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Polynucleotides. XL.<sup>1</sup> Synthesis and properties of poly 2'-azido-2'-deoxyadenylic acid

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**ABSTRACT**

Poly 2'-azido-2'-deoxyadenylic acid (Poly Az) was synthesized from 2'-azido-2'-deoxyadenosine diphosphate by polynucleotide phosphorylase. Poly (Az) has U.V. absorption properties similar to poly (A) and hypochromicity of 40% at 0.1 M Na<sup>+</sup> and neutrality. CD curve also resembled to that of poly (A), but has smaller ellipticity. Titration of poly (Az) with HCl gave a transition at pH 5.5, but exact structure of the acid-form complex was not elucidated. Upon mixing with poly (U), poly (Az) forms a 1:1 and 1:2 complexes having T<sub>m</sub>'s somewhat higher than that of poly (A)·poly (U) complex in the same condition.

A number of polynucleotides containing analogs of pyrimidine and purine nucleosides have been reported.<sup>2</sup> Among these polynucleotides, ones which have 2'-substituted nucleosides are especially interesting because they have natural 3'-5'-phosphodiester linkages and are suitable for elucidating physical and biological properties of polynucleotides. However, up to the present they have been limited to only 2'-substituted pyrimidine nucleotides, i.e. 2'-halogeno-,<sup>3</sup> azido-,<sup>4</sup> methoxy-,<sup>5</sup> and ethoxy-,<sup>6</sup> compounds. We have found a new method for the synthesis of 2'-azido and 2'-amino-2'-deoxyadenosine and guanosine<sup>7</sup> by way of purine cyclonucleosides,<sup>8</sup> which are readily available from the naturally occurring nucleosides. In this communication we report the synthesis of poly 2'-azido-2'-deoxyadenosine (poly (Az)) and its physical properties, such as UV, C.D., T<sub>m</sub> and hybridization with poly (U).

**MATERIAL AND METHODS**

**2'-Deoxy-2'-azidoadenosine 5'-diphosphate**

2'-Azido-2'-deoxyadenosine (Ia) (63 mg, 0.21 mmole) was dissolved in a mixture of POCl<sub>3</sub> (0.5 ml) and triethylphosphate

(2 ml) with cooling to 0°. The solution was stirred for 2 hr at 0° and poured in ice-water.<sup>9</sup> The aqueous solution was applied to a column of charcoal, which was washed with water and eluted with 50% EtOH containing 5% conc. ammonia. Eluents were evaporated in vacuo and the residue was dissolved in water. The aqueous solution was applied to a column (1.5 x 15 cm) of Dowex 1 x 2 (formate). After the water-wash the column was eluted with 0.1N formic acid to give 2'-azido-2'-deoxy AMP (2160 OD<sub>260</sub> units, 0.15 mmole) in a yield of 73%. UV:  $\lambda_{\text{max}}^{\text{pH}2}$  257 nm,  $\lambda_{\text{max}}^{\text{pH}12}$  259 nm.

2'-Azido-2'-deoxy AMP (0.15 mmole) was dissolved in a mixture of H<sub>2</sub>O (1.5 ml), t-BuOH (1.5 ml) and morpholine (0.058 ml, 0.6 mmole). A solution of dicyclohexylcarbodiimide (124 mg 0.6 ml) in t-BuOH (2.25 ml) was added dropwise into the solution while refluxing.<sup>10</sup> The refluxing was continued for 2 hr, the mixture was evaporated in vacuo, and the residue was equilibrated in a H<sub>2</sub>O-ether (1:1) mixture. Insoluble material was filtered off and the aqueous layer was separated and evaporated. The residue was azeotropically dried with pyridine several times. To the residue, inorganic phosphate (0.04 ml, of 95%), which was previously dried by azeotropical evaporation with pyridine together with tri-n-butylamine (0.14 ml) and dissolved in pyridine (1 ml), was added. The reaction mixture was kept at 30° for 5 days. The reaction mixture was evaporated in vacuo, the residue dissolved in H<sub>2</sub>O, and applied to a column of charcoal. The column was washed with H<sub>2</sub>O and eluted with 50% EtOH containing 5% conc. ammonia. Eluents were concentrated and applied to a column (1.0 x 17.5 cm) of DEAE-Sephadex A 25. Elution was performed with 0.1-0.3 M triethylammonium bicarbonate buffer (total 2 l) in a linear gradient and 15 ml fractions were collected. Fractions No. 65-85 were pooled and evaporated. 2'-Azido-2'-deoxy ADP (760 OD<sub>260</sub> units, 35%) was obtained. UV:  $\lambda_{\text{max}}^{\text{pH}2}$  257 nm,  $\lambda_{\text{max}}^{\text{pH}7}$  259.5 nm,  $\lambda_{\text{max}}^{\text{pH}12}$  260 nm. Paper electrophoresis:  $R_{\text{AMP}}$  1.44.

### Poly (2'-azido-2'-deoxyadenylic acid)

2'-Azido-2'-deoxy ADP (4 mM), polynucleotide phosphorylase (2.2 units /ml), Tris-HCl (pH 8.5) 100 mM and MgCl<sub>2</sub> or MnCl<sub>2</sub> 0.4 mM were adjusted to 0.25 ml with H<sub>2</sub>O and incubated at 37°

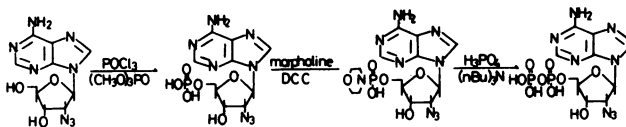
for 6 hrs. Pi release was 0.27  $\mu\text{mole}$  (34%) or 0.33  $\mu\text{mole}$  (41%) in the presence of  $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$  respectively.

A large scale incubation was performed in a total volume of 7.3 ml containing the same ingredients in same concentrations. After 20 hrs the mixture was deproteinised with isoamylalcohol- $\text{CHCl}_3$  (1:3) and the aqueous layer was evaporated. The residue was dissolved again in water and applied to a column of Sephadex G-50. Poly (Az) was eluted as a symmetrical peak in the void volume. The yield was 51.3  $\text{OD}_{260}$  units (5.5  $\mu\text{moles}$ , 18%). AzDP (17.2  $\mu\text{moles}$ , 57%) was recovered. UV:  $\lambda_{\text{max}}^{\text{pH } 7.0}$  256 nm ( $\epsilon = 8,900$ ). This sample was completely hydrolyzed with snake venom phosphodiesterase to give only 2'-azido-2'-deoxy A5'p. UV spectra were taken with a Hitachi 124 spectrophotometer in the presence of 0.1 M NaCl and 0.05 M Na cacodylate (pH 9.0) at 12°. CD spectra were taken with a JASCO ORD/UV-5 spectrometer equipped with a CD attachment in the presence of 0.1M NaCl and 0.05M Na cacodylate (pH 7.0) at 16°. Melting temperature was measured in the presence of 0.1M NaCl and 0.05M Na cacodylate at pH 7.0 with a Hitachi spectrophotometer equipped with a thermostated cell. The temperature inside the cell was measured with a thermocouple. Mixing curves were obtained by measuring the absorbance of mixture which contained 0.04 mM total concentration of poly (Az) and poly (U) in the ratios indicated in Fig 6 . Salt concentration was 0.1 M NaCl and pH was adjusted to 7.0 with 0.05M Na cacodylate.

Poly (U) was purchased from Miles Laboratories Ltd. Snake venom phosphodiesterase was purchased from Boehringer Mannheim, Ltd.

#### RESULTS AND DISCUSSION

As in the case with 2'-substituted pyrimidine nucleoside 5'-diphosphates<sup>4</sup> the polymerization reaction of AzDp proceeds better in the presence of  $\text{Mn}^{++}$  than in the presence of  $\text{Mg}^{++}$ . The large scale preparation was thus performed with  $\text{Mn}^{++}$  ion and the time course is presented in Fig 1. The inorganic phosphate liberated was more than 50% after 20 hrs incubation, but the isolated yield of poly (Az) was only 18%. Probably



Synthetic route to 2'-azido-2'-deoxyadenosine 5'-diphosphate

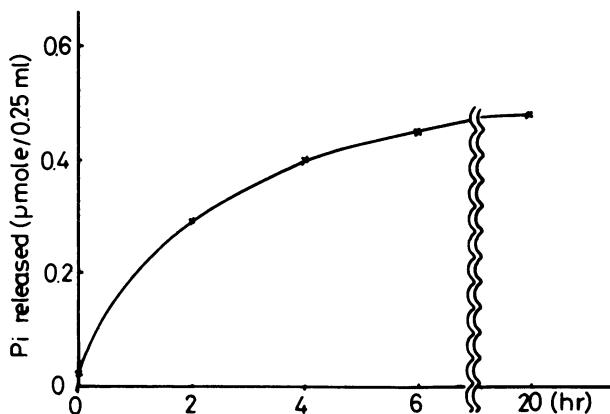


Fig. 1. Time course of polymerization of AzDP

some of the material was lost during the deprotection, because an intractable fluffy mass was observed between the two phases.

#### UV Absorption of Poly (Az)

UV absorption of poly (Az) is shown in Fig 2. At pH 7.0 in the presence of 0.15M Na<sup>+</sup>, it shows  $\lambda_{max}$  at 256 nm. A blue shift of 4 nm of the  $\lambda_{max}$  compared to that of monomer was observed. Hypochromicity at neutrality was calculated as 40% assuming  $\epsilon$  of the monomer, 2'-azido-2'-deoxyadenosine as 14,800.<sup>7</sup> This hypochromicity is larger than that of poly (A) (37%) under the same conditions.<sup>11</sup> It is also larger than those of poly (2'-O-methyl A)<sup>12</sup> and poly (2'-O-ethyl A).<sup>13</sup> Since poly (dA) has hypochromicity of 41%,<sup>14</sup> the effect of 2'-substitution on hypochromicity follows the order, H > N<sub>3</sub> > OH > OMe > OEt. This cannot be explained solely in terms of sterical distortion by 2'-groups of the vertical stacking

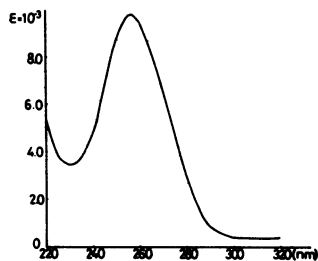


Fig.2. UV absorption spectrum of poly (Az) at pH 7.0.

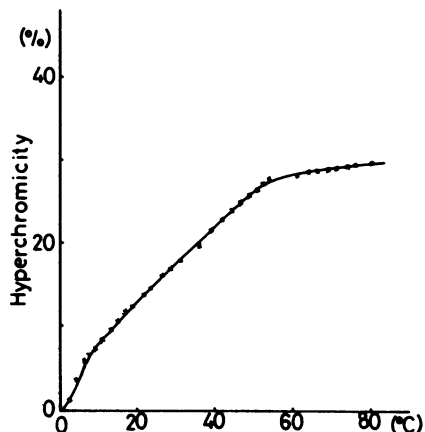


Fig.3. Temperature-absorption profile of poly (Az) at pH 7.0.

of adjacent adenines.<sup>13</sup> This tendency is in agreement with that observed when comparing poly (U) and poly (Uz).<sup>4</sup> A big increase in hypochromicity was observed from 9.2% for poly (U) to 14.1% for poly (Uz). Therefore, influences of the azido group on base stacking give rise to similar effects both in pyrimidine and purine polynucleotides.

#### Temperature-absorbance profile

The temperature-absorbance profile of poly (Az) in neutral conditions is shown in Fig 3. The UV absorption increased gradually on heating from 0° to 80° without showing any steep increase indicative of cooperative melting. As was observed for poly (A)<sup>11</sup> and poly (m<sup>2</sup>A),<sup>15</sup> this curve suggests a flexible structure of poly (Az), which is only stabilized by the vertical stacking. This phenomena is in sharp contrast with that observed for poly (Uz).<sup>4</sup> In the latter case a steep rise of the temperature-absorbance profile was observed and the T<sub>m</sub> was reported to be 12° in the presence of 0.01M MgCl<sub>2</sub>. This different influence of the azido group when introduced to the 2'-position of polynucleotides of purine and pyrimidine may be interpreted as indicating a difference in conformations of both types of polynucleotides stabilized by

different stacking interactions.

#### CD spectra of poly (Az)

A CD curve was obtained in the presence of 0.15 M Na<sup>+</sup> at neutrality and 16°. As shown in Fig. 4 , it shows a peak

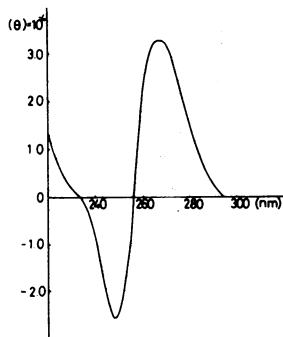


Fig. 4 . CD spectrum of poly (Az) in the presence of 0.15M Na<sup>+</sup> at pH 7.0 and 16°.

at 268 nm ( $[\theta]=39,000$ ) and a trough at 248 nm ( $[\theta]=30,000$ ). The cross-over point was at 256 nm, close to the absorption maximum of poly (Az). Although the CD profile itself is very similar to those of poly (A)<sup>16</sup>, poly (m<sup>2</sup>A)<sup>12</sup> and poly (Ae)<sup>13</sup>, the molecular ellipticity  $[\theta]$  is significantly different. The  $[\theta]_{\text{peak}}$  of poly (Az) is only two thirds that of poly (A) and one half that of poly (m<sup>2</sup>A). It was even somewhat smaller than that of poly (Ae). The magnitude of the  $[\theta]_{\text{trough}}$  also shows the same tendency.

As was discussed previously<sup>17</sup>, the magnitude of rotatory strengths reflects both the stacking tendency and direction of adjacent bases. It may be deduced that in the molecule of poly (Az), adjacent bases stack rather strongly, but their arrangement is not similar to the case of poly (A). This unusual stacking conformation might be ascribed to steric distortion by azido groups.

#### Acid titration of poly (Az)

Poly (Az) was titrated with 0.1N HCl in the presence of 0.1 M sodium chloride (Fig. 5). The absorption curve is

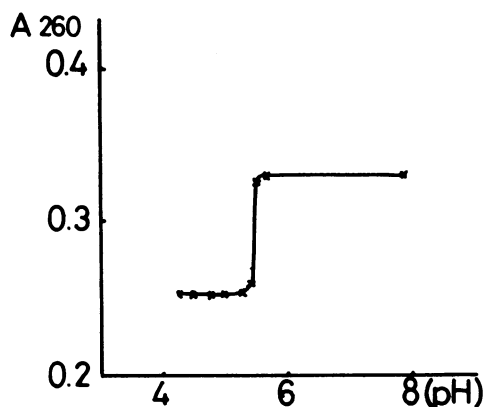


Fig 5. Acid titration curve of poly (Az).

almost flat at pH 4.2 to 5.3 and then a steep rise at pH 5.5 was observed. This hyperchromic change may indicate the formation of an acid form complex similar to the case of poly (A)<sup>18</sup> where a similar transition occurs at pH 5.

#### Hybridization experiments of poly (Az) with poly (U)

It is well known that poly (A) forms double or triple stranded complexes, according to the salt concentration, upon mixing with poly (U). Poly (dA), poly (Am) and poly (Ae) also show similar complex formation.

We examined the complexing of poly (Az) with poly (U) by the continuous variation method. 0.04 mM base concentrations of poly (Az) and poly (U) were mixed in various ratios (as indicated in Fig. 6) in the presence of 0.04M Na<sup>+</sup> at pH 7.0. The curves at 250, 260 and 270 nm clearly showed formation of a 1:1 complex, poly (Az)·poly (U) as in the case of poly (A)·poly (U). Raising the Na<sup>+</sup> concentration to 0.15M, inflection points were observed at the concentration of poly (Az) : poly (U) equal to 1:2. (Fig. 7) It seems therefore, that a complex, poly (Az)·2poly (U) was formed in this conditions.

As shown in Fig. 8, this complex formation by poly (Az) and poly (U) was also supported by measurements of CD before

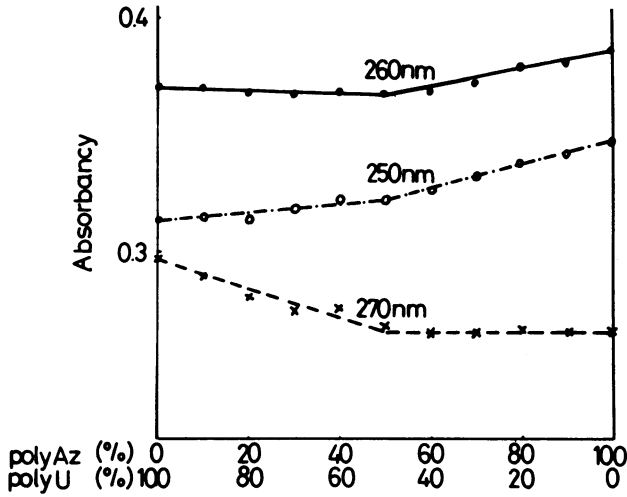


Fig. 6. Mixing curves of poly (Az) and poly (U) at 0.04M Na<sup>+</sup>.

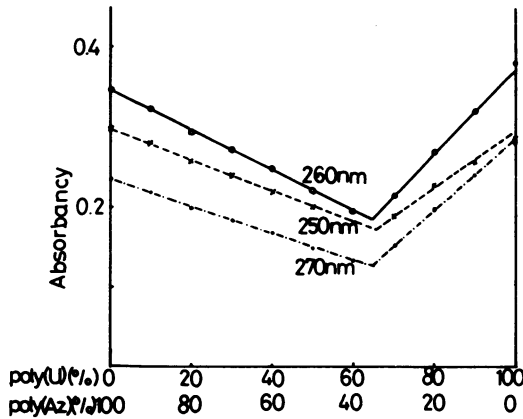


Fig. 7. Mixing curves of poly (Az) and poly (U) at Na<sup>+</sup> concentration of 0.15M.

and after the mixing of two components. The CD curve before the mixing (----) showed a peak at 273 nm and a trough at 246 nm. After the mixing the curve changed to a completely different one (—), which had a peak at 263 nm and a



trough at 242 nm. This fact suggested the formation of a complex. The overall shape of the CD curve resembled that of poly (A)·2 poly (U), except that the trough has a much smaller ellipticity.

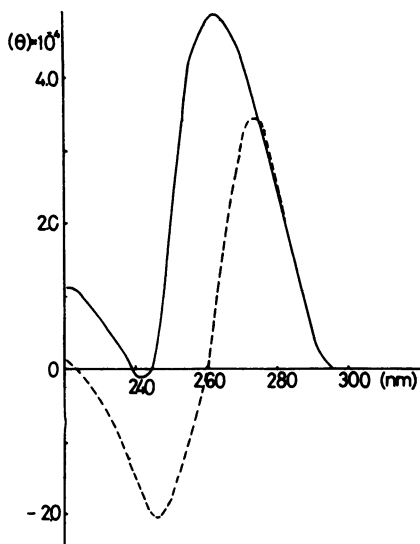


Fig. 8. CD curves of poly (Az) plus poly (U) before and after mixing at 0.15 M  $\text{Na}^+$  concentration.

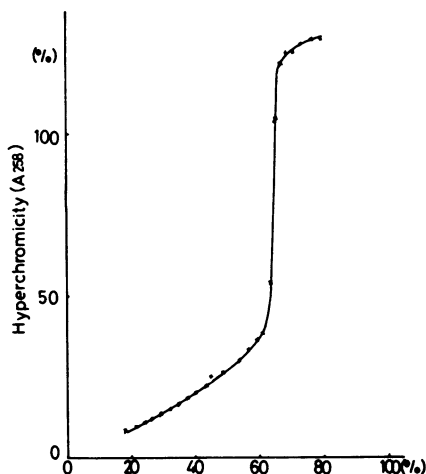


Fig. 9. Temperature-absorption profile of poly (Az)·poly (U) in the presence of 0.15M  $\text{Na}^+$ .

#### Melting of poly (Az)-poly (U) complexes

When the absorbance of poly (Az)·poly (U) complex was measured at 0.04 M  $\text{Na}^+$  concentration and pH 7.0, it rose gradually from 20° to 43° and then steeply until 48°. The curve tapered off after 50°. The hyperchromicity reached around 40%. This melting curve gave a  $T_m$  value 46° for the poly (Az)·poly (U) complex. As it was reported previously the poly (A)·poly (U) complex has a  $T_m$  of 47° in the same salt concentration<sup>19</sup>, this value of poly (Az)·poly (U) complex seems to be reasonable. In this comparison it may be deduced that the effect of an azido group for the stability of a double strand complex is same as an OH group.

The temperature-absorption profile of the poly (Az)·

2 poly (U) complex was then measured in the presence of 0.15 M Na<sup>+</sup> and at pH 7.0 (Fig. 9 ). The total concentration was again 0.04 mM. The absorbance gradually increased from 17° up to around 60°, rose steeply after 63° and reached a plateau after 68°. This shows a T<sub>m</sub> at 65° and a hyperchromicity as large as 46%.

This T<sub>m</sub> value of poly (Az) · 2poly (U) is somewhat higher than that observed in poly(A) · 2poly(U) (60°).<sup>19</sup> Since the T<sub>m</sub> of poly (dA) · 2poly (U) was reported to be 46°, a comparable effect of 2'-substitution by OH or N<sub>3</sub> for stabilization of the complex can be noted. It is known that the methyl or ethyl group or the 2'-OH of poly (A) reduced the thermal stability of heteroduplexes.<sup>13</sup> Therefore, not only the size of the 2'-substituent, but also its hydrophilicity must be taken into account for the stabilization of the complex.

### Concluding Remarks

By the introduction of the azido group at the 2'-carbon of 2'-deoxyadenylic acid in the polynucleotide chain, it becomes possible to compare the nature of polynucleotides of purine nucleotides with various 2'-substituents. The adenine bases in poly (Az) are rather well-stacked in the neutral form maybe because of the hydrophilic nature of this group. The space which is filled by an azido group must be smaller than an O-methyl or O-ethyl group and the sterical distortion might be comparable to that of a hydroxyl group.

Comparing the CD spectrum of poly (Az) with that of poly (A), it may be concluded that the stacking arrangement of bases in the former polynucleotides is somewhat different to that of poly (A). If we adopt Tinoco's theory,<sup>17</sup> the angle between transition moments of adjacent bases may be smaller in poly (Az) than in poly (A). Tilting of the base planes may also account for this.

When poly (Az) form a complex with poly (U), it forms a 1:2 complex in 0.15M Na<sup>+</sup> solution. This complex has a T<sub>m</sub> higher than that of poly (A) · 2poly (U). The stabilising effect of the azido group seems to be invariable by complexing. This may be because 2'-substitutions are working as a hydrogen-

bond acceptor by virtue of its polarizable nature.

Introduction of other 2'-substituents to the purine polynucleotides may provide more information about the factors which stabilise secondary structures of these polynucleotides and their complexes.

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