). That is, the reduction of $T_{2\to3}$ by 50% glycol is ~5 times as great as $(T_{2\to1}{}^{H_2O} - T_{3\to1}{}^{50\%\text{glycol}})$ for poly($2NH_2A$)·poly(U) and about ~2.5 times as great as for poly(A)·poly(U). These results are consistent with the conclusion that stacking of the single-stranded purine polymer plays a major role in driving the disproportionation reaction and that the greater effect of glycol in the poly($2NH_2A$) system arises from more extensive stacking of this polymer.

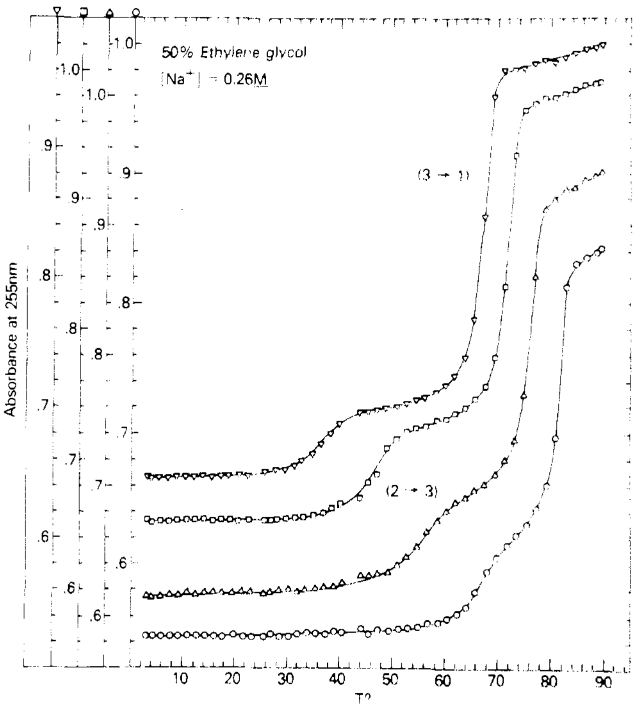

FIGURE 13: Ultraviolet melting curves of 1:1 mixtures of poly(A,$2NH_2A$)(X, $100 - X$) [X = 67 (▽), 48 (□), 25 (△), and 0 (○)] and poly(U) (8×10^{-5} M in total polymer P; 0.002 M sodium pyrophosphate, pH 8.0; [Na^+] = 0.26 M; 50 vol % ethylene glycol]. The first transition of each curve arises from the disproportionation (2 → 3 transition) of 2 mol of the double helix, resulting in formation of the triple helix and single-stranded copolymer. The second transition represents dissociation of the triple helix to single strands (3 → 1 transition).

Supplementary Material Available

Experimental data which show the linear dependence of the extinction coefficient upon composition (Figure 1S), UV mixing curves (Figures 2S–9S), CD mixing curves (Figures 10S and 11S), CD spectra (Figures 12S and 13S), UV melting curves (Figure 14S), and values used for statistical tests (Table 1S) (15 pages). Ordering information is given on any current masthead page.

References

Armitage, P. (1977) *Statistical Methods in Medical Research*, p 141, Blackwell Scientific Publications Ltd., Oxford, England.

Bloomfield, V. A., Crothers, D. M., & Tinoco, I. (1974) *Physical Chemistry of Nucleic Acids*, pp 19–52, Harper and Row, New York.

Bush, C. A. (1974) *Basic Princ. Nucleic Acid Chem. 2*, 91–169.

Bush, C. A., & Brahms, J. (1973) in *Physico-Chemical Properties of Nucleic Acids* (Duchesne, J., Ed.) pp 147–186, Academic Press, New York.

Crothers, D. M., & Kallenbach, N. K. (1966) *J. Chem. Phys. 45*, 917–927.

Crothers, D. M., Kallenbach, N. K., & Zimm, B. H. (1965) *J. Mol. Biol. 11*, 802–820.

Gray, D. M., & Tinoco, I., Jr. (1970) *Biopolymers 9*, 223–244.

Gray, D. M., Tinoco, I., Jr., & Chamberlin, M. J. (1972) *Biopolymers 11*, 1235–1258.

Gray, D. M., Ratliff, R. L., & Williams, D. L. (1973) *Biopolymers 12*, 1233–1245.

Gray, D. M., Morgan, A. R., & Ratliff, R. L. (1978) *Nucleic Acids Res. 5*, 3679–3695.

Heppel, L. A., Oritiz, P. J., & Ochoa, S. (1957) *J. Biol. Chem. 229*, 695–710.

Howard, F. B., Frazier, J., & Miles, H. T. (1966) *J. Biol. Chem. 241*, 4293–4295.

Howard, F. B., Frazier, J., & Miles, H. T. (1969) *J. Biol. Chem. 244*, 1291–1302.

Howard, F. B., Frazier, J., & Miles, H. T. (1971) *J. Biol. Chem. 246*, 7073–7086.

Howard, F. B., Frazier, J., & Miles, H. T. (1976) *Biochemistry 15*, 3783–3795.

Howard, F. B., Hattori, M., Frazier, J., & Miles, H. T. (1977) *Biochemistry 16*, 4637–4646.

Inman, R. B., & Baldwin, R. L. (1962) *J. Mol. Biol. 5*, 172–184.

Inman, R. B., & Baldwin, R. L. (1964) *J. Mol. Biol. 8*, 452–469.

Kallenbach, N. R. (1968) *J. Mol. Biol. 37*, 445–466.

Kirk, R. E. (1968) *Experimental Design: Procedures for the Behavioral Sciences*, pp 79–81, 88–90, Brooks/Cole Publishing Co., Belmont, CA.

Lazurkin, Yu. S., Frank-Kamenetskii, M. D., & Trifonov, E. N. (1970) *Biopolymers 9*, 1253–1306.

Madison, V., & Schellman, J. (1972) *Biopolymers 11*, 1041–1076.

Marmur, J., & Doty, P. (1962) *J. Mol. Biol. 5*, 109–118.

Miles, H. T. (1971) *Proced. Nucleic Acid Res. 2*, 205–232.

Miles, H. T., & Frazier, J. (1978) *Biochemistry 17*, 2920–2926.

Pullman, B. (1968) in *Molecular Associations in Biology* (Pullman, B., Ed.) pp 1–19, Academic Press, New York.

Riley, M., Maling, B., & Chamberlin, M. J. (1966) *J. Mol. Biol. 20*, 359–389.

Ross, P. D., & Scruggs, R. L. (1965) *Biopolymers 3*, 491–496.

Scruggs, R. L., & Ross, P. D. (1970) *J. Mol. Biol. 47*, 29–40.

Shapiro, M., & Schultz, A. (1971) *Anal. Chem. 43*, 398–405.

Singer, M. F., & Guss, J. K. (1962) *J. Biol. Chem. 237*, 182–189.

Sprecher, C. A., & Johnson, W. C. (1977) *Biopolymers 16*, 2243–2264.

Stahl, S. J., & Chamberlin, M. J. (1976) in *RNA Polymerase* (Chamberlin, M. J., & Losich, R., Eds.) pp 429–440, Cold Spring Harbor Press, Cold Spring Harbor, NY.

Stahl, S. J., & Chamberlin, M. J. (1978) *J. Biol. Chem. 253*, 4951–4969.

Swierkowski, M., Szer, W., & Shugar, D. (1965) *Biochem. Z. 342*, 429–436.

Tinoco, I., Jr. (1964) *J. Am. Chem. Soc. 86*, 297–298.

Tinoco, I., Jr., & Cantor, C. R. (1970) *Methods Biochem. Anal. 18*, 81–203.

Yang, J. T., & Samejima, T. (1969) *Prog. Nucleic Acid Res. Mol. Biol. 9*, 223–300.

Appendix: Calculation of Copolymer Probabilities

Given that $A,2NH_2A$ copolymers are formed randomly with fractional molarity p for A and $1 - p$ for $2NH_2A$, the fraction of A bases occurring in runs of length k or more can be approximated by considering the negative binomial distribution which assigns probability $(1 - p)p^j$ to the formation of a fragment $ApAp...Ap2NH_2A$ (j A bases) within a copolymer strand. That is, each copolymer strand is regarded as an outcome of some number of negative binomial Bernoulli trials obtained by cutting the copolymer strand to the right of each $2NH_2A$ base. This approximation neglects a correction needed for the effect of poly(A) fragments occurring at the end of copolymer chains. However, this correction is negligible if $1 - p$ is sufficiently large so that most copolymer strands contain many $2NH_2A$ bases. For the above negative binomial distribution for the fragments, the expected length $E(\mathrm{L})$ and the expected number $E(\mathrm{C}_k)$ of A bases in runs of length k or more are given by

$$E(\mathrm{L}) = \sum_{j=0}^{\infty}(j + 1)(1 - p)p^j \qquad E(\mathrm{C}_k) = \sum_{j=k}^{\infty} j(1 - p)p^j$$

Repeating this negative binomial experiment in the Bernoulli trials leads to an expected sum of fragment lengths of $nE(\mathrm{L})$ and an expected number of A bases in runs of length k or more given by $nE(\mathrm{C}_k)$. So, the ratio $nE(\mathrm{C}_k)/nE(\mathrm{L})$ of the expected number of A bases in runs of length k or more to the total length is independent of the number of Bernoulli trials. Note that the MacLaurin series, convergent for $0 < p < 1$, is given by

$$1/(1 - p)^2 = \sum_{i=0}^{\infty}(i + 1)p^i$$

Substituting by this formula, the following equations are obtained:

$$E(\mathrm{L}) = 1/(1 - p)$$

$$E(\mathrm{C}_k) = p(1 - p)[1/(1 - p)^2 - \sum_{k=0}^{k-2}(i + 1)p^i]$$

This leads to the formula shown under Circular Dichroism:

$$E = E(\mathrm{C}_k)/E(\mathrm{L}) = p[1 - (1 - p)^2\sum_{i=0}^{k-2}(i + 1)p^i]$$

Computer Monte Carlo generation of random chains of length 20 000 on two bases yielded results within 2% of predicted values for a range of p and k arguments.

This analysis can also be applied to polymers formed from three or more bases. Divide all the types of bases into two disjoint classes. Let p be the sum of fractional molarities of bases of the first class and $1 - p$ be the corresponding sum for the second class. Then the expected fraction of bases of the first class which occur in runs of length k or more of bases all belonging to the first class is approximated by the formula for E.