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Nucleotides. XX.[1] Synthesis and Properties of Poly-2-alkyladenylic Acids. III. Interactions with Poly(X)

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Poly-2-methyladenylic acid (poly(m^2A)), poly-2-ethyladenylic acid (poly(e^2A)) and poly-2-isopropyladenylic acid (poly(iso-pr^2A)), which are polyadenylic acid (poly(A)) analogues bearing alkyl groups at the C(2)-position of the adenine moiety, form 1 : 1 complexes with polyxanthylic acid (poly(X)) under the same conditions where poly(A) forms both 1 : 1 and 1 : 2 complexes. In contrast to the base pairing between poly(A) and poly(X), these poly-2-alkyladenylic acids cannot, for steric reasons, form normal Watson–Crick type complexes involving the amino group and the N(1)-atom, but coordinate instead with the N(7)-position. Relative rates of complex formation between poly-2-alkyladenylic acids and poly(X) vary with the nature of the alkyl substituents. The half-times for the complex formation were determined for the poly(m^2A)–poly(X), poly(e^2A)–poly(X) and poly(iso-pr^2A)–poly(X) systems as 10 min, ~7 h and ~9 h, respectively. In the presence of 0.1 M Na^+, pH 7.0, the poly(m^2A)·poly(X) complex does not dissociate below 95 °C, but the poly(e^2A)·poly(X) and poly(iso-pr^2A)·poly(X) complexes show cooperative dissociation at T_m's of 84.2 and 69.7 °C, respectively. The ultraviolet and circular dichromic spectra of the complexes are also presented and compared with those of poly(A)·poly(X).

Keywords——poly-2-methyladenylic acid; poly-2-ethyladenylic acid; poly-2-isopropyladenylic acid; polyadenylic acid; polyxanthylic acid; non-Watson–Crick base pairing; polynucleotide interaction

In preceding papers,[1–4] we have reported on the synthesis of poly-2-alkyladenylic acids, poly-2-methyladenylic acid (poly(m^2A)), poly-2-ethyladenylic acid (poly(e^2A)) and poly-2-isopropyladenylic acid (poly(iso-pr^2A)), and their interactions with poly-5-bromouridylic acid (poly(br^5U)) or polyinosinic acid (poly(I)). The former two polyadenylic acid analogues form only 1 : 1 complexes with poly(br^5U), obviously in a Hoogsteen-type base pairing fashion,[5] due to steric interference of the bulky alkyl substituents at the C(2)-position in the normal Watson–Crick hydrogen bonding arrangement.[6] On the other hand, poly(m^2A) associates with poly(I) to form a 1 : 2 complex, whereas poly(e^2A) again shows a 1 : 1 stoichiometry for the same steric reason. These results reflect a complex interaction of polynucleotide chains sensitive in their aggregation to minor structural changes at the aglycone moiety. In order to get a more general view of such intermolecular interactions we investigated further the properties of the complexes between poly-2-alkyladenylic acids and polyxanthylic acid (poly(X)) as another interesting complementary polynucleotide. Construction of mixing curves and studies on the kinetics, thermal stabilities and spectroscopic properties of these complexes have been included in our program of investigations.

Materials and Methods

Poly(m^2A) and poly(e^2A) were synthesized as reported in the previous papers,[3,4] whereas poly(iso-pr^2A) was

obtained by a slightly modified procedure as described below. Poly(X) was synthesized according to the method of Michelson *et al.*[7] except that the polymerization was carried out in the presence of manganese ion instead of magnesium ion. All polynucleotide preparations were subjected to dialysis against the following buffer solutions before use: (i) 0.5 M NaCl–0.01 M Tris-HCl (pH 7.3)–0.001 M ethylenediaminetetraacetate (EDTA), (ii) 0.1 M NaCl–0.01 M Tris-HCl (pH 7.3), (iii) distilled water (twice). The chain length of these polynucleotides was over 100 residues, as determined by alkaline digestion, followed by quantitative analysis of nucleoside and 2′(3′)-nucleotides. The $\varepsilon(p)$'s of the polymers were as follows: poly(m^2A), 9100 at 257 nm; poly(e^2A), 8900 at 256 nm; poly(iso-pr^2A), 9300 at 256 nm; poly(X), 8000 at 250 nm in 0.1 M NaCl, pH 7.3, at 25 °C.

Polynucleotide phosphorylase (EC 2.7.7.8) from *Micrococcus luteus* was purchased from Boehringer Mannheim (Germany) and a standard phosphate solution (1 μmol/ml) for quantitative phosphate analysis was obtained from Serva Feinchemica (Germany).

Synthesis of poly(iso-pr^2A): A reaction mixture (10 ml) containing 2-isopropyladenosine-5′-diphosphate (4×10^{-5} M), $MnCl_2$ (5×10^{-6} M), Tris-HCl (pH 8.5, 1×10^{-3} M), EDTA (2×10^{-6} M), albumin (5 mg) and polynucleotide phosphorylase (0.2 mg) was incubated for 24 h at 37 °C and then extracted 4 times with the same volume of a $CHCl_3$–isoamyl alcohol mixture (3 : 1, v/v) to remove proteins. After centrifugation, the water layer was continuously dialyzed against (i) 2 l of 0.5 M NaCl–0.01 M Tris-HCl–0.01 M EDTA, (ii) 2 l of 0.1 M NaCl–0.01 M Tris-HCl (pH 7.3) and (iii) 2 l of distilled water. Each process was repeated twice for 24 h in a cold room (at 4 °C). Finally 2.2×10^{-5} mol of poly(iso-pr^2A) was obtained in a yield of 55% (calculatd from nucleotide residues incorporated into the polymer). The ultraviolet (UV) spectrum and the extinction coefficient at λ_{max} were identical with those reported previously.[4]

UV spectra were measured with a Cary 15 spectrophotometer and circular dichroism (CD) wih a Cary 60 spectropolarimeter equipped with a CD attachment.

Mixing curves were constructed in various salt concentrations, pH 7.0, at 25 °C by varying the ratio of each

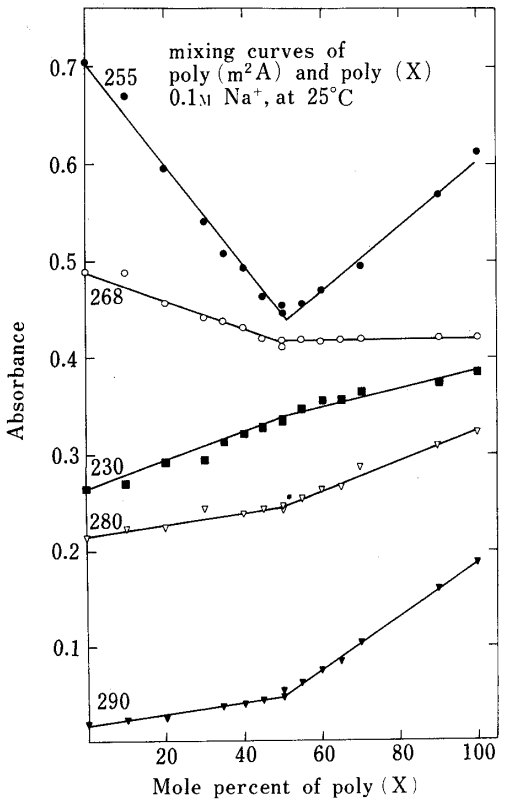


Fig. 1. UV Mixing Curves of Poly(m^2A) and Poly(X) in 0.1 M Na^+ and 0.01 M Phosphate, pH 7.0 at 25 °C

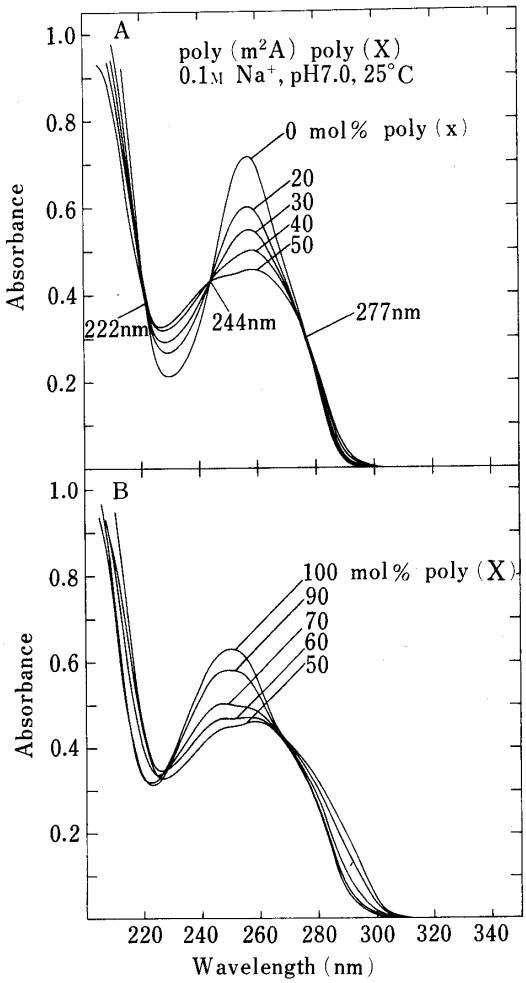


Fig. 2. UV Spectra of Mixtures of Poly(m^2A) and Poly(X) under the Same Conditions as Described in the Legend to Fig. 1

component in a separate solution for each point.

Melting temperatures (T_m) of polynucleotide complexes were measured with a Cary 15 spectrometer equipped with a cuvette holder in which the temperature was controlled by circulating an ethylene glycol–water mixture.

Results

Stoichiometry of the Complexes between Poly-2-alkyladenylic Acids and Poly(X)

The mixture of poly(m^2A) and poly(X) formed a 1 : 1 complex in the presence of 0.11 M Na^+, pH 7.0, at 25 °C as seen from absorbance plots at 230, 255, 268, 280 and 290 nm, showing only one break at 50 mol% poly(X) (Fig. 1). Further support was provided by the isosbestic behavior of the UV spectra of the mixtures containing 0 to 50 mol% or 50 to 100 mol% of poly(X) (Fig. 2). Critical judgement, however, of the latter ratio revealed some irregularities in the lower wavelength region, which may be accounted for by the existence of small amounts of multi-stranded structures.[8-10]

Similar behavior was found with poly(e^2A), which also formed a 1 : 1 complex with poly(X) as demonstrated by the mixing curves at 230, 255, 274, 280 and 290 nm, showing one break at 50 mol% of poly(X) after 48 h (Fig. 3). The UV spectra of various mixtures containing 0 to 50 mol% of poly(X) were isosbestic at 221, 243.5 and 274 nm, whereas those of mixtures containing 50 to 100 mol% of poly(X) again did not coincide exactly in isosbestic point, as in the case of poly(m^2A)–poly(X).

The third example, poly(iso-pr^2A), also associated to form a 1 : 1 complex with poly(X) in the presence of 0.2 M Na^+, pH 7.3, at 25 °C, but the complex formation was very slow. The UV spectrum of the 1 : 1 mixture changed slightly at almost all wavelengths just after mixing and progressed slowly with time to reach an equilibrium state after 60 to 70 h. The mixing

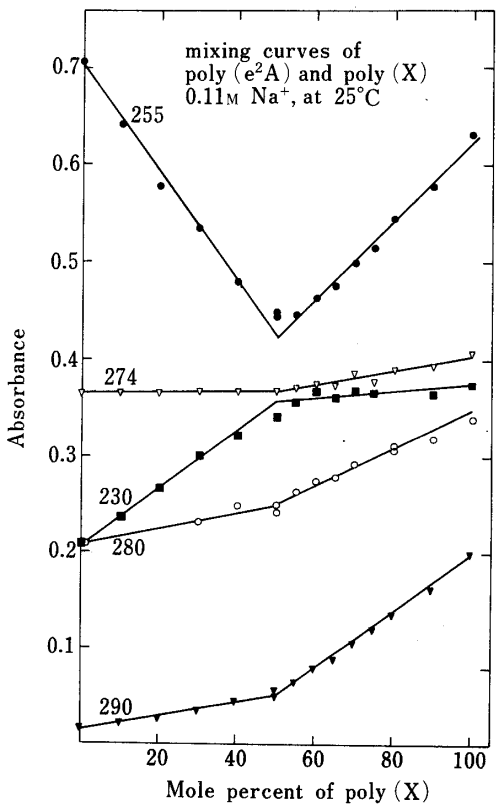


Fig. 3. UV Mixing Curves of Poly(e^2A) and Poly(X) in 0.1 M Na^+ and 0.01 M Phosphate, pH 7.0, at 25 °C

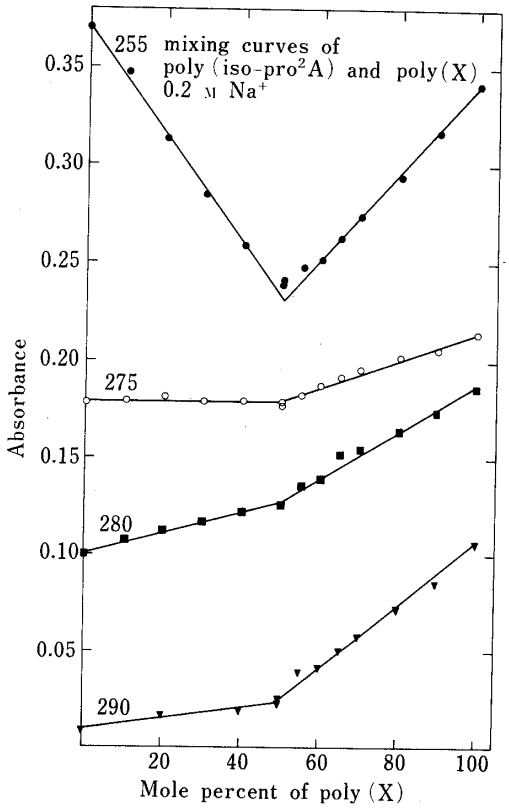


Fig. 4. UV Mixing Curves of Poly(iso-pr^2A) and Poly(X) in 0.2 M Na^+ and 0.01 M Phosphate, pH 7.0, at 25 °C

curves again showed only one break at 50 mol% of poly(X) at wavelengths of 255, 275, 280 and 290 nm (Fig. 4).

Kinetics of the Complex Formation

In order to study the kinetics of the complex formation, two complimentary polynucleotides were mixed together in the presence of 0.1 M Na^+, pH 7.0, at 25 °C. Complex formation was then monitored by measuring the decrease of UV absorbance at various time intervals as shown in Fig. 5. Poly(A) interacted fairly fast with poly(X), and the reaction was characterized by a half-time of 3 min for the complex formation. Under the same conditions, poly(m^2A) interacted somewhat more slowly with poly(X), with an estimated half-time of 10 min. On the other hand, complex formations of poly(e^2A) and poly(iso-pr^2A) with poly(X) were very slow, as is clear from the time courses. The half-times of complex formation in these cases were *ca.* 7 h for the poly(e^2A)–poly(X) system and *ca.* 9 h for the poly(iso-pr^2A)–poly(X) system. These findings were in accordance with the structural features of the poly-2-alkyladenylic acids indicating that the rates of complex formation with poly(X) were strongly affected by the substituent at the C(2)-position of the poly(A) analogues. Increase of the bulkiness of the substituent resulted in a decrease of the relative rate of complex formation.

UV and CD Spectra of the Complexes

The interaction of the poly-2-alkyladenylic acids with poly(X) was also detectable by comparing the UV spectra of the complex and the summation of each component (Fig. 6). Since the relative rates of complex formation of poly(e^2A) and poly(iso-pr^2A) with poly(X) were extremely slow, the UV spectra of the complexes were measured only when no further appreciable changes occurred. The complex formation was then seen as a splitting of the absorption band at 254 nm in the 1 : 1 mixtures into double bands with maxima at 245 and 261 nm. The extinction coefficients at λ_{max} drastically decreased with hypochromicities of 38, 33 and 32% in poly(m^2A)·poly(X), poly(e^2A)·poly(X) and poly(iso-pr^2A)·poly(X), respectively. Under the same conditions, a poly(A)·poly(X) duplex was characterized by a maximum at 259 nm and a shoulder at 245 nm, whereas a poly(A)·2poly(X) triplex showed

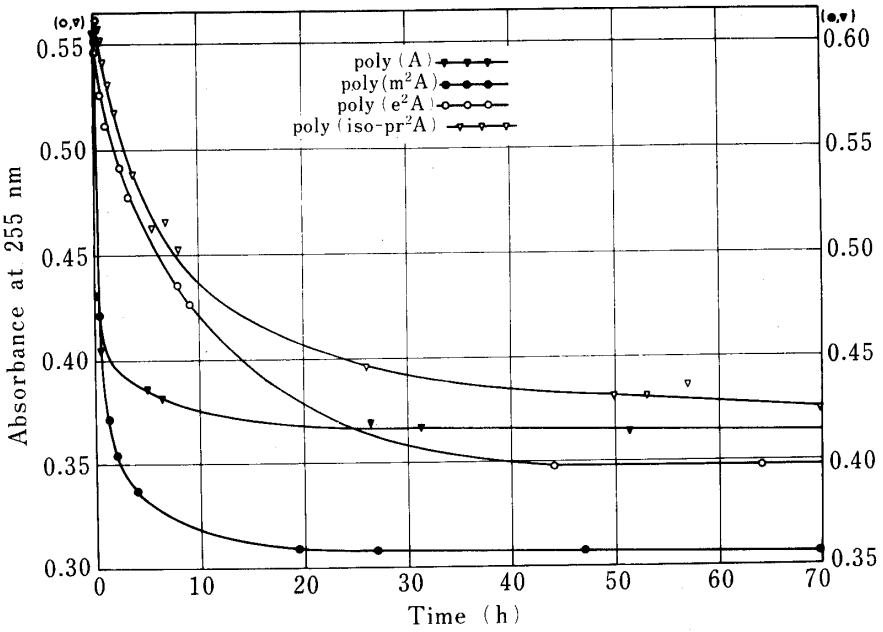


Fig. 5. Kinetics of Complex Formation in 0.1 M Na^+, pH 7.0, at 25 °C

▼—▼, poly(A)+poly(X); ●—●, poly(m^2A)+poly(X); ○—○, poly(e^2A)+poly(X); ▽—▽, poly(iso-pr^2A)+poly(X).

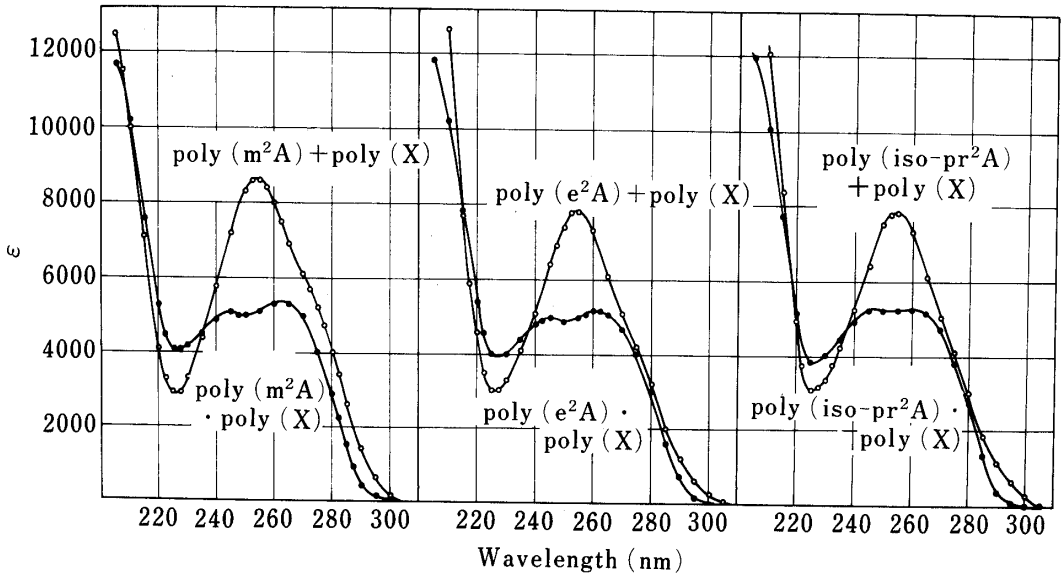


Fig. 6. UV Spectra of Complexes and Summation of Each Component in 0.1 M Na^+, pH 7.0, at 25 °C

TABLE I. Spectroscopic Data for Polynucleotide Complexes in 0.1 M Na^+, pH 7.0, at 25 °C

Complexes	UV spectra		Hypochromicity[a]
	λ_{max} (nm)	ε(p)	
Poly(A)·poly(X)	(245)	5500	
	259	6100	28%
Poly(A)·2poly(X)	240	5100	
	262	5700	31%
Poly(m^2A)·poly(X)	244	5100	
	261	5300	38%
Poly(e^2A)·poly(X)	245	5000	
	262	5200	33%
Poly(iso-pr^2A)·poly(X)	245	5300	
	260	5400	32%

a) Hypochromicity at λ_{max} $h=(1-\varepsilon_{poly}/\varepsilon_{mono})\times 100$.
( ), shoulder.

λ_{max} at 240 and 262 nm (Table I).

Further information on the molecular interactions of the polynucleotides could be derived from comparisons of the CD spectra of the complexes and the summation of the spectra of poly-2-alkyladenylic acids and poly(X) (Fig. 7). Complex formation was associated with a significant change of the CD spectrum especially in the long wavelength region. The shoulder at 235 nm and peak at 265 nm, which reflect the molecular features mainly of the poly-2-alkyladenylic acids, changed to an apparent peak at *ca.* 237 nm and a trough at 272 nm, respectively, as a result of complex formation. The general spectral features of the complexes were fairly similar to each other in shape, but the magnitude of each peak or trough of the complex varied with the alkyl substituents introduced. There was a tendency for a decrease of the ellipticities at the 272 nm transition and an increase at 237 nm, in the order of poly-

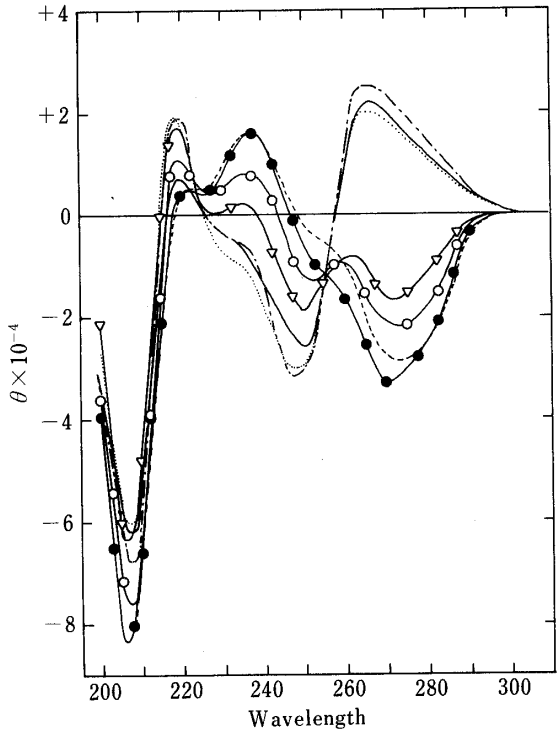


Fig. 7. CD Spectra of Complexes and Summation of Each Component in 0.1 M Na^+, pH 7.0, at 25 °C

——, poly(m^2A)+poly(X); – – – –, poly(e^2A)+poly(X); ------, poly(iso-pr^2A)+poly(X); – – – –, poly(A)·poly(X); ●—●, poly(m^2A)·poly(X); ○—○, poly(e^2A)·poly(X); ▽—▽, poly(iso-pr^2A)·poly(X).

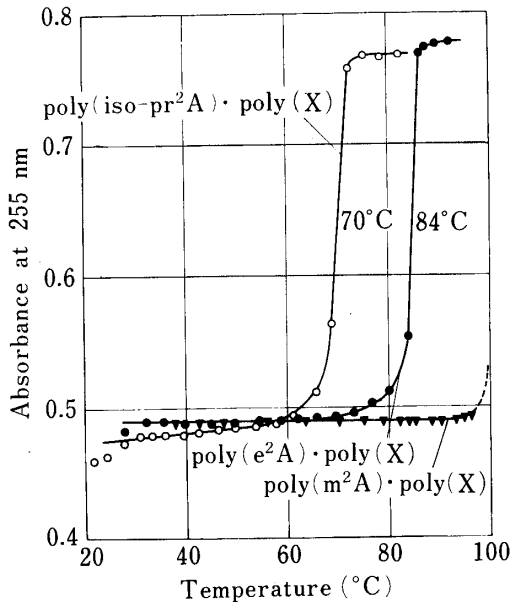


Fig. 8. UV-Temperature Melting Profiles of the Complexes in 0.1 M Na^+ at pH 7.0

(m^2A), poly(e^2A) and poly(iso-pr^2A).

Thermal Dissociation of the Complexes

UV-temperature melting profiles of poly(e^2A)·poly(X) and poly(iso-pr^2A)·poly(X) were measured in the presence of 0.1 M Na^+, pH 7.0, (Fig. 8). The profiles consisted of sharp, sigmoid and monophasic curves with breadths of *ca.* 5 °C, showing that the complexes dissociated cooperatively to the corresponding separate chains. The midpoints of the thermal helix-coil transitions of poly(e^2A)·poly(X) and poly(iso-pr^2A)·poly(X) were found at 84.2 and 69.7 °C, respectively. On the other hand, poly(m^2A)·poly(X) did not dissociate below 95 °C under the same conditions, but at a lower salt concentration (0.02 M Na^+, pH 7.3) a T_m of 93 °C was measured. This result again was in agreement with the fact that increase of the bulkiness of the 2-alkyl substituent in the sequence methyl, ethyl, isopropyl group leads to a decrease of the thermal stabilities of the complexes in that order, as has been observed in the case of the complexes with poly(br^5U) or poly(I).[1)]

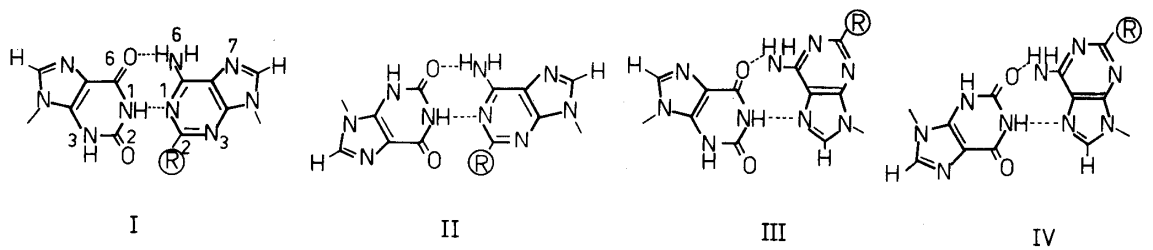
Discussion

Michelson *et al.*[11)] have reported that poly(A) forms both 1:1 and 1:2 (1(A) to 2(X)) complexes with poly(X) at pH 7 within 2 min after mixing, followed by a slower process involving a change to only the 1:2 complex after 24 h. Fiskus and Shugar[12)] have also reported primarily on the basis of melting profiles that at equilibrium only a 1:2 complex is formed either in poly(A)+poly(X) or in poly(A)+2poly(X) mixtures, irrespective of the ratio of the two components, though they suggested that a 1:1 complex is present as an

intermediate in the formation. Contrary to the above observations, Torrence *et al.*[13] have recently concluded that both poly(A)·poly(X) duplex and poly(A)·2poly(X) triplex are formed at their stoichiometric endpoints in 0.1 M Na^+, pH 7, at 20 °C on the basis of mixing curve experiments in which they observed two breaks at 48—50 and 30—33 mol% poly(A). They also observed that at temperatures between 50 and 80 °C in 0.1 M Na^+, pH 7, only the duplex exists regardless of the initial ratio of polymers.

Our own investigations have been concentrated on the interactions between a series of newly synthesized poly-2-alkyladenylic acids and poly(X) by studying the mixing curve profiles. Under conditions similar to those where poly(A) forms both 1 : 1 and 1 : 2 complexes with poly(X) as mentioned above,[13] all poly-2-alkyladenylic acids were demonstrated to form only 1 : 1 complexes at the stoichiometric endpoint. However, with respect to the rates of complex formation, these poly(A) analogues are quite different from one another. The complex formation of either poly(e^2A) or poly(iso-pr^2A) with poly(X) is extremely slow in rate compared to those of poly(A) and poly(m^2A), in contrast with the previously reported cases of the interaction of poly-2-alkyladenylic acids with poly(br^5U) or poly(I).[1] This slower process of complex formation can be explained in terms of steric interaction, *i.e.*, a bulky alkyl substituent introduced at the C(2)-position of the adenine moiety of poly(A) kinetically interferes to some extent with association of the complementary polynucleotide chain to form a double-stranded helical structure. The bulkiness of the alkyl substituents also significantly affects the thermal stabilities of the complexes. T_m's of these complexes tend to decrease in the order of poly(m^2A)·poly(X), poly(e^2A)·poly(X) and poly(iso-pr^2A)·poly(X) depending on the bulkiness of the alkyl substituent introduced. Nevertheless, the structures of these complexes seem to be essentially quite similar to one another by the criteria of the similarities of mixing curves, UV, CD spectral features and monophasic melting profiles. Of the plausible combinations of base pairing between poly-2-alkyladenylic acids and poly(X) (Fig. 9, I—IV), only the latter two (III and IV) look reasonable because an alkyl substituent, a methyl, ethyl or isopropyl group, in the poly-2-alkyl adenylic acids could interfere with normal Watson–Crick or reverse Watson–Crick type base pairing (I or II) by repulsive contact with the C(2)- or C(6)-carbonyl oxygen atom as observed in a CPK space-filling model. A distinction between the base pairings III and IV, where the N(7)-ring atom and the NH_2 group of the adenine base take part in the hydrogen bonding with the C(2)- or C(6)-carbonyl oxygen atom of the xanthine moiety, cannot be made at this stage, leaving the real structures of the 1 : 1 complexes to some extent uncertain.

In comparison with the duplex of poly(A)·poly(X), which has been proposed to have Hoogsteen-like base pairing (Fig. 9, III) by Michelson *et al.*,[11] though they have never offered any direct evidence, poly(m^2A)·poly(X) is quite similar in CD spectral features (Fig. 7) but different in thermal stability and hypochromicity. That is, the thermal stability and the value of percent hypochromicity of poly(m^2A)·poly(X) (T_m > 95 °C in 0.1 M Na^+, at pH 7.0 and



I II III IV

Fig. 9. Plausible Hydrogen-Bonding Schemes of the 1 : 1 Complexes of Poly 2-Alkyladenylic Acids with Poly(X)

I, Watson–Crick type arrangement. II, reverse Watson–Crick type arrangement. III, Hoogsteen type arrangement. IV, reverse Hoogsteen type arrangement.

38% hypochromicity) are greater than those of poly(A)·poly(X) (T_m=83 °C,[13] and 28% hypochromicity), under comparable conditions. On the other hand, poly(e^2A)·poly(X) and poly(iso-pr^2A)·poly(X) are slightly greater in hypochromicity (33 and 32%, respectively) but similar or lower in thermal stability (84.2 and 69.7 °C, in 0.1 M Na^+, at pH 7.0, respectively) compared to poly(A)·poly(X). These findings indicate that if poly(A)·poly(X) is assumed to possess the same basic structure as the poly-2-alkyladenylic acids·poly(X) complexes (which is partly supported in this experiment by the similarity of the CD spectral features), introduction of a methyl substituent at the C(2)-position of the adenine moiety significantly stabilizes the complex in comparison with poly(A)·poly(X), analogous to the cases of acid forms of poly(m^2A)[4] and poly-5-methylcytidylic acid (poly(m^5C)),[14,15] a neutral ordered form of poly-ribothymidylic acid (poly(rT))[16,17] and the complexes of poly(I)·poly(m^5C)[14,15] and poly(A)·poly(rT))[16-18] except poly(m^2A)·2poly(I)[19] relative to poly(A)·2poly(I), in which all of the methyl substituents are located in positions not involved in base–pair hydrogen bonding. Furthermore, the introduction of an ethyl substituent does not appreciably influence the apparent T_m of the complex in consequence of the balance between the stabilizing effect of hydrophobicity[15] and the destabilizing effect of increased bulkiness (steric hindrance), and the introduction of an isopropyl substituent destabilizes the complex because of the latter effect exceeding the former.

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