Effects of Salt Concentration and pH on the Absorption Spectra of Polynucleotides with Various S-Values

Yoshiki KATOH, Akira KUNINAKA and Hiroshi YOSHINO

Research Laboratory, Yamasa Shoyu Co., Ltd., Choshi

Received April 2, 1975

Poly (A) took stable double-stranded form in 0.02 M phosphate buffer pH 5.8 when its S-value was higher than 3.5. Poly (C) also took stable double-stranded form in 0.01 M acetate buffer pH 3.9 containing 0.1 M NaCl if its S-value was higher than 4.8.

Poly (I) with S-values higher than 6.5 stably formed triple-stranded form. Poly (A) with S-values over 3S and poly (I) with S-values over 7S were observed to aggregate in the presence of high concentration of salt, but poly (C) and poly (U) did not aggregate under the same condition. Effect of salt concentration differed with different polynucleotides and with different salts.

Polynucleotides were synthesized enzymatically by Grunberg-Manago *et al.* in 1955.¹⁾ Since then various biochemical activities thereof have been observed. Especially, interferon-inducing activities of double stranded polynucleotides seem to promise pharmaceutical uses. To investigate the relation between the molecular size or conformation and the activities inducing interferon and/or host resistance to viral infection, we synthesized various sizes of polynucleotides. Molecular size requirements of poly (I)·poly (C) for the induction of interferon and/or viral resistance have been already reported.²⁾ This paper is concerned with the relationship between the UV absorption spectra and S-values of polynucleotides in various environments. Such relationship has not so precisely been described, although many reports on physicochemical characteristics of polynucleotides have been published.³⁾

MATERIALS AND METHODS

Polynucleotides. Various sizes of polynucleotides (purity: more than 99 %) were synthesized in our laboratory according to the method described previously.^{4,5)} Nucleoside-5'-monophosphates were Yamasa's commercial products.

Ultracentrifuge. $s_{20,w}^0$ values of polynucleotides were determined according to the method described

previously.⁴⁾

Spectra. UV absorption spectra of polynucleotides and 5'-mononucleotides were determined by means of a Hitachi model 124 double-beam spectrophotometer and model 056 recorder at 23°C.

pH. pH was determined by means of a Hitachi-Horiba model F-7 pH meter at 23°C.

RESULTS

Poly (A)

Absorption spectra of poly (A) (5.9S and 9.6S) at various pH were carefully investigated in 0.02 M buffers. Marked difference was not observed between both preparations. As shown in Fig. 1, steep change of absorption spectrum was observed at pH value around 6. Thus, absorption spectra of poly (A) with various S-values were investigated around pH 6 (Fig. 2). At pH 6.8, where poly (A) was reported to be a flexible single-strand helix with stacked bases,^{6~9)} only small difference of absorption spectra between poly (A) with 1.4S and those with over 1.8S was observed. At pH 5.8, where poly (A) was reported to be a double-stranded structure,^{6~9)} marked difference of absorption spectra was observed between poly (A) with 1.4S and that with over 1.8S: The E_{max} of poly (A) was observed to decrease gradually accompanied by a gradual hypsochromic shift of

FIG. 1. The Effect of pH on the Absorption Spectrum of Poly (A) with 5.9S.

Three ml of poly (A) in water (100 $\mu\text{g}/\text{ml}$) was mixed with *ca.* 6 ml of a buffer and filled up to 10 ml. The final concentration of poly (A) was 30 $\mu\text{g}/\text{ml}$, and the final buffer concentration was 0.02 M. Absorption spectra were immediately scanned against the 0.02 M buffer. Scan speed was 60 nm/min and chart speed was 120 mm/min for the determination of O.D. Scan was repeated carefully with the scan speed of 30 nm/min and chart speed of 240 mm/min for the determination of λ_{max} and λ_{min} . Buffers used were HCl-NaCl (pH 2~3.2), acetic acid-Na acetate (pH 3.5~4.5), KH_2PO_4 - Na_2HPO_4 (pH 5.0~8.2), NH_4OH - NH_4Cl (pH 8.5~10.7), Na_2HPO_4 -NaOH (pH 11.6) and NaCl-NaOH (pH 9.6~12.1). $E(p)$ was determined by hydrolyzing a sample of known absorbancy to mononucleotide with 0.3 N KOH for 18 and 24 hr at 37°C. $\circ$, E_{max} ; $\bullet$, E_{min} ; $\triangle$, λ_{max} ; $\blacktriangle$, λ_{min} ; $\square$, E_{260} .

λ_{max} with increase of its S-value until 3.5S. E_{max} and λ_{max} of poly (A) with 1.4S at pH 5.8 were substantially the same as those at pH 6.8. Absorption spectrum of 5'-AMP at pH 6.8 (λ_{max} : 258.8 nm, λ_{min} : 226.5 nm, E_{max} : 15.5×10^3 , E_{min} : 2.6×10^3) was much differed from that of poly (A) with 1.4S. These results indicate that the double-stranded form of poly (A) was stabilized in sizes with S-value larger than 3.5S in 0.02 M phosphate buffer, pH 5.8, and this value was much greater than S-value which was required for the stabilization of ordered single-stranded structure in 0.02 M phosphate buffer, pH 6.8 (1.4S or less).

Next, the effect of salt concentration on the

FIG. 2. Absorption Spectra of Poly(A) with Various S-Values.

Absorption spectra were determined in the same manner as described in Fig. 1.

$\times$ --- $\times$, 0.02 M phosphate buffer (pH 5.8); $\times$ --- $\times$, 0.02 M phosphate buffer (pH 6.8); $\circ$ --- $\circ$, 0.1 M NaCl (pH 5.8~6.1); $\bullet$ --- $\bullet$, 1 M NaCl (pH 5.5~5.8); $\triangle$ --- $\triangle$, 1 M KCl (pH 5.8~6.4); $\blacktriangle$ --- $\blacktriangle$, 2 M KCl (pH 5.9~6.6); $\square$ --- $\square$, 0.5 M NH_4Cl (pH 5.2~5.4); $\square$ --- $\square$, 1 M NH_4Cl (pH 5.0~5.2); $\blacksquare$ --- $\blacksquare$, 2 M NH_4Cl (pH 4.8~4.9).

absorption spectra of various sizes of poly (A) was carefully investigated (Fig. 2). The E_{max} values of poly (A) with S-values ranging from 1.8S to 7.9S in 0.1 M (pH 5.8~6.1) and 1 M NaCl (pH 5.5~5.8) were smaller than those in 0.02 M phosphate buffer at pH 6.8 and greater than those in 0.02 M phosphate buffer at pH 5.8, indicating that the secondary structure of poly (A) was partially formed in 0.1 and 1 M NaCl. The solution of poly (A) with 10.1S in 1 M NaCl became slightly turbid indicating that the aggregation occurred, and the E_{max} and E_{min} became greater and the λ_{max} and λ_{min} became longer than those in

0.1 M NaCl. The $E_{\max}$, $E_{\min}$, $\lambda_{\max}$ and $\lambda_{\min}$ of poly (A) in 1 M KCl were nearly equal to those in 0.02 M phosphate buffer, pH 6.8. The solution of poly (A) with S-value over 3S in 2 M KCl became turbid indicating that the aggregation occurred. In such solution owing to the light scattering the $E_{\max}$ and $E_{\min}$ were larger, and $\lambda_{\max}$ and $\lambda_{\min}$ were longer than those in 1 M KCl. The spectra in 2 M NaCl were similar to those in 2 M KCl (The data are not shown). The spectra of poly (A) in 0.5 M NH_4Cl were similar to those in 0.02 M phosphate buffer, pH 5.8, except that $\lambda_{\max}$ of poly (A) with 1.8S was shorter and $E_{\max}$ of this was smaller in 0.5 M NH_4Cl than those in 0.02 M phosphate buffer, pH 5.8. Even the solution of poly (A) with S-value over 3S in 2 M NH_4Cl did not become turbid, but the $E_{\max}$ and $E_{\min}$ were larger and $\lambda_{\max}$ and $\lambda_{\min}$ were longer than those in 0.5 M NH_4Cl . Strange to say, the $E_{\max}$ of poly (A) with S-value over 8S in 2 M NH_4Cl were smaller than those in 1 M NH_4Cl and substantially the same as those in 0.5 M NH_4Cl . These results indicate that poly (A) with S-value larger than 3S aggregated in 2 M KCl and NaCl perhaps by the result of interruption of intramolecular base-stacking in the presence of high concentration of alkali metal ion which can not be replaced by ammonium ion.

Poly (C)

Absorption spectra of poly (C) (9.9S and 5.2S) at various pH were carefully investigated in 0.02 M buffers. As only small differences were observed between absorption spectra of poly (C) with 5.2S and those of 9.9S, the data from poly (C) with 5.2S were plotted in Fig. 3. As steep changes of absorption spectra were observed at pH around 6 and pH around 4, absorption spectra of poly (C) with various S-values were investigated at pH 9 and pH 3.9 (Fig. 4). At pH 9.0, where poly (C) was reported to be single-strand helix with stacked bases,¹⁰⁻¹²⁾ gradual decrease of $E_{\max}$ of poly (C) was observed with increase of its S-value, and marked difference was observed between $E_{\max}$ of 5'-CMP and that of poly (C) with

FIG. 3. The Effect of pH on the Absorption Spectra of Poly(C) with 5.2S.

Absorption spectra were determined in the same manner described in Fig. 1.

○, $E_{\max}$; ●, $E_{\min}$; △, $\lambda_{\max}$; ▲, $\lambda_{\min}$; □, E_{280} .

1.3S. At pH 3.9 in 0.01 M acetate buffer, $E_{\max}$ of poly (C) was observed to be substantially constant over the S-value range 1S to 10.5S. While 1 M NaCl did not affect the absorption spectra of poly (C) at pH 9.0, NaCl of 0.1 M and 1 M greatly decreased $E_{\max}$ of poly (C) at pH 3.9 probably by stabilizing double-stranded structure. Under this condition gradual decrease of $E_{\max}$ was observed with increase of its S-value until 4.8S. Unlike poly (A) and poly (I), poly (C) did not aggregate even in the presence of high concentration of NaCl. These results indicate that the base-stacked secondary structure of single-stranded form of poly (C) was stabilized in sizes with a S-value larger than about 1S, and double-stranded structure of poly (C) was stabilized in sizes with S-value larger than 4.8S, and that the secondary structure of poly (C) was more stable than that of poly (A) in the presence of high concentration of salt.

Poly (I)

Absorption spectra of poly (I) (10.5S) at various pH were carefully investigated in 0.02 M buffers (Fig. 5). $E_{\max}$ was constant for pH ranging from 4 to 10. It was reported

FIG. 4. Absorption Spectra of Poly(C) with Various S-Values.

Absorption spectra were determined in the same manner as described in Fig. 1.

×---×, 0.01 M acetate buffer (pH 3.9); ×—×, 0.01 M ammonium buffer (pH 9.0); ○—○, 0.1 M NaCl-0.01 M acetate buffer (pH 3.9); ●—●, 1 M NaCl-0.01 M acetate buffer (pH 3.9); ●—●, 1 M NaCl-0.01 M ammonium buffer (pH 9.0).

Zero point of abscissa indicates 5'-mononucleotide.

that poly (I) was a random, single-stranded structure in low salt concentration, and was triple-strand helix in 1 M NaCl.^{13,14} In Fig. 6, data of absorption spectra of poly (I) with various S values are illustrated. In 0.02 M phosphate buffer, pH 6.8, $E_{\max}$ of poly (I) was substantially constant regardless of S-value, and marked difference was observed between $E_{\max}$ of 5'-IMP and that of poly (I) with 1.1S. In 1 M NaCl, 0.5 M and 2 M NH_4Cl and 0.6 M and 2 M KCl, marked decrease of $E_{\max}$ was observed with increase of S-value

FIG. 5. The Effect of pH on the Absorption Spectra of Poly(I) with 10.5S.

Estimation condition and symbols are the same as employed in Fig. 1.

FIG. 6. Absorption Spectra of Poly(I) with Various S-Values.

Absorption spectra were determined in the same manner as described in Fig. 1.

×---×, 0.02 M phosphate buffer (pH 6.8); ○—○, 0.5 M NaCl (pH 5.6~6.3); ●—●, 1 M NaCl (pH 5.7~6.4); △—△, 0.6 M KCl (pH 6.0~6.2); ▲—▲, 2 M KCl (pH 6.2~6.5); □—□, 0.5 M NH_4Cl (pH 5.1~5.3); ■—■, 2 M NH_4Cl (pH 4.7~4.9).

until 3S and gradual decrease of $E_{\max}$ was then observed until S-value reached 6.6S. This hypochromic effect, which would be due to formation of triple-strand helix, was observed to be the most conspicuous in the presence of KCl. The solution of poly (I) with S-value over 6.6S in 1 M NaCl became turbid indicating that aggregation of poly (I) occurred, and owing to the light scattering the $E_{\max}$ and $E_{\min}$ were observed to increase gradually with increase of its S-value. These results indicate that the triple-stranded structure of poly (I) seems to be formed in sizes with S value greater than about 3S, and to be stabilized in sizes with S-value over about 6.5S, and that structure of poly (I) with S-value over 6.6S in 1 M NaCl was less stable than that in 2 M KCl.

Poly (U)

Absorption spectra of poly (U) with various S-values in various concentrations of salts were investigated. (The data are not shown). There was no marked difference of absorption spectra among poly (U) with various S-values in 0.1 to 2 M NaCl, KCl and NH_4Cl . Poly (U) did not aggregate even in the presence of high concentration of salt, and this characteristic was resemble to poly (C).

DISCUSSION

Marked difference of absorption spectra was observed between polynucleotide with large S-value and that with small S-value in multistranded form. In general, $E_{\max}$ decrease with increase of S-value. The similar result was observed in the case of double-stranded conformation composed of two components, poly (I) and poly (C).¹⁵⁾ These results indicate that the stability of multistranded structure strongly depended on the molecular size of polynucleotides. Furthermore, molecular size required for the stability of multistranded form seemed much larger than that required for the stability of single-stranded base-stacked form, because in single-stranded form the difference of absorption

spectra among polynucleotides with various S-values examined was observed to be smaller than that between 5'-mononucleotide and the smallest polynucleotide examined (around 1S). Singer⁶⁾ and Michelson¹⁶⁾ indicated that the smallest chain length giving rise to a double helical structure which was fully formed at or above 0°C in the presence of 0.1 M NaCl was heptamer in series of oligo (A) by the titration study. Brahms¹⁰⁾ and Michelson¹⁶⁾ also indicated that the smallest chain length required for double-helix formation of oligo (C) was 7. Our results indicate that S-value required for the stabilization of double-stranded form of poly (A) was 3.5S in 0.02 M phosphate buffer, pH 5.8, and 1.8S in the presence of 2 M NH_4Cl , pH 4.8. For the time being, we shall assume that each polynucleotide would follow the equation described by Niblack.¹⁷⁾ Under this assumption 3.5S and 1.8S are equal to molecular weight *ca.* 1.1×10^5 and 1.3×10^4 respectively, indicating chain length of *ca.* 320 and 40 nucleotides respectively. Our results also indicate that S-value required for the stabilization of double-stranded form of poly (C) was 4.8S, and this is calculated chain length of *ca.* 430 nucleotide pairs.

In the presence of high concentration of salt, poly (I) or poly (A) with large S-value aggregated and owing to the light scattering the $E_{\max}$ increased gradually with increase of S-value. This phenomenon was not observed in the case of poly (C) or poly (U). Thus, the stability of the secondary structure of polynucleotides in the presence of high concentration of salt would be affected by molecular size, kind of salt, salt concentration and kind of polynucleotide.

At acid pH, where poly (A) and poly (C) would form double-stranded structure, poly (C) showed a red shift of its absorption maximum while poly (A) showed a blue shift of its absorption maximum. There is not yet any clear explanation about this interesting phenomenon.

REFERENCES

- 1) M. Grunberg-Manago and S. Ochoa, *J. Amer. Chem. Soc.*, **77**, 3165 (1955).
 - 2) H. Machida, Y. Katoh, A. Kuninaka and H. Yoshino, *Virus*, **23**, 358 (1973); *idem*, *Jap. J. Microbiol.*, submitted for publication.
 - 3) B. Janik, "Physicochemical Characteristics of Oligonucleotides and Polynucleotides," Plenum, New York, 1971.
 - 4) Y. Katoh, A. Kuninaka and H. Yoshino, *Agr. Biol. Chem.*, **37**, 1537 (1973).
 - 5) K. Rokugawa, Y. Katoh, A. Kuninaka and H. Yoshino, *ibid.*, **39**, 1455 (1975).
 - 6) M. F. Singer, L. A. Heppel, G. W. Rushizky and H. A. Sober, *Biochem. Biophys. Acta*, **61**, 474 (1962).
 - 7) M. Leng and G. Felsenfeld, *J. Mol. Biol.*, **15**, 455 (1966).
 - 8) J. Brahms, A. M. Michelson and K. E. Van Holde, *ibid.*, **15**, 467 (1966).
 - 9) D. N. Holcomb and I. Tinoco, *Biopolymers*, **3**, 121 (1965).
 - 10) J. Brahms, J. C. Maurizot and A. M. Michelson, *J. Mol. Biol.*, **25**, 465 (1967).
 - 11) E. O. Akinrimisi, C. Sander and P. O. P. Ts'o, *Biochemistry*, **2**, 340 (1963).
 - 12) G. D. Fasman, C. Lindblow and L. Grossman, *ibid.*, **3**, 1015 (1964).
 - 13) J. Brahms and Ch. Sadron, *Nature*, **212**, 1309 (1966).
 - 14) P. K. Sarker and J. T. Yang, *Biochemistry*, **4**, 1238 (1965).
 - 15) Y. Katoh, A. Kuninaka and H. Yoshino, *Agr. Biol. Chem.*, **39**, 1327 (1975).
 - 16) A. M. Michelson and C. Monny, *Biochim. Biophys. Acta*, **149**, 107 (1967).
 - 17) J. F. Niblack, *Nature New Biology*, **233**, 52 (1971).
-