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Polynucleotides. L. Synthesis and properties of poly (2' - chloro - 2' - deoxyadenylic acid) and poly (2' - bromo - 2' - deoxyadenylic acid)

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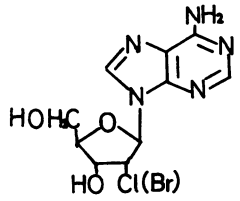
ABSTRACT

Poly (2'-chloro-2'-deoxyadenylic acid) and poly (2'-bromo-2'-deoxyadenylic acid) were synthesized from the corresponding diphosphates with the aid of polynucleotide phosphorylase from E. coli. UV, CD, acid titration and mixing with poly (U) were investigated. Comparing these properties with those of poly (A) and poly (2'-azido-2'-deoxyadenylic acid), it was found that 2'-substituents exert significant effects on the thermal stability of these polynucleotides, though the overall conformational structure was not greatly changed.

INTRODUCTION

Recently we have developed a versatile method for synthesizing 2'-azido[2,3], 2'-amino[2,3], 2'-chloro[3] and 2'-bromo[4]-2'-deoxyadenosine starting from 8,2'-O-cycloadenosine.[5] Using these 2'-substituted 2'-deoxyadenosines as starting materials, poly (2'-azido-2'-deoxyadenylic acid)[poly (Az)][6], poly (2'-amino-2'-deoxyadenylic acid)[poly (Aa)][7] and poly(2'-azido-2'-deoxyinosinic acid) [poly (Iz)][8] were synthesized.



From studies of the physical properties of these polynucleotides, it was found that the 2'-azido-polynucleotides showed only small differences in physical properties to those of poly (A) in contrast to pyrimidine 2'-azido-polynucleotides which showed marked increases in thermal stability relative to their 2'-OH counterparts. Furthermore, the poly (Iz)·poly (C) complex showed an enhanced interferon inducing activity

relative to the known poly (I)·poly (C).[9]

In this paper we report synthetic methods for the preparation of poly (2'-chloro-[poly (Acl)] and 2'-bromo-2'-deoxyadenylic acid) [poly (Abr)] and physical properties of these polynucleotides in comparison to those of poly (A) and poly (Az). It is concluded that 2'-halogeno substituents exert significant effects on the thermal stability although overall conformations are not greatly affected.

MATERIAL AND METHODS

General Procedure

UV absorption spectra were taken with a buffer containing 0.1M NaCl and 0.05M Na Cacodylate (pH 7.0) at 24-26° with a Hitachi Model 200-10 spectrophotometer. The concentrations of nucleotides were determined by phosphate analysis and are presented as per residue values. Hypochromicity was obtained by measuring UV absorption at λ max before and after the digestion of polynucleotides. CD spectra were taken with a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment using 10 mm path-length cell. The concentration of nucleotides was 0.5-1.0 OD_{260}. The solution contained 0.1M NaCl and 0.05M Na Cacodylate (pH 7.0) and was measured at 24-26°. Mixing curves and Tm's were measured with a Hitachi 124 spectro-photometer equipped with a Komatsu thermostated cell SPD-H-124. The temperature inside the cell was measured with a Cu-constantan termocouple. Solutions containing 0.04M or 0.15 M NaCl, 0.05M Na Cacodylate (pH 7.0) and each component polynucleotide were heated once at 60° for 10 min after mixing and measured at 30 min (in case of 0.09M Na^+) to 10 hrs (in case of 0.15M Na^+) after cooling to 24-26°.

Poly (A) and poly (U) were purchased from Miles Laboratories.

Paper-chromatography (PPC) was performed in solvent systems : A, isopropanol-conc. ammonia-water (7:1:2); B, n-butanol-acetic acid-water (5:2:3); C, sat. $(NH_4)_2SO_4$-water-isopropanol (79:19:2); D, n-propanol-conc. ammonia-water (55:10:35), by the descending technique. Paper electrophoresis was performed in 0.05M triethylammonium bicarbonate buffer (pH 7.5) at

900V/40 cm. Migration ratios are presented by R_{PA-A}, which corresponds to migration distance divided by distance between adenosine (0.0) and adenosine 5'-phosphate (1.0).

2'-Chloro-2'-deoxyadenosine 5'-phosphate

2'-Chloro-2'-deoxyadenosine (32.1 mg, 0.11 mmole) was dissolved in a mixture of $POCl_3$ (50 µl, 0.54 mmole) and triethyl phosphate (2 ml) at 0°. The reaction mixture was stirred at 0° for 6 hrs. The mixture was poured in ice-water and absorbed on a column of charcoal. The column was washed thoroughly with water and eluted with 50% EtOH containing 5% ammonia. Eluents were evaporated in vacuo and the residue was dissolved in water (20 ml), and applied to a column of Dowex 1x2 (formate form). After a water-wash, the column was eluted with 0.1N HCOOH. The yield of Acl 5'-MP was 1012 OD_{260} (61%). UV: λ_{max}^{H2O} 259 nm. PPC: Rf (A) 0.17, Rf (B) 0.35. PEP: R_{pA-A} 0.96. This sample was hydrolyzed completely with snake venom 5'-nucleotidase to give Acl and inorganic phosphate.

2'-Bromo-2'-deoxyadenosine 5'-monophosphate

2'-Bromo-2'-deoxyadenosine (27.4 mg, 83 µmoles) was treated with $POCl_3$ (0.1 ml, 1.1 mmole) in triethyl phosphate (1 ml) as described above. Yield of Abr 5'-MP was 790 OD_{260} (63%). UV: λ_{max}^{H2O} 259 nm. PPC: Rf (B) 0.28, Rf (C) 0.54. PEP: R_{pA-A} 0.96. This sample was hydrolyzed completely with sanke venom 5'-nucleotidase to give Abr and inorganic phosphate.

2'-Chloro-2'-deoxyadenosine 5'-diphosphate

Acl 5'-MP (61 µmoles) was dissolved in a mixture of H_2O (1 ml), t-BuOH (1 ml) and morpholine (30 µl, 0.35 mmole). The solution was heated at refluxing temperature and a solution of DCC (72 mg) dissolved in t-BuOH (1.5 ml) was added dropwise in 40 min. Refluxing was maintained for 2 hrs and dicyclohexyl urea was filtered off. Water and ether were added to the filtrate and water-layer was evaporated in vacuo. The residue was rendered anhydrous by evaporation several times with added pyridine. To the residue a pyridine solution (1 ml) of 80% H_3PO_4 (0.018 ml, 0.26 mmole) and $(nBu)_3N$ (0.062 ml, 0.26 mmole) were added. The solution was evaporated and the residue was dissolved in pyridine (1 ml). The reaction mixture was kept at room temperature for 3 days. The reaction was quenched by

the addition of water, the solvent was removed by evaporation in vacuo, and the residue was dissolved in water. The aqueous solution was brought to ca. pH 5 and applied to a charcoal column. The nucleotidic material was eluted with 50% EtOH containing 5% conc.NH_4OH and evaporated in vacuo. The residue was taken up in water and applied to a column (1.7x15 cm) of DEAE-Sephadex A-25(bicarbonate form). Elution was carried out with 0-0.25 triethylammonium bicarbonate buffer (2 l+2 l) in a linear gradient. The yield of Acl 5'-DP was 330 OD_{260} (35%). PPC: Rf (B) 0.08, Rf (D) 0.54. PEP: R_{pA-A} 1.25.

2'-Bromo-2'-deoxyadenosine 5'-diphosphate

Abr 5'-MP (790 OD_{260}) was treated with morpholine (75 µl) dissolved in t-BuOH (1 ml) and water (1 ml) and DCC (176 mg, 0.86 mmole) in t-BuOH (1.5 ml) as described above. After the appropriate work up, the residue was allowed to react with 80% H_3PO_4 (30 µl, 0.4 mmole) and $(n\text{-}Bu)_3N$ (0.1 ml, 0.4 mmole) in DMF (1 ml). After 3 days at room temperature the reaction mixture was applied to a column of DEAE-Sephadex A-25 as described above. The yield of Abr 5'-Dp was 377 OD_{260} (48%). PPC: Rf (B) 0.05, Rf (C) 0.50. PEP: R_{pA-A} 1.27.

Poly (2'-chloro-2'-deoxyadenylic acid)

A solution (4.5 ml) containing Acl 5'-DP (4 mM), $MgCl_2$ (2.2 mM), Tris-HCl (pH 8.5, 66mM) and E.coli polynucleotide phosphorylase[10] (3.9 units/1 ml) was incubated at 37° for 24 hrs. The mixture was deproteinized with isoamyl alcohol-chloroform (1:3, vol/vol)mixture and the water-layer was lyophilized. The residue was dissolved in water and filtered through a column (2.6 x 80 cm=425 ml) of Sephadex G-50 gel. The flow rate was 5 ml/20 min/fraction. The polynucleotide was eluted in the void volume and the yield was 45 OD_{260} (25 %, ignoring hypochromicity). The fact suggests that the poly (Acl), thus obtained, has a chain length greater than 50 nucleotide units.

Poly(2'-bromo-2'-deoxyadenylic acid)

A solution (6 ml) containing Abr 5'-DP (4 mM), $MgCl_2$ (2 mM), Tris-HCl (pH 8.5, 80 mM) and E. coli polynucleotide phosphorylase (4.5 units/ml) was incubated at 37° for 24 hrs. After deproteinization with isoamyl alcohol-chloroform (1:3, col/vol), the polynucleotide was subjected to gel filtration

through a column of Sephadex G-50 as described above. The yield was 33 OD_{260} (13%, ignoring hypochromicity).

Enzymatic digestion of polynucleotides

i) Polynucleotides (ca. 2 OD_{260}) were incubated with ribonuclease M^{11} (2 mg/ml) 2 ul in water 50 ul containing 1M NH_4OAc (pH 7.5) 2 ul at 37° for 150 min. While poly (A) was hydrolyzed completely in these conditions, poly (Acl) and poly (Abr) were resistant to hydrolysis. This fact confirms that nucleosides in poly(Acl) and poly(Abr) were substituted at their 2'-positions.

ii) Polynucleotides (ca. 2 OD_{260}) were incubated with snake venom phosphodiesterase (5 mg/ml) i ul in water 50 ul containing 1M Tris-HCl (pH 8.5) 3 ul at 37° for 30 min. While poly(A) was completely hydrolyzed after 30 min, poly (Acl) and poly(Abr) were only hydrolyzed to extents of 8% and 9% to give Acl 5'-MP and Abr 5'-MP, which were identified directly with authentic samples, after 2 hrs incubation. This fact suggests that large electronegative substituents inhibit hydrolysis catalyzed by snake venom phosphodiesterase to some extent.

RESULTS AND DISCUSSION

UV absorption properties

The UV absorption spectra of poly (Acl) in the presence of 0.15M Na^+ at 24-26° are shown in Fig. 1. The spectrum at pH 7.0 showed a maximum at 257 nm smilar to that of poly (A) in the same conditions in our hands. For poly(Abr) also, the same λmax at 257 nm was found. λmin's were 230 nm for both 2'-halogenated polynucleotides.

Molecular extinctions (ε) at λmax were 10,500 and 10,700 for poly(Acl) and poly(Abr), respectively. These values are in the same range as that for poly(A)(10,000). Hypochromicity obtained by the digestion of polynucleotides were 32% and 29% for poly (Acl) and poly (Abr), respectively. This may suggest that stacking tendency of poly(Acl) is somewhat larger than that of poly(Abr) as Alderfer et al.[12] suggested with 2'-O-alkylated poly(A) analogous that the degree of hypochromicity was inversely proportional to the size of 2'-substituents.

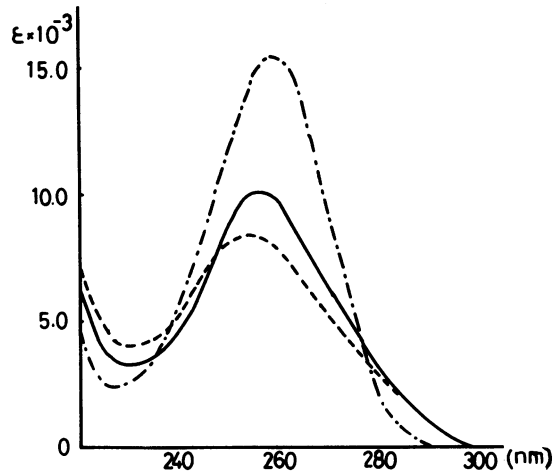


Fig. 1. UV absorption spectra of poly (Acl). ——at pH 7.0. ..o..o..o at pH 4.5, -.-.- Acl 5'-MP.

CD spectra

CD spectra of poly(Acl) and poly(Abr) are illustrated in Fig. 2. together with that of poly (A). Although the overall shapes of the curves are very similar to each other, the magnitude of $[\theta]_{max}$ at long wavelengths and $[\theta]_{min}$ are different in each polynucleotide (Table I).

If we compare the $[\theta]$ max at around 263-265 nm, which could presumably be assigned to a positive splitting band of B_{2u} transition,[13] the order of magnitude is Acl > Abr > A > Az. In the $[\theta]_{min}$ at around 237-238 nm assigned to the negative splitting bands the order is Acl > A > Abr > Az. Alderfer et al.[12] showed in the case of 2'-O-alkylated polyriboadenylic acid that the amplitude of $[\theta]_{max}$ increased with hypochromicity and deduced that the hypochromicity would reflect the degree of stacking of bases. However, in the case of the poly (A) analogs shown here the order of magnitude of hypochromicity is not always paralleled to the magnitude of the $[\theta]$ value. Although among polynucleotides with the halogenated 2'-position, Acl and Abr, this relationship held good, introduction of the extremely polarized N_3 group changed the nature

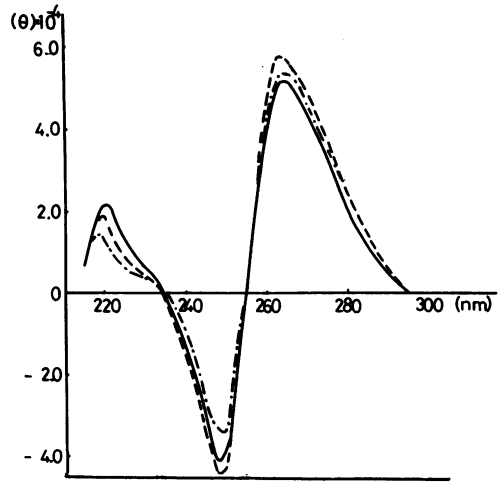


Fig. 2. CD Spectrum of Poly (Acl), Poly (Abr) and Poly(A).
——— Poly (A), ---- Poly (Acl), —.—.— Poly (Abr).

Table I Molecular Ellipticity of Polynucleotides

Polynucleotides	$[\theta]_{max}$	$[\theta]_{min}$	Total
Poly (A)	52,000	41,000	93,000
Poly (Acl)	57,000	44,000	101,000
Poly (Abr)	53,000	34,000	87,000
Poly (Az)[6]	31,000	26,000	57,000

of the stacking interaction which was manifested in exceptionally small [θ] values in poly (Az).[6]

Protonated Forms of Poly (Acl) and Poly (Abr)

If we titrated poly (Acl) and poly (Abr) with 0.1N HCl in the presence of 0.15M Na^+ at 24-26°, transition points from the random single stranded form to the protonated, double stranded, acid form as was observed in poly (A),[14] were observed. As summarized in Table II, pH values of the transition were in the range of 5.0-6.0 and increased with

Table II Acid Titration of Polynucleotides

Polynucleotides	Transition pH	Tm at pH 4.5
Poly (A)	6.0	80°
Poly (Acl)	5.5	63°
Poly (Abr)	5.0	56°
Poly (Az)	5.5	38°

decreasing size of the 2'-substituent, except for the azido group, which showed again an abnoramality reflecting its unusual properties. The thermal transition temperatures of these polynucleotides measured at pH 4.5 in the presence of 0.15M Na^+ are included in Table II. Tm's increased in the order of Az < Abr < Acl < A, the same order as for the transition pH's, again with Az as an exception. Although at the present stage it is difficult to draw any conclusion, the thermal stabilities of 2'-substituted polyribonucleotides in the acid form again seem to reflect the size of the 2'-substituents except for the azido group. Alderfer et al.[12] suggested that stacking forces in the single stranded forms of ribopolynucleotides work as negative factors as regards the stability of double-stranded forms. In the present case, however, the order of stability is parallel in both single- and double-stranded forms.

Formation of Complexes with Poly (U)

The formation of double- and triple-stranded complexes of Poly (Acl) and poly (Abr) with poly (U) was investigated using continuous variation method.

As shown in Fig. 3a, poly (Acl) clearly showed inflection points at a ratio of poly (Acl) : Poly (U) equal to 1:1 in the presence of 0.09M Na^+ ion as observed at 250, 260 and 270 nm. This fact suggests the formation of a complex, poly (Acl)·poly (U) as was found in the case of poly (A)-poly (U).[15] However, after the prolonged storage of this mixture at room temperature these mixing curves changed to more complicated ones, suggesting partial transition from the 1:1 to 1:2

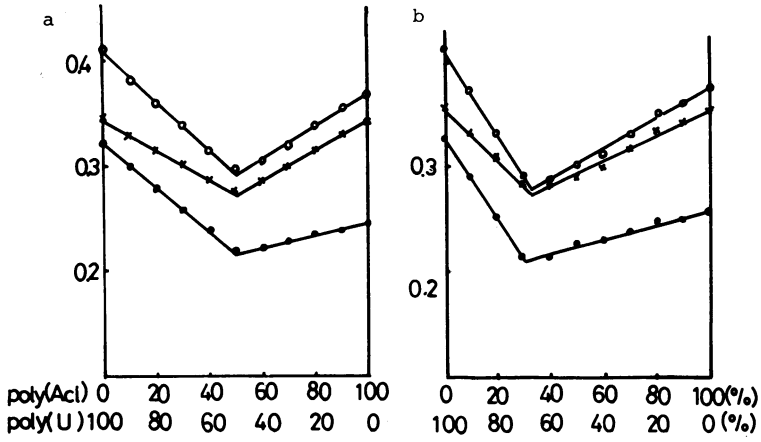


Fig. 3. Complex Formation of Poly (Acl) with Poly (U).

complex described below.

As shown in Fig. 3b, at ionic concentration of 0.15M poly (Acl) showed inflection points at a ratio of poly (Acl) : poly (U) equal to 1:2 as observed at 250, 260 and 270 nm, suggesting the formation of 1:2 complex between them. Fig. 4a and 4b show the same type of complex formation between poly (Abr) and poly(U). At Na^+ ion concentration of 0.09 M (Fig. 4a) they showed the formation of a 1:1 complex, poly (Abr)·poly (U) and at 0.15M a triple-stranded complex, poly (Abr)·2 poly (U) was formed. These facts indicate that 2'-substituents such as halogen did not change the complex forming properties as compared to that of poly (A).

Thermal Transition of Complexes

The thermal transition points (Tm) of these 1:1 and 1:2 complexes between poly (Acl), poly (Abr) and poly (U) are summarized in Table III.

It is observed that the Tm's of double helical complexes increase in the order Abr < Acl = Az < A. This tendency is more clearly observed in the case of triple-stranded complexes as Abr < Acl < A < Az. Therefore, we may conclude that the thermal stability of complexes such as poly (A)·poly (U) or poly (A)·2 poly (U) is determined by the size of 2'-substituent atoms

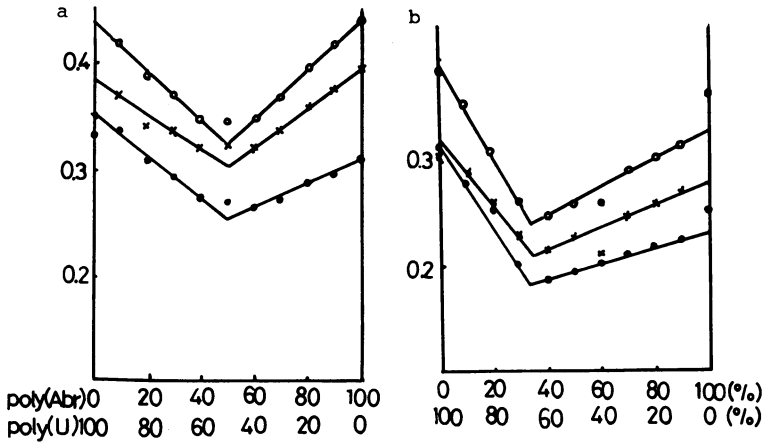


Fig. 4. Complex Formation of Poly (Abr) with Poly (U).

Table III. Thermal Transition Points of Complexes.

Polynucleotides	Tm (°C)	
	At 0.09M Na^+	at 0.15M Na^+
Poly (A)-poly (U)	51	62
Poly (Acl)-poly (U)	46	56
Poly (Abr)-poly (U)	45	53
Poly (Az)-poly (U)	46	65

as observed in the case of acid duplex forms. Again poly (Az) behaves exceptionally presumably due to high polarity of the azido group.

CONCLUSIONS

From the experiments described in this paper, several interesting points may be emphasized.

The hypochromicity of poly (A) analogs should reflect the tendency for overlapping and stacking interaction of adenine bases. Comparing poly (Acl) and poly (Abr), the hypochromicity is larger in the former polynucleotide presumably because of a smaller substituent in the 2'-position. Since the hydroxyl group in poly (A) and the azido group in poly (Az) are polar groups, the stacking may be enhanced by these groups

to bring about the larger hypochromicities of poly (A) and poly (Az) when compared to poly (Acl) and poly (Abr). Observing the CD spectra it is also reasonable to state that poly (Acl) has a more strongly stacked conformation than that of poly (Abr), but the stacking of poly (A) seems to be intermediate between them. However in the case of poly (Az), the magnitude of $[\theta]$ is extremely small and association of solvent molecules to the polar azido group which labilize the stacking conformation of poly (Az), may be the reason.[6]

Poly (A) is known to form the so-called acid structure at pHs below 4.5.[14] The transition pHs (5.5-6.0) to form the acid structure of the polynucleotides discussed here are in almost the same range and it may be deduced that 2'-halogeno or azido substituents do not significantly affect the pK values of these polynucleotides. However, as regards their stability these substituents have large effects. For 2'-halogeno compounds the decrease in thermal stability relative to poly (A) is in the range of 20-25° and for poly (Az) it is more than 40°. This destabilizing effect may be due to size and polarity of these substituents.

In the case of the complexes formed between poly (U) and these poly (A) analogs, again the 2'-substituents did not inhibit the formation of double- ot triple-stranded complexes, though the thermal stability was affected. The Cl, Br and N_3 substituents at the 2'-position significantly lowered the thermal stabilities of the double helical complexes, poly (Acl)·poly (U), poly (Abr)·poly (U) and poly (Az)·poly (U), to the extent of 5-6°. This tendency was also observed in the case of the triple-stranded complexes, poly (Acl)·2poly (U) and poly (Abr)·2poly (U). However, in the case of poly (Az)·2 poly(U), the Tm was increased 3° relative to poly (A)·2poly (U). It seems reasonable to assume that these substituents in the 2'-position may affect the thermal stability of polynucleotides not only for steric reasons, but also by their polarizability causing association of solvent molecules. These effects in the anti-parallel double stranded poly (A)·poly (U) analogs and Watson-Crick-Hoogsteen (antiparallel) arrangements in poly (A)·2 poly(U) type complexes may be some-

what different from case to case.

The fact that poly (2'-azido-2'-deoxyinosinic acid)[8,16] and poly (2'-chloro-2'-deoxyinosinic acid)[16], when complexed with poly (C), are active as interferon inducers is a very interesting reflection of the structure-function relationship of such polynucleotides.

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