Poly(2-aminoadenylic acid): Interaction with Poly(uridylic acid)†

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ABSTRACT: Poly(2-aminoadenylic acid) forms both double and triple helices with poly(uridylic acid) [poly(U)]. The 2-amino group forms a third hydrogen bond, elevating the 2 → 1 transition temperature by 33 °C. The third strand, however, has about the same stability as poly(A)·2poly(U), as measured by T_m 3 → 2. This selective stabilization of the two-stranded helix results in a much greater resolution of the different thermal transitions than that observed in analogous polynucleotide systems. In contrast to other A, U systems 3 → 1 and 2 → 3 transitions are not observed under any conditions, and the triple helix always undergoes a 3 → 2 transition even at very high ionic strength. A 1:1 mixture of poly($2NH_2A$) and poly(U) exhibits no transient formation of 1:2 complex, unlike similar mixtures of poly(A) with poly(U) and poly(T). This difference is evidently due to a more rapid displacement reaction: [poly($2NH_2A$) + poly($2NH_2A$)·2poly(U) → 2 poly($2NH_2A$)·poly(U)] with poly($2NH_2A$) than with poly(A). We describe a method for establishing the combining ratios of polynucleotide complexes which uses a computer to calculate the angles of intersection of mixing curves as explicit and continuous functions of the wavelength. The wavelength dispersions of the angles of intersection determine optimum wavelengths for establishing stoichiometry and can also provide reliable negative evidence that presumably plausible complexes are not formed. Analogous computer procedures have been developed to determine wavelengths which are selective for the formation of both 1:1 and 1:2 complexes. Infrared spectra of the 1:1 and 1:2 complexes resemble those of other A, U homoribopolynucleotide helices in having two and three strong bands, respectively, in the region of carbonyl stretching vibrations. CD spectra of the two complexes are unusual in having negative first extrema of moderate intensity. We attribute these extrema to intrastrand interactions of strong, well-resolved transitions at 278 nm (B_{2u}) of the 2-aminoadenine residues. The CD spectra are correlated with those of other polynucleotide helices.

Investigations of polynucleotide model systems have greatly aided our understanding of the structural and energetic properties of these synthetic polymers and of the naturally occurring nucleic acids. Selective variation of chemical properties often offers the best approach for a separation and analysis of the different factors contributing to the observed properties of these macromolecules. In this paper we examine the consequences of perturbing poly(riboadenylic acid) by introduction of an amino group into the 2-position of the purine ring. A previous report (Howard et al., 1966a) described the formation of a 1:1 helix between poly($2NH_2A$)[1] and poly(U) and attributed the high stability of the complex to formation of a third hydrogen bond by the 2-NH_2 group.

In this study we demonstrate formation of poly($2NH_2A$)·2poly(U) and present a detailed characterization of the system defined by the interaction of poly($2NH_2A$) with poly(U). The thermal behavior of this system is dominated by differential elevation of transition temperature of the two-stranded helix and an unchanged (i.e., from poly(A)·2poly(U)) stability of the third strand. This differential change causes a wide separation of the curves defining the dependence on cation concentration of 2 → 1 and 3 → 1 transition temperatures and produces a phase diagram quite different from those of analogous systems. In systems defined by the interaction of poly(A) with poly(U), poly(T), and poly(BrU), the four transitions, 2 → 1, 3 → 1, 2 → 3, and 3 → 2, are crowded relatively close together in the phase diagram. Two transitions may overlap or coincide, obscuring some phenomena and making interpretation difficult. By producing much greater resolution of the different transitions poly($2NH_2A$) provides a well-documented reference system for interpretation of other polynucleotide interactions.

Experimental Section

For mixing curves, we prepared a separate solution for each point, using the technique described previously (Howard et al., 1971). Spectra were measured with a Cary Model 118 spectrophotometer as a function of time until absorbance became constant. The spectrophotometer was connected on line to a Honeywell Model DDP-516 computer (Shapiro and Schultz, 1971).

UV melting curves were measured with a Zeiss Model PMQII spectrophotometer, as described in a previous report (Howard et al., 1971).

Circular dichroic spectra were measured with a Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroism accessory. Data were collected on line and processed with the Honeywell Model DDP-516 computer.

Infrared spectra were recorded with a Beckman Model IR7 spectrophotometer which was on line to the same computer as the other instruments. The spectra were normalized to a molar absorbance basis (cf. Miles, 1971, and references cited therein) by the computer and drawn by a peripheral plotter. The spectroscopic procedures have been described (Miles, 1971). We are indebted to Mrs. Marie Chang for writing most of the programs required for data processing.

Poly(U) was purchased from Schwarz Bioresearch, Inc. (No. 6701). Poly(A) was purchased from P-L Biochemicals, Inc. (No. 179-14). The sedimentation coefficient of this polymer was reported by the manufacturer to be 7.73 ± 0.15.

2-Aminoadenosine was obtained from Cyclo Chemical Corporation.

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[1] Abbreviations used are: poly ($2NH_2A$), poly(2-aminoadenylic acid); poly(U), poly(uridylic acid); poly($2NH_2A$)·poly(U), 1:1 complex of these components; poly($2NH_2A$)·2poly(U), 1:2 complex of these components; poly(C), poly(cytidylic acid); poly(MeC), poly(5-methylcytidylic acid); poly(BrC), poly(5-bromocytidylic acid); poly(I), poly(inosinic acid); poly(A), poly(adenylic acid); uv, ultraviolet; ir, infrared.

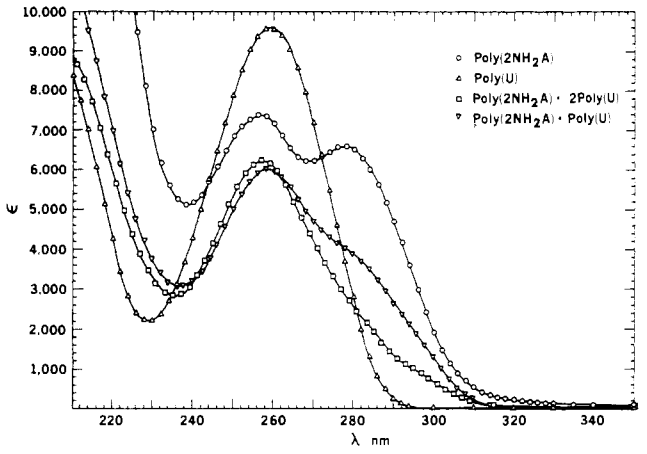

FIGURE 1: Ultraviolet spectra at 25.0 °C of poly($2NH_2A$) (○) (5.27×10^{-5} M, 0.01 M pyrophosphate buffer, pH 8.0, 0.1 M Na^+), poly(U) (△) (3.78×10^{-5} M, 0.02 M sodium cacodylate buffer, pH 7.0), poly($2NH_2A$)·2poly(U) (□) and poly($2NH_2A$)·poly(U) (▽) (each 1×10^{-4} M, 0.002 M pyrophosphate buffer, pH 8.0, 0.1 M Na^+). Spectra of poly($2NH_2A$)·poly(U) and poly($2NH_2A$)·2poly(U) were measured 34 and 740 h, respectively, after the components were mixed. Outlines of equilibrium mixing curves may be constructed from these spectra at any wavelength (cf. text).

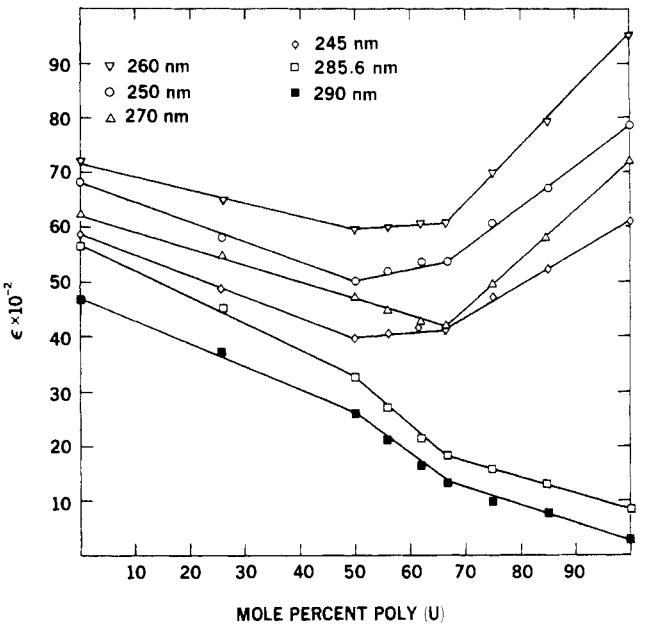

FIGURE 2: Ultraviolet mixing curves for the interaction of poly($2NH_2A$) with poly(U) in 0.002 M pyrophosphate buffer, pH 8.0, 0.1 M Na^+ at 260 (▽), 250 (○), 270 (△), 245 (◇), 285.6 (□), and 290 nm (■). Breaks in the curves occurring at 1:1 and 1:2 ratios of poly($2NH_2A$) to poly(U) demonstrate formation of poly($2NH_2A$)·poly(U) and poly($2NH_2A$)·2poly(U). Mixing curves plotted at 250 and 270 nm represent maximum detectability of the 1:1 and 1:2 complexes, respectively (see Figures 1 and 6).

Polynucleotide phosphorylase was prepared by the method of Singer and Guss (1962).

Tetraethylammonium salts of polymers and of pyrophosphate were prepared by methods described previously (Howard et al., 1971).

2-Aminoadenosine 5′-diphosphate and poly($2NH_2A$) were synthesized and characterized by methods described in detail in the supplementary material (see paragraph concerning supplementary material at the end of this paper). Molar absorbance of the polymer (25 °C; pH 8; Na^+, 0.1 M) was found to be $\epsilon_{256.7}$ 7390 by analysis for phosphorus and 7355 by enzymic hydrolysis. We have used the value 7370 in the physical measurements.

The polymer was completely degraded by venom to 2-aminoadenosine, identified by chromatography and electrophoresis.

Ultraviolet titration of poly($2NH_2A$) was carried out in degassed 0.1 M NaCl solution with 0.05 M HCl. pK of the polymer is ~6.9, but precipitation at pH ≤5 interfered with measurements at lower pH. At pH 5.9 the polymer has λ_{max} 258 nm (ϵ 7600) and 287 nm (ϵ 5200). The increase in pK and decrease in ϵ_{max} of the polymer with respect to the monomer indicate formation of a stable acid helix. For comparison neutral 2-aminoadenosine (Davol and Lowy, 1951) has λ_{max} 256 nm (ϵ 9450) and 280 nm (ϵ 10 000) at pH 6.3. The protonated nucleoside (pH 2.1) has λ_{max} 252 nm (ϵ 11 300) and 291 nm (ϵ 9800). pK of the nucleoside is 4.3 and isosbestic points were observed at 236, 263, and 286 nm. No isosbestic points were observed in titration of the polymer.

Results and Discussion

Stoichiometry. Investigation of new polynucleotide complexes should begin with determination of the combining ratio of the components. This property is most fundamental in that virtually all other properties depend upon it. An error in stoichiometry will lead to a series of consequent errors in design of experiments and interpretation of results.

The experimental technique most commonly applied to establish stoichiometry has been the method of continuous variations (Job, 1928; Felsenfeld and Rich, 1957). The stoichiometry is obtained from a mixing curve in which a physical property sensitive to changes in composition, usually uv absorbance, is plotted against mole fraction of the polynucleotide constituents. Discontinuities in mixing curves occur at mole fractions corresponding to the combining ratio of the components. The method is especially valuable for complexes which do not crystallize from solution and has, in part for this reason, been extensively employed in inorganic coordination chemistry (Katzin and Gebert, 1950; Woldbye, 1955). Its applications and limitations in that field have been critically reviewed (Rossotti and Rossotti, 1961).

The method has had a number of successful applications in polynucleotide chemistry, but in many cases the results have been ambiguous or erroneous. One of our objectives in making a detailed spectroscopic examination of the present system was to develop a general method which we believe is capable of reducing or eliminating erroneous conclusions about stoichiometry and of providing objective evidence that the results it produces are correct. We introduce in a later section the measurement of wavelength dispersion of the angles of intersection of mixing curves. This method examines as an explicit dependent variable the quantity actually employed in reaching conclusions on stoichiometry.

Before constructing a mixing curve we have found it convenient to prepare preliminary outlines of mixing curves employing only mixtures of the suspected combining ratios. It is first necessary to follow the time course of the reaction, often for a number of days, to be sure that equilibrium has been reached. When this condition of equilibrium is met, we consider the ultraviolet spectra of the complexes to be physical constants, which are largely invariant with experimental conditions (below the melting range) and quantitatively reproducible with different preparations of the polymers. From these equilibrium spectra (Figure 1, 1:1 and 1:2 ratios) we can determine the form of the mixing curves at different wavelengths (Figure 2)

and estimate from these the number of mixtures required for convincing evidence of the stoichiometry. We anticipate the final result (Figure 2) and defer until later sections the analysis of spectra and method of selecting wavelengths. The full mixing curves for the present system (Figure 2) were based upon seven mixtures. The minima at $X_U = 0.50$ and $X_U = 0.67$ establish the existence of both 1:1 and 1:2 complexes.

An alternative method of establishing stoichiometry from the ultraviolet spectra is shown in Figure 3 (supplementary material). Uv spectra of experimental mixtures are shown to be reproduced with high precision by linear combinations of the four spectra in Figure 1, indicating that these components can account quantitatively for mixtures of any composition.

We now consider the question of determining the most favorable wavelengths for studying a complex of any composition. Our approach is effectively to use all wavelengths. The method has been briefly outlined previously (Howard et al., 1971), but it has now been refined and developed for automatic solution by computer. The method considers the angles between the limbs of mixing curves as explicit dependent variables, with wavelength as the independent variable. The resulting wavelength dispersion of angle systematically and clearly reveals all of the information the spectra contain with respect to the property of interest. We define the angle, θ_1, between the first limb (0 to 50 mol % poly(U)) and the second limb (50 to 66.7 mol % poly(U)), θ_1 being measured in a clockwise manner from the first limb to the second. Similarly, we define θ_2 to be the angle measured clockwise from the second limb (50 to 66.7 mol % poly(U)) to the third (66.7 to 100 mol % poly(U)). The wavelength dispersions of θ_1 and θ_2 for the poly($2NH_2A$)–poly(U) system are shown in Figure 4. Obviously, additional angles can be defined if they are needed. Examination of these dispersion curves for maxima and minima allows one to determine by inspection the most favorable wavelength for detection of a complex. The curve for detection of poly($2NH_2A$)·poly(U), for example, has a minimum at 250 nm. The mixing curve plotted at 250 nm, accordingly, is the most favorable for detecting the 1:1 complex. At 231 and 269 nm, on the other hand, $\theta_1 = 180°$. The 1:1 complex, therefore, cannot be detected at all at these wavelengths and only with difficulty at wavelengths close to these. Detectability, thus, is greatest when $|180° - \theta_1|$ has its maximum value. θ_2, the angle for detection of poly($2NH_2A$)·2poly(U), has a minimum at 270 nm in the dispersion curve. The 1:2 complex, therefore, can best be detected by the mixing curve plotted at 270 nm.

To show how angle dispersion curves differ from one another in related systems, we have calculated these curves for the extensively studied poly(A),poly(U) system (Figure 5, supplementary material). In this case no attempt was made to determine experimentally the complete mixing curves. The calculation rather is based upon spectra of the components and of the 1:1 and 1:2 complexes. Dispersion curves for the poly(A),poly(U) system are quite distinct from those characteristic of the poly($2NH_2A$),poly(U) system. θ_1 has maxima at 236 and 280 nm, and a minimum at 254 nm. $|180° - \theta_1|$ has its largest value (22°) at 280 nm, and so this is the most favorable wavelength for detection of poly(A)·poly(U). At 259 nm, $\theta_1 = 180°$ and, therefore, the 1:1 complex is undetectable at this wavelength and would be difficult or impossible to detect over a range (257 to 263 nm) where $|180° - \theta_1| \leq 2.5°$. In early studies of the poly(A),poly(U) system, most mixing curves were measured near 260 nm at moderate cation concentration, where only a single break occurs at 66.7 mol % poly(U). Failure to observe a break at 50 mol % poly(U) led to much discussion as to whether the 1:1 complex can exist

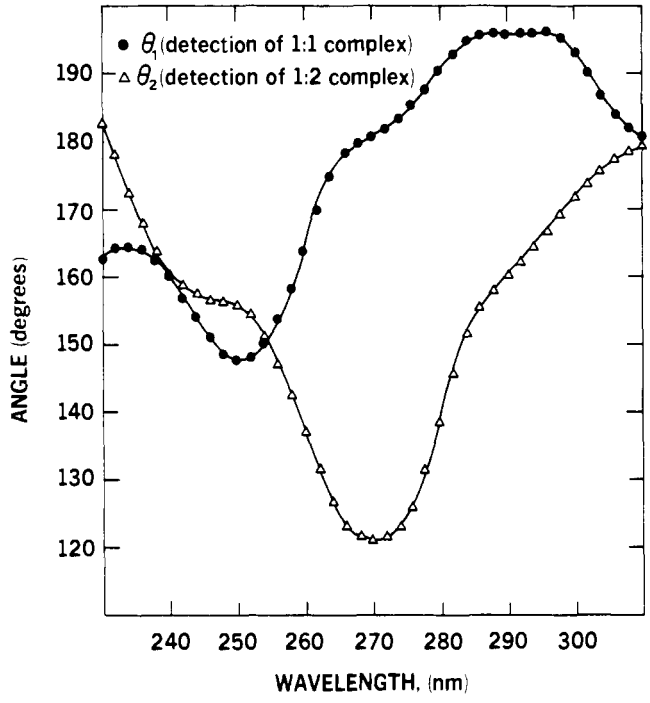

FIGURE 4: Dependence of θ_1 (●), and θ_2 (△) on wavelength. θ_1 and θ_2 are the angles between the first and second limbs (0 to 50 and 50 to 66.7 mol % poly(U)) and between the second and third limbs (50 to 66.7 and 66.7 to 100 mol % poly(U)) of poly($2NH_2A$),poly(U) mixing curves. We define θ_1 and θ_2 to be the positive angles measured in a clockwise manner from the first to the second and from the second to the third limbs, respectively. Wavelengths most favorable for detection of a complex occur at maxima or minima of these curves. Detectability increases with $|180° - \theta|$ ($\theta = \theta_1$ or θ_2). The curve for poly($2NH_2A$)·poly(U) (θ_1) has a minimum at 250 nm, and the mixing curve plotted at this wavelength is the most favorable for detecting the 1:1 complex. For poly($2NH_2A$)·2poly(U) a minimum occurs at 270 nm, and, accordingly, the 1:2 complex can best be detected by a mixing curve plotted at 270 nm. θ_1 and θ_2 were calculated by computer from uv spectra of poly($2NH_2A$),poly(U) mixtures of varying composition (Figures 1 and 2). Maxima, minima, and inflection points are independent of the coordinates, but numerical values of the angles depend upon the coordinates selected. The coordinates employed for θ_1 and θ_2 in this case are those of Figure 2.

under these conditions when poly(A) and poly(U) are mixed at the 1:1 ratio. Later, formation of the 1:1 complex was demonstrated, first by infrared spectroscopy (Miles and Frazier, 1964), and then by ultraviolet spectroscopy at 280 nm (Stevens and Felsenfeld, 1964; Blake et al., 1967). The dispersion curve for detecting poly(A)·2poly(U) has a minimum at 262 nm, and, accordingly, the 1:2 complex can best be detected at this wavelength.

Procedures for determining wavelengths which are selective for the 1:1 or 1:2 complex have been discussed in previous publications (Blake et al., 1967; Howard et al., 1971). We describe here the application of a method of intercepts (Howard et al., 1971) to the poly($2NH_2A$),poly(U) system. This procedure has now been adapted to automatic determination of selective wavelengths by computer. For a wavelength to be selective for the 1:2 complex, for example, it must meet the following conditions. First, the extinction coefficients of poly($2NH_2A$), poly($2NH_2A$)·poly(U), and poly(U) must be colinear; that is, the intercept of the first limb of the mixing curve ($0 \leq X_U \leq 0.5$) on the right ordinate must equal the extinction coefficient of poly(U) at that wavelength. If no such wavelength exists, then there is no wavelength selective for the 1:2 complex. A second condition is that $\theta_2 \neq 180°$; that is, the extinction coefficient of poly($2NH_2A$)·2poly(U) must not be

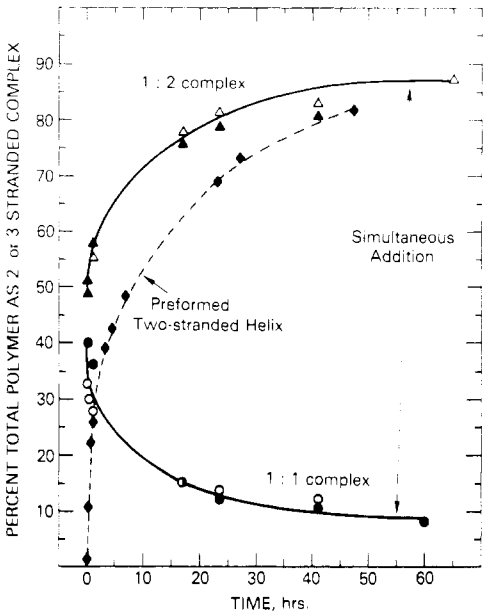

FIGURE 7: Composition changes during formation of poly($2NH_2A$)·2poly(U) for different orders of polymer addition. Conditions of reactions were: 1×10^{-4} M total polymer P in 0.002 M pyrophosphate buffer, pH 8.0, 0.1 M Na^+, 25.0 °C. In one set of observations all polymers were mixed simultaneously (△, ○), and in the other poly(U) was added to preformed 1:1 complex (♦). Formation of 1:2 complex (△, ♦) was followed at 285.6 nm, and disappearance of 1:1 complex (●) at 297.6 nm, these wavelengths being selective for the 1:2 and 1:1 complexes, respectively. Computer calculations (▲, ○) (see text) of the percent polymer present as 1:1 complex (○) or 1:2 complex (▲) agree well with the composition calculated from selective wavelengths.

colinear with those of the other three components. If the first condition is met, but only at wavelengths for which $\theta_2 = 180°$, then again there will be no wavelength selective for the 1:2 complex. The procedure we have adopted in searching for a wavelength selective for the 1:2 complex has been to plot as a function of wavelength the extinction coefficient values determined by the intercept of the first limb of the mixing curve on the right ordinate ($X_U = 1$), and then superimpose this plot on the absorption spectrum of poly(U) (Figure 6, supplementary material). Since $\theta_1 \neq 180°$, the wavelength at which the two curves intersect, 285.6 nm, is selective for the 1:2 complex (Figure 6, supplementary material). An analogous plot designed to determine wavelengths selective for the 1:1 complex resulted in intersection of the two curves at 297.6 nm, which is therefore selective for poly($2NH_2A$)·poly(U). We should point out that a selective wavelength is not an intrinsic property of the complex for which it is selective. It represents, rather, a contrived degeneracy designed to reduce the system, at some point, from one of four components to one of a single apparent component. On account of its obvious dependence on spectra of unassociated polymers, it may be quite dependent on temperature, which should therefore be controlled and specified when selective wavelengths are determined. The usefulness of a selective wavelength, moreover, may vary with the polymer system under investigation. If the selective wavelength happens to occur at a wavelength where the molar absorbance of a complex is very small, or if the molar absorbance differs from colinearity with those of the other components in the system in only a small degree, the sensitivity of detection of the complex may be reduced, and the selective wavelength may be of limited practical value.

Kinetics. Our primary purpose in observing kinetics of complex formation in the poly($2NH_2A$),poly(U) system was to establish conditions necessary to achieve equilibrium, both in order to determine stoichiometry and in order to make spectroscopic and other measurements on a defined equilibrium species. We find that the two-stranded helix is formed rapidly and completely. When the polymers are mixed (0.1 M Na^+, pH 7.8, 25 °C) absorbance becomes constant in 5 h and reaches more than 95% its eventual total change within 4 min (ϵ_{259}, 8440, 6070, 6020, 6020 at 0, 0.07, 5, and 70 h, respectively). Formation of the three-stranded helix, however, is significantly slower than the two stranded. With simultaneous addition of poly($2NH_2A$) and poly(U) at a 1:2 ratio (Na^+, 0.1 M, temperature 25.0 °C), the molar absorbance at 257.8 nm reached values of 6417, 6363, 6309, 6283, and 6250, at 23.6, 65, 209, 401, and 497 h, respectively. We assume that the spectrum at 500 h does not differ from the equilibrium value by more than the experimental uncertainty of the absorbance measurements. We have followed the rate of formation of triple helix at the selective (but less sensitive) wavelength 285.6 nm when the polymers are added simultaneously, and the rate of disappearance of the double helix at its selective wavelength, 297.6 nm. The compositions calculated from fractional change of absorbance at these selective wavelengths are plotted in Figure 7. Good agreement (Figure 7) with these values was obtained by an independent method of calculation in which a computer was used to find a least-squares best fit to the experimental spectra (235–300 nm) in terms of catalog spectra of the four possible components, shown in Figure 1. This method of calculation is an adaptation to ultraviolet spectra of that developed previously for infrared spectra (Miles and Frazier, 1964; Howard et al., 1966b). We find that in simultaneous addition the triple helix is approximately 50% formed in 0.5 h and 80% formed in 25 h. When poly(U) is added to preformed double helix, the rate of triple helix formation is initially slower than in the case of simultaneous addition of polymers (Figure 7). As measured at 257.8 nm the triple helix poly($2NH_2A$)·2poly(U) is ~10% formed in 0.5 h, 42% in 7.2 h, and ~70% in 24 h. Blake et al. (1968) reported that formation of poly(A)·2poly(U) is much slower in 0.01 M Na^+ (by a factor of 10^{-4} to 10^{-6}) when poly(U) is added to preformed double helix than when all polymers are added simultaneously. The authors suggested the more rapid reaction in the latter case may be due to intramolecular reaction of a poly(U) strand with the unpaired side of poly(A) whenever it finds itself overlapping some poly(A)·poly(U) that had previously formed further along the chain. When poly(U) is added to preformed double helix, on the other hand, a rate-determining nucleation step would be required, leading to a much slower rate of formation of triple helix. Our observation of somewhat more rapid formation of poly($2NH_2A$)·2poly(U) on simultaneous addition of polymers in 0.1 M Na^+ is qualitatively consistent with these proposals, but the rate difference is far smaller than that observed by Blake et al. (1968). We assume that the much more unfavorable electrostatic potentials involved in a nucleation reaction in 0.01 M Na^+ may be responsible for the difference. To determine whether the moderately rapid formation of triple helix from preformed double helix may be due to specific properties of poly($2NH_2A$) we have observed the reaction of poly(A)·poly(U) in 0.1 M Na^+ with the same preparation of poly(U) used in the experiments with poly($2NH_2A$). When poly(U) was added to preformed (allowed to stand 9 days) poly(A)·poly(U) (in 0.1 M Na^+, 0.002 M phosphate buffer, pH 7.6, 25 °C) the following extents of reaction were observed at 260 nm: 3 min, 53%; 40 min, 66%; 1 h, 71%; 6 h, 83%; 23 h, 90%; 55 h, 93%. At this ionic strength initial rate of formation is evidently moderately rapid, though completion of the reac-

tion appears to be quite slow. The rate of triple helix formation from preformed double helix and poly(U) is thus somewhat faster with poly(A) than with poly($2NH_2A$), but the two are qualitatively similar.

Kinetic measurements also provide additional information concerning formation of the 1:1 complex. We have observed that no intermediate 1:2 complex is formed when poly($2NH_2A$) and poly(U) are mixed at the 1:1 ratio, in contrast to what has been found in similar systems (see following sections for further details). In the poly(A)–poly(U) system, though the elementary rate of two-stranded helix formation is very rapid (Ross and Sturtevant, 1962; Blake et al., 1967, 1968), there is concomitant formation of a smaller amount of three-stranded helix in the interaction of poly(A) with poly(U) (Blake et al., 1968) or poly(T) (Howard et al., 1971) at the 1:1 ratio. This small amount (~15%) of 1:2 complex imposes a very slow (and, for the overall reaction, rate determining) strand displacement reaction as the only route to complete formation of poly(A)·poly(U) at equilibrium, a process requiring several days. The difference in behavior of the poly($2NH_2A$)–poly(U) system may arise from one of two causes (or from a combination). One possibility is that the rate of three-stranded complex formation is slower relative to the 1:1 complex for the poly($2NH_2A$)–poly(U) system than for poly(A)–poly(U). The data cited above indicate that the rate differences are in the suggested direction. An alternative explanation of the difference is that the rate of the displacement reaction is faster for poly($2NH_2A$) than for poly(A). By using the difference between the spectra of the 1:1 complex and the summation poly($2NH_2A$)·2poly(U) + poly($2NH_2A$) (Figure 8, supplementary material), we have measured the rate at which poly($2NH_2A$) reacts with preformed triple helix in 0.1 M Na^+ (Figure 9, supplementary material). We find that the displacement reaction is half complete in 3 min with poly($2NH_2A$) but in 108 min with poly(A). This difference is sufficient to account for our failure to detect transient formation of triple helix at the 1:1 ratio, though relatively slow addition of poly(U) to the double helix presumably also contributes to the observed result.

Derivative Spectra. Before discussing circular dichroism of polynucleotides investigated in this report we shall consider the first derivatives of their ultraviolet spectra. Weak, unresolved electronic transitions are difficult to detect in polynucleotide absorption spectra and estimates of their wavelengths often rely heavily on subjective judgement. Derivative spectra, however, reveal these transitions more clearly than the ultraviolet spectra and permit more objective estimates of wavelength. The derivative spectra, $d\epsilon/d\lambda$ vs. λ, were calculated by a computer, sampling at 0.2-nm intervals and drawn by a peripheral plotter (Figure 10, supplementary material, and Figure 11).

When an absorption spectrum has only clearly resolved peaks, the derivative spectrum is straightforward: maxima and minima of $d\epsilon/d\lambda$ vs. λ determine inflection points of the absorption spectrum, and $d\epsilon/d\lambda = 0$ at maxima and minima of the spectrum (in Figure 10 (supplementary material) and 11, these are located on segments of the curves with negative and positive slopes, respectively). In the region of the absorption spectrum on the low energy side of the last maximum $d\epsilon/d\lambda$ has only negative values. Maxima and minima in this region of the derivative curve correspond to inflections in the spectrum, and inflections in the derivative curve to local relative maxima and minima in the absorption spectrum. We take the midpoint between a maximum and minimum to be an inflection point in the derivative curve, corresponding to a local

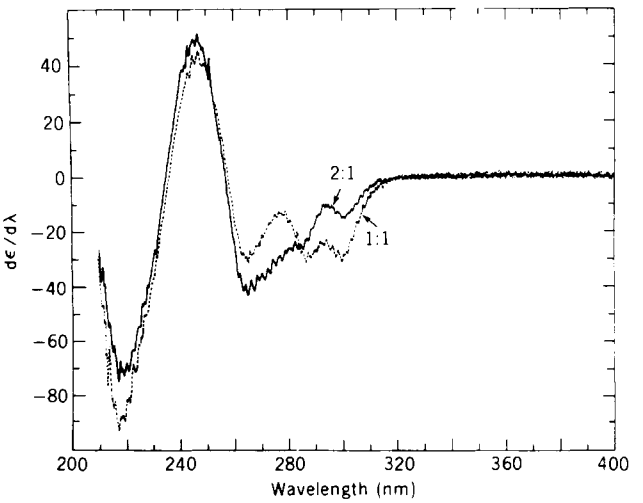

FIGURE 11: Derivative spectra of poly($2NH_2A$)·poly(U) (- - -) and poly($2NH_2A$)·2poly(U) (—). Spectra were calculated by computer as described in the legend of Figure 10 (supplementary material). Inflection points at about 284 and 297 nm in the derivative spectra of the 1:1 and 1:2 complexes, respectively, indicate the presence of unresolved bands in the uv spectra.

maximum or minimum in the absorption spectrum. In principle these inflection points could be determined by taking the second derivative, but this procedure would require denser sampling by the computer and at present may be unwarranted because of uncertainty inherent in locating a weak transition under a much stronger one.

In addition to the absorption maximum at 256 nm the derivative spectrum of poly(A) has an inflection at ~268 nm and a less distinct, though definite, one at ~280 nm (Figure 10, supplementary material). Voelter et al. (1968) have observed intense magnetic CD bands at 253 and 272 nm and assigned these to B_{2u} and B_{1u} transitions of adenosine. The transitions detected at 256 and ~268 nm in the derivative spectrum of poly(A) may be given the same assignments. As we note in a later section, however, different assignments of the adenosine transitions have been proposed on the basis of other observations. The observation of a transition at 280 nm may be relevant to an assignment by Bush and Scheraga (1969) of an n–π^* transition in poly(A) and poly(dA) at 280 nm. The latter polymer has a CD maximum at this wavelength, and in poly(A) skewing on the high wavelength side of the first CD maximum was also attributed to an n–π^* transition at 280 nm. We have also measured the derivative spectrum of poly(dA) and found that it is virtually identical with that of poly(A) in Figure 10 (supplementary material). In the region of the derivative curve above 250 nm they are completely superimposable.

Poly($2NH_2A$) has a "simple" derivative spectrum (Figure 10, supplementary material), revealing no unresolved transitions at higher wavelength. The 1:1 complex of this polymer with poly(U) (Figure 11) has two unresolved transitions: at ~283 nm, corresponding to an obvious shoulder in the absorption spectrum (presumably the B_{2u} transition observed at 278 nm in poly($2NH_2A$)), and at ~297 nm, where no shoulder is otherwise discernible in the absorption spectrum. The triple helix has unresolved transitions at ~284 and ~298 nm. In these cases shoulders can, with difficulty, be detected in the absorption spectrum (Figure 1), but estimation of wavelength would be largely subjective.

Circular Dichroism. Circular dichroism of polynucleotides is in many cases highly sensitive to molecular geometry and

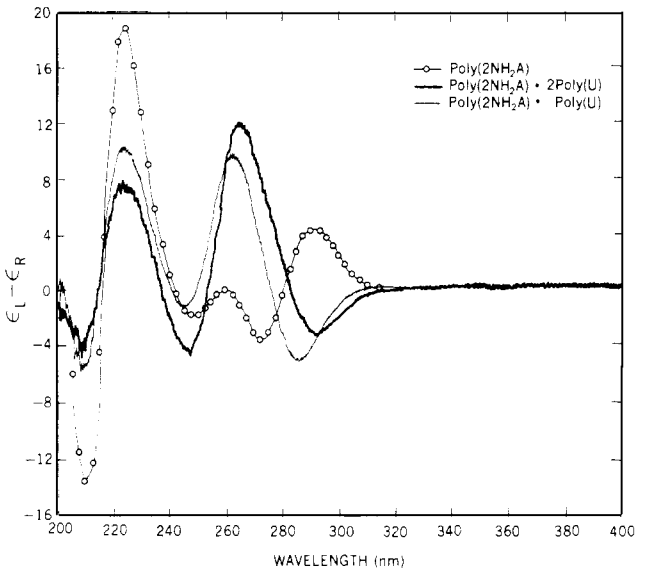

FIGURE 12: CD spectra of poly($2NH_2A$) (○), poly($2NH_2A$)·poly(U) (—) and poly($2NH_2A$)·2poly(U) (—). The spectra of poly($2NH_2A$)·poly(U) and poly($2NH_2A$)·2poly(U) are the average of three and two experimental runs, respectively. Data were collected, processed, and plotted by computer and are unsmoothed. Polymer concentration (in repeating units) was 1×10^{-4} M for poly($2NH_2A$) and the 1:1 complex and 2×10^{-4} M for the 1:2 complex. Sodium pyrophosphate concentrations (pH 8, 25 °C) were 0.005 M for the homopolymer and 0.002 M for the two complexes. Na^+, 0.1 M for all solutions.

has often proved useful in studies of molecular interactions (for general reviews, see Yang and Samejima (1969), Bush and Brahms (1973), Bush (1974), and Bloomfield et al. (1974)). There is at present, however, inadequate knowledge of the number and nature of electronic transitions in the purine and pyrimidine bases and of the relationship between interaction of these transitions and the geometry of polynucleotide helices. In our view one source of difficulty in interpreting CD spectra is the fact that electronic absorption spectra of the common bases are so similar to each other, making it difficult to identify and sort out the different transitions and their interactions. One approach to this problem is perturbing the electronic structure of polynucleotides by introducing chemical substituents into the basic ring systems. By this means it is possible to achieve greater resolution or altered relationships between transitions of different bases. Beyond the practical usefulness of this approach in specific cases we anticipate that, from a variety of modified polynucleotides, patterns and regularities may emerge that would be more difficult to recognize in interactions of the naturally occurring bases.

The CD spectrum of poly($2NH_2A$) has six extrema above 200 nm (Figure 12), and we interpret these as being primarily due to exciton splitting (Tinoco, 1964) of the three strong ultraviolet transitions at 214, 256.5, and 278 nm (Figure 1). We assign the conservative pair at high wavelength (positive at 291 nm and negative at 273 nm) to splitting of the transition at 278 nm, the pair at 249, 260 nm to the 256.5-nm transition, and the pair at 210, 225 nm to the transition at 214 nm. Other weak or unobserved transitions presumably also contribute to the observed CD bands, but we assume for the present that such contributions are not predominant.

We shall consider next the spectrum of poly(U) since it has evident similarities to the spectra of the complexes. Miles et al. (1969b) observed CD extrema of uridine in aqueous solution at 267 nm ($\epsilon_L - \epsilon_R$ +2.57), 240 ($\epsilon_L - \epsilon_R$ −1.12), and 215 ($\epsilon_L - \epsilon_R$ −1.33). These transitions are usually designated B_{2u}, B_{1u}, and E_{1ua}, respectively, by analogy with nomenclature of the π–π^* transitions of benzene (Clark and Tinoco, 1965), though physical similarities of uracil to benzene are small. Ishikawa et al. (1972) reported the CD spectrum of helical poly(U) (−0.3 °C, Na^+, 0.5 M, pH 7.9), with extrema at 265 nm ($\epsilon_L - \epsilon_R$ +20.4), 246 (−3.7), and 220 (+5.7). We attribute these extrema to the B_{2u}, B_{1u}, and E_{1ua} transitions, respectively, the wavelengths being little displaced from the monomer values. The magnitudes are greatly enhanced in the helix, however, and the sign of the 220-nm extremum is reversed.

The CD spectrum of poly($2NH_2A$)·poly(U) has extrema at 286 nm ($\Delta\epsilon$ −5.4), 262 (+9.4), 245.5 (−1.4), 224 (+9.7), and 209 (−5.8) (Figure 12 and Table I). The spectrum of the 1:2 complex is similar (extrema at 291 and 264.5 nm, Figure 12 and Table I), and we shall consider them together.

Before discussing further the CD spectra of the polynucleotide helices described in this paper we shall review briefly certain relevant aspects of spectra of the natural nucleic acids and of selected synthetic polynucleotides. CD spectra of nearly all natural DNAs are conservative at moderate ionic strength, with a positive extremum at ~276 nm ($\epsilon_L - \epsilon_R$ ~2.6) and a negative extremum at ~247 nm ($\epsilon_L - \epsilon_R$ ca. −3), and with crossover near the absorption maximum. (See reviews cited above and Johnson and Tinoco, 1969; Allen et al., 1972; Gratzer et al., 1970). Spectra of most (though not all) synthetic deoxypolynucleotide helices are qualitatively similar (Allen et al., 1972; Wells, 1975; Gray and Ratliff, 1975). Natural RNAs, however, have intense positive extrema near the absorption maximum. They have also weak negative extrema near 295 nm (cf. reviews cited above), which we consider in more detail in a later section. Yang and Samejima (1969) proposed that the difference between the two types of spectra was due to different geometries of the two kinds of nucleic acids: in the B form of DNA the bases are perpendicular to the helix axis, whereas all RNAs have bases tilted with respect to the axis. The authors also suggested that the form of DNA observed by Brahms and Mommaerts (1964) in 80% ethanol resulted from a B to A conversion in the presence of ethanol. A similar observation, more clearly delineated with defined synthetic polynucleotides, has been reported recently by Gray and Ratliff (1975). d(A-C)·d(G-T) has a CD spectrum like other DNAs, but in 60–80% ethanol the spectrum changes to resemble that of most RNAs. The same authors find that r(A-C)·r(G-T) has a CD spectrum like RNA (though the negative first extremum is more intense, and a clear shoulder appears at ~280 nm), and that the spectrum is essentially unchanged by ethanol. They conclude that the ethanol-induced change of the DNA spectrum results from a B → A conversion. Tinoco (1968) and Johnson and Tinoco (1969) have attributed the nonconservative nature of the RNA spectrum to the tilt of the bases and the effect of this tilt on the contribution of the far ultraviolet transitions to the circular dichroism (cf. Studdert and Davis, 1974).

We shall next discuss CD spectra of several ribopolynucleotides whose properties are relevant to our interpretation of the spectra of poly($2NH_2A$)·poly(U) and poly($2NH_2A$)·2poly(U). We have previously examined a ribopolynucleotide helix, poly(I)·poly(BrC), in which the strongest transitions of the purines and pyrimidines are widely separated ($\Delta\lambda_{max}$ 38 nm in the helix and 44 nm in the separate polymers) (Howard et al., 1969). Its CD spectrum is different in several respects from those of most other polynucleotide helices. The spectrum consists of a conservative pair of extrema at longer wavelength (301 nm, $\Delta\epsilon$ +3; 275 nm, $\Delta\epsilon$ −1.9), a moderately strong band at 245 nm ($\Delta\epsilon$ +7.9), and a weak minimum ($\Delta\epsilon$ ~0) at 214 nm.

TABLE I: Spectroscopic Data.[a]

1. Ultraviolet	λ_{max} (nm)	ϵ_{max}	λ_{min} (nm)	ϵ_{min}
Poly($2NH_2A$)	278	6 580	269	6210
	256.6	7 370	238	5120
	214	17 300		
Poly($2NH_2A$)·poly(U)	~282 (sh)	3 720	237	3080
	258.6	6 020		
Poly($2NH_2A$)·2poly(U)	~283 (sh)	2 020	234	2840
	~295 (sh)	1 020		
	257.2	6 230		

2. Circular Dichroism	Temp (°C)	λ_{max} (nm)	$\epsilon_L - \epsilon_R$	λ_{min} (nm)	$\epsilon_L - \epsilon_R$
Poly($2NH_2A$)	25.0	291	4.06	273	−3.63
		260	−0.03	249	−1.93
		225	18.67	210	−13.61
	0	298	2.2	276	−9.6
		257	−5.2	249	−5.8
		222	17.3	210	−3.0
Poly($2NH_2A$)· poly(U)		262	9.39	286	−5.40
		224	9.75	245.5	−1.36
				209	−5.82
Poly($2NH_2A$)· 2poly(U)		264.5	11.63	291	−3.42
		224	7.29	246	−4.45
				209	−3.91
Poly($2NH_2A$)· poly(T)[b]		270	5.68	290	−3.47
		225	7.99	251	−4.53
Poly(A)·poly(T)[c]		280	3.67	267	2.30
		258	3.72	247	−1.05
		226	2.61	208	−3.09
Poly(I)·poly- (MeC)[d]		289	5.11	268	−3.49
		240	7.73		
		222.5 (sh)	4.82		
Poly(MeC)[d]		286	16.39	262	−2.76
		219	8.61	245	−3.30
				212	5.46
Poly(BrC)[e]		300	8.88	277	0.46
		251	4.06	237	3.27
		232	3.67	216	−0.82

3. Infrared	Temp (°C)	λ_{max}	ϵ_{max}
Poly($2NH_2A$)	4.2	~1600.0 (sh)	482
		1619.0	800
		1626.0	823
	24.8	~1600.0 (sh)	587
		1618.0	965
	37.1	~1600.0 (sh)	677
		1615.5	993
	49.8	1612.5	1025
	65.9	1610.0	1070
	85.2	1608.5	1190
Poly($2NH_2A$)·poly(U)	28.7	1594.0	164
		~1612.0 (sh)	203
		1621.5	302
		1671.5	637
		1691.5	584
Poly($2NH_2A$)·2poly(U)	28.4	1592.5	63
		1619.0	73
		1657.0	586
		1674.5	364
		1695.0	554

[a] Experimental conditions are given in figure legends unless otherwise indicated. [b] In 0.005 M sodium pyrophosphate, pH 8.0; 0.1 M Na^+; 25 °C. [c] In 0.005 M sodium cacodylate, pH 7.0; 0.2 M Na^+; 25 °C. [d] In 0.005 M sodium cacodylate, pH 7.0; 0.1 M Na^+; 25 °C. [e] In 0.005 M sodium cacodylate, pH 7.5; 0.1 M Na^+; 25 °C.

The mean of the wavelengths of the first two extrema is 288 nm. The conservative pair of extrema arise from exciton splitting of a BrC transition observed at 289 nm in the uv spectrum of the homopolymer and at 283 nm in the I·BrC complex. The CD spectrum of poly(BrC) itself has its first two extrema at essentially the same wavelengths (300 and 277 nm) as does the complex with poly(I) (Table I), and again the mean of these wavelengths corresponds to λ_{max} of the uv spectrum. Exciton splitting of the long wavelength transition (B_{2u}) also occurs in the homopolymer, but in this case the simple conservative shape of the first pair of bands is obscured by transitions at lower wavelengths. The strong band at 245 nm in the CD spectrum of r(I)·r(BrC) occurs at λ_{max} of the absorption spectrum of poly(I) in both the homopolymer and the complex, though a BrC transition may also make a contribution to this band (cf. Table I). The first point of importance in the spectrum of the complex is that the longest wavelength BrC transition contributes to the spectrum independently, essentially unperturbed by those of the other base. The 245-nm band appears to arise largely from a poly(I) transition unperturbed by BrC and evidently does not undergo exciton splitting. That this independent contribution of each base to the long wavelength CD bands does not result merely from the wide separation of the strongest I and BrC transitions is indicated by CD spectra of r(I)·r(5MeC) (Table I) and r(I)·r(C) (Wells, 1975; cf. Mitsui et al., 1970). Poly(5MeC) has uv λ_{max} at 277 nm and the CD spectrum has its first two extrema at 286 and 262 nm, probably resulting from splitting of the 277-nm transition. In the double helix the first pair of extrema (289 and 268 nm; mean, 277 nm) are conservative and arise from splitting of the 277-nm transition, essentially unperturbed by poly(I). The positive band at 240 nm ($\Delta\epsilon$ +7.7) presumably arises from the B_{1u} transition of poly(I) at 245 nm. The 5-nm decrease in λ_{max} may result from interaction with poly(5MeC), or merely from subtraction by a negative MeC contribution at 245 nm ($\Delta\epsilon$ −3.4, Table I; note λ_{max} of 245 nm for r(I)·r(C); r(C) has a crossover at 243 nm and a minimum at 234 nm ($\Delta\epsilon$ −2.3) (Wolfe et al., 1969)). A similar interpretation can be made of poly(I)·poly(C), as we have suggested earlier on the basis of ORD data (Howard et al., 1969). The mean of the first pair of extrema at 277 nm ($\Delta\epsilon$ +6.3) and 262 nm ($\Delta\epsilon$ −4.2) (Wells, 1975; cf. Mitsui et al., 1970) is 270 nm, near the uv maximum at 268 nm. The third band occurs at 245 nm ($\Delta\epsilon$ +6.2) and is again apparently due to the poly(I) B_{1u} transition. The CD spectrum of poly(C) itself has λ_{max} at 276 (like the complex with poly(I)). It appears that the pattern of long wavelength CD extrema noted above in this series of I·C helices arises to a large extent from interactions within the strands rather than between the strands. The strictly repeating ribopolynucleotide helix poly[r(I-C)]·poly[r(I-C)] (Wells, 1975; cf. Mitsui et al., 1970) has a quite different CD spectrum, presumably as a result of intrastrand I–C interactions, though a geometry differing from that of the homopolymer helices may also contribute to the difference in CD spectra.

Another notable feature of the CD spectra of poly(I)·poly(BrC) and poly(I)·poly(5MeC) is that the high wavelength parts of the spectra appear quite "DNA-like", with a conservative pair of extrema of magnitude comparable to that observed in DNA ($\Delta\epsilon$ ~3). This property in DNA is believed to result from the B geometry in which the bases are perpendicular to the helix axis; the conservative spectrum of DNA is lost when the geometry is changed by ethanol or high salt (see foregoing references and discussion). Since all ribopolynucleotide helices are believed to have an A geometry with tilted bases, however, poly(I)·poly(BrC) and poly(I)·poly(5MeC)

presumably also have this geometry.

Johnson and Tinoco (1969) concluded that it is cancellations among the large number of different base-base interactions that cause the anomalously low intensity of the long wavelength circular dichroism extrema of DNA. In the present cases, however, only two bases are present in each helix, and the transitions of I and BrC or I and MeC do not undergo significant interaction. The low intensity of the first two extrema in the CD spectra evidently arises only from BrC–BrC or MeC–MeC interactions and not from cancellation of CD bands resulting from interactions of unlike bases.

The CD spectra of the 1:1 and 1:2 complexes (Figure 12) have some resemblance to typical RNA spectra (cf. Johnson and Tinoco, 1969) in being nonconservative and in having negative first extrema. They differ, however, in that the magnitudes of the first extrema are roughly eightfold (1:1 complex) and fivefold (1:2 complex) greater than in RNA. The ratios of $\Delta\epsilon$ of the second to the first extremum in the two complexes is 1.74 (1:1) and 3.40 (1:2) compared with about 10 in RNA. Rather than having the same λ_{max} as the uv spectra, the CD maxima at 262 nm (1:1) and 264.5 nm (1:2) differ from λ_{max} of the most intense uv peaks by 5 and 7 nm, respectively. The negative sign and relatively large magnitude of the first extremum are unusual features of the spectra of both 1:1 and 1:2 complexes. The majority of polynucleotide helices have positive first extrema, though natural RNA molecules usually have weak extrema ($\Delta\epsilon < 1$) at ~295 nm (cf. reviews cited above). Bush and Brahms (1973) concluded on theoretical grounds (Johnson and Tinoco, 1969; Bush, 1974; Mitsui et al., 1970) that, for DNA-like geometry with 5 to 12 residues per turn and bases nearly perpendicular to the helix axis, the longest wavelength band is positive for right-handed helix and negative for a left-handed helix. The prediction assumes only one absorption band per monomer, and Bush and Brahms (1973) have noted that failure of actual polynucleotides to satisfy assumptions of the theory could negate the prediction. Ribopolynucleotide helices, for example, do not satisfy the assumption of perpendicularity of bases to the helix axis, and the theory of Johnson and Tinoco (1969) does not account for the negative first extremum in RNA helices. Poly[d(I-C)]·poly[d(I-C)] was assigned a left-handed helical sense, in part on the basis of a negative long wavelength CD band (Mitsui et al., 1970), but the assignment has subsequently been questioned (Arnott et al., 1974). Poly($2NH_2A$)·poly(U) and poly($2NH_2A$)·2poly(U) are, like other ribopolynucleotide helices, presumably right handed.

The spectra reported in this paper resemble those of another 2-aminoadenine polyribonucleotide complex in having a relatively strong first negative extremum (Ikeda et al., 1970). Poly($2NH_26NMeA$)·poly(U) has extrema at 285 nm ($\Delta\epsilon$ −5.0), 265 (+3.8), 252 (+0.8), and 232 (+8.4). The wavelengths of the first two extrema are essentially the same as those of poly($2NH_2A$)·poly(U), but the negative extremum is more intense than the positive band at 265 nm. The 1:1 helix formed by 2-amino-6-*N*-methyladenosine and poly(U) also has a CD spectrum very similar to that of the corresponding polymer–polymer helix (Ikeda et al., 1970). We have also examined the double helix formed by poly($2NH_2A$) and poly(T) and find that its CD spectrum is similar to that of poly($2NH_2A$)·poly(U) (Table I). Like all the other 2-aminoadenine helices it has a negative first extremum, and the wavelength (290 nm) and magnitude ($\Delta\epsilon$ −3.5) are about the same as those referred to above. Though a 2-amino group on the adenine residues is not essential for a negative first extremum (see prior references and discussion), this substitution does lead to a moderately intense negative band in the region ~285–295 nm in all cases so far examined. In considering the possible relation of a 2-amino group to a negative first extremum at 290 ± 5 nm we observe that none of the ribo- or deoxyribopolynucleotide double helices containing only unmodified A and U or A and T residues have negative first extrema: poly(A)·poly(U) (Brahms, 1965; Gray et al., 1972); poly(A)·poly(T) (Table I); poly[r(A-A-U)]·poly[r(A-U-U)], poly[d(A-A-T)]·poly[d(A-T-T)], poly[d(A-T)]·poly[d(A-T)] (Gray et al., 1973); and the double helical oligonucleotides (A_4U_4)·(A_4U_4), (A_6U_6)·(A_6U_6), and (A_4UAU_4)·(A_4UAU_4) (Borer et al., 1973). The only exception is the strictly alternating (AU) polymer, which appears to have an extremely weak negative extremum ($\Delta\epsilon$ −0.15) at 290 nm (Gray et al., 1972). We observe further that, though many of the polynucleotide double helices containing G do not have CD spectra with negative first extrema (e.g., B-form DNA), virtually all of those composed of the common bases with such extrema do contain G. These bands are usually weak, as in RNA or in most of the 12 G-containing oligonucleotide helices reported by Borer et al. (1973). In some cases, however, the bands are considerably more intense: poly[r(A-C)]·poly[r(G-U)] (Gray and Ratliff, 1975) has a negative band of $\Delta\epsilon$ −1.3 at 296 nm; poly[r(G-C)]·poly[r(G-C)] has a band of $\Delta\epsilon$ −3.0 at 290 nm (Gray et al., 1972); and (A_2CGU_2)·(A_2CGU_2) has a band of $\Delta\epsilon$ −1.8 at 297 nm (Borer et al., 1973). To these examples of negative first extrema in complexes containing unmodified G we may add that of the Watson–Crick helix formed by poly($8NH_2G$) with poly(C) (Hattori et al., 1975). The monomer, $8NH_2GMP$, has a strong uv transition at 294 nm (CD λ_{min} 292 nm), and at pH 10 the polymer (believed to be four stranded) has a peak at 290 nm. The first extremum of the CD spectrum of poly($8NH_2G$)·poly(C) is negative with λ_{min} 297 nm and $\Delta\epsilon$ −3. This CD band presumably arises from the B_{2u} transition of $8NH_2G$ at about 294 nm.

In our view appearance of a negative first extremum in double helices containing both 2-aminoadenosine and guanosine (or 2-aminoinosine) residues is not coincidental but results from analogous strong transitions of the two bases at about 280 nm. Mason (1954) showed that a 2-amino substitution in purines has the effect of intensifying, red shifting, and increasing the resolution of the longest wavelength transition from the next lower one. Clark and Tinoco (1965) have assigned the longest wavelength bands of 2-aminoadenine and 9-ethylguanine in water at 280 and 275 nm, respectively, to B_{2u} transitions. Analogous B_{2u} transitions have been assigned in adenine and hypoxanthine (cf. Voelter et al., 1968; Clark and Tinoco, 1965), though there does not at present appear to be general agreement on assignments of the complex long wavelength absorption bands of these bases (for discussion and references, see, for example, Bush, 1974; Miles et al., 1969a; Kleinwachter et al., 1967; Stewart and Davidson, 1963). Whatever the detailed assignments may be, second components of the long wavelength bands of adenosine and inosine are much weaker than in $2NH_2A$ and G, are at lower wavelength, and are not clearly resolved from the B_{1u} transitions. With three exceptions (see above) double helices containing only adenine or hypoxanthine as the purine component do not have negative first extrema.

Our attempts to correlate the spectroscopic properties discussed above for different polynucleotide helices may be summarized in the following proposals:

(a) Negative first extrema near 290 nm arise from strong purine transitions near 280 nm (B_{2u}) in double-stranded polynucleotide helices.

(b) The predominant interaction is with the same transition (B_{2u}) and is primarily intrastrand rather than interstrand.

(c) The negative band is favored by a strong and well-resolved purine B_{2u} transition ($2NH_2A$ and, to a somewhat lesser extent, G) but may also occur (rarely) when the B_{2u} transition is weaker and not resolved from the B_{1u} transition (three examples of the latter have been reported: r(A-U)·r(A-U) (Gray et al., 1972), r(I-C)·r(I-C), and d(I-C)·d(I-C) (Mitsui et al., 1970)).

(d) Interaction between B_{2u} transitions which produces the negative bands is strongly dependent on helix geometry (as are other interactions) and is favored by A-form geometry.

Two helices in which the purine residues have electronic properties similar to those in poly($2NH_2A$) but quite different geometry are poly($2NMe_2A$)·poly(U) and poly($2NMe_2A$)·poly(BrU) (Ishikawa et al., 1972). Poly($2NMe_2A$) resembles poly($2NH_2A$) in having an intense, well-resolved, long-wavelength band (at 295 nm, ϵ 6230), which presumably results from a long-axis polarized (cf. Mason, 1954; Stewart and Davidson, 1963) B_{2u} transition. Because Watson–Crick pairing is blocked by the *N*-methyl groups, however, the helices formed with poly(U) and poly(BrU) are forced to assume a less stable Hoogsteen structure. The longest wavelength extrema of the helical complexes are positive in both cases, with λ_{max} 305 nm ($\Delta\epsilon$ +0.6) and 293 (+5.5), respectively (Ishikawa et al., 1972). The homopolymer does form a neutral self-structure, however, with a negative first extremum at 320 nm ($\Delta\epsilon$ −0.35), the negative member of a conservative pair (λ_{max} of the next band is 302 nm, $\Delta\epsilon$ +0.41). This form of the polymer melts, and the negative band disappears (Ishikawa et al., 1973) by ~45 °C. The structure of this ordered form is not known, but it apparently resembles the two helices reported in this paper in having geometry favorable for interaction of the purine B_{2u} transitions.

Lack of interaction between I and BrC, I and MeC, and I and C transitions, discussed in previous paragraphs, led us to consider that the $2NH_2A$ and U transitions may contribute independently to the CD spectra observed in Figure 12. Though the purine and pyrimidine transitions are more closely spaced than in I·BrC, it appears that a consistent interpretation can be made on a similar basis. The first extremum of the 1:2 complex, poly($2NH_2A$)·2poly(U), has the same wavelength, 291 nm, as neutral poly($2NH_2A$) (Figure 12 and Table I), approximately the same magnitude ($\Delta\epsilon$ −3.4 and +4.1, respectively), and opposite sign. In the 1:1 complex the corresponding minimum occurs at 286 nm ($\Delta\epsilon$ −5.4). A very similar change is observed in the 1:1 complex poly($2NH_26NMeA$)·poly(U) (Ikeda et al., 1970), which has a negative first extremum at 285 nm ($\Delta\epsilon$ −5.0); the homopolymer in this case has λ_{max} 293 nm ($\Delta\epsilon$ +13.3). We attribute the 291 nm (1:2) and 286 nm (1:1) extrema to the strong B_{2u} 2-aminoadenine transition near 278 nm, which undergoes exciton splitting to produce these bands. The intense bands of λ_{max} 264.5 nm (1:2) and 262 nm (1:1) are skewed on the high wavelength side, and we assume that the positive components of exciton splitting of the B_{2u} transition of 2-aminoadenine are in part responsible for the skewing. The intense extremum at 264.5 nm (1:2) and the minimum at 246 nm may have major contributions from the B_{2u} and B_{1u} uridine transitions, respectively. The helical form of poly(U) has extrema of the same wavelengths and sign and of comparable magnitudes (264 nm ($\Delta\epsilon$ +20.4); 246 (−3.7)). The corresponding bands of the 1:1 complex occur at 262 and 245.5 nm (Table I). We assume the 265-nm transition of poly($2NH_2A$) is split, with a weak positive component at about 260 nm and a weak negative component near 249 nm,

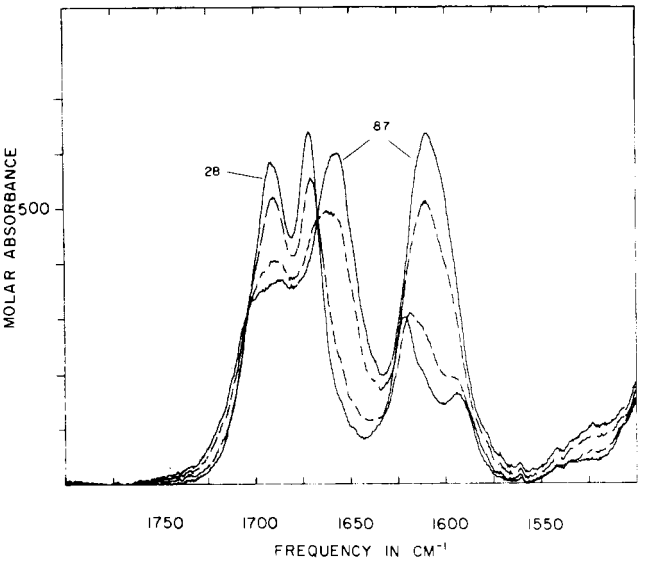

FIGURE 13: Infrared spectrum of poly($2NH_2A$)·poly(U) in 0.01 M phosphate buffer, pD 8.4, 0.15 M Na^+ at 28 °C. Concentration of each polymer was 0.02 M; path length 48.6 μm; scale expansion 6.0-fold. The spectra were measured at 28, 76, 83, and 87 °C. Data were transmitted to an on-line computer, and spectra were drawn by a peripheral plotter. Uridine carbonyl bands occur at 1692 and 1672 cm^{-1} and purine ring vibrations at 1622 and 1594 cm^{-1}. All absorbance above 1650 cm^{-1} is due to uracil residues, and isosbestic points at 1667 and 1703 cm^{-1} show that during melting these residues exist in only two states, helix and random coil (Howard et al. 1969, 1971; Miles, 1971).

or contributing to the observed minimum at 246 nm. Figure 12 shows that similar extrema in the homopolymer poly($2NH_2A$) are indeed very weak, in contrast to the intense bands of poly(A) (Brahms et al., 1966). It therefore appears reasonable that contributions of these bands would be obscured by strong uridine extrema at 246 and 264.5 nm. The pair of extrema at 209 and 225 nm (1:1 and 1:2), on the other hand, appear to be determined largely by a poly($2NH_2A$) transition at 214 nm. The magnitude of the 225-nm maximum has roughly half the value in the 1:1 complex and a third the value in the 1:2 complex that it does in the homopolymer. Since the spectra are normalized on the basis of polymer phosphate concentration, this relation is consistent with predominant contribution from poly($2NH_2A$). The E_{1ua} transition of poly(U) may make a contribution near 220 nm, as it does in poly(U), though presumably of smaller magnitude.

The foregoing interpretation reflects our view that purine and pyrimidine transitions may not be interacting with each other to a major extent in these two helices, but, clearly, other interpretations are possible, as we have noted in previous sections.

Infrared Spectra: Dissociation of Two-Stranded Helix. The infrared spectrum of poly($2NH_2A$)·poly(U) has strong uridine carbonyl bands at 1692 and 1672 cm^{-1} (Figure 13) and 2-aminoadenine ring vibrations at 1622 and 1594 cm^{-1}. The appearance of two carbonyl bands at approximately 1690 and 1670 cm^{-1} is characteristic of two-stranded A·U helices formed by homopolynucleotides and persists through structural changes of both A and U components (Miles and Frazier, 1964; Howard et al., 1966a, 1971; Ikeda et al., 1970; Ishikawa et al., 1972). Three-stranded helices, however, have a third strong band near 1657 cm^{-1}, in addition to two at higher frequencies (loc. cit. and following section). The two purine ring vibrations undergo a large decrease in intensity upon helix formation and are well-resolved in the helix. The weaker purine

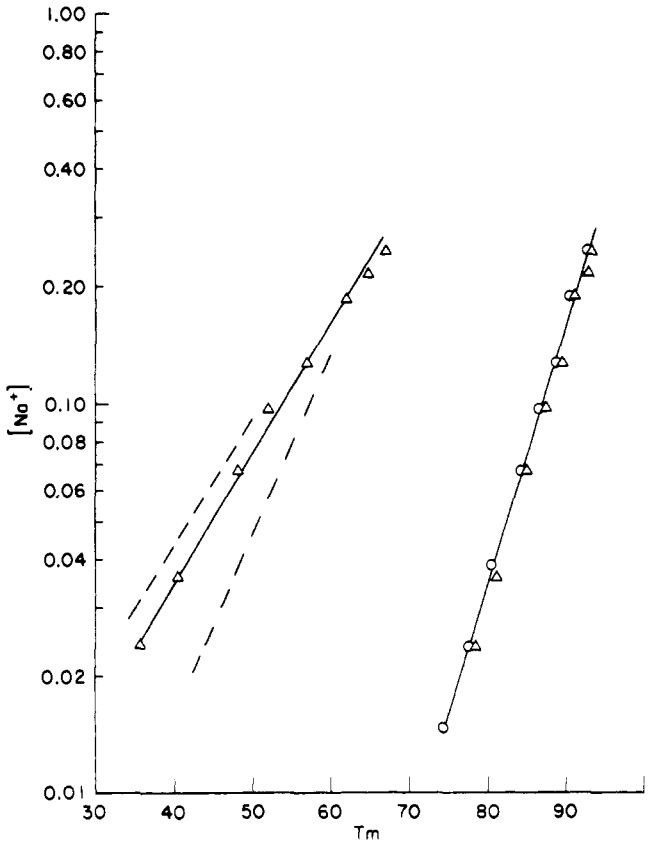

FIGURE 16: Dependence of T_m of the 3 → 2 and 2 → 1 transitions of the poly(2NH_2A),poly(U) system upon [Na^+]. Values are taken from melting curves measured at the 1:1 ratio (4.5×10^{-5} M total polymer P) (○) and at the 1:2 ratio (7.3×10^{-5} M total polymer P) (△) for solutions containing 0.005 M sodium phosphate, pH 7.5. The slope of the 3 → 2 transition (△; left) (dT_m/d log [Na^+]) is 30 °C; that of the 2 → 1 transition (△, ○; right) is 14.8 °C. Also included (dashed lines) are curves for the 3 → 2 transition of poly(A)·2poly(U) (left curve) and for the 2 → 1 transition of poly(A)·poly(U) (right curve).

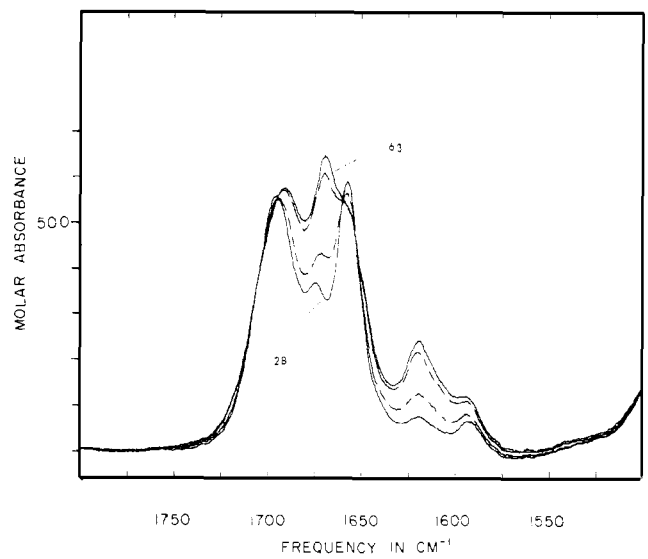

FIGURE 17: Infrared spectrum of 1:2 mixture of poly(2NH_2A) and poly(U) in 0.01 M phosphate buffer, pD 8.4, 0.15 M Na^+ at 28 °C. The concentration of poly(2NH_2A) was 0.0132 M, and poly(U), 0.03 M; path length 48.6 μm; scale expansion 4.5-fold. At 28 °C bands occur at 1695, 1675, 1657, 1619, and 1593 cm^{-1}, the first three being uracil vibrations, the latter two, 2-aminoadenine ring vibrations. With increasing temperature the spectrum undergoes two sets of changes, the first occurring within the temperature range 28–63 °C. At 63 °C the first set is complete, and the spectrum remains constant from 63 to 72 °C. The plateau spectrum is identical with a summation of the spectra of the 1:1 complex and poly(U), indicating that dissociation of a single strand of poly(U) from the three-stranded complex occurs during the first set of spectral changes. Spectra were measured at 28.0, 50.5, 56.5, and 63.3 °C. The apparent isosbestic point at 1659 cm^{-1} results from the following near-coincidence: $\epsilon_{1659}^{(3)} \simeq (\epsilon_{1659}^{(2)} + \epsilon_{1659}^{(1)})/2$. Dissociation of the triple helix (3) gives rise to equal amounts of two products (double helix (2) and poly(U) (1)) having an average value of ϵ_{1659} approximately equal to that of the triple helix.

band, which occurs at 1594 cm^{-1} in the helix and is not resolved in the single-stranded form, is evidently the analogue of the weak 1575-cm^{-1} band in poly(A).

Infrared temperature profiles show a single transition with T_m = 82 °C (Figure 14, supplementary material). Parallel melting at purine (1610 cm^{-1}) and pyrimidine (1691, 1672 cm^{-1}) frequencies demonstrates specific interaction between A and U residues (cf. Miles and Frazier, 1964; Howard et al., 1966a,b, 1971).

Ultraviolet Melting Curves. Ultraviolet melting curves (Figure 15, supplementary material) of poly(2NH_2A)·poly(U) also show a single transition over the range of sodium ion concentration studied (0.015 to 0.25 M, Figure 16). The transition temperature is 33 °C higher than that of poly(A)·poly(U) (in 0.03 M Na^+; 30 °C in 1.0 M Na^+), indicating that formation of a third hydrogen bond per base pair in the 1:1 complex results in a significant increase in helix stability. Since only a Watson–Crick bonding scheme can accommodate three hydrogen bonds, moreover, an elevation of about this magnitude may be used to distinguish Watson–Crick from Hoogsteen bonding.

Absence of (2 → 3) Transition. Ir spectra, which demonstrate the disproportionation reaction (2 → 3) transition for poly(A)·poly(U), poly(A)·poly(T), and poly(A)·poly(BrU) at moderate cation concentration (Miles and Frazier, 1964; Howard et al., 1971; Ishikawa et al., 1972), show that it does not occur in the poly(2NH_2A),poly(U) system (Figure 13). Ross and Scruggs (1965) concluded that disproportionation of poly(A)·poly(U) is driven by an increase in entropy resulting from release of poly(A) as a partially disordered single strand. We attribute the failure of poly(2NH_2A)·poly(U) to undergo disproportionation, in contrast to poly(A)·poly(U), to the additional stabilization provided by the third hydrogen bond. To illustrate a hypothetical disproportionation of poly(2NH_2A)·poly(U) we represent hydrogen bonds between A and U by the notations A≡U and A═U for associations having three and two hydrogen bonds, respectively. The (2 → 3) transition then can be written:

$$2U{\equiv}A \rightarrow U{\equiv}A{=}U + A$$

The overall process would result in the net loss of one hydrogen bond. The attendant increase in free energy if the 2 → 3 transition occurred would evidently be more than adequate to compensate for the increase in entropy resulting from release of single-stranded poly(2NH_2A). The third hydrogen bond is thus able to control the direction of the above reaction and cause a marked difference in the chemical behavior of this system from that of similar systems.

Infrared Spectra. Stepwise Dissociation of Three-Stranded Helix. The infrared spectrum of poly(2NH_2A)·2poly(U) has bands at 1695, 1675, 1657, 1619, and 1593 cm^{-1} (Figure 17). The first three are uracil vibrations, and those at 1619 and 1593 cm^{-1} are 2-aminoadenine ring vibrations. The spectrum closely resembles spectra of three-stranded helices formed between 2-aminoadenosine and poly(U) (Howard et al., 1966b), poly(A) and poly(U) (Miles and Frazier, 1964), poly(A) and

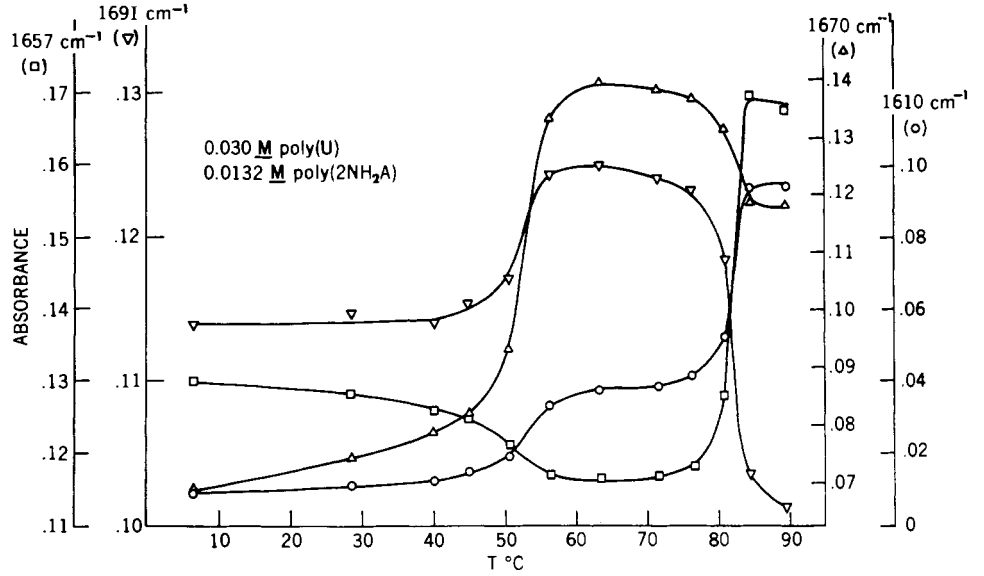

FIGURE 19: Infrared melting curves of the strand-wise dissociation of poly($2NH_2A$)·2poly(U). The first step (T_m 52 °C) arises from dissociation of a single strand of poly(U) from the 1:2 complex (3 → 2 transition). The second step is due to melting of the 1:1 complex (2 → 1 transition) and occurs with the same T_m (82 °C) as was measured for melting of poly($2NH_2A$)·poly(U). As before, simultaneous changes in separate purine (1610 cm^{-1}) and pyrimidine (1670, 1691 cm^{-1}) bands are a reflection of specific interactions between the bases. Conditions of Figure 17.

poly(T) (Howard et al., 1971), and poly(A) and poly(BrU) (Ishikawa et al., 1972); thus, there are three bands in the carbonyl region at frequencies quite similar to those occurring in the four cases cited, and the purine vibrations have undergone reduction in intensity in all of these helices even larger than those observed in the corresponding double helices. With increasing temperature, the spectrum undergoes two sets of changes corresponding to two separate thermal transitions (Figure 18, supplementary material). In the range of 40–63 °C the spectrum changes continuously until 63 °C, where the first process is complete (Figure 17). From 63 to 72 °C the spectrum remains constant as temperature increases and then begins a second set of changes from 72 to 85 °C (Figure 18, supplementary material).

We have shown in previous reports (Miles and Frazier, 1964; Howard et al., 1966b; Miles, 1971) that the composition of polynucleotide mixtures can be determined both qualitatively and quantitatively from their infrared spectra. In these infrared analyses a computer is used to obtain least-squares fits to standard library spectra, usually measured over the range 1500–1800 cm^{-1}. In this case the experimental spectrum measured at the plateau temperature (63 °C) was analyzed in terms of the following library spectra: poly($2NH_2A$), poly(U), poly($2NH_2A$)·poly(U), and poly($2NH_2A$)·2poly(U). The analysis showed the following composition: poly($2NH_2A$), 0%; poly(U), 39.4%; poly($2NH_2A$)·poly(U), 60.6%; and poly($2NH_2A$)·2poly(U), 0% (all expressed in terms of polymer phosphate or polymer repeating units). If only 1:1 complex and poly(U) are present at 63 °C, the calculated compositions are 38.6% poly(U) and 61.4% 1:1 complex. The close agreement demonstrates that the transition of T_m = 52 °C is in fact a 3 → 2 transition:

poly($2NH_2A$)·2poly(U)
→ poly($2NH_2A$)·poly(U) + poly(U)

The second set of spectral changes (Figure 18, supplementary material) shows that the second step of the melting curve (Figure 19) corresponds to a 2 → 1 transition.

In agreement with ir melting curves, the uv melting behavior of poly($2NH_2A$)·2poly(U) (Figure 20) exhibits a two-step process over the range of sodium ion concentration from 0.025 to 0.25 M (Figure 16).

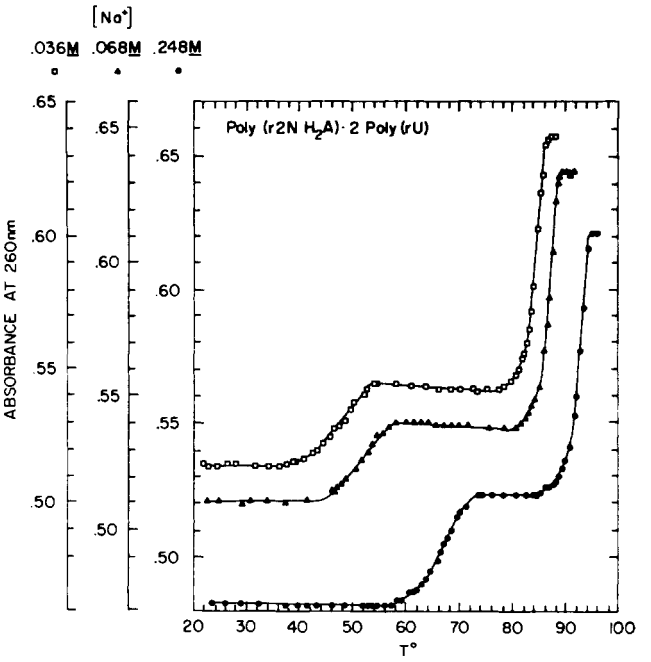

FIGURE 20: Ultraviolet melting curves of 1:2 mixtures (7.3×10^{-5} M in total polymer P) of poly($2NH_2A$) and poly(U) in 0.005 M sodium phosphate, pH 7.5, and at varying [Na^+]. The first transition of each curve arises from a strand dissociation reaction (3 → 2 transition), resulting in formation of poly($2NH_2A$)·poly(U) and excess poly(U). The second transition is due to dissociation of the 1:1 complex (2 → 1 transition). Mixtures were allowed to stand 15 (●) or 65 h (▲, □) before heating was begun.

Dependence on Cation Concentration. The wide range in sodium ion concentration over which strand-wise dissociation (3 → 2 transition) takes place (Figure 16) is in marked contrast to the behavior which has been reported for other polymer pairs. Simple dissociation (3 → 1 transition) is the only transition observed with poly(A)·2poly(U) above ~0.1–0.2 M Na^+ (Fresco, 1963; Miles and Frazier, 1964). For poly(A)·2poly(T)

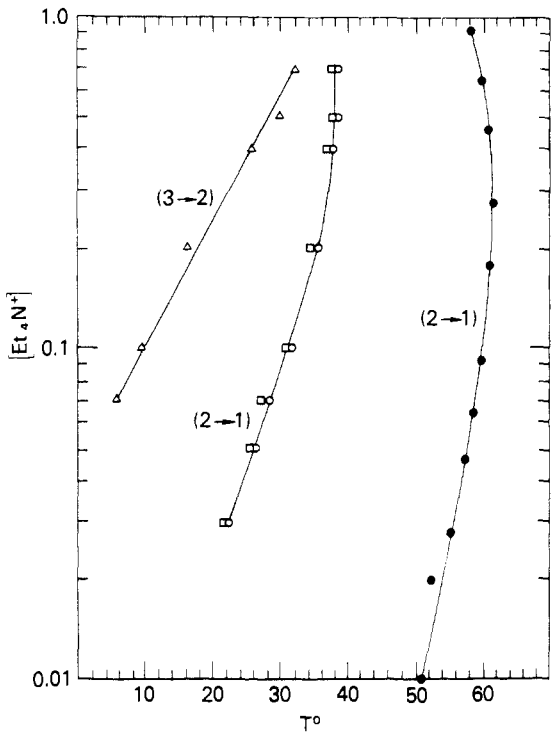

FIGURE 21: Phase diagram relating T_m and $[Et_4N^+]$. The two curves on the left (open symbols) show the cation dependence of the 3 → 2 and 2 → 1 transitions of the poly(A),poly(U) system and that on the right (solid dots), cation dependence of the poly($2NH_2A$),poly(U) system. dT_m/d log $[Et_4N^+]$ for the linear portions of these curves are 25.8 (△), 17.3 (○), and 9.5 °C (●), respectively. With poly(A)·2poly(U) Et_4N^+ causes a selective destabilization of the triple as compared with the double helix because of the higher charge density of the former complex (cf. Howard et al., 1971, and dashed lines, Figure 16). A triple helix was not formed by poly($2NH_2A$) and poly(U), and the double helix was destabilized by higher cation concentrations. The solutions were 0.002 M tetraethylammonium pyrophosphate, pH 8, and the polymers had been converted to the Et_4N^+ form by ion-exchange chromatography.

direct (3 → 1) dissociation to single-strand polymers is the only reaction observed for the range of sodium ion concentration 0.05–0.7 M (Howard et al., 1971).

As we have seen above, introduction of an amino group into the 2-position of poly(A) markedly increases the stability of the 1:1 complex formed with poly(U). This substitution, however, has little effect upon stability of the 1:2 complex formed when a second poly(U) is added. The difference in T_m of the 3 → 2 transition of poly($2NH_2A$)·2poly(U) and poly(A)·2poly(U) from 0.03 to 0.09 M Na^+ is only 3 °C (Figure 16), indicating that dissociation of a poly(U) strand from either 1:2 complex occurs with approximately equal facility. Of the two sets of binding sites on poly ($2NH_2A$), the Watson-Crick set is occupied first when the first poly(U) strand pairs with poly($2NH_2A$). The remaining Hoogsteen set is the only one available for the addition of a second poly(U) strand. Since the 2-position is distant from the bonding site at N7, it is not surprising that a substituent at the 2-position has little direct effect on the binding properties of the Hoogsteen site. Whether secondary structural alterations may affect the stability of third strand attachment, however, would have been more difficult to predict.

Destabilization of Two- and Three-Stranded Helices at Low and Moderate Concentrations of Tetraethylammonium Ion. We have shown in a previous report (Howard et al., 1971) that NEt_4^+ counterion destabilized both poly(A)·poly(T) and poly(A)·2poly(T), with respect to sodium counterion, but T_m of the latter complex was decreased much more than the former. These changes result in a selective destabilization of the triple helix with respect to the double helix, which we attribute to the higher charge density of the triple helix and to less effective screening by NEt_4^+ than by sodium ion.

The marked effect on the stability of poly($2NH_2A$)·poly(U) complex of changing the cation from Na^+ to Et_4N^+ is shown in Figures 16 and 21. Comparison of the two salt-dependence curves for poly($2NH_2A$)·poly(U) over the range of cation concentration where both curves are linear (0.015 to 0.07 M cation) shows that T_m of the 1:1 complex is about 24 °C lower in Et_4N^+ than in Na^+, reflecting a lower effectiveness of Et_4N^+ in screening electrostatic repulsion between the phosphates of the two polymer chains. Comparable depressions of T_m (2 → 1) are 26 °C for poly(A)·poly(U) (Figure 21) and 22 °C for poly(A)·poly(T) (Howard et al., 1971; this value was measured at 0.03 M cation concentration, since a 2 → 1 transition does not occur in this system above 0.035 M Na^+).

A detailed comparison of the effect of low and moderate concentrations of Et_4N^+ in three systems (r(A), r(U); r(A), r(T); and r($2NH_2A$), r(U)) is given in the supplementary material. Poly($2NH_2A$) and poly(U) do not form a triple helix at all with this cation, in contrast to the other two systems.

Supplementary Material Available

Figures 3, 5, 6, 8–10, 14, 15, and 18 plus additional experimental procedures and discussions as noted in the text (17 pages). Ordering information is given on any current masthead page.

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