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A·I and A·G Polynucleotide Pairing. Controlling Effect of Amino-Group Hydrogen Bonds to Solvent Water†

F. B. Howard, Masao Hattori, Joe Frazier, and H. Todd Miles*

ABSTRACT: Poly(2-aminoadenylic acid) (poly($2NH_2A$)) forms a well-defined double helix with poly(I), in contrast to unsubstituted poly(A), but does not form a triple helix under any conditions. This $2NH_2A$·I double helix has the same stability as that of the poly(A)·2poly(I) triple helix. Poly($8NH_2A$) also forms a complex with poly(I), less well-defined than the previous one because of its low stability. In the $2NH_2A$·I complex there is not enough space for a solvent water molecule to be hydrogen bonded to one of the 2-amino protons when I is on the N(1) side of A. Conversely, in the $8NH_2A$·I pair the hydrogen-bonding potential of the 8-NH_2 group could not be satisfied because of steric exclusion of water when pairing occurs at N(7) of A but could be satisfied when pairing occurs at N(1) of A. We conclude that it is the inability of 2-NH to make a hydrogen bond to water in a triple helix which restricts pairing to a 1:1 complex and that the structure of the 2-NH_2A·I complex involves bonding at N(7) of A rather than N(1). The possibility that base pairing is restricted by direct steric interference of the A 2-NH_2 group with poly(I) has been excluded by examining the interaction of poly(2MeA) with poly(I). The latter polymers form only a 1:2 complex, which has the same stability as poly(A)·2poly(I). Since relevant geometric features of A·G pairing are the same as those of the above $2NH_2A$·I systems (the 2-substituents being merely transposed), the same conclusion can be applied to A,G interaction. Base pairing of G to either the N(1) or the N(7) side of A is prevented by steric exclusion of a solvent water molecule, necessary to satisfy the hydrogen-bonding potential of the amino groups. The poly($2NH_2A$)·poly(I) helix is the first purine·purine heteroduplex helix to be described. The I is bonded to N(7) of A, and the two strands are presumably parallel. In A·I complexes of either 1:1 or 1:2 stoichiometry, bonding at N(7) is more stable than bonding at N(1), in striking contrast to A·U systems.

Interactions of A and I residues in polynucleotides are of interest in several contexts. Studies with A and I in polynucleotide model systems help to establish fundamental principles of polynucleotide structure and reactivity, with special reference to purine–purine interactions. There is a biological interest in the A·I pair because of the presence of I as the third base in the anticodon of many tRNA molecules and in the nature of its pairing with A in messenger RNA during protein synthesis. Finally, a possible role of poly(A)[1] and poly(I) as the basis of a primitive prebiotic self-replicating system has been suggested. In this paper, we approach A, I interactions in the first of these contexts and discuss the implications of our results for the other two.

Interaction of A and G would be of major importance if such pairing could be observed, but it has in fact never been demonstrated. Rich (1959) had suggested that guanine could replace hypoxanthine in an A·I pair, since the additional amino group attached to C(2) of the purine ring would not introduce any steric interference. In considering nonstandard base pairs to account for observed degeneracy in the third letter of mRNA codons, Crick (1966) proposed wobble pairings of U to G or I and of I to A. He concluded, however, that a G·A pair to N(1) of A would not occur, "because the NH_2 group of guanine cannot make one of its hydrogen bonds, even to water".

The possibility of G pairing to A at N(7) rather than at N(1)

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[1] Abbreviations used are: poly($2NH_2A$), poly(2-aminoadenylic acid); poly(I), poly(inosinic acid); poly(G), poly(guanylic acid); poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); poly(2MeA), poly(2-methyladenylic acid); poly($2NH_2A$)·poly(I), 1:1 complex of these components; poly(2MeA)·2poly(I), 1:2 complex of these components; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

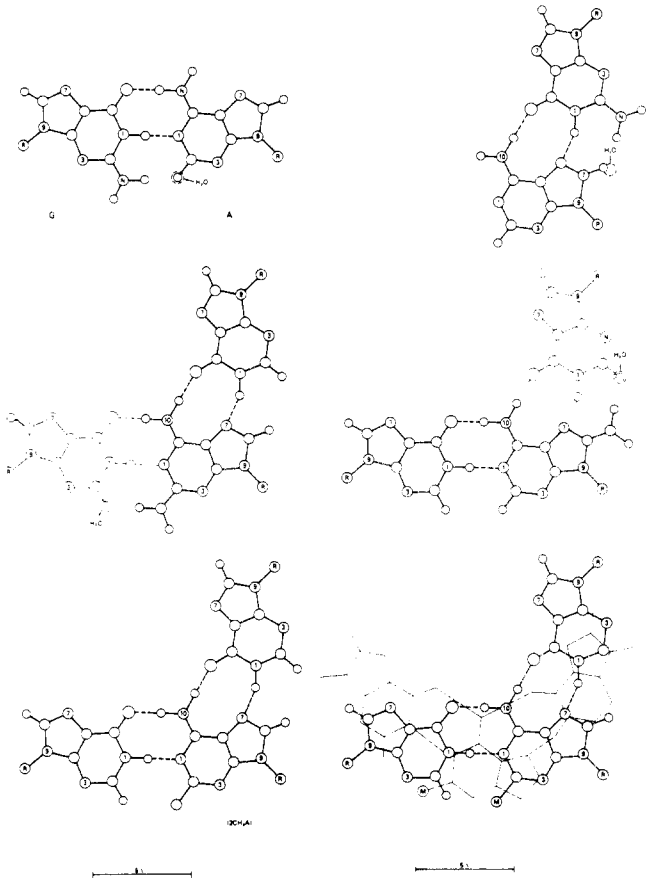

FIGURE 1: Hydrogen-bonding schemes of A·I and A·G pairs. Top left: A·G pairing at N(1) of A. If a water molecule was bonded to the second proton of the G NH_2 group, it would have to occupy the incidated position, prohibited by close contact with 2-CH of A. Top right: alternate A·G pair bonded at N(7); hydrogen bond of 2-NH of G to water molecule is prevented by close contact of A 8-CH. Center left: pairing of I with $2NH_2A$. The actual complex, bonded at N(7), is shown in heavy lines. Faint lines show hypothetical attachment of a second I strand at N(1) of A. The latter pairing is not made because water cannot occupy the indicated position for bonding to 2-NH of A. Center right: pairing of I with $8NH_2A$ occurs at N(1) (heavy lines) but not at N(7) (light lines). Lower left: triple helix formed by I and 2MeA. Lower right: Stacking of layers of triple helix poly(2MeA)·2poly(I), M = CH_3. The geometry would be similar for M = NH_2 but here N(1) bonding does not occur for reasons discussed in the text.

had been raised earlier by Donohue and Trueblood (1960) in a different context. They had proposed that this pairing (Figure 1, top right) with one base in a syn conformation may be inserted, in either order into the Watson-Crick structure for DNA.

The stereochemical problem of both N(1) and N(7) A·G pairs, and of related A·I pairs, is illustrated in Figure 1. Whether G is paired to A at N(1) or at N(7), a water oxygen placed at ~2.85 Å from G N(2) to form a linear hydrogen bond would be only about 1 Å distant from C(2) of A and nearly coincident with H(2) (Figure 1, top row, left and right structures, respectively). This spacing is obviously not allowed. Various perturbations of this structure (e.g., nonlinear hydrogen bonds, rotation of 2-NH_2 perpendicular to the plane, dihedral twist of the A·G pair) do not improve the situation significantly if accepted criteria of hydrogen bonds and van der Waals contacts are observed. It is clear that the indicated hydrogen bond to water cannot be made. Any effect this restriction has would be an indirect one mediated by the energetics of the helix–coil equilibrium. It is less obvious whether there may also be direct steric interference of G NH_2 with A 2-CH.

Formation of ordered, base-paired structures from separate polynucleotide chains results from a balance of opposing factors. Since magnitudes of relevant energetic factors are large and not precisely known, the position of equilibrium depends on differences which are smaller than uncertainties in the basic energetic parameters. For this reason, we consider that it is not possible to make a reliable prediction of the net effect of blocking a hydrogen bond to solvent and that an experimental resolution of the problem is required. We report here our study of the effect of blocking a specific water–NH_2 hydrogen bond and of possible steric interference of purine amino groups with a CH of a potentially complementary base.

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). Wavelength dispersions of the angles of intersection of the mixing curve and use of the computer to calculate these dispersions have been described (Howard et al., 1976). Computer calculation of derivative spectra was described in the same paper.

Ultraviolet melting data were obtained automatically and transmitted to the on-line computer in the following manner. A Cary 118 spectrometer with an automatic sample changer equipped with jacketed turrets was employed. A temperature programmer (NIH Biomedical Engineering and Instrumentation Branch) was designed to provide a number of discrete temperatures steps which could be set to provide large increments in the flat parts and small increments in the steeply rising parts of the melting curve. All measurements were made at thermal equilibrium. Temperature measurements were made with a Yellow Springs Instrument Thermivolt thermometer whose thermistor was inserted in a cuvette and attached to a Digitec Model 226 digital voltmeter. A remote operator's console (ROC, designed by John Powell of the NIH Division of Computer Research and Technology) mediated communication between the spectrometer, its accessories, and the computer, forming together a closed-loop system. Operation of the spectrometer is controlled entirely by the ROC, which directs the sequential scanning of each sample over a preset wavelength range. Completion of a series of up to five scans sends a signal to the ROC, which then signals the temperature programmer. When the preset temperature increment has been reached in the Lauda circulator bath, a previously selected thermal equilibration time (usually 10 min) was allowed before a signal from the programmer to the ROC permits the ROC to direct the sample changer and spectrometer to initiate another series of scans. At the end of each scan the computer must signal the ROC that is has closed out the current file. Before the new scan can begin the computer must signal the ROC automatic file increment (AFI) that it is ready to receive spectral and temperature data under the new file number.

Estimates of $dT_m/d \log [Na^+]$ and of standard deviations are derived from linear least-squares regression lines fitted by computer to the data.

Circular dichroic spectra were measured with a Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroic 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 on a molar

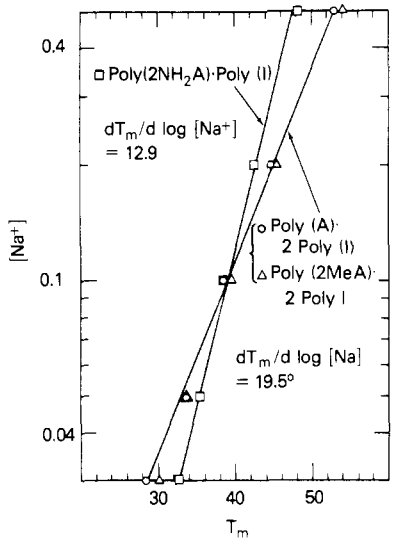

FIGURE 3: Salt-dependence curve of poly($2NH_2A$)·poly(I) (□), poly(A)·2poly(I) (○), and poly(2MeA)·2poly(I) (△). Curves for the latter two triple helices are coincident with $dT_m/d \log [Na^+] = 19.7 \pm 0.5$ °C. The double helix has a smaller dependence on $[Na^+]$.

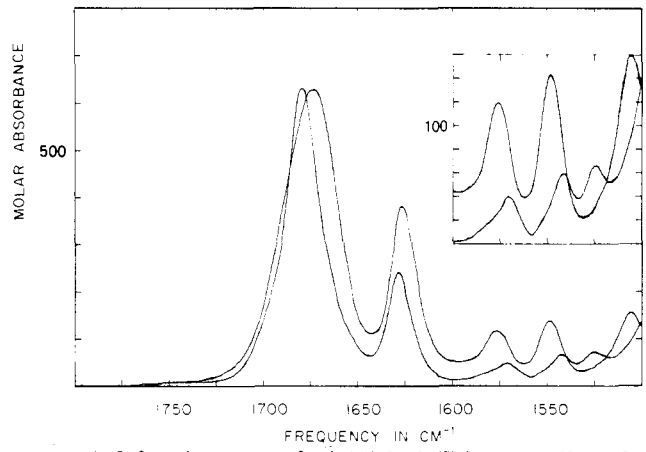

FIGURE 4: Infrared spectrum of poly(A)·2poly(I) has ν_{max} 1680, 1630, 1571, 1541, 1525, and 1496 cm^{-1} (30 °C, Na^+ 0.1 M, pD 7). When the helix is melted (50 °C) ν_{max} is observed at 1673, 1626, 1576, 1548, and 1505.5 cm^{-1}.

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.

Synthesis and characterization of poly($2NH_2A$) have been described by Howard et al. (1966, 1976). The sedimentation coefficient was 8.1 S in 0.005 M sodium pyrophosphate, pH 8.0, 0.1 M sodium chloride at 19.9 °C. Poly(2MeA) was prepared by the method of Ikehara et al. (1972), except that polynucleotide phosphorylase from *Micrococcus luteus* (P-L Biochemicals, Type 15) was employed rather than enzyme from *Escherichia coli*. To confirm the identity of this preparation of poly(2MeA), we degraded 0.14 mmol of the polymer in 0.031 M Tris buffer, pH 8.1, with crude venom of *Crotalus adamanteus* (3.1 mg/mL) (total volume 0.32 mL) for 21 h at 23 °C. Thin-layer chromatography of the digest (1-butanol–water (86:14) with ammonia vapor in the developing chamber) on a cellulose sheet (J. T. Baker Chemical Co.) revealed a single, UV-absorbing product (R_f 0.57). Adenosine had R_f 0.38 in this system. Poly(I) was purchased from Miles Chemical Co. (lot no. 33411) and poly(A) from P-L Biochemicals (lot. no. 179-14, reported to have a sedimentation coefficient 7.7 S). All polymers were purifed and dialyzed as described previously (Howard et al., 1971).

The substrate 8-aminoadenosine 5′-diphosphate was prepared from the corresponding 5′-phosphomorpholidate (Moffatt and Khorana, 1961). The corresponding 5′-phosphate was prepared by phosphorylation of the nucleoside with $POCl_3$ in redistilled trimethyl phosphate at 0 °C (Yoshikawa et al., 1969). Synthesis of the nucleoside 8-aminoadenosine was described by Holmes and Robins (1965). Details of the synthesis and the characterization of the phosphorylated intermediates are presented in the Supplementary Material.

Synthesis of Poly(8-aminoadenylic acid). A solution (1.55 mL) containing 0.02 M 8-aminoadenosine 5′-diphosphate, 0.006 M magnesium chloride, 0.1 M Tris buffer, pH 9.0, 2×10^{-4} M ethylenediaminetetraacetate, 0.01 M dithiothreitol, and 11.6 units (as defined by Singer and Guss, 1962) of polynucleotide phosphorylase (P-L Biochemicals, Type 15) was incubated at 37 °C. After 2 h, 45% of the substrate was polymerized, as measured by release of inorganic phosphate. Poly($8NH_2A$) was purified by phenol extraction and ethanol precipitation and dialyzed as described previously (Howard et al., 1971). The polymer (8.83×10^{-3} mmol, 28.5%) was recovered in water and stored frozen.

Poly($8NH_2A$) (6.92×10^{-3} M, 0.195 mL) in 0.05 M Tris buffer, pH 8.1, was degraded with crude venom of *C. adamanteus* (5.1 mg/mL) for 12 days at room temperature and the digest examined by paper chromatography (same solvent as above). The polymer was converted to a single, UV-absorbing compound with the same R_f (0.59) as authentic 8-aminoadenosine.

The extinction coefficient of poly($8NH_2A$) was determined by analysis for phosphorus by the procedure described previously (Howard et al., 1971). The mean of three determinations gave a value of 14 640 at 270 nm (0.02 M sodium pyrophosphate, pH 8.0) with a standard deviation of 40.

Results and Discussion

Poly(a), Poly(I)

Stoichiometry. In order to clarify the previous literature and establish a baseline for comparison with other A, I interactions we have recently reexamined the stoichiometry and kinetics of the poly(A), poly(I) system (Howard and Miles, 1977). The study clearly showed that a double helix is not formed at any polymer ratio or salt concentration, contrary to earlier reports (Rich, 1958, 1959), and that the triple helix is the only species existing at equilibrium. Observation of ultraviolet spectra of mixtures as a function of time also indicated that there is no objective evidence supporting the formation of a double helix as a transient intermediate.

Other properties of poly(A)·2poly(I) are reported below for comparison with those of the new complexes described in this paper.

Helix–Coil Transition. Salt Dependence. Ultraviolet temperature profiles of poly(A)·2poly(I) are cooperative monophasic curves with a transition breadth of 5 °C (distance between intercepts of upper and lower plateaus with line of steepest ascent) and T_m 38.5 °C in 0.1 M Na^+ (Figure 2, Supplementary Material). Dependence of T_m on log $[Na^+]$ is linear, and $dT_m/d \log [Na^+] = 19.7 \pm 0.5$ °C (Figure 3). This value of the slope is the same as that of many double helices and somewhat lower (~3–5 °C) than those of most purine–pyrimidine triple helices.

Infrared Spectra. The triple helix poly(A)·2poly(I) has infrared bands at 1680, 1630, 1571, 1541, 1525, and 1496 cm^{-1} (Figure 4). The 1680-cm^{-1} band is assigned to the car-

bonyl-stretching vibration of I (Howard and Miles, 1965), which is shifted from the random-coil value of 1674 cm^{-1} and narrowed when the helix is formed. The A ring vibration at 1630 cm^{-1} decreases by about one-third in absorbance in the ordered form. Both of these changes are smaller than those usually observed in polynucleotide interactions (cf. Miles, 1971). The ring vibrations between 1500 and 1600 cm^{-1} (Figure 4, inset) show larger relative changes when the helix is formed. We make the following assignments of these ring vibrations to the individual bases in the helix and in the random-coil polymers after melting. The band at 1571 cm^{-1} in the helix occurs at 1576 cm^{-1} after melting and is composed primarily of an A ring vibration at 1576 cm^{-1} ($\epsilon \sim 175$) but also of an I band at 1579 cm^{-1} ($\epsilon \sim 75$). The band at 1544 (helix) and 1548 cm^{-1} (coil) is entirely an I ring vibration and increases nearly threefold in intensity on melting. The origin of the 1524-cm^{-1} band (helix) is less obvious, since neither polymer has a perceptible band near this frequency under the usual conditions of measurement. An expanded spectrum of 0.1 M poly(A) solution, however, shows a shoulder at about 1518 cm^{-1}, and a faint shoulder detectable at $\sim$1522 cm^{-1} is apparent in the expanded 46 °C spectrum in Figure 4. This band is greatly intensified in the AI_2 helix (Figure 4). We have not observed a similar intensification in other complexes containing poly(A). The band at 1496 cm^{-1} (helix) shifts to 1505 cm^{-1} on melting, with little change in intensity. This band disappears on heating to 80–90 °C as a result of deuterium exchange at the 8 position, and a new band is observed at 1480 cm^{-1}. This isotopic shift indicates that the vibration corresponds to a normal mode involving motion of C(8), to which the exchanged proton was bound.

By selecting resolved vibrations corresponding to the separate bases it is possible to monitor simultaneously and independently the melting of both polymers. The parallel temperature profiles (Figure 5, Supplementary Material) clearly demonstrate specific base-pairing interaction (for detailed discussion, cf. Miles, 1971).

Poly(2NH_2A), Poly(I)

Test of the Role of a Specific Hydrogen Bond to Water in Controlling Polynucleotide Reactivity and Structure. The proposal that the inability of G-NH_2 to form a hydrogen bond to water prevents A·G pair formation is important in its original context of condon–anticodon interaction (Crick, 1966). The principle involved is also significant more generally for any polynucleotide structure which would place an amino group in close (but not sterically repulsive) proximity to an atom which cannot serve as a hydrogen-bond acceptor. There has so far, however, been no direct evidence that such an effect exists.

Amino protons in the random-coil form of polynucleotides are hydrogen bonded to solvent water. When steric restriction prevents a water molecule from making a hydrogen bond in the helix (cf. Figure 1), the effect of the restriction is clearly destabilizing to the helix. It is less clear in general, however, where the energy balance would lie between placing two bases in the helix and foregoing a hydrogen bond to water as opposed to unstacking and looping out the two bases for each such bond not formed.

The helix $\rightleftarrows$ coil equilibrium will involve a hydrogen bond from the relevant 2-amino proton to a water molecule on the coil side of the equation and no compensating hydrogen bond on the helix side. This is in contrast to the hydrogen bonds of the ring NHs, which are made to water on one side of the equilibrium and to a complementary base on the other side, the H-bond contribution to the equilibrium being determined by the difference of free energy of the bonds rather than by the magnitude of either. The free energy of a hydrogen bond present only in the coil, however, contributes its entire value to destabilization of the helix. Among the opposing factors favoring the helix will be the positive free energy required to unstack and loop out those bases which are not paired as the result of not making a hydrogen bond in the helix. Reliable values of the appropriate free energies would provide a clear basis for predicting whether the helix or coil will be favored, but available data do not appear to be adequate for the purpose.

"The Hydrogen Bond" by Pimentel and McClellan (1960) contains a comprehensive tabulation of hydrogen-bond energies from the literature. Many of the values reported for similar systems, however, show rather wide variation. In their critical review of the data the authors state that the most outstanding conclusion is that additional accurate and systematic studies are needed. Many of the reported enthalpy values for O-H···O and N-H···N bonds fall in the range 3–6 kcal/mol. For acids and hydroxyl compounds, there is a monotonic increase of $-\Delta S$ with $-\Delta H$, so that higher values of enthalpy are reduced by larger $T\Delta S$ terms, and the free energy or equilibrium constant is not particularly sensitive to change in ΔH (Pimentel and McClellan, 1960). The effect of going from organic solvents (in which most of the solution measurements were made) to water would presumably be to weaken the hydrogen bond. The magnitude of the stacking free energy is also not well known, but an estimate of 7 kcal/mol has been made by Crothers and Zimm (1964), who discuss the underlying assumptions and probable sources of error in the estimate. If the value is this large in a system having potential A·G pairing, it seems unlikely that the energy of a single hydrogen bond to water would be large enough to force the equilibrium in the direction of the coil. It appears to us, however, that none of the relevant parameters are known with sufficient accuracy to permit an objective prediction of the position of equilibrium. The experiments described below were designed to obtain information on the effect of helix structure and stability of hydrogen bonds to solvent which are present in the coil form but sterically precluded in the helix.

Because the high stability of the poly(G) helix prevents or seriously impedes the formation of heterocomplexes, possible A·G pairing cannot be usefully investigated by mixing poly(A) and poly(G). By transposing the 2-substituents of A and G, however, we obtain a pair which has precisely the same geometry with respect to hydrogen bonding to water by the 2-amino group (Figure 1, middle row, left). In this case, neither poly(2NH_2A) (Howard et al., 1966, 1976) nor poly(I) has a self-structure sufficiently stable to prevent formation of a heterocomplex. If inability to form the relevant hydrogen bond can control pairing at the N(1) bonding site of A, the prediction is that either a two-stranded A·I helix will be formed, or, possibly, that there will be no interaction. If this specific hydrogen bond to solvent does not play the proposed role, then a triple helix analogous to poly(A)·2poly(I) will be formed.

Infrared Evidence for Complex Formation. The infrared spectrum of a 1:1 mixture of poly(2NH_2A) and poly(I) in the region of double-bond vibrations shows large changes from the spectra of the components (Figure 6), characteristic of base-pairing interactions (for references and discussion, cf. Miles and Frazier (1964), Howard et al. (1969), and Miles (1971)). The inosine carbonyl vibration (cf. Howard and Miles, 1965) has shifted to 1689 cm^{-1} in the helix ($\Delta\nu$ = 15.5 cm^{-1}) and the half-bandwidth has decreased from 35 to 20 cm^{-1}. The A ring vibration at 1612.5 cm^{-1} (50 °C) shifts to 1622 cm^{-1} in the helix, and the band visible as a shoulder at $\sim$1600 cm^{-1} de-

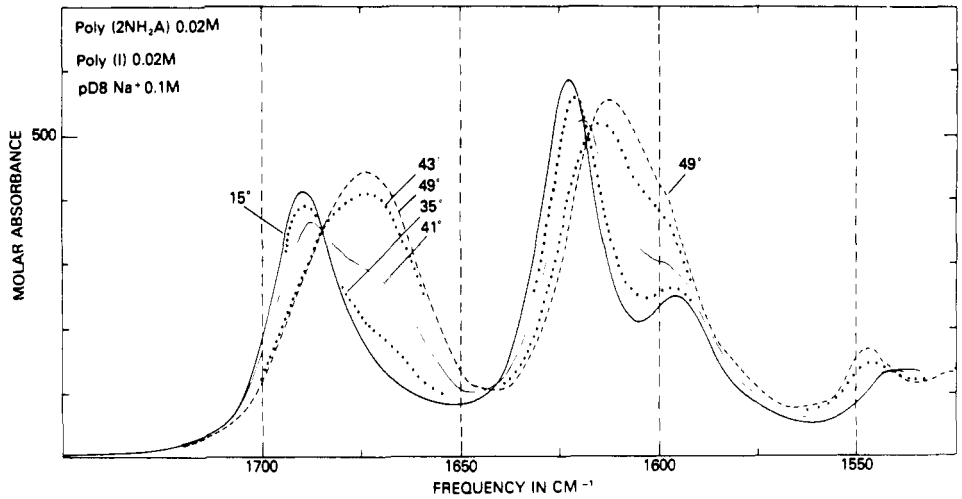

FIGURE 6: Infrared spectra of poly(2NH_2A)·poly(I) (15 °C) and of mixtures formed by thermal dissociation of the double helix. The large change in I carbonyl-stretching frequency on going to the ordered form (1673 → 1689 cm^{-1}) is similar to that observed in many helices. The isosbestic point in the carbonyl region occurs at 1684 cm^{-1}. The 2NH_2A ring vibration (1613 cm^{-1}) shows the same frequency increase observed in the 1:1 complex with poly(U) (Howard et al., 1976) but, in marked contrast to the large loss of intensity in the latter helix, exhibits little change in ϵ_{max}.

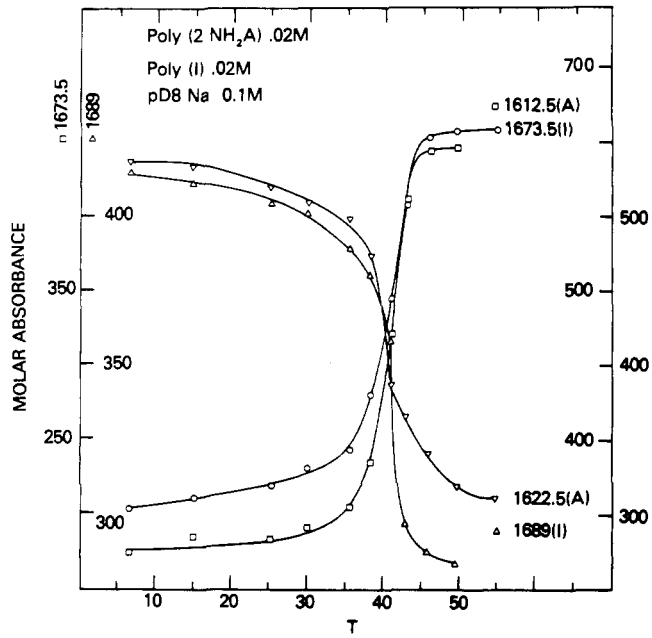

FIGURE 7: Infrared melting curves of poly(2NH_2A)·poly(I). Parallel temperature dependence of A and I bands shows that spectroscopic changes are due to a specific A·I pairing interaction.

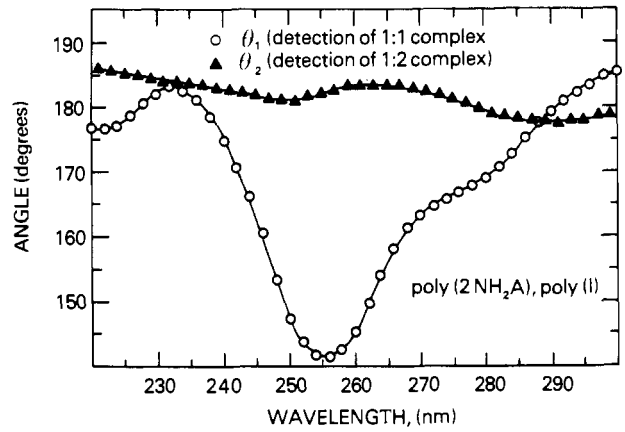

FIGURE 8: Wavelength dispersion of the angles of intersection of the mixing curves corresponding to 50% poly(I) (θ_1) and 67% poly(I) (θ_2). The most sensitive wavelength for detecting a 1:1 complex is indicated by the minimum at 256 nm. The fact that $\theta_2 \simeq 180°$ over the observed spectral range indicates that a 1:2 complex is not formed. These dispersion curves are quite distinct in different interacting polynucleotide systems and constitute an additional means of characterizing such systems.

creases in intensity and appears as a clearly resolved band at 1595.5 cm^{-1} in the helix.

Infrared temperature profiles of bands assigned exclusively to A (1612.5, 1622 cm^{-1}) and I (1673.5, 1689 cm^{-1}) vibrations, respectively, show parallel temperature dependence (Figure 7), demonstrating that the spectral changes result from specific base-pairing interactions rather than from self-structures of either component (for previous infrared studies and detailed discussion of this point, see references cited above).

Stoichiometry. We have pointed out in previous discussions that determination of the combining ratio of polymers is always important, since the interpretation of virtually all other properties of the complex eventually depends upon it (e.g., Howard et al., 1971, 1976). The present example further emphasizes the point, since a major conclusion of the study is based entirely and directly upon the stoichiometry.

We have recently developed new methods of analyzing spectroscopic data which eliminate most of the ambiguity and uncertainty frequently associated with studies of stoichiometry in the past. The first method employs the ultraviolet spectra (200–400 nm) of mixtures ofpolymers and the second their derivative spectra, $d\epsilon/d\lambda$ vs. λ, both collected and processed by a digital computer (Howard et al., 1976). Angles of intersection of the mixing curves are defined as explicit dependent variables θ_1 and θ_2 and calculated by the computer as a continuous function of wavelength. Maxima and minima in these dispersion curves correspond to optimum wavelengths for detecting a complex of a particular composition. Conversely, when $\theta_i = 180°$ over the entire spectrum, there is no break in the mixing curve at any wavelength, and we conclude that a complex corresponding to mole fraction X_i is not formed. The dispersion curve for θ_1 (Figure 8) shows that 256 nm is the optimum wavelength for detecting a 1:1 complex, and mixing curves at this and other wavelengths exhibit a clear break at this ratio but none at 67% poly(I) (Figure 9). $\theta_1 \simeq 180°$ between 220 and 240 nm and between ~275 and 300 nm, indicating lack of detectability in these ranges. The angle disper-

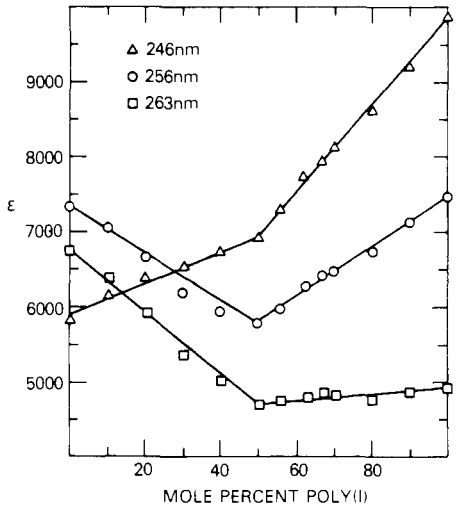

FIGURE 9: Clear discontinuities in the mixing curve at 50% poly(I) show that a 1:1 complex is formed.

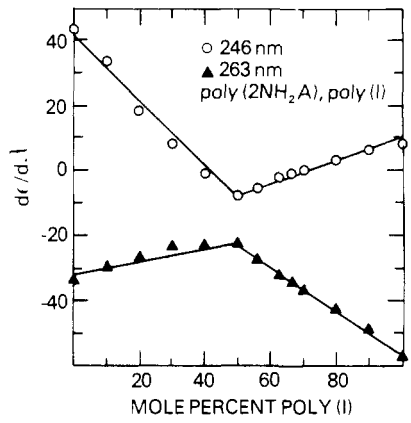

FIGURE 10: Derivative mixing curves are obtained from derivatives of the ultraviolet spectra ($d\epsilon/d\lambda$ vs. λ). They confirm the formation of a 1:1 complex between poly(2NH$_2$A) and poly(I) and the absence of a 1:2 complex. Ordinate values have been multiplied by 100.

sion curve for detection of the 1:2 complex shows that $\theta \simeq 180°$, at all wavelengths (Figure 8), indicating that there is no discontinuity in the mixing curve at any wavelength, and that a 1:2 complex is not formed. Confirmatory evidence was obtained by differentiating the ultraviolet spectra and applying the same analysis to the derivative spectra. The wavelength dispersion of the angles of intersection of the derivative mixing curves has a minimum for θ_1 (1:1 complex) at 246 and maxima at 263 and 286 nm, which are therefore the wavelengths of choice for the derivative mixing curves. The first two of these correspond also to suitable wavelengths for ultraviolet mixing curves, but at 286 nm the UV spectra do not detect the 1:1 complex (Figure 8). The derivative mixing curves intersect sharply at 50% poly(I) and exhibit negligible scatter of data from the least-squares straight lines (Figure 10). $\theta_1 \simeq 180°$ at 235 ± 5 nm, at 252 nm, and at 275 nm, indicating lack of detectability at these wavelengths. θ_2 (1:2 complex) $\simeq 180°$ over the entire wavelength range of the derivative spectra (220–300 nm), again showing that a triple helix is not formed.

The evidence is thus clear that the 2-amino group of A has prevented formation of a 1:2 A·I complex which, in the absence of this group, is the only complex formed. We therefore conclude that the bonding site for the single poly(I) strand in the double helix demonstrated above must be N(7) of A. This conclusion is confirmed in experiments with poly(8NH$_2$A) and poly(2MeA), described in subsequent sections.

Helix–Coil Transition. Salt Dependence. Like the infrared temperature profiles (Figure 7) the ultraviolet melting curves are sigmoid, with a breadth of 9 °C and T_m = 39 °C in 0.1 M Na^+ (Figure 11, Supplementary Material). It is noteworthy that this value is virtually identical with that of the triple helix poly(A)·2poly(I), and implications of this result are discussed in a later section. The salt-dependence curve (Figure 3) is linear and shows $dT_m/d \log [Na^+] = 12.9 \pm 0.4$ °C. This slope is appreciably lower than the usual value for double helices (18–20 °C) and slightly less than that of poly(2NH$_2$A)·poly(U) (14.8 °C; Howard et al., 1976).

Poly(2MeA), Poly(I)

Having shown that a purine 2-NH$_2$ group will prevent hydrogen bonding to a base which lacks a complementary hydrogen-bond acceptor for this group, we wish to raise two further questions about the reason for this effect. In addition to its destabilizing exclusion of a hydrogen bond to water, an indirect effect, does the 2-NH$_2$ group interfere sterically with the 2-CH of the opposing base (a direct effect) and does it exclude water from the surface of the opposing base in a way that is destabilizing for reasons other than lack of hydrogen bonding specifically to the 2-NH group (a differert indirect effect)? An experimental approach to both of these questions is available through the use of poly(2MeA), first synthesized by Ikehara et al. (1972). By direct steric interference of the 2-CH$_3$ group of A with the 2-CO group of U, the 2-CH$_3$ substituent has been shown to block Watson–Crick A·U pairing, permitting the less stable Hoogsteen double helices to be formed (Ikehara et al., 1972; Hattori et al., 1974).

The CH$_3$ group is an appropriate steric model for NH$_2$ with reference to the questions cited above. It is slightly larger than NH$_2$ and, if it can be shown to offer no direct steric interference to the other base, clearly, NH$_2$ would offer none in the same position. Our tests for possible steric effects of the 2-CH$_3$ group are the combining ratio of the modified polymer with poly(I) and thermal denaturation behavior of the complex.

Ikehara and Hattori (1972) found in unpublished experiments that poly(2MeA) interacts with poly(I) and concluded from ultraviolet mixing curves that a 1:2 complex was formed (Ikehara and Hattori, personal communication). Our demonstration of the combining ratio of the two polymers was carried out by three methods, described below.

Stoichiometry. The wavelength dispersions of θ_1 (1:1) and θ_2 (1:2) for mixtures of poly(2MeA) and poly(I) (Figure 12, Supplementary Material) are closely similar to those of poly(A) and poly(I). Wavelengths of greatest detectability for the 1:2 complex (θ_2) are 220, 250, and 288 nm, and mixing curves at these wavelengths (Figure 13) show clearly the formation of a triple helix. At 229 and 275 nm no interaction can be detected. The wavelength dispersion of θ_1 (1:1 complex) shows $\theta_1 \simeq 180°$ over the entire range of the spectra, showing no formation of a 1:1 complex. The derivatives of all spectra in the mixing were calculated and plotted by computer (Figure 14a,b, Supplementary Material). In the range 0–67% poly(I) isosbestic points are observed at 224, 259, and 287 nm and in the range 67–100% poly(I) at 217, 243, 267, and 286 nm. The wavelength dispersion of θ_2 for the derivative spectra (Figure 15, curve 1, Supplementary Material) shows that the most favorable wavelengths are 227, 261, 276, and 295 nm. Except for slight wavelength shifts, this dispersion curve is remarkably similar to that of poly(A), poly(I) (Figure 15, curve 2). Derivative mixing curves at these wavelengths exhibit sharp intersections and negligible scatter of data (Figure 16). $\theta_1 \simeq$

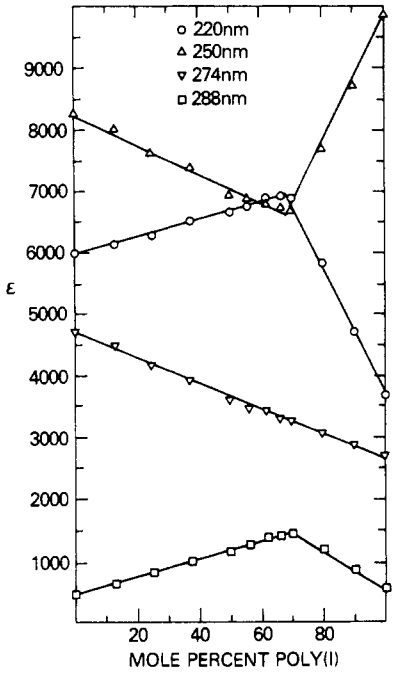

FIGURE 13: Ultraviolet mixing curves show that a 1:2 complex but not a 1:1 complex is formed between poly(2MeA) and poly(I). Conditions were: 0.002 M sodium cacodylate, pH 7; 0.1 M Na^+; 25.0 °C.

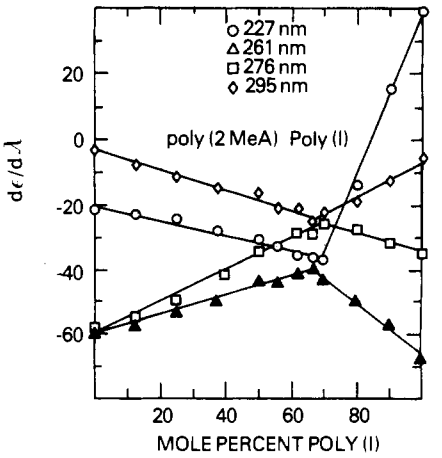

FIGURE 16: Derivative mixing curves demonstrate that a 1:2, and only a 1:2, complex is formed between poly(2MeA) and poly(I). At three of these wavelengths (227, 276, and 295 nm) stoichiometry could not be demonstrated from ultraviolet mixing curves themselves (cf. Figure 12 in Supplementary Material). Values of the ordinate have been multiplied by 100.

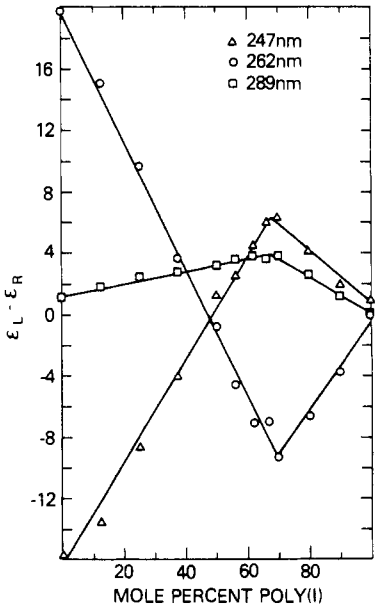

FIGURE 19: CD mixing curve of poly(2MeA) and poly(I). Large changes in circular dichroism (cf. Figure 17) provide a clear demonstration of 1:2 stoichiometry by a method independent of the ultraviolet spectra.

180° over the observed spectral range of 200–400 nm, indicating no 1:1 complex is formed.

Finally, CD spectra were measured for all mixtures (Figure 17a,b, Supplementary Material), and CD wavelength dispersions of θ_1 and θ_2 and CD mixing curves were prepared. The most favorable wavelengths for θ_2 are 247, 262, and 289 nm (Figure 18, Supplementary Material) and mixing curves at these values clearly demonstrate a 1:2 complex (Figure 19). As before, $\theta_1 \simeq 180°$ over the range 220–300 nm (Figure 18, Supplementary Material), again demonstrating that a 1:1 complex is not formed.

from these results it follows that a 2-CH_3 group does not block pairing at the N(1) bonding site of poly(A), nor does it destabilize such pairing to an extent that would permit independent existence of a double helix at a 1:1 polymer ratio. In the following section, we shall see that the thermal stability of the AI_2 triple helix is unchanged by the 2-CH_3 group over the observed range of sodium ion concentration (0.03–0.5). Consequently, the observed blocking effect of the 2-NH_2 group, reported above, is due neither to direct steric interference with the complementary base nor to an indirect effect of excluded water other than that of a specific hydrogen bond of a water molecule to 2-NH.

Helix–Coil Transition. Salt Dependence. Ultraviolet melting curves of poly(2MeA)·2poly(I) are sigmoid curves with breadth = 7.5 °C and T_m = 39°C in 0.1 M Na^+. The slope of the salt-dependence curve is identical with that of poly(A)·2poly(I) and $dT_m/d \log [Na^+] = 20.2 \pm 0.6$ °C (Figure 3).

Poly(8NH_2A), Poly(I)

The foregoing evidence documents the formation of a two-stranded A·I helix bonded at N(7) of poly(2NH_2A) and shows that a specific hydrogen bond to solvent has a controlling role in the interaction. We next investigated 8-NH_2 substitution of poly(A) to see whether a two-stranded helix bonded at N(1) of A could be formed and to obtain evidence on feasibility of the alternative A·G pairing hydrogen bonded at N(7) (Figure 1, top right). Previous experiments with 8NH_2GMP and poly(8NH_2G) Hattori et al., 1975, 1976) have shown that an 8-amino group does not interfere with Watson-Crick G·C pairing and that stability of triple helices is greatly enhanced when hydrogen bonding to the 8-NH_2 group can occur. With poly(8NH_2A) and poly(I) the geometrical relation of A 8-NH to I 8-H is similar to that of A 2-NH to I 2-H in that there is no hydrogen-bond acceptor on I for the 8-NH group and no space for a water molecule to which a bond could be made (Figure 1, center right). Any complex formed by these two polymers should therefore be double stranded and bonded at N(1) of A rather than N(7).

Infrared spectra of a 1:1 mixture of poly(8NH_2A) and poly(I) (Figure 20, Supplementary Material) indicate that interaction occurs at low temperature but that melting of the A·I helix is complicated by formation and subsequent melting of the poly(I) self-structure. Exclusive formation of an A·I complex is probably not observed at any temperature. An A

ring vibration occurs at 1634 cm^{-1} in the A,I mixture at −2 and +9 °C and has the same intensity at both temperatures. Poly($8NH_2A$), in contrast, has ν_{max} 1637 cm^{-1} at +4 °C and shows a monotonic change with temperature of frequency and intensity (Figure 21, Supplementary Material). A similar contrast between constant intensity of the 1590 cm^{-1} ring vibration in the mixture (A,I −2 and +9 °C) and variation of this band in the homopolymer is also observed. Infrared temperature profiles (Figure 22, Supplementary Material) plotted at A (1631.5, 1591 cm^{-1}) and I (1673.5, 1680 cm^{-1}) frequencies show a transition of T_m about 16 °C in which melting of A and I residues occurs in a parallel manner, indicating a specific interaction of these residues. The I band at 1680 cm^{-1} shows a second transition occurring over the same range as the final part of the poly(I) transition (25–35 °C) under these conditions (Howard et al., 1966). Though the results are by no means as clear cut as those with poly($2NH_2A$), they support the conclusion that a specific A·I complex is formed to a major extent at low temperature. This A·I helix melts quite cooperatively and some of the poly(I) released from the complex forms the poly(I) helix, which subsequently melts in a less cooperative manner.

Though reliable mixing curves cannot be obtained with this pair of polymers, the data show that the 8-NH_2 group has blocked formation of an A·I complex of "normal" stability (cf. Figures 3 and 22).

We have noted above that there is clearly not space in the alternative A·G or $8NH_2A$·I base pairs (Figure 1, top and center, right) for a water molecule bonded to the C2-NH or the A8-NH protons. The experiments with poly($2NH_2A$) have shown that such a stereochemical limitation prevents base-pair formation at the bonding site on the same side of the purine molecule as the amino group. Conversely, the $8NH_2A$·I complex which is formed is hydrogen bonded at N(1) of A and not at N(7) of A, since the N(7) side is restricted whereas the N(1) side is not.

We reach from these studies a general conclusion that the hydrogen-bonding potential of both protons of the base amino groups in nucleic acids and polynucleotides must be satisfied in the helix if a base pair is to be formed. Any steric restraint excluding a water molecule from a position in which it can accept a hydrogen bond from the NH protons not bonded to a complementary base will cause the bases to remain unpaired. Though this study has dealt explicitly with that proton of the NH_2 groups which lies in the direction of the complementary base, it also follows that the protons directed toward the minor (G and $2NH_2A$) and major (C and A) grooves of the helix must also be hydrogen bonded to water. Any restriction of access to a water molecule here would also prevent base pairing.

Stability of A·I Complexes. The same two bonding sites of poly(A) are involved in pairing with either poly(U) or poly(I): N(1), NH_2; and N(7), NH_2. We find in this study, however, a striking reversal with poly(I) of the relative stabilities of complexes formed at the two bonding sites of poly(A) from that observed with poly(U). Previous investigations have shown that two-stranded A·U helices with Hoogsteen (N(7)) bonding are capable of independent existence if pairing at N(1) is blocked, but that the stability is much lower than that of the corresponding Watson–Crick helix (ΔT_m 35–50 °C) (Ishikawa et al., 1972; Ikehara et al., 1972; Hattori et al., 1974).

With A,I systems, in contrast, it is the N(7) bonding site which leads to the more stable complex. The two-stranded helix formed by poly($2NH_2A$) with poly(I) has the same T_m as that of poly(A)·2poly(I) in 0.1 M Na^+ (Figure 3). The similarity suggests that in the triple helix it is the pair bonded at N(7) which determines the stability of the A·2I complex. The converse relationship exists in A·2U and A·2T helices, with the Watson–Crick pair determining the stability of the triple helix of which it is a component part (in moderate to high salt concentrations). The low stability of the complex formed by poly($8NH_2A$) with poly(I) is fully consistent with the idea that it is the pair bonded at N(7) which determines the stability of the triple helix.

It is remarkable that the 2-Me substituent of A has no effect in either direction upon the stability of the A·2I triple helix. A 5-methyl substituent in pyrimidine polynucleotides elevates the transition temperatures of the complexes formed by these polymers by ~15–20 °C (Szer and Sugar, 1966; Massoulié et al., 1966; Howard et al., 1971) and might have had such an effect in the present case. On the other hand, the (A)2-CH_3···H(I) contact is close, and might be considered slightly destabilizing. A KPC space-filling model shows direct but not repulsive contact when the C–H and the CH_3 are in a staggered relationship. Despite considerations which might favor either an enhanced or a reduced stability, however, the transition temperatures of A·2I and 2MeA·2I are identical at all salt concentrations (Figure 3). The methyl groups in the helix do not lie directly above the purine rings in the preceding layer (Figure 1, bottom right), and lack of stabilization by stacking interactions is consistent with this geometry. As for a possible destabilizing CH_3–HC contact, minor stereochemical adjustments might be involved in producing an optimum fit of the 2MeA·I pair. That significant adjustments from the usual A, I values are not necessary, however, is indicated by the stability and spectroscopic properties of the complex.

We recognize that, in principle, positive and negative effects of the 2-CH_3 group might simply cancel in the present instance, but consider this possibility unlikely. A similar stereochemical location and lack of effect on T_m is observed in the double-helix poly($2NH_2A$)·poly(I). The approximate position of the 2-NH_2 group with respect to the purine in the next layer of the helix can be seen in Figure 1 by letting M = NH_2 and shortening the M–C(2) bond by about 0.15 Å. There is still no overlap of the base in the next layer by the 2-substituent and both protons of the 2-NH_2 group in the double helix are free to make hydrogen bonds to water.

We propose the term neutral substituent to describe the A 2-CH_3 group with respect to the interaction with poly(I). It can evidently fit into the helical structure without perturbing or interacting significantly with other parts of the molecule and has no effect on the stability. The A 2-CH_3 substituent is obviously not neutral with respect to poly(U), with which it blocks interaction. The A 2-NH_2 substituent could be considered neutral with respect to the double helix it forms with poly(I), since it does not appear to change the stability, though with respect to the A, I triple helix it is a destabilizing and with respect to the A, U double helix a stabilizing substituent.

Structure of Poly($2NH_2A$)·Poly(I). This complex is the first heteroduplex helix containing only purine bases. Hydrogen bonding occurs at N(7) rather than N(1) (Figure 1, middle row, left). Two distinct structures may be considered for the helix: (a) a helix with parallel strands and all bases in the anti conformation, (b) a helix with antiparallel strands, one strand with syn bases and one with anti bases. Structure a would presumably resemble two strands of the triple helix poly(A)·2poly(I). Arnott and Bond (1973) have published a molecular model for this triple helix in which there is very little change of the conformation dihedral angles from those of conventional RNA (purine–pyrimidine pairs). Such a structure for a double helix is clearly feasible.

The alternative antiparallel structure b requires that one of the bases have a syn conformation. Though the common purine nucleosides have been found with this uncommon conformation in a number of crystal studies, there has been much less information about the properties of syn polynucleotides because they were not available for study. One syn polynucleotide has been described, however, and its properties provide information relevant to other systems. Poly(8BrA) forms a highly stable double helix with itself and is strongly stacked (by UV and NMR criteria) both in the helix and in the single-stranded form after melting (Howard et al., 1974, 1975).

Both NMR observations and classical potential energy (CPF) calculations indicate that the dihedral torsional angles describing the structure are quite similar to those of poly(A), with the exception of the glycosidic angle (Govil et al., 1977). Since the energy barrier to rotation about purine glycosidic bonds is generally considered to be low and since acceptable backbone torsional angles can be employed in a helical structure with a syn polymer, there appears to be no compelling argument against the antiparallel structure b. The close similarity in stability of the $2NH_2A{\cdot}I$ and the A·2I helices, nevertheless, makes it appear more likely that the glycosidic bond conformations are anti and the strand polarity is parallel in the two-stranded complex. The only heteroduplex helices with parallel strand polarity reported previously are the A·U Hoogsteen complexes in which Watson–Crick pairing is sterically blocked (Ishikawa et al., 1972; Ikehara et al., 1972; Hattori et al., 1974).

Codon–Anticodon Pairing. The A·I wobble pairing proposed by Crick is bonded at N(1) of A and resembles the first two (antiparallel) strands of the A·2I triple helix. The fact that the glycosidic distance (C(1)′–C(1)′ ≈ 13 Å) is greater than standard pairings (~10.8 Å) causes no difficulty in a homopolymer helix but would be destabilizing in polymers of mixed sequence, requiring the short and long distances to be accommodated in the same helix. Most of this destabilization can be avoided by I in the anticodon, since it is only a terminal position of the short helical region which would have the longer glycosidic distance. The question of having a standard 10.8-Å separation on either side of a pair having a 13-Å separation, thus, does not arise.

In discussing application of the wobble hypothesis to the presence of hypoxanthine in tRNA, Sakore and Sobel (1969) made an interesting alternative proposal for A·I pairing. They suggest that instead of pairing at N(1) of A the I residues may bond at N(7), giving the same glycosidic distance (10.8 Å) as the standard Watson–Crick purine–pyrimidine pair, and therefore causing "less distortion in the trinucleotide interaction". Since the anticodon and messenger have an antiparallel association, the adenylic acid residue must have a syn conformation. The glycosidic distance would be the same whether the A is syn or anti.

A CPF study of poly(8BrA), which is entirely syn, has indicated that a BrA·A model can be constructed in which a strand of poly(BrA) is hydrogen bonded to an antiparallel strand of poly(A) at N(7) (Govil et al., 1977). (We would not expect and do not observe such a complex because of the high stability of BrA self-association.) The structure would be sterically allowed but would have relatively high single-strand conformational energy. While a syn A·I paired at N(7) of A is probably feasible for codon–anticodon pairing, it does not appear that our knowledge is sufficiently precise at present to decide between this scheme and that of Crick on energetic grounds.

Possible Primitive Coding Systems. In discussions of possible routes of evolution of the genetic apparatus, Orgel (1968) and Crick (1968) have considered the possibility that the familiar four-letter code arose from a primitive two-letter code. The suggested candidate bases were A and I, since adenine is formed more readily than the other purines or pyrimidines in simulated prebiotic experiments, and hypoxanthine could be formed from adenine by deamination. Orgel (1968) posed the question, "Is a replicating A-I double helix possible?", and Arnott and Bond (1973) concluded, "Our studies suggest that nucleic acid double helices containing only the longer (A·I) pairs [i.e. those bonded at N1 of A] could well have evolved to contemporary species with purine-pyrimidine pairs with no major discontinuity of molecular geometry." For the (A·I) wobble pairing of anticodon and messenger these authors considered the N(7) bonding site to be not as good a candidate as N(1).

Our experiments provide information relevant to some of the questions raised here. First, poly(A) and poly(I) do not form a double helix at any ratio of polymers or any salt concentration (Howard and Miles, 1977). One could consider the possibility that A·2I triple helices might have evolved into double helices containing the standard bases. This appears unlikely, however, since the pyrimidine bases have no equivalent of the purine N(7) position for attachment of a third strand. An alternative possibility is that A, I copolymers, in contrast to the homopolymers, could form a double helix. On this point we have no direct evidence, but consideration of a further finding leads us to consider it also unlikely as a route in biochemical evolution. It is the pairing at N(7) of A which is more stable rather than that at N(1). If an A·I double helix of varied sequence should be formed, it would presumably have the standard Watson–Crick bonding site of A unpaired and would have parallel strand polarity (unless one of the strands had its bases in syn conformations). These considerations appear to make a purely A, I evolutionary route unattractive, though it is quite possible that I may have played a prominent role in a primitive code. We may note that two results of replacing I with G during an evolutionary progression would be an increase in selectivity and in regularity of helical structures as the pyrimidines became involved in nucleic acid coding. Thus, I and G would have very similar properties with respect to pairing with C and with respect to dimensions of the I·C and G·C pairs. G, however, being unable to pair with A as well as C, would have greater selectivity than I. It is possible, in fact, that the lower pairing selectivity of I may have had an evolutionary advantage in certain specific situations and may account for its presence in the initial position of several tRNA anticodons. If it is necessary to recognize C, U, and A in the terminal codon position, as with arginine in *E. coli,* for example, I is the only base which can fill this function, since G cannot pair with A. In this case a single tRNA molecule recognizes six different codons.

Relevance to Model for Mutagenesis by BrU and Mispairing of BrU to G. An interesting model to account for mutagenesis by BrU and mispairing of this base to G has been proposed by Bugg et al. (1971). They postulate that a tendency of Br to stack over specific regions of adjacent bases is sufficiently favorable to cause a rotation and translation of the BrU residue to give the G·U geometry indicated in Figure 23 (Supplementary Material). In this position, with a presumably favorable stacking interaction between Br and the adjacent base, there is a single hydrogen bond from G N(1) to Bru O(4). While the stability of this one-bond pair may be questionable, there is a further serious problem in satisfying the hydrogen-bonding potential of two protons to water. These are the N(3) proton of BrU and one of the amino protons of G. There is not space for a water molecule to accept a hydrogen bond from

either proton (Figure 23, Supplementary Material). A water oxygen bonded to G N(2)H would need to be roughly 1.5 Å from N(3) of BrU and oxygen bonded to N(3)H of BrU would be less than 2.3 Å from N(2) of G in the structure proposed by Bugg et al. (1971). Variations in structure which retain the N(1)···O(4) hydrogen bond and the approximate stacking arrangement proposed may increase the water–base distances somewhat, but not enough to allow both base protons to form hydrogen bonds to water. We therefore conclude, for reasons set forth in previous sections, that the proposed G·T or G·U pairings (unlike the wobble pairings) would not be formed.

Supplementary Material Available

Figures 2, 5, 11, 12, 14, 15, 17, 18, and 20–23 plus details of the synthesis of 8-aminoadenosine 5′-diphosphate (11 pages). Ordering information is given on any current masthead page.

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