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Poly(8-aminoguanylic acid): Formation of Ordered Self-Structures and Interaction with Poly(cytidylic acid)†

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ABSTRACT: Poly(8-aminoguanylic acid) has in neutral solution a novel ordered structure of high stability. The 8-amino group permits formation of three hydrogen bonds between two residues along the "top", or long axis, of the purines. The usual hydrogen bonding protons and Watson–Crick pairing sites are not involved in the association. The bonding scheme has a twofold rotation axis and is hemiprotonated at N(7). Poly($8NH_2G$) is converted by alkaline titration (pK = 9.7) to a quite different ordered structure, which is the favored form over the range ~pH 10–11. The bonding scheme appears to be composed of a planar, tetrameric array of guanine residues, in which the 8-amino group does not participate in interbase hydrogen bonding. Poly($8NH_2G$) does not interact with poly(C) in neutral solution because of the high stability of the hemiprotonated G·G self-structure. Titration to the alkaline plateau, however, permits ready formation of a two-stranded Watson–Crick helix. In contrast to the monomer $8NH_2GMP$, poly($8NH_2G$) does not form a triple helix with poly(C) under any conditions. The properties of the ordered structures are interpreted in terms of a strong tendency of the 8-amino group to form a third interbase hydrogen bond, when this possibility is not prevented by high pH.

In ordered structures of nucleic acids and polynucleotides the amino groups perform a vital function in forming the hydrogen bonds upon which specificity of pairing depends. We have investigated the role of amino groups in determining the geometry and stability of base-pairing interactions by introducing additional amino groups into the purine rings of both monomers and polynucleotides (Howard et al., 1966a,b; Ikeda et al., 1970; Hattori et al., 1975a). These studies have shown that when a new hydrogen bond can be formed by the introduced amino group there is a marked elevation of the transition temperature of the complex formed by the modified purine (Howard et al., 1966a,b; Ikeda et al., 1970; Hattori et al., 1975a). Conversely, when a new hydrogen bond can be formed in only one of several possible bonding schemes, formation of such a bond can be used to elucidate the geometry of pairing (Ikeda et al., 1970; Hattori et al., 1975a).

We have introduced an 8-amino group into poly(G) and report here the synthesis of the polymer and the effect of this chemical perturbation on the ordered structures which

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Table I: Spectroscopic Data.

(a) Ultraviolet

Material	λ_{max}	ϵ_{max}	λ_{min}	ϵ_{min}
$8NH_2GMP$[a]	251.5	16900	224	4700
(pH 1)	289.5	10000	272.5	7600
(pH 7.4)	258	16700	226	3800
	294.5	9700	281.5	7800
(pH 13)	260	14600	230	4400
	~280	11800 (sh)		
$8NMe_2GMP$[a]	264.5	17300		
(pH 1.2)[a]	~294 (sh)			
(pH 7.3)[b]	263.5	16600		
	~294 (sh)			
(pH 12.4)	270	15400		
Poly($8NH_2G$)	253	11400	225	3000
(pH 7)[b]	295.5	4100		
(pH 9.76)[c]	256	12800	222.4	3810
	290	5800		
Poly($8NH_2G$)·poly(C) (pH 9.76)[c]	261.8	9970	228	4600

(b) Circular Dichroism

Material	λ_{max}	$\epsilon_L - \epsilon_R$	λ_{min}	$\epsilon_L - \epsilon_R$
$8NH_2GMP$	258	+0.190	292	−0.447
(pH 7)[d]	218	+1.46	~200	~−1.7
$8NMe_2GMP$	294	+0.868	266	−1.52
(pH 7.3)[e]	~249 (sh)	~−0.4		
	219	+4.05	<205	~−5
8-Dimethylaminoguanosine	292	+0.527	271	−0.175
(pH 7.3)[e]	249	+0.50		
	217	+3.97	<205	~−5
Poly($8NH_2G$)[f]	311	+2.33	291	−0.43
(pH 5.4)	266	+3.52	244	−3.52
	215	+3.03		
(pH 7.0)	312	+2.06	291	+0.10
	266	+3.20	244	−2.91
	217	+4.08		
(pH 9.76)	305	+9.37	282	+0.69
	274	+1.36	257	−5.43
	221	+3.29		
(pH 10.1)	306	+9.69	285	+0.90
	275	+2.15	254	−6.44
Poly($8NH_2G$)·poly(C) (pH 10.1)[f]	272.5	+8.71	297	−2.92

(c) Infrared

Material	ν_{max}	ϵ_{max}
$8NH_2GMP$	1659	1430
(pD 6.5; 36°)[g]	1609	656
	1574	1060
	1558	1420
Poly($8NH_2G$)	1708.5	770
(pD 7.5; 10°)[h]	1607	740
	1584.5	1070
	1560	180
Poly($8NH_2G$)	1665.5	1650
(pD 10.6; 57°)[i]	~1605 (sh)	1055
	1584.5	1555
	1560	1130
Poly($8NH_2G$)·poly(C)	1682.5	800
(pD 10.5; 23°)[j]	(1670)	(615)
	1647	640
	1621	490
	~1585 (sh)	280
	1573	385
	1558	340
	~1527 (sh)	130
	1497	285

[a] Na^+, 0.2 *M*; pH adjusted to alkaline range with NaOH, to acid with HCl; 25°. [b] 0.1 *M* Sodium cacodylate; 25°. [c] 0.13 *M* Na^+; 0.05 *M* glycine; 25°. [d] 0.02 *M* Sodium phosphate buffer. [e] 0.01 *M* Sodium phosphate buffer. [f] 0.13 *M* Na^+; 0.05 *M* glycine, pH adjusted with HCl or NaOH (the solution was buffered only at pH 9.7); 25°. [g] 0.3 *M* Na^+. [h] 0.1 *M* Na^+. [i] 0.2 *M* Na^+; 0.05 *M* sodium borate buffer. [j] 0.27 *M* Na^+; 0.03 *M* sodium borate buffer.

poly($8NH_2G$) forms with itself and with poly(C).[1] We have previously examined the same 8-amino substitution in the monomer $8NH_2GMP$ and described two self-structures of the nucleotide (Hattori et al., 1975b) and the triple helix which it forms with poly(C) (Hattori et al., 1975a). In both the polymer–polymer system (present work) and in the monomer–polymer system (Hattori et al., 1975a) the 8-amino group plays a dominant role, yet the contrast between the two systems is almost complete. Both the monomer and the polymer form a hemi-protonated self-structure, of low stability in the former case, and of extraordinarily high stability in the latter. $8NH_2GMP$ forms a very stable triple helix with poly(C) but forms no 1:1 complex (Hattori et al., 1975a). Poly($8NH_2G$), in contrast, forms a two-stranded Watson–Crick G·C helix at pH 10 but does not form a three-stranded G·2C helix under any conditions, because of the high stability of the hemiprotonated G·G self-structure. The information provided by study of the monomer and the polymer is thus complementary, permitting us to determine the effect of the 8-amino group in both two- and three-stranded helices.

Experimental Section

Poly(C), polynucleotide phosphorylase isolated from *Micrococcus luteus* ("Type 15"), and polynucleotide phosphorylase isolated from *Escherichia coli* B ("Type 2") were obtained from P-L Biochemicals. Quantities of both enzyme preparations are expressed as polymerizations units as defined by Klee and Singer (1967).

GDP was obtained from P-L Biochemicals and was further purified by DEAE-Sephadex column chromatography to remove inorganic phosphate.

8-Dimethylamino-5′-guanylic Acid. $8NMe_2GMP$ was prepared by a direct displacement reaction with dimethylamine on 8BrGMP, as described by Ikehara et al. (1969). The nucleotide was purified by gradient elution from Bio-Rad AG1X8 resin and DEAE-Sephadex A-25 and the extinction coefficient determined by inorganic phosphate analysis (Fiske and Subba Row, 1925) after ashing by the procedure of Howard et al. (1971). The extinction coefficients are given in Table I.

8-Aminoguanosine 5′-Diphosphate. 8-Aminoguanosine 5′-monophosphate (Hattori et al., 1975b) (morpholinium salt) (0.72 mmol) was dissolved in 15 ml of aqueous (50% v/v) *tert*-butyl alcohol. A solution of 600 mg (2.9 mmol) of dicyclohexylcarbodiimide in 10 ml of *tert*-butyl alcohol was added dropwise while refluxing. After 3 hr of refluxing the reaction mixture was kept overnight at room temperature. 8-Aminoguanosine 5′-monophosphate was converted almost quantitatively to the phosphomorpholidate. R_{8NH_2GMP} = 5.6 on Eastman chromogram 6065 in *n*-BuOH–concentrated NH_3–H_2O (55:10:35). The reaction mixture was evaporated to small volume in vacuo and added to 10 ml of water. The precipitate was filtered, and the aqueous phase was extracted with ether and evaporated to dryness in vacuo. The morpholidate was further dried by repeated evaporation with dry pyridine. 8-Aminoguanosine 5′-phosphomorphol-

[1] Abbreviations used are: poly($8NH_2G$), poly(8-aminoguanylic acid); $8NH_2GMP$, 8-aminoguanosine-5′-guanylic acid; poly(C), poly(cytidylic acid); poly($8NH_2G$)·poly(C), 1:1 complex of these components; $8NH_2GMP$·2poly(C), 1:2 complex of these components; G·C, 1:1 hydrogen bonded association of guanine and cytosine residues; G·2C, 1:2 hydrogen bonded association of guanine and cytosine residues; G·G, hydrogen bonded association of unspecified number of guanine residues.

idate was insoluble in either pyridine or dimethylformamide, making the pyrophosphate synthesis considerably more difficult than usual. The morpholidate was suspended in 8 ml of Me_2SO or tetrahydrothiophene 1′1′-dioxide. To the suspension was added 4 equiv of tri-*n*-octylamine (or tri-*n*-butylamine) and 4 equiv of phosphoric acid which were dried by repeated evaporation with dry pyridine. The reaction mixture was stirred for 7 days at room temperature. After adding an excess of ether the nucleotide was collected by decanting the ether, dissolved in water, and applied to a column of DEAE-Sephadex (A-25) (2 cm × 30 cm) (bicarbonate form). The column was washed with water and eluted by linear gradient of 1 l. of 0.5 *M* triethylammonium bicarbonate buffer and 1 l. of water. Tubes containing the diphosphate were pooled and concentrated to dryness in vacuo. 8-Aminoguanosine 5′-diphosphate was obtained in a yield of 0.216 mmol (30% from the monophosphate). The product was chromatographically homogeneous and had the following properties: Eastman chromogram sheet 6065 cellulose; R_f 0.07 (2-PrOH–concentrated NH_3–H_2O, 55:10:35); R_f 0.07 (*n*-BuOH–AcOH–H_2O, 5:2:3); uv (0.1 *N* HCl λ_{max} 251.4, 289.2 nm; pH 7.0 λ_{max} 257, 293 nm; 0.1 *N* NaOH λ_{max} 259.5 ~280 nm (sh).

Enzymatic Polymerization by Polynucleotide Phosphorylase. Preliminary experiments indicated that $8NH_2GDP$ was a poorer substrate for polynucleotide phosphorylase than the common nucleotide diphosphates, including GDP. In seeking suitable conditions for polymerization we observed the effect of oligonucleotide primers (Singer et al., 1960), though our enzyme preparation was primer independent for ADP. For a review of GDP polymerization, see Thang and Grunberg-Manago (1968), and other papers there cited. We shall describe briefly several of these preliminary experiments for information they may provide about substrate properties of the nucleotide diphosphate. Since our primary objective was obtaining polymer for physical investigation, we did not pursue the enzymology further after that goal was achieved.

Experiments with E. coli B Enzyme ("Type 2", Described by the Supplier as Suitable for Poly(G) Synthesis). Parallel solutions contained 10 units/ml of enzyme, $MnCl_2$ (5×10^{-4} *M*), Tris-HCl (pH 8.5) (5×10^{-2} *M*), nucleoside diphosphate (4×10^{-3} *M*), and primer if indicated. The reactions were carried out at 60° (Singer et al., 1960) and followed by P_i release. The percent of theoretical P_i release is indicated for each composition at 20 hr (extent of reaction remained nearly constant from 6 to 30 hr). GDP (no primer), 62%; $8NH_2GDP$, ApApA, (1.1×10^{-4} *M*, expressed as trimer), 29%; $8NH_2GDP$, UpUpU, (1.1×10^{-4} *M*), 24%; $8NH_2GDP$, no primer, 14%. The experiment indicated that polymerization was possible, though not favorable, for $8NH_2GMP$ with this preparation of enzyme. GDP was readily polymerized under the same conditions.

Experiments with M. luteus Enzyme ("Type 15"). Polymerization of GDP and $8NH_2GDP$ was compared in the presence of primer. The solutions contained nucleoside diphosphate (4×10^{-3} *M*), $MnCl_2$ (5×10^{-4} *M*), Tris-HCl (pH 8.5) (5×10^{-2} *M*), ApApA (1×10^{-4} *M* in trimer), and 18 units/ml of enzyme (polymerization units) and were incubated at 50°. In 3 hr there was 83% phosphate release with GDP and 35% with $8NH_2GDP$. These values were unchanged in 23 hr.

In a separate experiment with GDP under the same conditions but without primer both P_i release and absorbance at 287 nm were observed. Decrease in absorbance followed increase in P_i. Both became constant in 3 hr, at which time P_i release had reached 82%. These experiments suggested that with this enzyme, as with the *E. coli* enzyme, polymerization proceeds more readily with GDP than with $8NH_2GDP$, and that GDP polymerization is not aided by primer under these conditions.

Polymerizations of $8NH_2GDP$ carried out in the presence of ApApA primer gave 54% yield of isolated, dialyzed polymer in one experiment and 41% in another, though the conditions were the same in both ($8NH_2GDP$ (4×10^{-3} *M*), $MnCl_2$ (5×10^{-4} *M*), Tris-HCl (pH 8.5) (5×10^{-4} *M*), dithiothreitol (8×10^{-3} *M*), albumin (3.5 g/ml), ApApA (10^{-4} *M* in trimer), and *M. luteus* enzyme (18 polymerization units/ml) in a total volume of 5.0 ml). The solution was incubated 17 hr at 50°. A parallel experiment under the same conditions but without primer gave 26% yield. It is the latter preparation of homopolymer that is characterized in this paper. Though there were often variations in quantitative values (whether of P_i release or yield of isolated, dialyzed polymer) with the same batch of enzyme used on different days, we observed no qualitative inconsistencies and conclude that yield of polymer is roughly twice as high in the presence of primer under these conditions.

Synthesis of Poly($8NH_2G$). The reaction mixture contained the following components: $8NH_2GDP$ (4×10^{-3} *M*), $MnCl_2$ (5×10^{-4} *M*), Tris-HCl buffer (pH 8.5) (5×10^{-2} *M*), dithiothreitol (8×10^{-3} *M*), albumin (3.5 mg/ml), and 225 units of *Micrococcus lysodeikticus* polynucleotide phosphorylase ("Type 15") in a total volume of 12.5 ml. The reaction mixture was incubated at 50° for 17 hr and extracted several times by $CHCl_3$–isoamyl alcohol (3:1 v/v) and dialyzed against 2 l. of 0.5 *M* sodium chloride–0.001 *M* EDTA–0.01 *M* Tris-HCl (pH 7.3), 2 l. of 0.5 *M* sodium chloride–0.01 *M* Tris-HCl (pH 7.3), 2 l. of 0.1 *M* sodium chloride–0.01 *M* Tris-HCl) (pH 7.3), and 2 l. of distilled water two times. The aqueous solution was freeze-dried to give 13 μmol (26% yield). Before measuring the physicochemical properties the polymer was further purified by passing it through Sephadex G-25 (2 × 110 cm) and freeze-dried to yield 9.8 μmol of purified polymer. Extinction coefficients were determined by analysis for inorganic phosphate (Fiske and Subba Row, 1925) after digestion of polymer by a procedure described previously (Howard et al., 1971); λ_{max} 253 (11400) and 295 nm (4100) in 0.1 *M* sodium cacodylate buffer (pH 7.0).

Poly($8NH_2G$) was subjected to alkaline hydrolysis by incubating in 0.3 *M* KOH at 37° for 18 hr. The solution was neutralized with HCl and buffered at pH 7.0 with sodium cacodylate. Spectra of the polymer and of the neutralized alkaline hydrolysate are shown in Figure 1. The ultraviolet spectrum had λ_{max} 257.5 nm (ϵ_{max} 16340); λ_{max} 294 nm (ϵ_{max} 9180). These values for 2′(3′)-$8NH_2GMP$ resulting from alkaline hydrolysis of the polymer may be compared with those of 5′-$8NH_2GMP$ observed previously (5): λ_{max} 258 nm (ϵ_{max} 16700); λ_{max} 294.5 nm (ϵ_{max} 9730). As with AMP and GMP the molar absorbance of the 2′(3′)-nucleotide is slightly lower than that of the 5′-nucleotide (2.6% for AMP and 4.4% for GMP (Pabst Laboratories, Circular OR-17). The absorbances in the present case are 2.1% lower for the 2′(3′) nucleotide at 258 nm and 5.6% lower at 295 nm. The agreement confirms the chemical composition of the polymer and the values of molar absorbance determined by phosphate analysis.

The general methods of preparing solutions for measuring infrared spectra have been described previously (Miles,

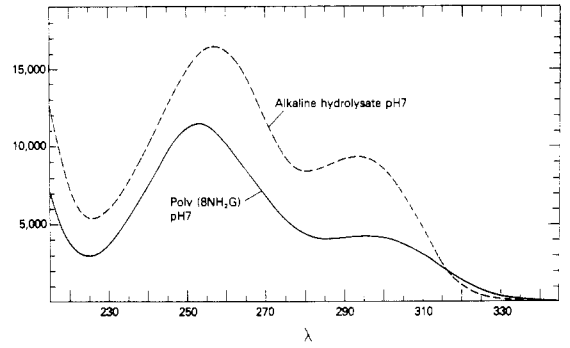

FIGURE 1: Ultraviolet spectra of poly($8NH_2G$) and of its alkaline hydrolysate, readjusted to pH 7 (see Experimental Section). Absorbance of the longer wavelength peak increases by 120% on hydrolysis of the polymer and that of the more intense transition by 43%. Plotted as molar absorbance (ϵ) vs. wavelength (λ) in nm.

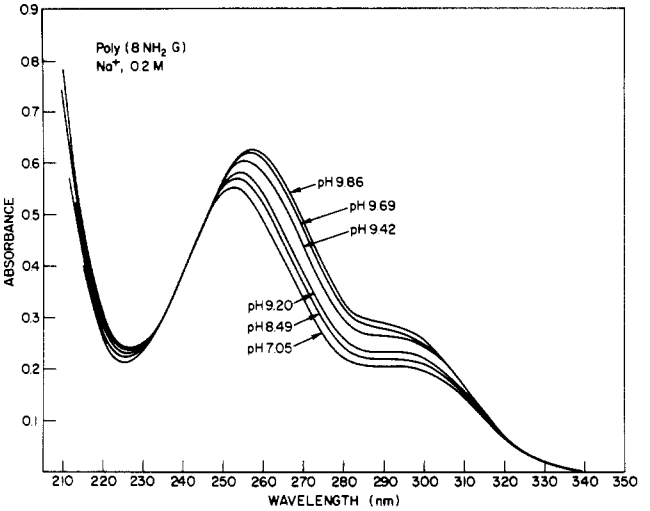

FIGURE 2: Ultraviolet spectra at 25° of poly($8NH_2G$) solutions of increasing pH. Polymer concentration, 4.89×10^{-5} *M*, Na^+, 0.2 *M*.

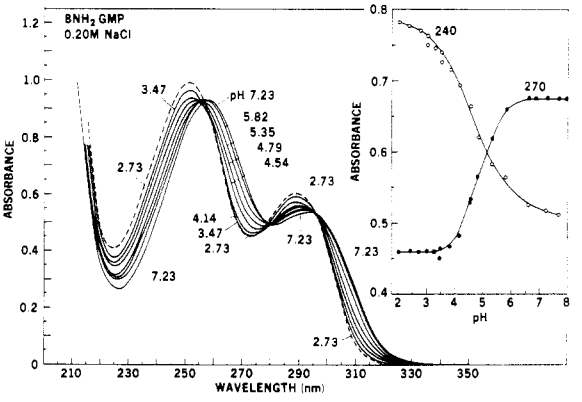

FIGURE 3: Ultraviolet titration of $8NH_2GMP$ in the acid range. Protonation at N(7) results in a small decrease in λ_{max} and slight increases in intensity for both transitions (cf. Table I). Isosbestic points are observed at 279 and 296 nm, and, with the exception of the highest and lowest pH values, at 255 nm. p*K* = 4.8 in 0.2 *M* Na^+.

1971 and references there cited), but were somewhat modified to measure poly($8NH_2G$) in alkaline D_2O. Because of the sharp dependence of G·G structures on pH and the relatively narrow plateau between two transitions, we carried out optical titrations at 260 nm in D_2O with a Zeiss PMQII spectrometer and Radiometer No. 26 pH meter. In this way we could be sure that a given pD value measured with the pH meter corresponded to the desired part of the titration range. The midpoints of the optical titration curves in D_2O (0.19 *M* Na^+) corresponded to pH meter readings of 9.3 and 11.2 or pD values (Glascoe and Long, 1960) of 9.7 and 11.6. Borate buffer was prepared by titrating boric acid with NaOD to a pD of 10.7. Helium was passed through the solutions during addition of base. The solutions were closed with Teflon stoppers during optical measurements. For the G·C experiments the same procedure was followed, but poly(C) was added before alkaline titration. After addition of buffer the solutions were lyophilized and redissolved in D_2O for spectroscopic measurement. The final concentrations of polymers, salt, and buffer are given in the figure legends.

Ultraviolet spectra were measured with a Cary 15 spectrometer. Circular dichroism was measured with a Cary 60 spectropolarimeter equipped with a Model 6001 CD attachment using square 1-cm cuvettes. Temperature was controlled by circulating water through a brass block constructed to fit the chamber and insulated with foamed polyethylene. Data were transmitted on-line to a Honeywell DDP-516 computer. Data reduction, scaling to a molar basis, averaging of multiple runs, and summation of spectra were carried out by the computer.

Infrared spectra were measured with Beckman IR7 spectrometer, using calcium fluoride cells and D_2O solutions, as described previously (Miles, 1971). The spectra were transmitted on-line to the computer referred to above to be normalized, smoothed, and drawn by a peripheral Calcomp plotter. Molar absorbance (ϵ), expressed in terms of molarity of repeating units in the polymer chains, is calculated from the expression $\epsilon = A/ClE$, where *A* is absorbance, *l* is path length in cm, and *E* is ordinate scale expansion factor.

Attempts to detect interaction of poly($8NH_2G$) with poly(C) at or near neutral pH were entirely negative. In order to define objectively this lack of reactivity, we summarize here the experimental conditions and methods of detection employed: (a) total polymer concentration, 8×10^{-5} *M*, mole fraction poly(C) from 0.10 to 1.0; pH 7; 25°; reaction times, 20 hr, 3 days, 8 days; mixing curves at 253 nm, 260 nm, 300 nm showed strictly linear dependence on composition; (b) 1:1 mixture of polymers, conditions of (a) but heated to 93° for 30 min and recooled; uv spectrum identical with summation of components; (c) 2:1 mixture of poly(C) and poly($8NH_2G$) and sodium cacodylate, 0.1 *M*, pH 6.5; after 8 days both uv and CD curves indistinguishable from computer-generated summations of spectra of the components; (d) same as (c) but heated to 90° for 1.5 hr, cooled slowly, and allowed to stand 20 hr; uv spectrum was the same as before heating.

We are indebted to Mrs. Marie Chang for writing many of the necessary programs for reducing the circular dichroism and infrared data.

Results and Discussion

(*a*) *The Homopolymer Poly(8NH₂G). Ultraviolet Spectra.* Poly($8NH_2G$) has ultraviolet maxima at 253 nm (ϵ 11400) and 295.5 nm (ϵ 4100) at 25° and pH 7 (Figures 1 and 2; Table I). These molar absorbances are much lower than those of the unassociated monomer $8NH_2GMP$ (λ_{max} 258 (ϵ_{max} 16700); λ_{max} 294.5 (ϵ_{max} 9700) (Table I; Figures 3 and 4; Hattori et al., 1975b), suggesting the presence of an ordered structure. We defer a more detailed comparison of the spectra and their pH dependence to a later paragraph

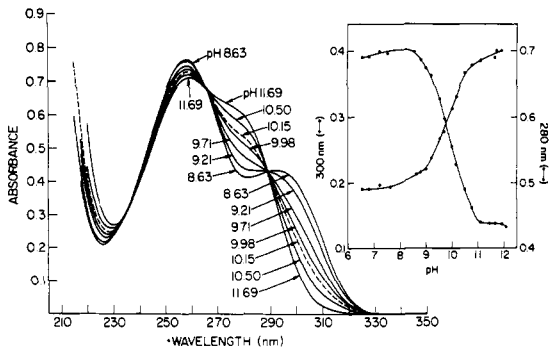

FIGURE 4: Ultraviolet titration of $8NH_2GMP$ in the alkaline range. Loss of the proton at N(1) causes a small decrease in absorbance of the 256-nm peak, with little change of wavelength. The peak at 293 nm increases significantly and shifts to ~280 nm (shoulder) with a significant increase in absorbance. Isosbestic points are observed at 289 and 265.5 nm. pK = 9.7 in 0.2 M Na^+.

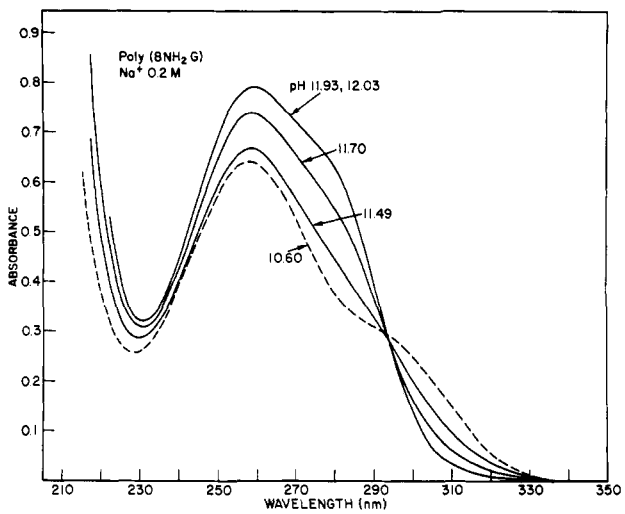

FIGURE 5: Ultraviolet spectral changes of poly($8NH_2G$) in the alkaline range. The process occurring here, N(1) deprotonation, is the same as that observed in the monomer in less basic solution (Figure 4) and the spectral changes are similar. Here an isosbestic point occurs at 293 nm.

and consider now the temperature dependence at neutral pH.

The ultraviolet spectrum of poly($8NH_2G$) at pH 7, 0.1 M Na^+ remains essentially unchanged over the range 10–90°. Temperature profiles at 272.5, 252.5, and 300 nm show no melting, indicating an extraordinarily stable structure. The same result was observed in 0.001 M Na^+.

We now turn our attention to the state of ionization of the guanine residues in the stable self-structure of the polymer which is present in neutral solution. A major chemical effect of the $8NH_2$ group is a large increase in basicity of the base. Protonation occurs at N(7) of the imidazole ring in the mononucleotide (Hattori et al., 1975b; Miles et al., 1963). The pK of $8NH_2GMP$ determined by ultraviolet titration is 4.7 (Figure 3), and the maxima shift from 257.5 and 293.2 at pH 7.2 to 251.3 and 289.1 in the protonated base at pH 3.5 (Figure 3). Isosbestic points occur at 279 and 296 nm. Alkaline titration of the nucleotide, corresponding to loss of N(1)–H and formation of the enolate anion (Miles et al., 1963), shows a pK of 9.9 (Figure 4).

In the acid range poly($8NH_2G$) shows no cooperative pH transition, though there is a gradual, relatively small increase in absorbance between pH 5 and pH 3 at 250 and 290 nm.

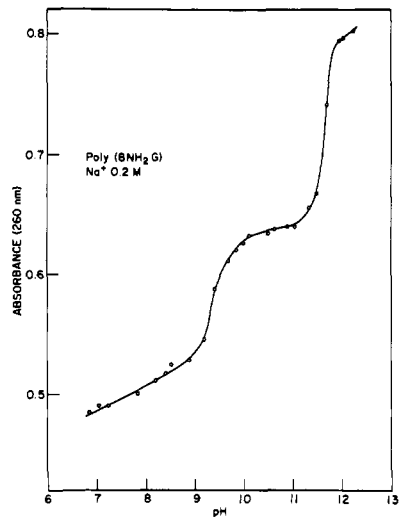

FIGURE 6: Ultraviolet titration of poly($8NH_2G$) in 0.2 M Na^+. The pK at 9.4 corresponds to loss of N(7)–H and that at 11.7 to loss of N(1)–H. See Figure 7 for the equations and text for discussion.

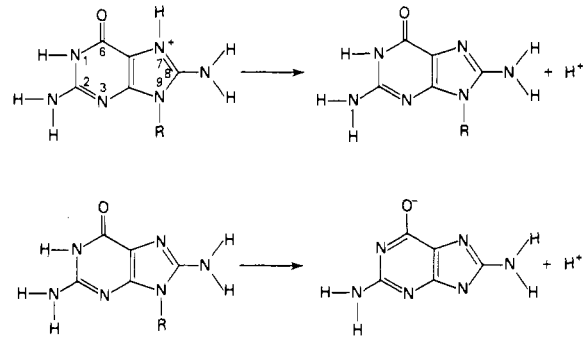

FIGURE 7: The first equation shows loss of N(7)–H from $8NH_2G$ residues and the second dissociation of N(1)–H, with formation of enolate anion (cf. text).

In the alkaline range poly($8NH_2G$) shows two cooperative pH transitions, the first at 9.4 and the second at 11.7 (Figures 2, 5, and 6).

Beginning with the maximally protonated guanine ring there are two deprotonation steps to be accounted for as the pH is increased (Figure 7). We assign the lower of the observed pK's (9.4) to loss of a proton from N(7) and the higher pK (11.7) to deprotonation at N(1). The first assignment implies a remarkable increase of 4.7 pH units in the pK for titration at N(7) and constitutes additional evidence of very high stability of the ordered form. The structural basis of this large pK shift is presented in a later section.

(*b*) *Circular Dichroism.* The monomer 5′-$8NH_2GMP$ has a maximum at 258 nm ($\Delta\epsilon$ +0.190) and a minimum at 292 nm ($\Delta\epsilon$ −0.447) (Figure 8). The nucleoside 8-aminoguanosine has essentially the same spectrum above 240 nm but differs in having a maximum at 218 nm ($\Delta\epsilon$ +1.46) and a minimum near 200 nm ($\Delta\epsilon$ ~−1.7). Ikehara et al. (1972) reported that introduction of a 5′-phosphate group into syn nucleosides (8-bromoadenosine, 8-(2-hydroxypropyl)-2-adenosine, and 8-bromoguanosine) caused major changes in the circular dichroism of the longer wavelength transitions, in contrast to the anti nucleosides, which show little change with the same substitution. The authors proposed that an

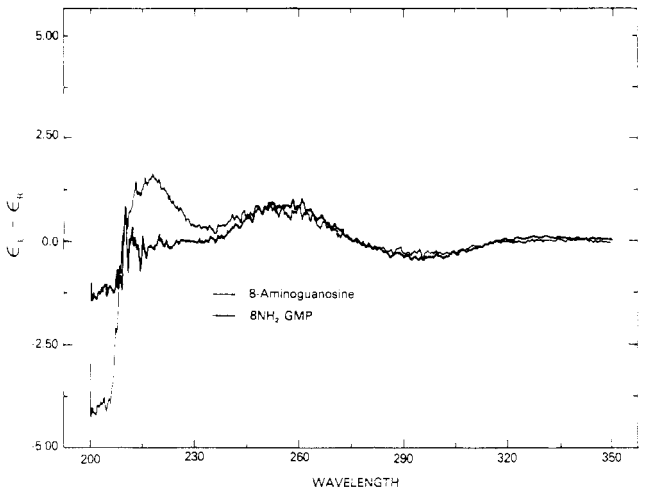

FIGURE 8: Circular dichroism of 8-aminoguanosine (light line) and $8NH_2GMP$ (heavy line). The similarity of the spectra above ~245 nm is consistent with an anti conformation of both molecules (cf. text). Each spectrum was measured five times, then averaged and smoothed by computer.

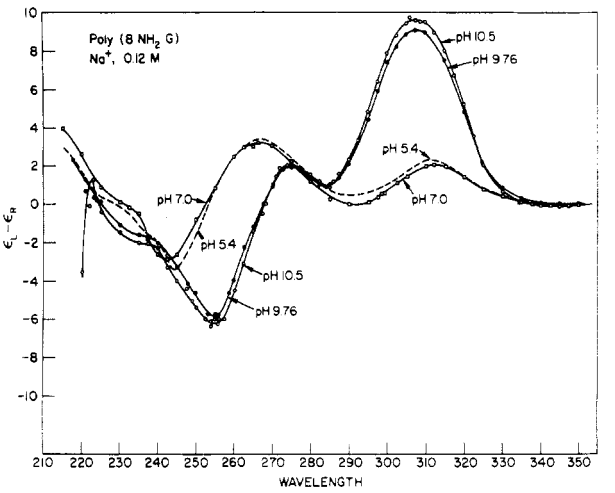

FIGURE 9: Circular dichroism of two ordered forms of poly($8NH_2G$) in 0.12 *M* Na^+. The spectra observed at pH 5.4 and 7.0 are characteristic of the hemiprotonated self-structure (A, Figure 12) and those at pH 9.76 and 10.5 of a second ordered form, probably structure B, Figure 14. See text for discussion of spectra.

observed change in circular dichroism on 5′-phosphorylation of a purine nucleoside can be used to distinguish syn from anti conformations. It is reasonable to expect a greater perturbation of the electronic transitions of the base by a syn than by an anti 5′-phosphate because of the greater proximity of the phosphate to the base in the former. The nature and magnitude of the perturbation are, however, difficult to predict in advance. We have observed an additional pair, 8-dimethylaminoguanosine and its 5′-monophosphate, which is closely related electronically to the subject of our present study, but which has an 8-substituent sufficiently bulky to exclude the anti range of conformations. We find that the spectra are indeed quite different (Table I). The nucleoside has a weak minimum at 271 nm ($\Delta\epsilon$, −0.175) whereas the nucleotide has a minimum of much larger magnitude at 266 nm ($\Delta\epsilon$, −1.52). The long wavelength maximum of the nucleotide is roughly 65% more intense than those of the nucleoside. Circular dichroism of the nucleoside 8-dimethylaminoguanosine has been published (Miles et al., 1971), but that of the nucleotide has not been reported. On the basis of the criterion proposed earlier (Ikehara et al., 1972) and the data reported here (Figure 8; Table I), it appears that 8-aminoguanosine and $8NH_2GMP$ have anti conformations.

The circular dichroism spectrum of poly($8NH_2G$) in neutral solution has maxima at 312 and 266 nm and minima at 291 and 244 nm (Figure 9; Table I). The pair of extrema at 266 and 244 nm are consistent with exciton splitting (Tinoco, 1964) of a transition observed at 255 nm in the ultraviolet spectrum (Figure 2). The higher wavelength extrema do not appear to have such an origin since the negative extremum (291 nm) occurs near the ultraviolet maximum at 295 nm. The maximum at 312 nm in the circular dichroism curve may arise from a transition too weak to be observed in the ultraviolet spectrum. The spectrum changes little between pH 5 and pH 7 but shows large changes above pH 9. There is a cooperative pH transition with pK = 9.2 in 0.1 *M* Na^+ (Figure 6) and an essentially constant plateau spectrum is reached above pH 9.7. The maxima of this spectrum occur at 305, 274, and 221 nm and the minima at 282 and 257 nm. A large increase in amplitude of the extrema is observed on going from pH 7 to 10 (Figure 9). There is a loss of exciton splitting of the 258-nm transition, which appears to be present at pH 7 (CD extrema 266 and 244 nm) on going to pH 10 (uv_{max} 258 nm, CD_{min} 257 nm). At pH 10 the pair of extrema at 305 and 282 nm may result from splitting of a transition at 294 in the ultraviolet spectrum.

(*c*) *Infrared Spectra.* The spectrum of poly($8NH_2G$) has strong bands at 1710, 1607, and 1584 cm^{-1} in neutral D_2O solution (Figure 10). The highest frequency band is assigned to a carbonyl stretching vibration (Hattori et al., 1975b; Miles et al., 1963; Howard and Miles, 1965) and the others to guanine ring vibrations. A frequency increase is characteristic of most carbonyl vibrations when the base is incorporated into a helix (Miles, 1971 and references there cited), but the magnitude of the shift in this case is the largest we have observed. The unassociated monomer $8NH_2GMP$ has ν_{max} 1659 cm^{-1} in neutral solution and 1684 cm^{-1} when the base is protonated at lower pH (Hattori et al., 1975b), frequencies which are 51 and 26 cm^{-1}, respectively, lower than ν_{max} of the polymer. The unusually high frequency of the polymer carbonyl vibration (1710 cm^{-1}) supports the conclusion that the G ring is protonated.

When the solution of poly($8NH_2G$) is heated the infrared spectrum does not change significantly. Even at 101° the changes are slight (about 10% in intensities of the strong peaks). The very weak absorbance centered at about 1687 cm^{-1} in the spectra at low and intermediate temperature disappears by 90° and is replaced by a very weak broad peak at 1663 cm^{-1}. This weak band remains on cooling to 25° and is not further changed on heating again to 100°. We do not know whether this minor irreversible change results from creation of slight disorder on heating or from the elimination of slight disorder by annealing. The other minor changes observed in Figure 10 are reversible on cooling.

A solution of poly($8NH_2G$) was titrated to an alkaline pD (10.5) chosen to correspond to the plateau region between the two transitions shown in Figure 6. The infrared spectrum at 12° has bands at ~1682 (sh), 1666.5, ~1605 (sh), 1584.5, and 1560 cm^{-1} (Figure 11). We assign the bands at ~1682 (sh) and 1666.5 cm^{-1} to carbonyl stretching vibrations (Hattori et al., 1975a; Howard and Miles, 1965) and the lower frequency bands to guanine ring vibra-

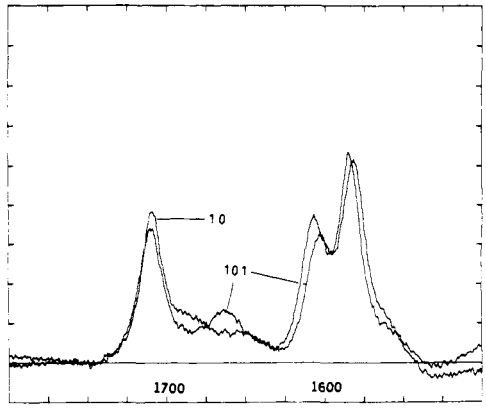

FIGURE 10: Infrared spectra of poly($8NH_2G$) in D_2O solution, pD 7.5, Na^+ 0.1 *M*, polymer 0.01 *M*, temperature 10 and 101°. Ordinate is molar absorbance, and abscissa is frequency in cm^{-1}. Ordinate index marks are 200 units apart, and the molar absorbance is taken to be zero at 1750 cm^{-1}. The spectrum changes little with temperature, but above 90° shows a slight decrease in absorbance near 1685 cm^{-1} and increase at 1665^{-1}. The experiment shows the very high stability of the ordered form of poly($8NH_2G$). The spectra are interpreted structurally in the text.

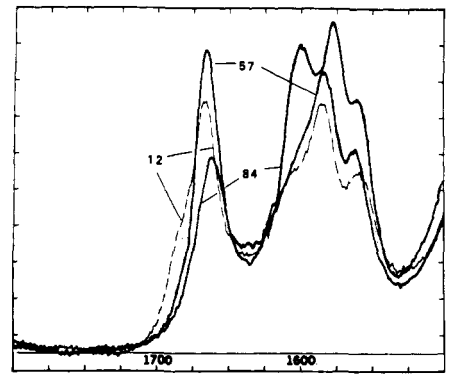

FIGURE 11: Infrared spectra of poly($8NH_2G$) in D_2O solution, pD 10.7, Na^+ 0.2 *M* at 12, 57, and 84°. Concentration of polymer, 0.01 *M* with an uncertainty of approximately ±10%. Ordinate is molar absorbance, and abscissia is frequency in cm^{-1}. Ordinate index marks are 200 units apart. The baseline is taken to be zero at 1750 cm^{-1}, and the first mark has a value of 100. The spectrum characteristic of the predominant ordered form is that observed at 57°. At 12° a minor component is observed with $\nu \simeq 1685$ cm^{-1} (shoulder), and this is converted to the major species by heating above 50°, as reflected by the increase in absorbance and narrowing of the carbonyl band at 1666 cm^{-1} (57°). Further heating causes dissociation to a single-stranded form with the carbonyl band at 1661.5 cm^{-1} and enolate anion bands at 1600 and 1580 cm^{-1}. We estimate that the single-stranded polymer is roughly 60% in the un-ionized form (see text).

tions. Titration to this alkaline pH has completely abolished the intense band seen at 1708.5 cm^{-1} in Figure 10 by removing a proton from N(7) of G and breaking up the stable structure which depends upon this proton (see below). The N(1) proton has, nevertheless, not been removed at pD 10.5. Titration of the N(1) proton in guanosine, inosine, and their derivatives converts these molecules to enolate anions which lack carbonyl bands and have no appreciable absorbance above ~1625 cm^{-1} (Miles et al., 1963; Howard and Miles, 1965). The helix observed at pH 7 has been destroyed by titration but another ordered structure (in fact, two structures; see below) has been created. The band at 1665.5 cm^{-1} is 6 cm^{-1} higher than the monomer value (Hattori et al., 1975b) and the p*K* for the removal of the N(1) proton is 11.7, 2.2 pH units higher than the p*K* for this process in $8NH_2GMP$. Both results indicate the existence of an ordered self-structure. When the solution is heated from 25 to 57° the shoulder at ~1680 cm^{-1} disappears, with a corresponding increase in intensity of the 1665.5 peak (Figure 11), as well as in the ring vibrations at 1584 and 1562 cm^{-1}. We suggest the parallel decrease of absorbance at ~1680 cm^{-1} and increase at 1665.5 cm^{-1} result from thermal conversion of a small amount of one ordered form of poly($8NH_2G$) to the predominant form, having ν_{max} 1665.5 cm^{-1}. The carbonyl band at 1665.5 cm^{-1} remains constant at 70°, then finally shifts to 1661.5 cm^{-1} at 85°. The G·G helix existing at the alkaline plateau has a p*K* of 11.7 (Figure 6), 2 p*K* units higher than the corresponding value for the monomer (Figure 4). As the helix undergoes thermal transition to single-stranded polymer, the p*K* for dissociation of N(1)-H (Figure 7, second equation) will shift downward toward the monomer value. There will, consequently, be partial ionization of G residues at higher temperature, to an extent determined by the experimental conditions and the p*K* value for the single-stranded polymer under those conditions. In Figure 11 the carbonyl band at 1661.5 cm^{-1} observed at 84° is due to un-ionized G residues, and the ring vibrations at 1600 and 1580 cm^{-1} are due to the enolate anion (Table I; compare guanosine anion vibrations at 1591 and 1571 cm^{-1}, Howard and Miles (1965). If we assume that the molar absorbance of the well-resolved carbonyl band (1661.5 cm^{-1}, 84°) is the same as that of the unassociated monomer (1430, Table I, Hattori et al., 1975b) we can estimate that roughly 60% of the G residues are un-ionized under these conditions. The polynucleotide undergoes progressive chain scission caused by the high temperature, high pH, and relatively long time required by the experiment of Figure 11. Since the chemical change is irreversible, the spectrum does not return to its original appearance when the solution is cooled from 85 to 25°.

Structure of Poly($8NH_2G$) in Neutral Solution. We propose structure A (Figure 12) for the ordered form of poly($8NH_2G$) in neutral solution. We advance arguments in the following paragraphs in support of this proposal and apply the experimental evidence to this and possible alternative structures.

We begin with a consideration of the fine structure of the guanine residues, in particular the number and location of the hydrogen bonding protons. The tautomeric form of the guanine is keto-amino on the basis of earlier spectroscopic studies[2] (Hattori et al., 1975a; Miles et al., 1963; Howard and Miles, 1965) and those reported above, permitting us to exclude from consideration those bonding schemes based on enolic or imino forms. The next significant point of chemistry is that ring protonation of G is essential to formation of the poly($8NH_2G$) helix at neutral pH. When this proton is removed by alkaline titration the "neutral" helix is destroyed and converted to a different ordered form, characterized spectroscopically in the previous sections. Finally, the 8-amino group is also essential to formation of the "neutral" helix. Poly(G), which lacks this group, also forms a

[2] The "ortho-quinonoid" form of G, with the proton transferred from N(1) to N(3) and the N(3)-C(2) double bond placed between C(2) and N(1), can also be ruled out by observing the infrared spectrum of a D_2O solution of 3-methylguanine (Townsend and Robins, 1962; Elion, 1962). We find a strong peak at 1628 cm^{-1}, one of moderate intensity at 1610 cm^{-1}, and weak ring vibrations at 1540 and 1516 cm^{-1}. There is no absorbance above ~1640 cm^{-1}, suggesting that the C(6)-O(5) bond has much less double bond character than it does in guanosine.

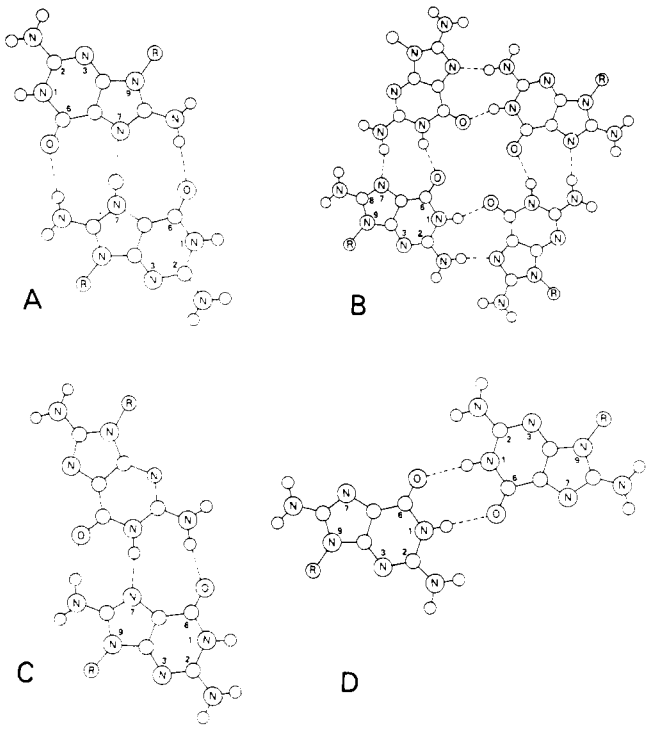

FIGURE 12: Possible hydrogen bonding schemes of ordered forms of poly(8NH_2G). Structure A is that of the polymer at neutral pH and B is probably the major species at pH ~10. A minor species also occurs at pH ~10, possibly with the nonsymmetrical structure C.

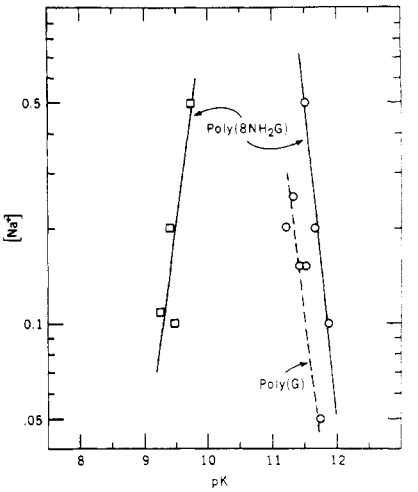

FIGURE 13: Salt dependence of pK of poly(8NH_2G) (solid lines) and of poly(G) (dashed line, data of Pochon and Michelson, 1965). The pK for N(7)-H dissociation has a slight positive dependence on log [Na^+] and that for dissociation of the alkaline "plateau" structure (cf. Figure 6) a slight negative dependence. The parallel dependence of poly(G) at slightly lower pK values is consistent with the dissociation of similar structures in this pH range.

very stable ordered form (Miles and Frazier, 1964; Pochon and Michelson, 1965; Englander et al., 1972), but this self-structure is completely different from that of poly(8NH_2G) at pH 7, as indicated by the distinct spectroscopic properties (Miles and Frazier, 1964; Figure 11) and by lack of a pH transition below pH 11 (Pochon and Michelson, 1965; Figure 13). The structure which poly(8NH_2G) forms at pH 10, however, may be similar to that of poly(G) at neutral pH, as suggested in the following section. For this structure the 8-NH_2 group is not required and plays no essential role. From the evidence that ring protonation and the presence of the 8NH_2 group are both required for existence of the "neutral" structure we conclude that both are involved in hydrogen bonding between 8-aminoguanine residues. Of the possible G·G bonding schemes only structure A meets these conditions. N(7) protonation would prevent the tetrameric structure B from forming. C and d could allow protonation of one or two N(7) positions, respectively, but in neither case would the proton at N(7) play a role in hydrogen bonding. Structure A involves protonation of only one of the paired bases, and resembles in this respect the acid helix of poly(C) (Hartman and Rich, 1965; Akinrimisi et al., 1963). Further protonation of the second base would break up the paired structure, and it is presumably this reaction which is responsible for the gradual absorbance increase below pH 5.

In addition to bonding by the N(7) proton, both 8-amino groups are bonded to 6-carbonyl oxygens in a structure with a twofold rotation axis. We assume that the central hydrogen bond has a double minimum, with an equal probability of finding the proton at a covalent distance from either of the two N(7) atoms, rather than a symmetrical bond (for a discussion of the problems involved in distinguishing symmetrical single and double minima, see Hamilton and Ibers, 1968). A novel feature of this structure is that the three bonding sites lie along the "top" of the purine rings, with the bases offset, the imidazole rings opposite each other, and the pyrimidine rings relatively exposed. The usual Watson–Crick bonding sites and all of the usual hydrogen bonding protons are unpaired. The stereochemistry of the two helices, G·G and G·C, would not permit both to coexist in a four-stranded helix, but it is possible that poly(C) might be able to form a G·C helix at the expense of G·G structure A (Figure 12). In fact this displacement by poly(C) does not occur (see Experimental Section), even under annealing conditions which cause formation of a G·C helix from poly(C) and the stable poly(G) helix. Evidently the very high stability of the poly(8NH_2G) self-structure is responsible for its surprising failure to interact with poly(C) at neutral pH.

The Ordered Form of Poly(8NH_2G) at the Alkaline Plateau (pH 10–11). The foregoing experiments show that alkaline titration of poly(8NH_2G) removes a proton at N(7) (pK = 9.4) with concomitant destruction of one ordered structure and formation of another. The infrared spectrum (Figure 11; cf. discussion in previous section) shows that the polymer still retains its N(1) proton at pH 10–11 and that it has a hydrogen-bonded, ordered structure. Consideration of possible alternative structures (Figure 12, and Hattori et al., 1975b) leads us to suggest hydrogen bonding scheme B (Figure 12) for the predominant form of the polymer in alkaline solution, though the evidence is not direct. The carbonyl frequency observed in the polymer at pD 10.5 (1665.5 cm^{-1}) is the same as that of the ordered form of the nucleotide 5′-8NH_2GMP in neutral solution (Hattori et al., 1975b). The basic hydrogen bonding scheme is the same as that proposed for 3′-GMP (Gellert et al., 1962), poly(G) (Zimmerman et al., 1975), 5′-GMP (Miles and Frazier, 1972), and 5′-8NH_2GMP (Hattori et al., 1975b) in neutral solution. The 8-amino group in this case plays no role in hydrogen bonding, though it is not large enough to interfere with mutual approach of the G residues in structure B (Hattori et al., 1975b). This scheme has the advantage of making the maximum number of hydrogen bonds.

The nonsymmetrical structure C (Figure 12) is also fa-

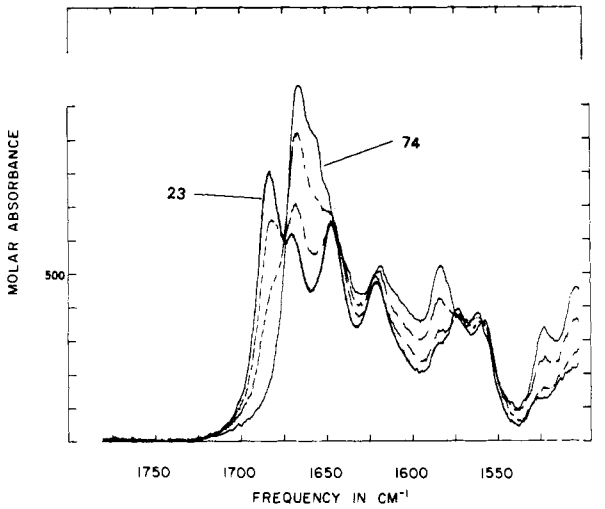

FIGURE 14: Infrared spectra of 1:1 complex formed by poly($8NH_2G$) and poly(C) at pD 10.5, Na^+ 0.27 *M* in D_2O solution, 0.02 *M* in each polynucleotide. We assign the strong bands at 1683 and 1647 cm^{-1} (23°) to a coupled pair of carbonyl vibrations (Howard et al., 1969). This coupling is characteristic of G·C and IC Watson–Crick pairs, and the frequency decrease of a C carbonyl band to ~1647 cm^{-1} is unique to such pairs. The ring vibrations at lower frequencies and the changes they undergo on heating are also characteristic of G·C helices. We attribute the band at 1670 cm^{-1} (23°) to a relatively minor amount of G·G self-structure. Spectra at the lowest (23°) and highest temperatures (74°) are plotted with solid lines and the intermediate temperatures (57.5 and 65°) with dashed lines. The poly(C) dissociates to a single strand, but the poly($8NH_2G$) is converted to an ordered G·G helix with its carbonyl band at 1665 cm^{-1} (cf. Figure 11). At still higher temperature (90°) this is dissociated to a single-stranded form.

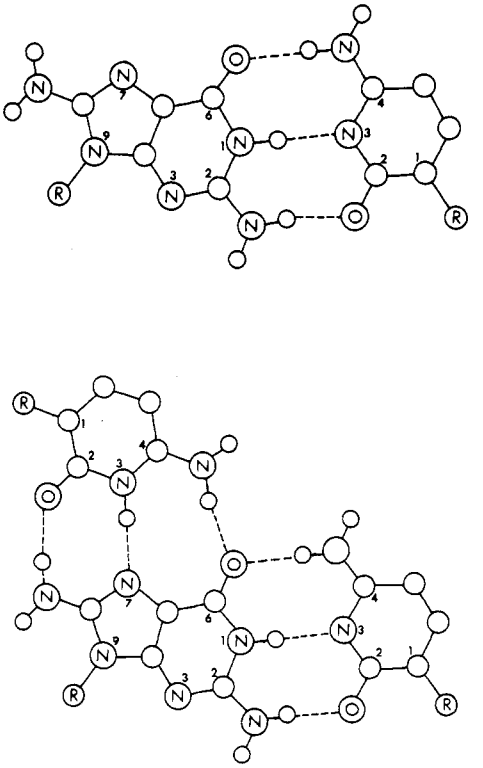

FIGURE 15: Hydrogen bonding schemes of a 1:1 complex formed by poly($8NH_2G$) and poly(C) (top) and of a possible 1:2 complex (bottom). In the two-stranded helix the 8-amino group plays no essential role in the pairing. In the three-stranded complex, however, the 8-amino group forms a third hydrogen bond. This bonding occurs in the monomer–polymer helix $8NH_2GMP$·2poly(C), with large elevation of T_m, but it is prevented from forming with poly($8NH_2G$) by the very stable G·G self-structure (A, Figure 12).

vored by the presence of three hydrogen bonds between the two bases. Possibly one of the minor components revealed in the infrared spectra (Figures 11 and 14) has this bonding arrangement. Structure D appears less attractive than B and C in that only a pair of hydrogen bonds is formed between two bases.

Detailed study of the alkaline form of poly($8NH_2G$) is made difficult by lability of the polymer under these conditions. As noted above, heating the solution for extended periods leads to chain scission.

Interaction of Poly($8NH_2G$) with Poly(C). (a) Neutral Solution. The great difficulty in achieving G·C interaction with poly($8NH_2G$) is that the highly stable hemi-protonated G·G self-structure must be disrupted before G·C helices can be formed. As we have noted above, although all of the Watson–Crick bonding sites remain available in the hemiprotonated G·G structure, this helix would be sterically incompatible with simultaneous pairing of poly(C) to form a Watson–Crick helix (Figure 15). A three-stranded G·2C helix would have a favorable bonding scheme (Figure 15) and would presumably be very stable since the monomer–polymer $8NH_2G$·2C complex is much more stable than the corresponding complex which lacks the 8-amino group (Hattori et al., 1975a). Like the 1:1 G·C complex, however, a polymer–polymer G·2C complex would require disruption of the G·G helix. It is clear from experiments (a) and (b) (Experimental Section) that this disruption does not occur at neutral pH.

Since a three-stranded G·2C helix would have three interbase hydrogen bonds on the imidazole side of the guanine ring as well as on the pyrimidine side (Figure 15) the structure is potentially very favorable. We have prepared a triple helix having this structure by interaction of the monomer 5′-$8NH_2GMP$ with poly(C) and find that the transition temperature is elevated 60° above that of the G·2C complex formed by GMP under the same conditions (Hattori et al., 1975a). Our attempts (Experimental Section, experiments (c) and (d) in final summary), to obtain evidence for an analogous complex with poly($8NH_2G$), however, were entirely negative. Though both the G·C and G·2C helices would undoubtedly be intrinsically very stable (cf. following sections), the even higher stability of the hemi-protonated G·G self-structure evidently prevents G·C interaction at pH values near neutrality. The contrasting ready formation and high stability of the G·2C monomer–polymer complex (Hattori et al., 1975a) also support this interpretation. Further confirmation is provided by the experiments described below.

(b) Alkaline Solution. Having found no reaction of the hemiprotonated G·G helix with poly(C), we examined reactivity of the alkaline G·G self-structure toward poly(C) in the pH range 10–11. Titrating the solution to the precise region between the two transitions (cf. Figure 6) had the desired effect, permitting formation and characterization of a 1:1 G·C complex.

Ultraviolet Spectra and Mixing Curves. Poly($8NH_2G$) and poly(C) were mixed (0.1 *M* Na^+, pH 10, 25°) and ultraviolet spectra measured as a function of time. The spectrum became essentially constant in 5 hr, showing only negligible changes at 8 and 20 hr. The absorbance at 260 nm reached half of its final change in 20 min and three-quarters of its final change in about an hour. The ultraviolet spectrum of the G·C complex is shown in Figure 16 super-

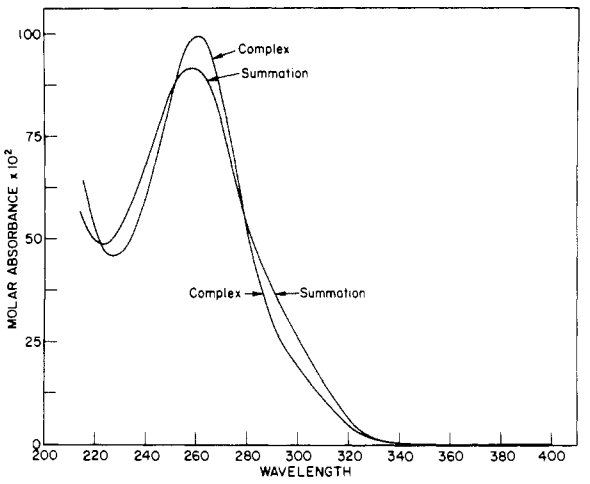

FIGURE 16: Ultraviolet spectrum of a 1:1 mixture of poly(8NH_2G) and poly(C) (pH 9.75, 0.13 *M* Na^+, 25°). Deviation from a summation of the components indicates that complex formation occurs at this alkaline pH.

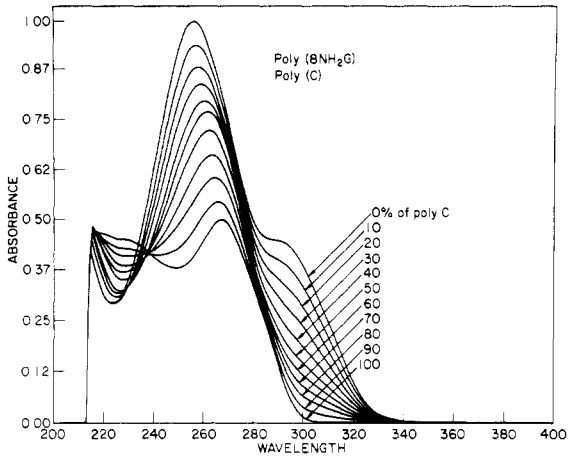

FIGURE 17: Ultraviolet spectra of mixtures of poly(8NH_2G) and poly(C) in 0.05 *M* glycine buffer (pH 9.75) (Na^+, 0.13 *M*; 25°; total polymer concentration of each solution, 7.74×10^{-5} *M* in repeating residues). The spectra exhibit isosbestic points at 238 nm for mole fraction of poly(C) ≥ 0.5 and at 229 nm for values ≤0.5. These results are consistent with their being on either side of $X_c = 0.5$ only two absorbing species: a 1:1 complex and that polymer which is in excess.

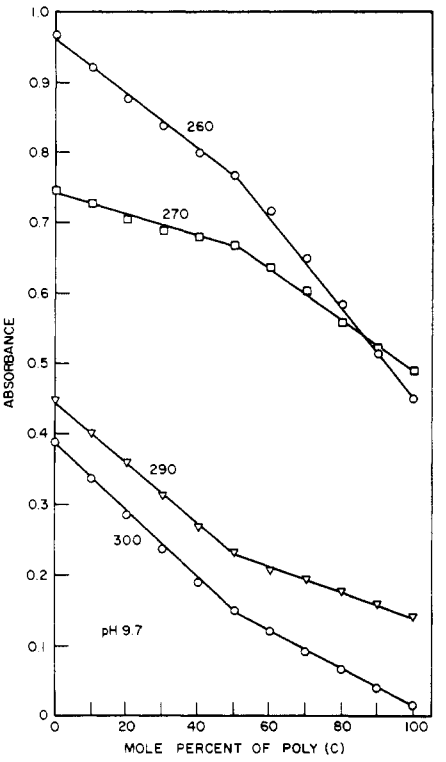

FIGURE 18: Ultraviolet mixing curves, conditions of Figure 17. A single discontinuity is observed at a mole fraction of poly(C) of 0.5, indicating formation of a 1:1 complex.

imposed on a summation spectrum of the components, measured under the same condition. The complex has a higher absorbance than the components near the maximum at 262 nm but a lower absorbance at higher wavelengths (>279 nm). This relation is the opposite of that observed with most polynucleotide systems, though in the present case the summation includes one component which is itself highly ordered and hypochromic.

An ultraviolet mixing experiment was carried out at pH 9.75 and 25° (Figures 17 and 18). The spectra, measured after 1 day (Figure 17), show a continuous and systematic progression as the mole fraction changes. Mixing curves show a clear discontinuity at all wavelengths at 50 mol % poly(C) and not at any other composition. We conclude that a 1:1 complex and only a 1:1 complex is formed.

Circular Dichroism. The circular dichroism of a 1:1 mixture of poly(8NH_2G) and poly(C) changes progressively as the pH is increased from 7 to 9.5 and then remains constant to pH 11 (Figure 19). Cooperative titration curves observed at 280 and 295 nm (Figure 19, inset) are parallel to those observed with poly(8NH_2G) alone (Figure 20), but the p*K* is ~0.2 pH unit lower in the mixture than in the poly(8NH_2G) alone.

At pH 9.5 the spectrum of the 1:1 complex has a minimum at 296.5 ($\Delta\epsilon$ − 3.14) and a maximum at 271.6 ($\Delta\epsilon$ 8.44) (Figure 19). We suggest the minimum is due to the 8NH_2G ultraviolet transition at ~295 nm in the monomer and polymer (Table I) and that exciton splitting of this transition does not occur to an appreciable extent. In contrast, this transition does appear to undergo splitting in the alkaline form of poly(8NH_2G) itself (cf. Figure 9 and preceding sections). The maximum at 271.6 nm presumably arises from both G and C transitions, but at present we have no objective means of resolving it.

Because the changes in circular dichroism that occur when the G·C helix is formed are greater than the ultraviolet changes (cf. Figures 16 and 19), the former method is more favorable for monitoring thermal transitions of the complex. The circular dichroism changes progressively with temperature (Figure 21), and the temperature profiles observed at the extrema of the complex (296 and 273 nm) show a cooperative thermal transition with $T_m = 66°$. The solution had been exposed to elevated temperatures for several hours by the end of the melting run because of relatively slow recording of the spectra and the time required for thermal equilibration. For this reason there was undoubtedly extensive chain scission of the polyribonucleotides under these conditions, and the observed spectroscopic changes were not reversed on cooling the solution.

Infrared Spectra. The infrared spectrum of a 1:1 mixture of poly(8NH_2G) and poly(C) at pD 10.5 and 23° (Figure 14) is in its principle features characteristic of a two-stranded, Watson-Crick G·C pair (Howard et al., 1969). The strong carbonyl bands at 1683 and 1647 cm^{-1} are members of a vibrationally coupled pair of G and C carbonyl stretch-

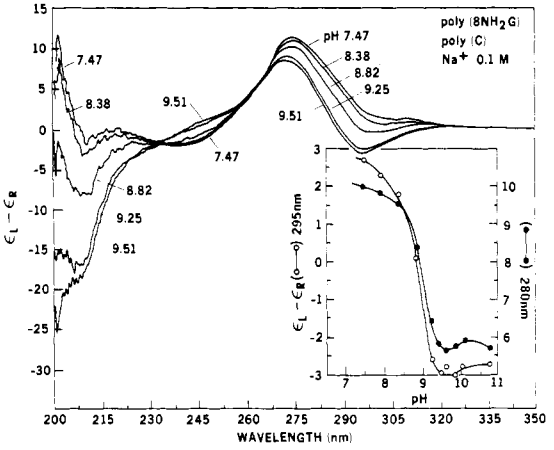

FIGURE 19: Circular dichroism of a 1:1 mixture of poly(8NH_2G) and poly(C) as a function of pH (Na^+, 0.1 *M*; borate buffer, 0.01 *M*; 25°). At neutral pH the spectrum is the same as a summation of its components, but as the pH increases the spectra change progressively, then become constant between pH 9.5 and 10.5 (for discussion of spectra, see text). pH profiles at 295 nm (inset, O) and 280 (●) correspond, respectively, to an 8NH_2G transition and primarily to a C transition (at 280 nm poly(8NH_2G) has at pH 9.76 and 10.5 $\Delta\epsilon \simeq +1.5$, whereas the value in this experiment is ~+10 at neutral pH and ~+6 at the alkaline plateau). The profiles are cooperative and parallel, showing a pH-dependent interaction of G and C with p*K* = 8.96.

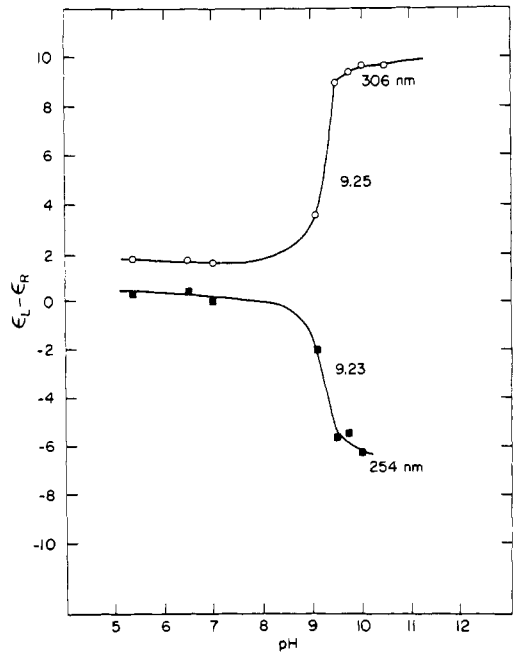

FIGURE 20: A circular dichroism titration of poly(8NH_2G) (0.12 *M* Na^+, 25°) in the absence of poly(C) may be compared with Figure 19 (inset), in which both polymers were present. Here the p*K* of the transition is slightly higher than in Figure 19, but the two titrations clearly indicate that alkaline dissociation of the "neutral" G·G self-structure is necessary for G·C pair formation.

ing vibrations (Howard et al., 1969). As a result of this coupling the frequency of one member of the pair is increased from its unperturbed value (to 1683 cm^{-1}) and the other is decreased (to 1647 cm^{-1}). A decrease from an unperturbed carbonyl frequency is unique to G·C and I·C pairs (Howard et al., 1969) and is thus an especially valuable diagnostic feature of the spectrum. Three-stranded G·2C helices do not show this decrease (Hattori et al., 1975a; Howard et al., 1964; Miles, 1968) and the lowest frequency carbonyl band

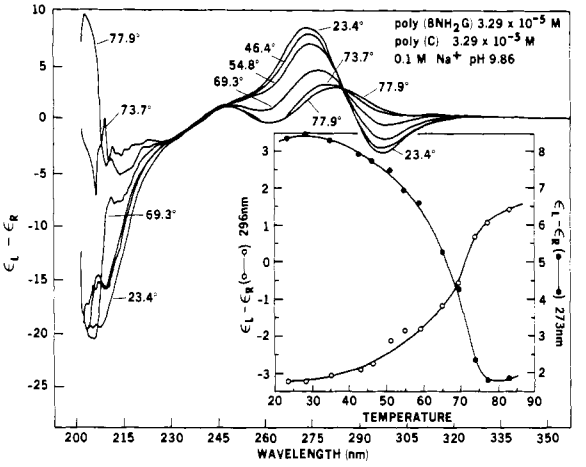

FIGURE 21: Temperature dependence of circular dichroism of poly(8NH_2G)·poly(C) in 0.01 *M* borate buffer, pH 9.86, 0.1 *M* Na^+. The transition temperature is 66° under these conditions. The temperature profiles at 296 and 273 nm monitor predominantly G and C transitions, respectively, as noted in Figure 22, showing that the spectral changes result from disruption of a G·C complex.

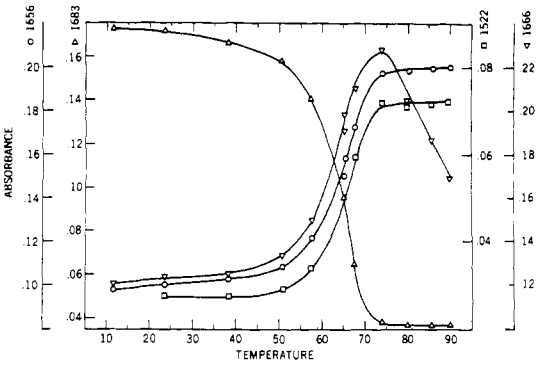

FIGURE 22: Infrared melting curves of poly(8NH_2G)·poly(C) at pD 10.6, conditions of Figure 14. At all frequencies the curves show cooperative melting with $T_m = 65 \pm 1°$. Absorbance of the following G and C vibrations changes in a parallel manner, showing that the changes result from thermal dissociation of specific G·C pairing: 1683 cm^{-1} (G carbonyl in G·C helix), 1666 cm^{-1} (G carbonyl in G·G helix; see text), 1656 cm^{-1} (C carbonyl in single strand), 1522 cm^{-1} (C ring vibration, more intense in single strand; cf. Figure 14). Decrease in absorbance at 1666 cm^{-1} above 75° results from dissociation of the G·G helix which is formed as the G·C helix dissociates.

of 8NH_2GMP·2poly(C) is 1654 cm^{-1}.

The band at 1670 cm^{-1} is not characteristic of a two-stranded helix, and we attribute it to a relatively minor amount of a G·G self-structure (cf. preceding sections). The temperature dependence of this and other bands is discussed in the following paragraph. Bands at 1621, 1527 (sh), and 1497 cm^{-1} (sh) are C ring vibrations (Miles, 1971) and those at ~1585 (sh), 1573, and 1558.5 cm^{-1} are G ring vibrations (Hattori et al., 1975b; Miles, 1971).

Temperature profiles of absorbance at characteristic frequencies (Figure 22) can be used to interpret the spectra and the course of thermal dissociation. At all frequencies the melting curves show cooperative transition, somewhat sharper than those observed by circular dichroism, but with essentially the same T_m. Parallel temperature dependence of bands assigned specifically to resolved G and C vibrations (see Figure 22) shows that the observed spectroscopic changes are due to specific G·C interaction rather than to independent melting of self-structures (for a more detailed

discussion, see Miles, 1971, 1968). Separate experiments with poly(C), moreover, show that it has no cooperative transition in this pH range. The G carbonyl band at 1666 cm^{-1} (74° spectrum) was shown in the preceding section to be characteristic of an ordered G self-structure at this pH (cf. Figure 11); a minor component with ν_{max} ~1685 cm^{-1} (sh, Figure 11) was converted to the predominant species by heating. Only at quite high temperature (85°) was the structure with ν_{max} 1666 cm^{-1} dissociated. The band at 1666 cm^{-1} in Figure 14 (74° and intermediate temperatures) is presumably due to the same G·G structure observed in Figure 11. The fact that the intensity of this band increases with temperature in a parallel manner to curves which reflect dissociation of the G·C helix (Figure 22, curves at 1683, 1656, 1522 cm^{-1}; see prior discussion) indicates that the G residues resulting from dissociation of G·C pairs are converted to an ordered G·G self-structure rather than to a single-stranded structure. At 86° absorbance at 1666 has decreased about 10% from the value at 74° and ν_{max} has shifted to 1658.5 cm^{-1} (broad). At 90° a further shift to 1656 cm^{-1} occurs to produce an asymmetric peak with a shoulder at about 1660 cm^{-1}. Upon recooling the solution there is only a small reversion to the original spectrum, for reasons discussed in a previous section.

Effect of the 8-Amino Group: Comparison of Ordered Structures. In separate reports (Hattori et al., 1975a,b) we have described ordered structures formed by 5′-$8NH_2$GMP with itself and with poly(C) and have, in the present paper, investigated helical complexes formed by poly($8NH_2$G). We shall now review briefly the chemical and structural consequences of introducing an 8-amino group into guanine residues at the monomer and the polymer level. Though the effects are markedly different in each of the ordered structures, most of the differences can be interpreted in terms of the strong tendency of the 8-amino group to form a third interbase hydrogen bond.

The structure of poly($8NH_2$G) in neutral solution is, as we have noted, unusual in several respects. The bases are offset, having only the imidazole rings directly opposed, with the pyrimidine rings and the usual Watson–Crick bonding sites exposed to the solution. None of the usual hydrogen bonding protons (N(1)H, $N(2)H_2$) are involved in pairing. By hemi-protonating a pair of N(7) positions and using the 8-NH_2 group as a donor to O(6), however, three hydrogen bonds can be formed between the pair of bases (A, Figure 12), and this structural feature appears to dominate the properties of the polymer. In addition to hydrogen bonding, other energetic factors (stacking of purine bases in the helix, electrostatic stabilization by the positive charge on the imidazole rings) undoubtedly make important contributions to overall helix stabilization. Without the three hydrogen bonds, however, a helix having this geometrical arrangement of the bases is not formed. A similar structure is formed by the monomer 5′-$8NH_2$GMP (Hattori et al., 1975b), but in this case a slightly acid pH (~4–5) is required, the complex is relatively unstable ($T_m \leq 25°$), and complete interaction is apparently not obtained at ~0°. As with other nucleotides, the less favorable stereochemistry of the monophosphate, compared to the phosphodiester linkage, and the less favorable entropy change on helix formation contribute to make the monomer helix less stable than the corresponding polymer helix. A practical consequence of this lower stability is that the G·G self-structure offers no impediment to formation of a G·2C triple helix (see below). The points discussed above can readily account for formation of the highly stable hemi-protonated structure, A (Figure 12). Poly($8NH_2$G), nevertheless, has the possibility of forming structure B (Figure 12), as well as structure A, since the 8-NH_2 group is not large enough to interfere sterically with the approach of the 2-NH_2 group of another base (Hattori et al., 1975b). In view of the known high stability of a poly(G) helix having a tetrameric structure analogous to A, the marked preference for the hemiprotonated structure is perhaps surprising. One measure of the higher stability of the hemi-protonated over the tetrameric structure is the necessity of raising the pH to ~10 before the former is destroyed by N(7) deprotonation, permitting the tetrameric structure to be formed. (In discussing the stability of the hemi-protonated form we should note that the elevation of pK of the monomer units by electronic effects of the 8-amino group ($\Delta pK \simeq 2.7$) facilitates protonation of $8NH_2$G at neutral pH but does not require it. If it were not for the ordered structure, the $8NH_2$G residues would not be significantly protonated at pH 7.) Once this structure is formed, it is quite stable, considering the high pH (cf. Figure 11). The stability of the poly($8NH_2$G) helix at pH 10 to dissociation by further addition of alkali is similar to that of poly(G) but requires a slightly higher pH for dissociation (Figure 13).

Interaction of poly($8NH_2$G) with poly(C) provides further information on the relative stabilities of structures A and B (Figure 12). While poly(C) can disrupt the very stable poly(G) or poly($8NH_2$G) (pH 10) self-structures to form G·C helices, the hemi-protonated form evidently cannot be disrupted under any conditions to form a G·C pair. This difference presumably results from the higher stability of the hemi-protonated structure.

The reaction between poly(C) and poly($8NH_2$G) evidently does not occur between two single-stranded polymers since the G·G helix (ν_{max} 1666 cm^{-1}), which we assume to be four-stranded, is the most stable form of the latter polymer under conditions of the reaction. Nor is the thermal dissociation of the G·C helix a simple helix → coil transition, since the poly($8NH_2$G) product at ~70° appears to be the same G·G helix (ν_{max} 1666 cm^{-1}) which reacted with poly(C) at ambient temperatures. The reaction at high temperature is thus a reversal of the one at ~25°. If the G·G helix is four-stranded, the processes at pH 10 could be written:

$$4\text{poly(C)} + [\text{poly}(8NH_2\text{G})]_4 \underset{\sim 65°}{\overset{\sim 25°}{\rightleftharpoons}} 4\text{poly(C)}\cdot\text{poly}(8NH_2\text{G})$$

The difference in stoichiometry of the complexes formed with poly(C) by the monomer (exclusively 1:2) and by poly($8NH_2$G) (exclusively 1:1) is an indirect result of the differing relative stabilities of the G·G self-structures. The rather low transition temperatures of the ordered forms of 5′-$8NH_2$GMP (Hattori et al., 1975b) mean that they will not interfere with formation of any heterostructures which are stable at ambient temperature. Poly(C) reacts with 5′-$8NH_2$GMP to form an extraordinarily stable triple helix (G·2C), hemiprotonated at N(7) (Figure 15; Hattori et al., 1975a). In this complex the third hydrogen bond formed by the 8-amino group is largely responsible for the 60° elevation of T_m. The preference for 1:2 stoichiometry is so strong that progressive elevation of pH does not lead to formation of a defined 1:1 complex (as it does with GMP and poly(C)) but, in sufficiently alkaline solutions, to disruption of the triple helix.

Failure of poly($8NH_2$G) to interact with poly(C) in neu-

tral solution is, as we have noted, due to the high stability of the G self-structure. Ready formation of a 1:1 G·C pair at pH 10 shows that this structure is intrinsically stable. The results cited above for the monomer-polymer helix, however, lead us to conclude that a triple helix would probably be the preferred form of interaction for poly($8NH_2G$) and poly(C) if other factors did not intervene. The factor which does intervene is again the very stable hemiprotonated G·G self-structure. Any reduction of pH below the plateau region (pH ~10–11) to achieve protonation essential to formation of the G·2C complex results instead in formation of the G·G helix.

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