STUDIES ON SYNTHESIS AND INTERACTION OF WATER SOLUBLE NUCLEIC ACID ANALOGS

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(水溶性核酸アナログの合成とその相互作用に関する研究)

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PREFACE

The work of this dissertation was done since 1982 under the guidance by Professor Kiichi Takemoto and many other members of Prof. Takemoto Laboratory at department of Applied Fine Chemistry, Faculty of Engineering, Osaka University.

The content of this dissertation is composed of the following papers:

1. Synthesis and properties of polyamino acid derivatives containing nucleic acid bases and nucleosides.
T. Wada, E. Mochizuki, Y. Inaki, and K. Takemoto.
Peptide Chemistry 1987, 41 - 44 (1988).

2. Synthesis and interaction studies on water soluble nucleic acid analogs: Polyethyleneimine derivatives containing cytosine and hypoxanthine.
T. Wada, Y. Inaki, and K. Takemoto.
Polymer J., 21, 11 - 18 (1989).

3. Synthesis and interaction studies on water soluble nucleic acid analogs: Polyethyleneimine derivatives containing thymine and adenine.
T. Wada, Y. Inaki, and K. Takemoto.
Polymer J., 20, 1059 - 1068 (1988).

4. Synthesis and interaction studies on water soluble nucleic acid analogs: Polyethyleneimine derivatives containing uracil and 5-fluorouracil.
T. Wada, Y. Inaki, and K. Takemoto.
J. Bioactive and Compatible Polymers, 4, No. 2 (April) (1989), in Press.

5. Interaction and conformational studies on water soluble nucleic acid analogs: Polyethyleneimine derivatives containing nucleic acid bases.
T. Wada, Y. Inaki, and K. Takemoto.
Polymer J., submitted.

6. Synthesis and properties of water soluble nucleic acid analogs: Poly-L-lysine derivatives containing thymine and hypoxanthine.
T. Wada, Y. Inaki, and K. Takemoto.
J. Polym. Sci., Polym. Chem. Ed., submitted.

7. Synthesis and properties of water soluble nucleic acid analogs: Poly-L-Lysine derivatives containing adenine and cytosine.
T. Wada, Y. Inaki, and K. Takemoto.
in preparation.

The related papers

1. Synthesis and properties of polyethyleneimine containing nucleosides and nucleic acid bases.
E. Harada, T. Wada, Y. Inaki, and K. Takemoto.
J.U.C. PHARM. Sci. '87, S219 (1987).

2. Nucleic acid analogs: their specific interaction and applicability.
K. Takemoto, E. Mochizuki, T. Wada, and Y. Inaki.
Polym. Mater. Sci. Eng., 58, 50 (1988).

CONTENTS

GENERAL INTRODUCTION

Nucleic acids play an important role in realizing replication and transcription of genetic codes for the protein synthesis. The essential function of nucleic acids is based on the higher structures and the specific base pairing through hydrogen bounding between purine and pyrimidine bases. DNA has a double-stranded structure consisting of two polynucleotide chains twisted about each other in a double helix.

Some synthetic polynucleotides are known to have biological activities. A synthetic double stranded polynucleotide complex of poly inosinic acid (Poly I) and poly cytidylic acid (Poly C) (Poly I - Poly C complex) is effective as an interferon inducer,[1] while it has a high level of toxicity. Very recently, it was reported that the 1:1 complex of poly uridylic acid (Poly U) and poly adenylic acid (Poly A) (Poly U - Poly A complex) was also effective as an interferon associated enzyme inducer, which had a low level of toxicity.[2] Therefore, a polymer complex of polynucleotides with the synthetic nucleic acid analogs is expected to have enhanced bioactivity.[3]

The chemistry of synthetic nucleic acid analogs has recently received much attention and a number of synthetic polymers containing nucleic acid bases have been prepared and their properties have been studied.[4-7] These synthetic nucleic acid analogs were almost insoluble in water at neutral pH values, because most of them consisted of a hydrophobic polymer backbone with pendant nucleic acid bases. The interactions between the synthetic nucleic acid analogs, and with polynucleotides were studied. However, these studies were limited to organic solvents or to water - organic mixed solvents. It is, therefore, very important to prepare the water soluble synthetic nucleic acid analogs for biomedical field applications.

The object of this study is to prepare water soluble nucleic acid analogs and to investigate the interaction between nucleic acid analogs and with polynucleotides in aqueous solution.

Chapter I deals with the synthesis and the interaction of the water soluble polyethyleneimine derivatives containing hypoxanthine and cytosine.

Chapter II deals with the synthesis and the interaction of the

water soluble polyethyleneimine derivatives containing adenine and thymine.

Chapter III deals with the synthesis and the interaction of the water soluble polyethyleneimine derivatives containing uracil and 5-fluorouracil. And the substituent effect at 5-position of pyrimidine base on base - base interaction is discussed.

Chapter IV deals with interaction and conformation studies of water soluble polyethyleneimine derivatives containing nucleic acid bases. The effect of the conformational change on interaction is discussed.

Chapter V deals with the synthesis and the interaction of the water soluble poly-L-lysine derivatives containing thymine and hypoxanthine.

References

1. J. H. Park, M. A. Gallin, A. Billiau, and S. Baron, *Arch. Ophthal.*, 81, 840 (1969).
2. H. B. Levy, *J. Bioact. Compat. Polym.*, 1, 348 (1986).
3. T. Sato, K. Kojima, T. Ihda, J. Sunamoto, and R. M. Ottenbrite, *J. Bioact. Compat. Polym.*, 1, 448 (1986).
4. J. Pitha, *Adv. Polym. Sci.*, 50, 1 (1983).
5. K. Takemoto and Y. Inaki, *Adv. Polym. Sci.*, 41, 1 (1981).
6. Y. Inaki and K. Takemoto, "*Current Topics in Polymer Science, Volume I*", R. M. Ottenbrite, L. A. Utracki and S. Inoue, Eds., Hanser Publisher, p 79, (1987).
7. K. Takemoto and Y. Inaki, "*Functional Monomers and Polymers*", K. Takemoto, Y. Inaki, and R. M. Ottenbrite, Eds, Marcel Dekker, Inc., p 149, (1987).

Chapter I SYNTHESIS AND INTERACTION STUDIES ON WATER SOLUBLE POLYETHYLENEIMINE DERIVATIVES CONTAINING CYTOSINE AND HYPOXANTHINE.

I - 1 INTRODUCTION

Synthetic polynucleotides are known to have biological activity. A synthetic double stranded polynucleotide complex of polyinosinic acid(Poly I) and polycytidylic acid(Poly C) (Poly I - Poly C Complex) is effective as an interferon inducer [1-5], while it has a high level of toxicity.[6] On the other hand, a number of synthetic polymers containing nucleic acid bases have been prepared and their properties studied. These synthetic nucleic acid analogs are very stable and have been found to have biological activity.[7-17] Therefore a polymer complex of polynucleotides with the synthetic nucleic acid analogs may be stable and be expected to have enhanced bioactivity.[18]

The synthetic nucleic acid analogs, however, were hardly soluble in water at neutral pH values, because most of them consist of a hydrophobic polymer backbone and pendant nucleic acid bases. It is, therefore, very important to prepare water soluble synthetic nucleic acid analogs in order to apply the analogs to biological fields.

The present article deals with the preparation of water soluble polyethyleneimine derivatives containing cytosine and hypoxanthine, interaction between these polymers and their interactions with Poly I or Poly C in aqueous solution. The bioactivity of polymer complex of the nucleic acid analogs and with polynucleotides will be shown elsewhere.[19]

I - 2 EXPERIMENTAL

Materials

Ethyl 3-(cytosyl-1-yl)propionate (1C)

To a suspension of N^4-acetyl cytosine [20] (27 g; 180 mmol) in ethanol (800 mL) with catalytic amount of sodium, ethyl acrylate (28

mL; 260 mmol) was added dropwise with stirring. The reaction was carried out at 90° C for 5 h to afford a clear solution. After the reaction, the solvent was removed under reduced pressure. The residue was recrystallized from ethanol to give ethyl 3-(cytosyl-1-yl)propionate (1C) in 94% yield (35 g); mp 199-201° C. IR (KBr,cm^{-1}): 1730 (ester), 1650, and 1610 (cytosine). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 1.10 (t, 3H), 2.50 (t, 2H), 3.90 (m, 4H), 5.55 (d, 1H) and 7.45 (d, 1H).
ANAL. Calcd for $C_9H_{13}N_3O_3$; C, 51.17%; H, 6.20%; N, 22.73%. Found; C, 51.06%; H, 6.26%; N, 22.77%.

3-(Cytosyl-1-yl)propionic acid (2C; 1-β-carboxyethyl cytosine)

The ester 1C (1.7 g; 6.7 mmol) was hydrolyzed by 1N NaOH aqueous solution at 60° C for 2 h. After the reaction, pH of the solution was adjusted to 5.0 by 1N HCl to give a white precipitate. The precipitate was collected and recrystallized from water to give 3-(cytosyl-1-yl)propionic acid (2C) in 80% yield (960 mg); mp 264-266 ° C(dec). IR (KBr,cm^{-1}): 1710 (C=O), and 1650 (cytosine). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 3.85 (t, 2H), 5.65 (d, 1H), and 7.55 (d, 1H).
ANAL. Calcd for $C_7H_9N_3O_3$; C, 45.90%; H, 4.95%; N, 22.94%. Found; C, 46.09%; H, 5.04%; N, 22.30%.

(±)-α-N-[3-(cytosyl-1-yl)propionyl]amino-γ-butyrolactone (4C; Cyt-Hse-L)

To a solution of the 1-β-carboxyethyl cytosine (1.3 g; 7.0 mmol) in N,N-dimethylformamide (40 mL), pentachlorophenyl trichloroacetate (4.5 g; 11.0 mmol) and catalytic amount of triethylamine were added. The mixture was stirred at 80° C overnight. The precipitate was filtered and washed with diethyl-ether to give 2.7 g of pentachlorophenyl 3-(cytosyl-1-yl) propionate (3C) in 90% yield. To a solution of (±)-α-amino-γ-butyrolactone hydrobromide (0.91 g; 5.0 mmol) in N,N-dimethyl formamide (20 mL), pentachlorophenyl 3-(cytosyl-1-yl)propionate (4C) (2.2 g; 5.0 mmol), triethylamine (0.7 mL; 5.0 mmol), and imidazole (340 mg; 5.0 mmol) were added and the reaction was carried out for one day at 50° C. After the reaction, the solvent was evaporated under reduced pressure. Acetone (150 mL) was added to the oily residue and

stirred for 4 h to give a light brown precipitate. The precipitate was washed with water (50 mL) and dried under reduced pressure. The yield was 1.2 g (88 %); mp 236-238° C. IR (KBr,cm^{-1}) : 1770 (lactone), 1650, 1530 (amide), 1670, and 1620 (cytosine). Thin Layer Chromatography(TLC): Rf. 0.44 (benzene:ethanol=3:1). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.50 (t, 2H), 3.90 (t, 2H), 4.40 (m, 4H), 4.70 (m, 1H), 5.60 (d, 1H), 7.55 (d, 1H), and 8.45 (d, 1H).
ANAL. Calcd for $C_{11}H_{14}N_4O_4$: C,49.63%; H,5.30%; N,21.04%. Found: C,48.93%; H,5.40%; N,21.31%.

(±)- α -N-[3-(hypoxanthyl-9-yl)propionyl]amino- γ -butyrolactone (4H; Hy-Hse-L)

To a solution of (±)- α -amino- γ -butyrolactone hydrobromide (910 mg; 5.0 mmol) in N,N-dimethylformamide (20 mL), pentachlorophenyl 3-(hypoxanthyl-9-yl)propionate [21](2.3 g; 5.0 mmol), triethylamine (0.7 mL; 5.0 mmol) and imidazole (340 mg; 5.0 mmol) were added. The mixture was stirred at 40° C for 20 h to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The residue was washed with acetone and then with water. Recrystallization from ethanol-benzene gave crystals in 79% yield (1.1g); mp 218-221° C. IR (KBr,cm^{-1}): 1770 (lactone), 1650, 1530 (amide), and 1680 (hypoxanthine). TLC: Rf. 0.53 (benzene:ethanol=3:1). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 4.20 (t, 2H), 4.50 (m, 4H), 4.70 (s, 1H), 7.80 (s, 1H), 8.00 (s, 1H), 8.50 (d, 1H), and 12.35 (s, 1H).
ANAL. Calcd for $C_{17}H_{13}N_5O_3$: C,38.83%; H,4.07%; N, 22.63%. Found: C, 38.65%; H, 3.98%; N,22.65%.

Polyethyleneimine (PEI)

Poly(2-ethyl-2-oxazoline) (PEOX presented by Dow Chemical Japan Co., M.W.= 50000, 40 g) was hydrolyzed in 6 N hydrochloric acid (200 mL) at 90° C for 48 h. After the reaction, the white precipitate was filtered and washed with 200 mL of ethanol to give polyethyleneimine hydrochloride. Then the polymer was dissolved in water (200 mL), and neutralized by NaOH (25 g; 0.36 mmol) at 90° C for 24 h. The solution was cooled in ice bath to give the polymer as a precipitate. The polymer was filtered and washed with 3 L of water and dried.

The hydrolysis was found to be complete from NMR and IR spectra.[22]

Poly-N- [{ *2-* [*3-(cytosyl-1-yl)propionyl*] *amino-4-hydroxy* } *butanoyl*] *ethyleneimine* (5C; PEI-Hse-Cyt)

To a suspension of Cyt-Hse-L (4C)(390 mg; 1.5 mmol) in water (5 mL), 1 N NaOH aqueous solution (1.5 mL; 1.5 mmol) was added. The mixture was stirred at 40° C for 1 h to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The resulting residue was washed with acetone (20 mL), then with diethyl ether (30 mL) to give a sodium salt of cytosine derivative (Cyt-Hse-Na) in 98 % yield(430 mg, powder). TLC: Rf. 0.00 (benzene : ethanol=3:1), (ethanol), (n-butanol : acetic acid : water =4:5:3)

To a suspension of Cyt-Hse-Na (430 mg; 1.5 mmol) in 10 mL of dry N,N-dimethylformamide, pentachlorophenyl trichloroacetate (910 mg; 2.2 mmol) and a catalytic amount of triethylamine were added. The mixture was stirred at 0° C for 2 h and then at 50° C until evolution of gas ceased to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The residue was washed with diethyl ether (30 mL) to give a powder of pentachlorophenyl ester of cytosine derivative (Cyt-Hse-PCP)(yield 92 %; 720 mg). TLC: Rf. 0.82 (benzene : ethanol = 3:1).

To a solution of polyethyleneimine (70 mg; 1.5 mmol) in N,N-dimethylformamide (10 mL), Cyt-Hse-PCP (720 mg; 1.5 mmol) and imidazole (90 mg; 1.5 mmol) were added and stirred at 60° C for 48 h. After the reaction, the solution was concentrated under reduced pressure and poured into excess acetone to precipitate the polymer.

The obtained polymer was purified by reprecipitation from N,N-dimethylformamide into excess acetone. The polymer was dissolved again in a small amount of water and freeze-dried to give a light brown powder. (360 mg, 78 %); mp 210-215° C. IR (KBr,cm^{-1}): 1650 and 1550 (amide). TLC: Rf. 0.00 (benzene : ethanol = 3:1), (ethanol). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.5 (t, 2H), 3.70 (m, 8H), 4.30 (m, 3H), 5.65 (d, 1H), 7.50 (d, 1H), and 8.60 (s, 1H).

Poly-N- [{ *2-* [*3-(hypoxanthyl-9-yl)propionyl*] *amino-4-hydroxy*} *butanoyl*] *ethyleneimine* (5H; PEI-Hse-Hyp)

This polymer was prepared from 4H (1.0 g; 3.6 mmol) and polyethyleneimine (160 mg; 3.6 mmol) according to a similar procedure described for PEI-Hse-Cyt (5C) (yield 68%; 820 mg; mp 166-172 ° C). IR (KBr,cm^{-1}): 1650 and 1550 (amide). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 3.70(m, 8H), 4.30 (m, 3H), 7.80 (s, 1H), 8.00 (s, 1H), and 8.60 (s, 1H).

Hydrolysis of the polymer

The polyethyleneimine derivatives were hydrolyzed in 6 N hydrochloric acid at 80 ° C for 48 h, into polyethyleneimine hydrochloride and the carboxyethyl derivatives of nucleic acid bases. Quantitative calculation was made using the corresponding carboxyethyl derivatives as standard samples. The nucleic acid base content in the polymers is tabulated in Table I -I.

Interactions between the polymers

Interactions of the polymers were determined from hypochromicity values in UV spectra as reported previously.[23]

The UV spectra were measured with a JASCO UV-660 spectrometer equipped with a temperature controller at 20 ° C. Poly I (sodium salt)($S^{\circ}_{20,w}$: 6-12), Poly C (sodium salt) ($S^{\circ}_{20,w}$: 6-12) were obtained from Yamasa Shoyu Co. Ltd. PEI-Hse-Cyt(5C), PEI-Hse-Hyp(5H) and polynucleotides were dissolved in Kolthoff buffer (pH 7.0) (1/10 M KH_2PO_4 - 1/20 M $Na_2B_4O_7 \cdot 10H_2O$). These solutions stocked for 2 days at 20 ° C, were mixed to give a polymer mixture of 10^{-4} M total concentration of nucleic acid base units in aqueous solution.

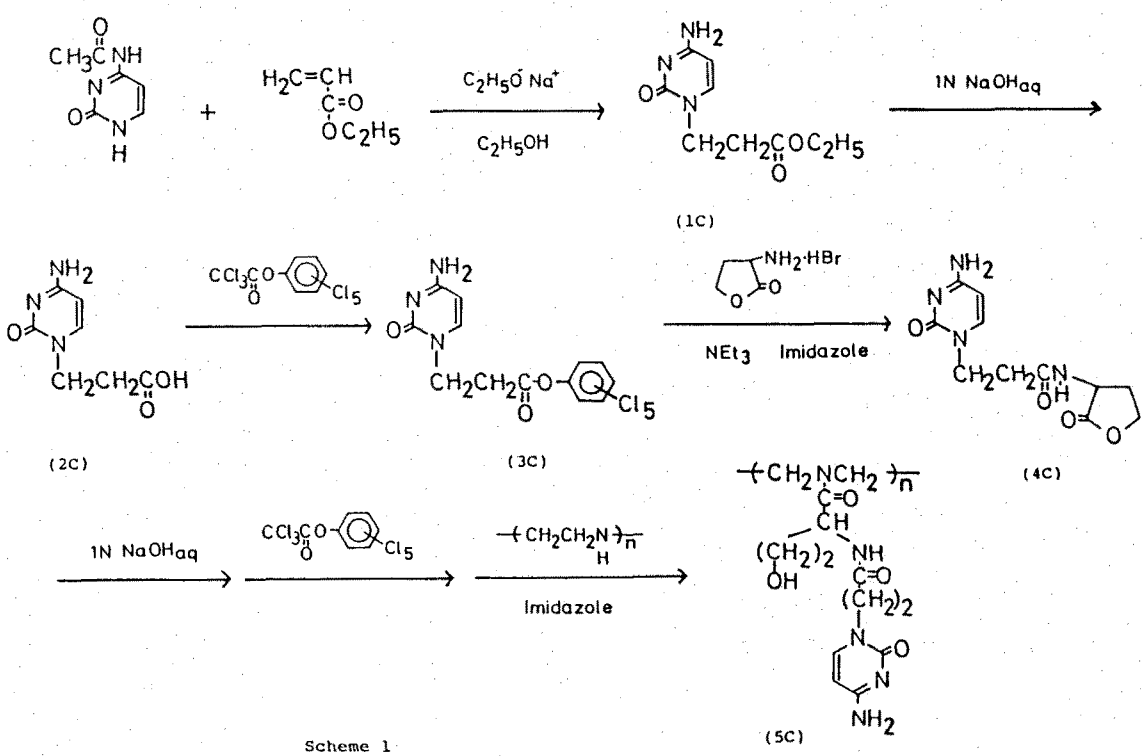
I - 3 RESULTS AND DISUCUSSION

Various kinds of synthetic nucleic acid analogs have been prepared. Most of these analogs, however, are not soluble in water, while polynucleotides are freely soluble in water. Therefore, interaction studies on nucleic acid analogs are limited in organic solvents or in mixture of water and organic solvents. A water soluble nucleic acid analog makes it possible to study interactions of a polymer with polynucleotides in an aqueous solution and biological activity of the analogs.

Overberger and Inaki prepared polyethyleneimine derivatives containing nucleic acid bases and amino acids.[24] One of these derivatives was a polyethyleneimine having a nucleic acid base and serine soluble in water. In the present study, water soluble polyethyleneimine derivatives containing nucleic acid bases were prepared. The derivatives contained homoserine units as a spacer between the polyethyleneimine and the nucleic acid base (Scheme I -1 and Scheme I -2).

Homoserine derivatives of nucleic acid bases

Starting with cytosine or adenine, the carboxyethyl derivatives of the nucleic acid bases were prepared by Michael type addition reaction of ethyl acrylate followed by hydrolysis. In the case of cytosine, the 4-amino group was protected by the acetyl group because of low solubility in ethanol, although the acetyl group was removed during the reaction. In the case of hypoxanthine, the



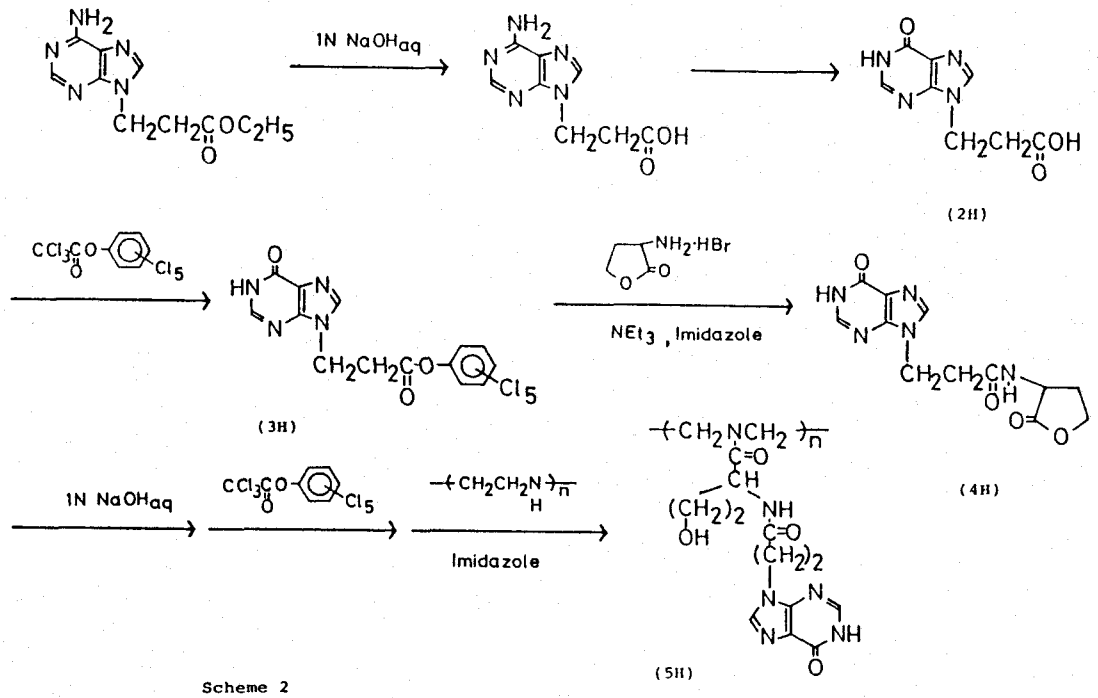
Scheme 1

addition occurred at the 7-position of the base. Therefore, the carboethoxyethyl derivative of hypoxanthine was prepared by the deamination reaction of carboxyethyl derivatives of adenine.[21]

The carboxyethyl derivatives of cytosine or hypoxanthine were reacted with (±)-α-amino-γ-butyrolactone hydrobromide to give γ-butyrolactone derivatives of cytosine or hypoxanthine. For the reactions, pentachlorophenyl ester derivatives were used with imidazole as a catalyst.[25] It was not necessary to protect the amino group of cytosine base.

Grafting onto polyethyleneimine

The grafting of nucleic acid base derivatives having hydroxyl group onto polyethyleneimine backbone was carried out by the activated ester method. Since the reactivity of the γ-lactone is low, direct reactions of the lactone derivative with polyethyleneimine hardly occurred. Therefore, the lactone derivatives were



Scheme 2

hydrolyzed to 3-hydroxybutylic acid derivatives, followed by condensation with polyethyleneimine using the activated ester method. The grafting reaction was carried out in N,N-dimethylformamide, where a small amount of 4-pyroridino pyridine was an effective catalyst. The nucleic acid base content of the polymer was determined by UV spectroscopy of the hydrolyzed sample. Quantitative calculations were made using the corresponding carboxyethyl derivatives as standard samples, and are tabulated in Table I-1.

Table I Contents of PEI-Hse-Cyt and PEI-Hse-Hyp.

	PEI-Hse-Cyt	PEI-Hse-Hyp
Contents(%)	86	92

Interactions of the polymers

Natural and synthetic polynucleotides are known to form polymer complexes by specific base - base interactions with nucleic acid bases. Synthetic nucleic acid analogs such as polyethyleneimine and polylysine derivatives containing nucleic acid bases were also found to form polymer complexes with polynucleotides by specific base - base interactions.[7,8,26-28] Most of these nucleic acid analogs are slightly soluble in water. Therefore, these studies were carried out in organic solvents or water - organic mixed solvents, such as dimethyl sulfoxide, ethylene glycol, and water - propylene glycol.

The water soluble polyethyleneimine derivatives of cytosine or hypoxanthine make it possible to study interactions with polynucleotides in neutral aqueous solution. Formation of the polymer complex was observed for the PEI-Hse-Cyt - PEI-Hse-Hyp system, and for polyethyleneimine derivatives - polynucleotides (Poly I, Poly C) systems.

Interactions of PEI-Hse-Cyt and PEI-Hse-Hyp

Figure I -1 shows the mixing curve between PEI-Hse-Cyt(5C) and PEI-Hse-Hyp(5H) in the Kolthoff buffer solution (pH 7.0). The figure shows the highest hypochromicity value at a base unit ratio of about

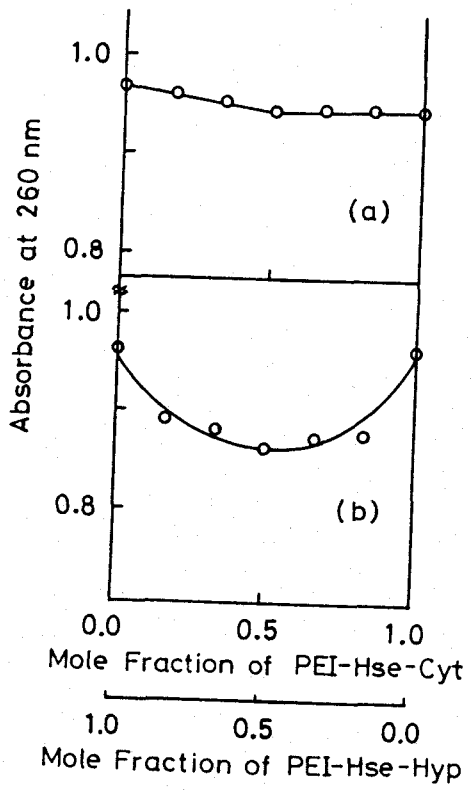


Figure I -1. Continuous Variation Curve of PEI-Hse-Hyp and PEI-Hse-Cyt.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PEI-Hse-Hyp] = 9.5 x 10^{-5} mol/L, and [PEI-Hse-Cyt] = 9.6 x 10^{-5} mol/L.

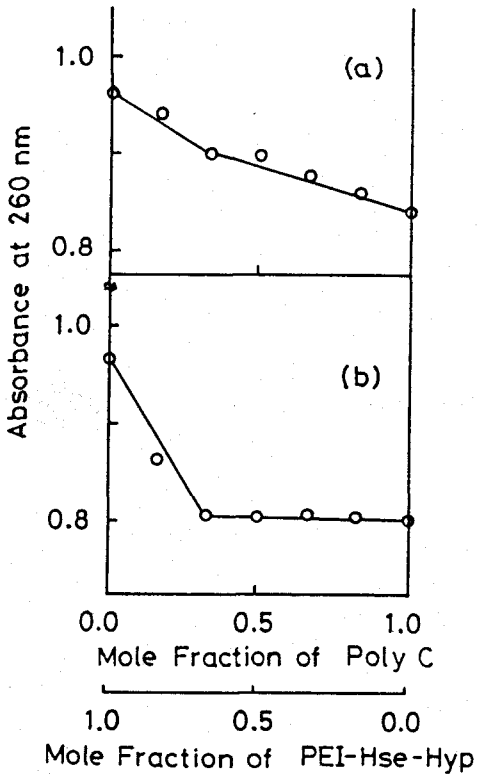


Figure I -2. Continuous Variation Curve of PEI-Hse-Hyp and Poly C.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PEI-Hse-Hyp] = 9.5 x 10^{-5} mol/L, and [Poly C] = 1.2 x 10^{-4} mol/L.

1:1 (hypoxanthine : cytosine), suggesting the formation of a stable 1:1 polymer complex due to complementary nucleic acid base interactions. The hypochromicity value (21%) was higher than that of the PEI-Ade - PEI-Thy system in the literature.[26] As shown in Figure 1, time dependence of the hypochromicity value was observed. Therefore the conformational change of synthetic polymers may be important for formation of a stable polymer complex.[21,29]

Interactions of PEI-Hse-Hyp with Poly C

Interactions between PEI-Hse-Hyp(5H) and Poly C which contains the complementary nucleic acid base can be clearly observed in aqueous solution at pH 7.0, as shown in Figure I -2. The overall

stoichiometry of the complex based on the nucleic acid base units was approximately 2:1 (hypoxanthine : cytosine) under the conditions used. The maximum hypochromicity value (26 %) was smaller than that of the Poly I - Poly C (33%) system but higher than the values of other synthetic polymer analog - polynucleotides systems.[7,26]

Formation of the Poly I - Poly C polymer complex was also studied under the same conditions used in this study (Kolthoff buffer solution). The stoichiometry of the complex based on the nucleic acid base units was 2:1 (hypoxanthine : cytosine) as in Figure I -3. The maximum hypochromicity value of this system was 33 %. The stoichiometry of Poly I - Poly C complex is reported to be 1:1 in tris - HCl buffer solution.[30] It is known that the concentration of salt affects the stoichiometry of the Poly I - Poly C complex.[30-31] Therefore, the salt concentration may affect the stoichiometry observed for the PEI-Hse-Hyp - Poly C system in our case.

Another factor controlling the stoichiometry of the complex may be the structure of the polymers. The base - base distance in PEI-Hse-Hyp is different from that in Poly C. This fact also affects the abnormal stoichiometry of the PEI- Hse-Hyp - Poly C complex. Self association of the nucleic acid bases in the polymer is also an important factor for the formation of the polymer complex. The rate of complex formation for polyethyleneimine derivatives was slow compared with that of polynucleotide systems. The absorbance of a mixed solution of the polymers decreased slowly and became constant after 3 days. This was caused by self association of the nucleic acid bases in the polyethyleneimine derivatives, which dissociated slowly to form the intermolecular polymer complex.

Interactions of PEI-Hse-Cyt and Poly I

Figure I -4 shows the mixing curve between PEI-Hse-Cyt(5C) and Poly I after 3 h and 3 days at pH 7.0 in the Kolthoff buffer aqueous solution. The stoichiometry of the complex was 2:1 (hypoxanthine : cytosine) and the hypochromicity value was 7 %. The stoichiometry of the PEI-Hse-Cyt - Poly I complex was similar to that of PEI-Hse-Hyp - Poly C system.

The hypochromicity value was small compared to that of the PEI-Hse-Hyp - Poly C system. Polynucleotides containing purine base form very stable structures by self association in aqueous

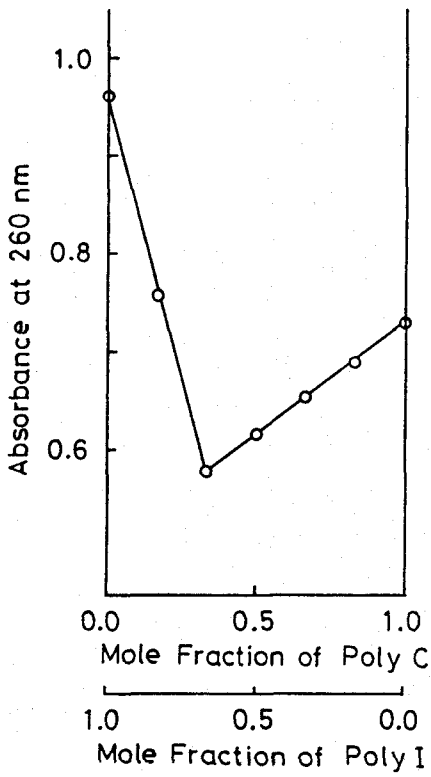


Figure I -3. Continuous Variation Curve of Poly I and Poly C.

Absorbance at 260 nm in 0.05 M Kolthoff buffer solution(pH 7.0) at 20° C. [Poly I] = 1.2 x 10^{-4} mol/L, and [Poly C] = 1.1 x 10^{-4} mol/L.

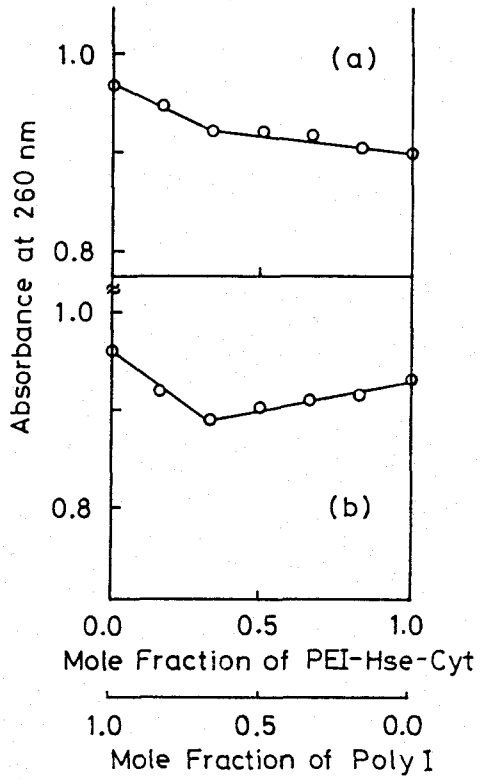


Figure I -4. Continuous Variation Curve of PEI-Hse-Cyt and Poly I.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly I] = 1.2 x 10^{-4} mol/L, and [PEI-Hse-Cyt] = 9.6 x 10^{-5} mol/L.

solution, while the structures of polynucleotides containing pyrimidine bases are not stable in aqueous solution. Poly I forms stable structures by self association in aqueous solution. Therefore, the intermolecular interactions of Poly I with PEI-Hse-Cyt become small because of enhanced intramolecular interactions of hypoxanthine bases in Poly I.[32]

I-4 REFERENCES

1. J. H. Park, M. A. Gallin, A. Billiau, and S. Baron, *Arch. Ophthal.*, 81, 840 (1969).

2. H. B. Levy, *J. Bioactive and Compatible Polymers*, 1, 348 (1986).

3. A. K. Field, A. A. Tytell, G. P.Lampson, and M. R. Hillman, *Proc. Nat. Acad. Sci. U.S.A.*, 58, 1004 (1967).
4. M. M. Nemes, A. A. Tytell, G. P. Lampson, A. K. Field, and M. R. Hillman, *Proc. Soc. Exp. Biol. Med.*, 132, 776 (1969).
5. V. DeVita, G. Canellos, P. Carbone, H. Levy, and H. Gralnick, *Proc. Am. Assoc. Cancer Res.*, 11, 21 (1970).
6. M. Absher and W. R. Stinebring, *Nature*, 223, 715 (1969).
7. J. Pitha, *Adv. Polym. Sci.*, 50, 1 (1983).
8. K. Takemoto and Y. Inaki, *Adv. Poly. Sci.*, 41, 1 (1981).
9. C. G. Overberger and Y. Morishima, *J. Polym. Sci., Polym. Chem. Ed.*, 18, 1247, 1267 (1980).
10. A. G. Ludwick and C. G. Overberger, *J. Polym. Sci., Polym. Chem. Ed.*, 20, 2123, 2139 (1982).
11. K. A. Brandt and C. G. Overberger, *J. Polym. Sci., Polym. Chem. Ed.*, 23, 1981 (1985).
12. C. G. Overberger and S. Kikyotani, *J. Polym. Sci., Polym. Chem. Ed.*, 21, 525, 541 (1983).
13. C. G. Overberger and C. X. Lu, *J. Polym. Sci., Polym. Chem. Ed.*, 23, 1321 (1985).
14. C. G. Overberger and C. C. Chen, *J. Polym. Sci., Polym. Chem. Ed.*, 24, 167 (1986).
15. N. M. Brahme and W. T. Smith Jr., *J. Polym. Sci., Polym. Chem. Ed.*, 22, 813 (1984).
16. W. T. Smith, Jr. and N. M. Brahme, *J. Polym. Sci., Polym. Chem. Ed.*, 23, 879 (1985).
17. A. G. Ludwick, K. S. Robinson, and J. McCloud, Jr., *J. Polym. Sci., Polym. Symp.*, 74, 55 (1986).
18. T. Sato, K. Kojima, T. Ihda, J. Sunamoto, and R. M. Ottenbrite, *J. Bioactive and Compatible polym.*, 1, 448 (1986).
19. J. Sunamotoand K. Takemoto, to be published.
20. D. M. Brown, A. R. Todd, and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956).
21. Y. Inaki, S. Sugita, T. Takahara, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, 24, 3201 (1986).
22. C. G. Overberger and C. C. Chen, *J. Polym. Sci., Polym. Chem. Ed.*, 25, 373 (1987).
23. G. J. Thomas, Jr. and Y. Kyogoku, *J. Am. Chem. Soc.*, 89, 4170 (1967).

24. C. G. Overberger and Y. Inaki, *J. Polym. Sci., Polym. Chem. Ed.*, 17, 1739 (1979).
25. Y. Inaki, Y. Sakuma, Y. Suda, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, 20, 1917 (1982).
26. J. Pitha, P. M. Pitha, and E. Stuart, *Biochemistry*, 10, 4595 (1971); *Biopolymer*, 9, 965 (1970).
27. C. G. Overberger, Y. Inaki, and Y Nambu, *J. Polym. Sci., Polym. Chem. Ed.*, 17, 1759 (1979).
28. A. Ferro, T. Minaga, W. N. Piper, and E. Kun, *Biochim. Biophys. Acta*, 519, 299 (1978).
29. S. Fang, Y. Inaki, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, 22, 2455 (1984).
30. D. Thiele, W. Guschlbauer, and A Faver, *Biochim. Biophys. Acta*, 272, 22 (1972).
31. K. Fujioka, Y. Baba, A. Kagemoto, and R. Fujishiro, *Polym. J.*, 12, 843 (1980).
32. Y. Inaki and K. Takemoto, *Makromol. Chem. Supple.*, 14, 91 (1985).

Chapter II SYNTHESIS AND INTERACTION STUDIES ON WATER SOLUBLE POLYETHYLENEIMINE DERIVATIVES CONTAINING THYMINE AND ADENINE.

II-1 INTRODUCTION

Recently, a number of synthetic polynucleotide analogs have been prepared and their properties have been studied. These synthetic nucleic acid analogs were found to form polymer complexes by specific interaction between complementary nucleic acid bases [1-13].

Most of the nucleic acid analogs consist of hydrophobic polymer backbone and pendant nucleic acid bases, and are insoluble in neutral water. Therefore the interactions of these nucleic acid analogs were studied in organic solvents or in water-organic mixed solvents. On the other hand, polynucleotides are freely soluble in neutral water, and are insoluble in organic solvents. For the application of the nucleic acid analogs to biological fields, it is very important to study the interactions of the synthetic analogs with polynucleotides in neutral aqueous solution. For this purpose, in the previous paper, water soluble polyethyleneimine derivatives containing cytosine and hypoxanthine were prepared and the interactions of these polymers with polynucleotides were studied[14].

In the present study, water soluble polyethyleneimine derivatives containing thymine and adenine are prepared, and their interactions with polyuridylic acid (Poly U) or polyadenylic acid (Poly A) in aqueous solution are investigated. The bioactivity of the polymer complex of the nucleic acid analogs and with polynucleotides will be shown elsewhere[15].

II-2 EXPERIMENTAL

(±)- α -N-[3-(thymin-1-yl)propionyl]amino- γ -butyrolactone (4T; Thy-Hse-L)

To a solution of (±)- α -amino- γ -butyrolactone hydrobromide (910 mg; 5.0 mmol) in N, N-dimethylformamide (30 mL), pentachlorophenyl 3-(thymin-1-yl)propionate (3T)[16] (2.2 g; 5.0 mmol), triethylamine (0.7

mL; 5.0 mmol), and imidazole (340 mg; 5.0 mmol) were added in that order and stirred for one day at 50° C. After the reaction, the solvent was evaporated under reduced pressure. Acetone (150 mL) was added to the oily residue and stirred further for 4 h to give a light brown precipitate. The precipitate was washed with water (50 mL) and dried under reduced pressure. The yield was 1.4 g (97 %); mp 274-276 ° C. IR (KBr, cm^{-1}): 1770 (lactone), 1650, 1530 (amide), 1700, and 1670 (thymine). Thin layer chromatography (TLC): Rf. 0.48 (benzene:ethanol=3:1). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 1.70 (s, 1H),2.45 (t, 2H), 3.78 (t, 2H), 4.25 (m, 4H), 4.55 (m, 1H), 7.35 (s,1H), 8.45 (d, 1H) and 11.05 (s, 1H).

ANAL. Calcd for $C_{12}H_{15}N_3O_5$: C, 51.24%; H, 5.38%; N, 14.94%. Found: C, 50.95%; H, 5.37%; N, 14.90%.

(±)- α -N-[3-(N^6-diphenylphosphinothioyl-adenin-9-yl)propionyl] amino-γ -butyrolactone (4PptA; PptAde-Hse-L)

To a solution of (±)- α -amino- γ -butyrolactone hydrobromide (910 mg; 5.0 mmol) in N, N-dimethylformamide (20 mL), pentachlorophenyl 3-(N^6-diphenylphosphinothioyl-adenin-9-yl)propionate (3PptA)[17] (3.4 g; 5.0 mmol), triethylamine (0.7 mL; 5.0 mmol) and imidazole (340 mg; 5.0 mmol) were added. The mixture stirred at 60° C for 20 h to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The residue was washed twice with 30 mL of diethyl ether and then with 40 mL of ethyl acetate. The oily residue stirred with 40 mL of diethyl ether at room temperature for 48 h. Resulting precipitate was collected and washed with 30 mL of water and dried to give light yellow powder (2.5 g; 97 %) . mp 115-117° C. IR (KBr, cm^{-1}): 1770 (lactone), 1650, 1530 (amide), 1660, 1600, and 1580 (Ppt-adenine). TLC: Rf. 0.53 (benzene:ethanol=3:1). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.78 (t, 2H), 3.53 (t, 2H) 4.40 (m, 4H), 4.68 (m, 1H), 7.50 (s, 10H), 8.32 (s, 1H), 8.51 (s, 1H), and 8.60 (s, 1H).

ANAL. Calcd for $C_{24}H_{23}N_6O_3PS$: C, 56.92%; H, 4.56%; N, 16.59%. Found: C, 56.52%; H, 4.36%; N, 16.24%.

(±)- α -N-[3-(adenin-9-yl)propionyl]amino- γ -butyrolactone hydrobromide (4A; Ade-Hse-L)

PptAd-Hse-L (4PptA)(2.0 g; 4.0 mmol) was added to 10 mL of

trifluoroacetic acid. The mixture stirred at room temperature for 2 h to afford a light brown clear solution. Then 30 mL of 25 % hydrogen bromide acetic acid solution was added to this solution. The reaction was carried out at 0° C for 2 h and then at room temperature for 12 h. After the reaction, the mixture was evaporated under reduced pressure. The resulting residue was washed several times with diethyl ether. The precipitate was collected and washed with 20 mL of acetone to yield 1.2 g (78 %). mp 130-133 ° C. Rf. 0.05 (benzene:ethanol=3:1).

ANAL. Calcd for $C_{12}H_{14}N_6O_3$ HBr: C, 38.83%; H, 4.07%; N, 22.63%. Found: C, 38.15%; H, 3.81%; N, 22.65%.

Poly-N- [*{2-[3-(thymin-1-yl)propionyl]amino-4-hydroxy }butanoyl*] *ethyleneimine* (5T; PEI-Hse-Thy)

To a suspension of Thy-Hse-L (4T)(1.0 g; 3.6 mmol) in water (5.0 mL), 1 N NaOH aqueous solution (3.6 mL; 3.6 mmol) was added. The mixture stirred at 40° C for 1 h to afford a light brown clear solution.

After the reaction, the solvent was evaporated under reduced pressure. Resulting residue was washed with acetone (20 mL), then with diethyl ether (30 mL) to give a sodium salt of thymine derivative (Thy-Hse-Na) in 96 % yield(1.1 g, powder). TLC: Rf. 0.00 (benzene:ethanol=3:1), (ethanol), (n-butanol:acetic acid:water =4:5:3).

To a suspension of Thy-Hse-Na (1.1 g; 3.2 mmol) in 10 mL of dry N, N-dimethylformamide, pentachlorophenyl trichloroacetate (1.8 g; 4.5 mmol) and catalytic amount of triethylamine were added. The mixture stirred at 0° C for 2 h and then at 50° C until gas evolution was ceased to give a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The residue was washed with diethyl ether (30 mL) to give the pentachlorophenyl ester of thymine derivative (Thy-Hse-PCP)(yield 92 %; 1.8 g). TLC: Rf. 0.82 (benzene:ethanol = 3:1).

To a solution of polyethyleneimine [14] (160 mg; 3.5 mmol) in N, N-dimethylformamide (10 mL), Thy-Hse-PCP (2.1 g; 3.5 mmol) and imidazole (210 mg; 3.5 mmol) were added and stirred at 60° C for 48 h.

After the reaction, the solution was concentrated under reduced pressure and was poured into excess acetone to precipitate the polymer. The obtained polymer was purified by reprecipitation from N, N-dimethylformamide into excess acetone. The polymer was

dissolved in a small amount of water and freeze-dried to give light brown powder (860 mg, 78 %); mp 116-120° C. IR (KBr, cm^{-1}): 1650 and 1550 (amide). TLC: Rf. 0.0 (benzene:ethanol = 3:1), (ethanol). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 1.75 (s, 3H), 2.70 (t, 2H), 3.40 (m, 8H), 3.80 (m, 4H), 7.20 (s, 1H), 7.50 (d, 1H), and 8.40 (s, 1H).

Poly-N- 〔 *{2-[3-(adenin-9-yl)propionyl]amino-4-hydroxy}butanoyl*〕 *ethyleneimine* (5A; PEI-Hse-Ade)

The polymer was prepared by two methods.

I) The polymer was prepared from 4A (1.4 g; 3.6 mmol) and polyethyleneimine (160 mg; 3.6 mmol) according to a similar procedure described for PEI-Hse-Thy (5T) (yield 68%; 820 mg), mp 95-98 ° C. IR (KBr, cm^{-1}): 1650 and 1550 (amide). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 3.70(m, 8H), 4.30 (m, 3H), 7.40 (s, 1H), 7.60 (s, 1H), and 8.60 (s, 1H).

II) *Poly-N-* 〔 *{2-[3-(N6-diphenylphosphinothioyl-adenin-9-yl) propionyl]amino-4-hydroxy}butanoyl* 〕 *ethyleneimine* (5PptA; PEI-Hse-PptAde) was prepared from 4PptA (2.0 g; 4.0 mmol) and polyethyleneimine (180 mg; 4.0 mmol) according to the similar procedure described for PEI-Hse-Thy (5T). Resulting PEI-Hse-PptAde (5PptA, 1.6 g; 2.9 mmol; 73%) was dissolved in 20 mL of trifluoroacetic acid and 10 mL of 25% hydrogen bromide acetic acid solution was added. The reaction was carried out at 0° C for 5 h and then at room temperature over night. After the reaction, the mixture was concentrated under reduced pressure and poured into excess acetone to give PEI-Hse-AdeHBr. The obtained polymer was dissolved in 5 mL of water and the pH of the solution was adjusted to 7.0 by NaOH aqueous solution. After purification of the polymer from water-acetone, the polymer was dissolved in a small amount of water and freeze-dried to give 770 mg of PEI-Hse-Ade (80 %); mp 93-97° C. IR (KBr, cm^{-1}): 1650 and 1550 (amide). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 3.70(m, 8H), 4.30 (m, 3H), 7.40 (s, 1H), 7.60 (s, 1H), and 8.60 (s, 1H).

Hydrolysis of the polymer

The polyethyleneimine derivatives were hydrolyzed in 6 N hydrochloric acid at 80 ° C for 48 h, into polyethyleneimine

hydrochloride and carboxyethyl derivatives of nucleic acid bases. The quantitative calculation was made by using the corresponding carboxyethyl derivatives as standard samples. The nucleic acid base contents of the polymers are tabulated in Table II-I.

Interaction between the polymers

Interaction of the polymers was estimated by hypochromicity values in UV spectra as reported previously [18].

The UV spectra were measured with an JASCO UV-660 spectrometer equipped with a temperature controller at 20° C. Polyuridylic acid (Poly U) (sodium salt)($S^{\circ}_{20,w}$: 6-12) and polyadenylic acid (Poly A) (sodium salt) ($S^{\circ}_{20,w}$: 6-12) were obtained from Yamasa Shoyu Co. Ltd. PEI-Hse-Thy (5T), PEI-Hse-Ade (5A) and polynucleotides were dissolved in Kolthoff buffer (pH 7.0) (1/10 M KH_2PO_4 - 1/20 M $Na_2B_4O_7.10H_2O$). These solutions stocked for 2 days at 20° C were mixed to give a polymer mixture of 10^{-4} mol/L total concentration of nucleic acid base units in aqueous solution.

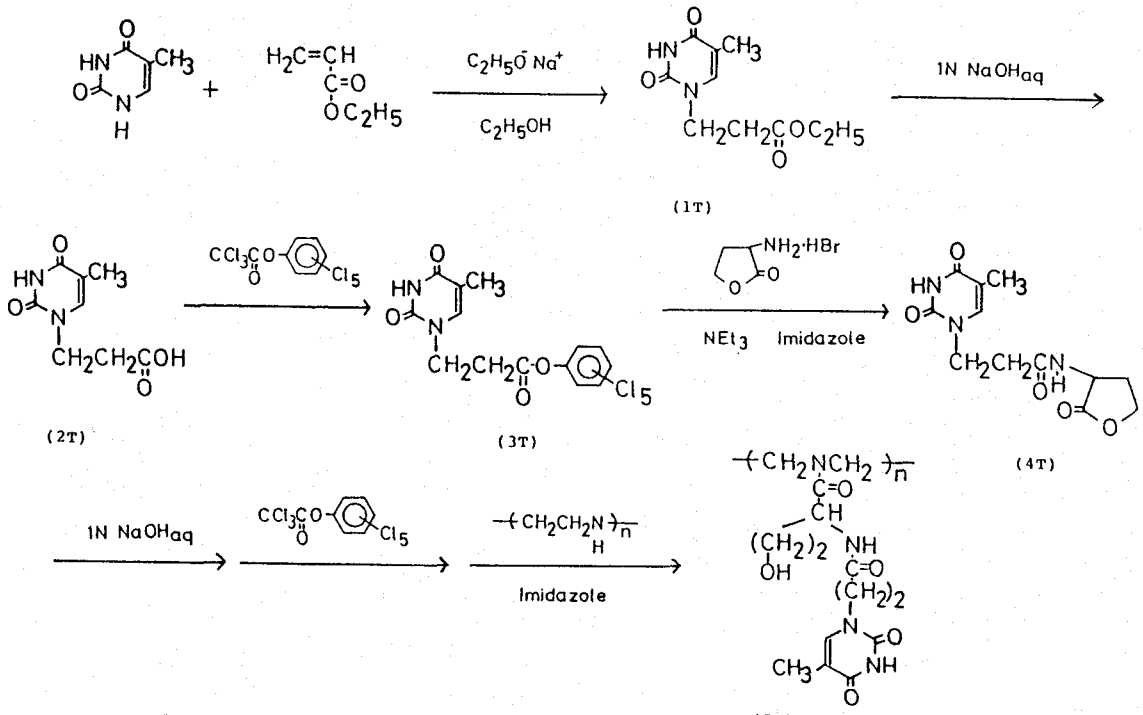
II-3 RESULTS AND DISCUSSION

Various kinds of synthetic nucleic acid analogs have been prepared by polymerization of the corresponding monomers or by grafting of the nucleic acid base derivatives on polymers[1]. Most of these analogs, however, are insoluble in water at neutral pH, while polynucleotides are freely soluble in water. Therefore the interaction studies of the nucleic acid analogs were limited in organic solvents or in the mixture of water and organic solvents. A water soluble nucleic acid analog makes it possible to study the interactions of the polymers with polynucleotides in aqueous solution and biological activity of the analogs.

The most convenient method of preparing the nucleic acid analog is the grafting of the nucleic acid base derivatives to functional polymers. By this method, the nucleic acid analogs have been prepared such as polyacrylate, polylysine, and polyethyleneimine derivatives containing nucleic acid bases. In order to prepare the water soluble polymers containing high content of nucleic acid bases, hydrophilic units should be introduced to the side chains.

Overberger and Inaki prepared polyethyleneimine derivatives containing nucleic acid bases, in which amino acids were used as the spacer[19]. One of these derivatives was the polyethyleneimine having nucleic acid base and serine as a spacer, which was soluble in water. For the preparation of the serine derivative, however, protections of the functional groups were necessary.

In the previous study, (±)- α -amino- γ -butyrolactone hydrobromide was used as a starting compound[14]. This lactone has a stable lactone ring where hydroxyl and carbonyl groups are protected, and gave homoserine unit by ring opening reaction. By using this lactone, polyethyleneimines containing nucleic acid bases and homoserine unit were successfully prepared in high yield. Schemes II-1 and II-2 show the preparation of the water soluble nucleic acid analogs containing thymine and adenine. The butyrolactone derivatives of thymine and adenine were obtained as stable solid in high yield.



(1T) (2T) (3T) (4T) (5T)

Scheme 1

Homoserine derivatives of nucleic acid bases

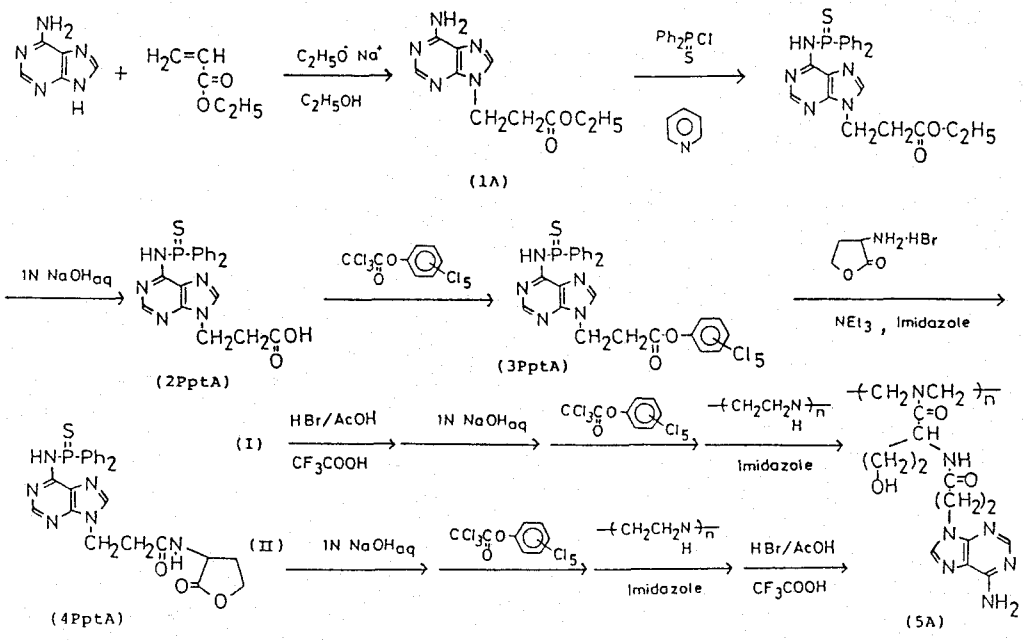
Starting with thymine or adenine, the carboxyethyl derivatives of the nucleic acid bases (1T and 1A) were prepared by Michael type addition reaction of ethyl acrylate. The N^6 amino group of the adenine base was protected by diphenylphosphinothioyl group in order to suppress the intramolecular catalytic activity of the adenine base for the activated ester derivative.

The carboxyethyl derivative of thymine (2T) or adenine (2PptA) was reacted with (±)- α -amino- γ -butyrolactone hydrobromide to give γ -butyrolactone derivative of thymine (4T) or adenine (4PptA).

For the reactions, pentachlorophenyl ester derivatives were used with imidazole as a catalyst[16].

Grafting onto polyethyleneimine

Since the reactivity of the γ -lactone is low, the direct reaction of the lactone derivative with polyethyleneimine was hardly occurred.



Scheme 2 Ph: 

Therefore the activated ester method was used. The lactone derivatives were hydrolyzed to the 3-hydroxybutyric acid derivatives, followed by the condensation with polyethyleneimine using the activated ester method. The grafting reaction was carried out in N, N-dimethylformamide, where a small amount of 4-pyrrolidinopyridine was used as an effective catalyst. The polymers containing thymine (PEI-Hse-Thy; 5T) was obtained in high yield. For the preparation of the polymers containing adenine (PEI-Hse-Ade; 5A), it is necessary to remove the protection group of adenine base. In the route (I) of Scheme II-2, the protecting group of the lactone derivative (4PptA) was removed before the grafting reaction. On the other hand, the protecting group was removed after the grafting reaction in route (II). The yield of the polymer in route (II) was higher than that of in route (I).

Nucleic acid base content of the polymer was determined by UV spectroscopy on their hydrolyzed samples. The quantitative calculation was made by using the corresponding carboxyethyl derivatives as standard samples, and nucleic acid base contents in the polymer (unit mole %) were tabulated in Table II-I.

Table I Contents of PEI-Hse-Thy and PEI-Hse-Ade.

	PEI-Hse-Thy	PEI-Hse-Ade
Contents(unit mol%)	97	92

Interaction of the polymers

Natural and synthetic polynucleotides are known to form a polymer complex by specific base-base interaction between nucleic acid bases. The synthetic nucleic acid analogs such as polyethyleneimine and polylysine derivatives containing nucleic acid bases were also found to form polymer complexes with polynucleotides by the specific base-base interactions [2,3]. As the solubilities of these nucleic acid analogs in water were low, the specific interactions were studied in organic solvents or water-organic mixed solvents, such as dimethyl sulfoxide, ethylene glycol, and water-propylene glycol[20-22].

On the contrary, the polyethyleneimine derivatives of thymine or

adenine having homoserine spacer unit were soluble in water over the entire range of pH values. This fact made it possible for the nucleic acid analogs to interact with polynucleotides in neutral aqueous solution. The complex formation of the nucleic acid analogs with polynucleotides in water is very important for the investigation of bioactivity of the polymers[23]. The interactions in water were thus studied between the polyethyleneimine derivatives, between the polymers and polynucleotides such as Poly U and Poly A.

Interaction of PEI-Hse-Thy and PEI-Hse-Ade

The interactions between water soluble polyethyleneimine derivatives containing thymine (PEI-Hse-Thy) and adenine (PEI-Hse-Ade) at pH 7.0 were studied with continuous variation techniques (Job plot[17]). As shown in Figure II -1b, the stoichiometry of the complex based on nucleic acid base units was determined to be 1:1 (thymine : adenine) from the base ratio at the maximum hypochromicity value (10.4 %). Hypochromicity has been widely used to indicate the interaction of nucleic acid base derivatives. The hypochromicity values for the PEI-Hse-Thy and PEI-Hse-Ade system (1:1 base unit) were determined at pH 2.2 and 5.5. At pH 2.2 the hypochromicity was negligible even after 3 days. On the other hand, the hypochromicity value at pH 5.5 was 7.1%, which was comparable to the value at pH 7.0. Adenine base has a pKa value at 4.15, and exists in a protonated form at pH 2.2. The protonated adenine base can not form a complex with the complementary thymine base[21]. This may be the reason for the negligible hypochromicity value at pH 2.2 and the high hypochromicity value at pH 5.5. From these facts, the polymer complex between PEI-Hse-Thy and PEI-Hse-Ade at pH 7.0 was concluded to be formed by the complementary nucleic acid base interaction.

In this system, time dependence of hypochromicity value was observed as shown in Figure II -1. The hypochromicity was not observed in 3 hours after mixing of the polymer solutions (Figure II -1a). The absorbance decreased (the hypochromicity increased) and then became constant after 3 days (Figure II -1b). Therefore, the conformational change of the synthetic polymers should be important for the formation of a stable polymer complex[24].

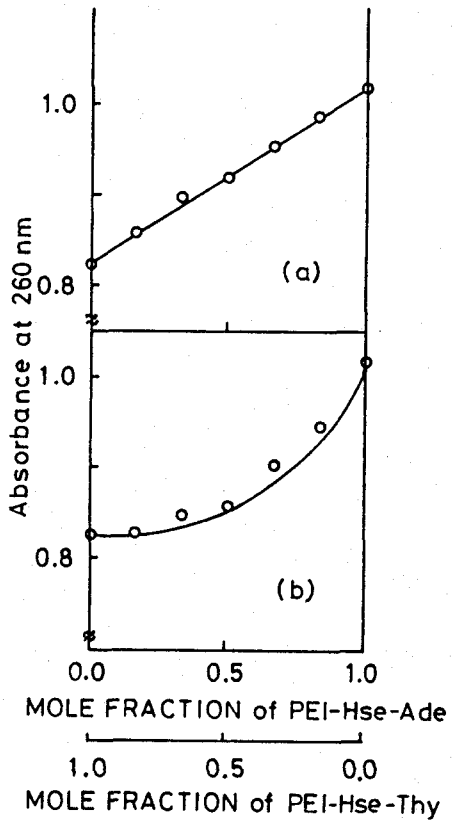


Figure II-1. Continuous Variation Curve of PEI-Hse-Thy and PEI-Hse-Ade.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PEI-Hse-Thy] = 1.0×10^{-4} mol/L, and [PEI-Hse-Ade] = 8.3×10^{-5} mol/L.

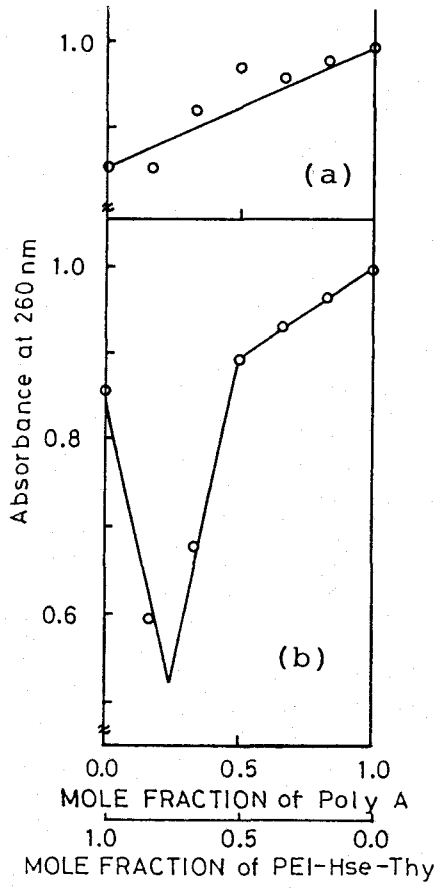


Figure II-2. Continuous Variation Curve of PEI-Hse-Thy and Poly A.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PEI-Hse-Thy] = 1.1×10^{-4} mol/L, and [Poly A] = 1.1×10^{-4} mol/L.

It has been known that formation of the polymer complexes between the nucleic acid analogs is influenced by several essential factors: nature of the polymers such as flexibility, stereoregularity, and electric charge, effect of solvent, and temperature. In studies of the interaction of the synthetic nucleic acid analogs, it was revealed that these factors closely related to the intramolecular and the intermolecular interactions of the nucleic acid bases in the polymer[16,17,24]. The nucleic acid bases in the synthetic nucleic acid analogs are self-associated intramolecularly in a solution. If the self-association of the bases is weak, the polymer forms a intermolecular polymer complex with the complementary polymer immediately after mixing, which is accompanied by a conformational change. However, for strong self-association of the nucleic acid

bases, it is necessary to break the association by heating, addition of good solvent, or change of pH value.

The time dependency in Figure II -1 suggests that the self-association of the nucleic acid bases in the polyethyleneimine derivatives dissociated slowly accompanying change of conformation, and formed the intermolecular polymer complex by the interaction between adenine and thymine.

Interaction of PEI-Hse-Thy with Poly A

The polyethyleneimine derivatives containing both the nucleic acid bases and the homoserine units are soluble in water, therefore it is possible to study the interactions of the polymers with nucleic acids and polynucleotides in aqueous solution. Figure II -2 shows the mixing curves for the PEI-Hse-Thy with Poly A at pH 7.0. The maximum hypochromicity value was obtained as 38.4 % at the base unit ratio of 2:1 (thymine:adenine) (Figure II -2b). The overall stoichiometry of the complex based on nucleic acid base units, therefore, was 2:1 (thymine : adenine). However, Figure II -2b indicates that the polymer complex contains both 2:1 and 1:1 complexes. The maximum hypochromicity value was higher than that of the PEI-Hse-Thy : PEI-Hse-Ade system. The value was also higher than the value both for the PEI-Hse-Hyp : Poly C system (26 %), and for the PEI-Hse-Cyt : Poly I (7 %) system in the previous paper[14].

The stoichiometry of the polymer complex PEI-Hse-Thy : Poly A, however, was different from that of the PEI-Hse-Thy : PEI-Hse-Ade complex. As a control experiment, the interaction between Poly A and Poly U was studied under the condition used here. As shown in Figure II -3, the complex formation was observed immediately after mixing of the polymer solutions, and the maximum hypochromicity (40.3 %) was observed at the base ratio of 2:1 (uracil:adenine). The reason of the formation of the 2:1 complex is known to be ability of single strand formation for Poly A. The 2:1 polymer complex of PEI-Hse-Thy : Poly A, therefore, may be formed by the same reason for the Poly A : Poly U complex.

To make sure that such complex formation is due to the complementary nucleic acid base interaction, the interaction of PEI-Hse-Thy with Poly U was measured under the same condition.

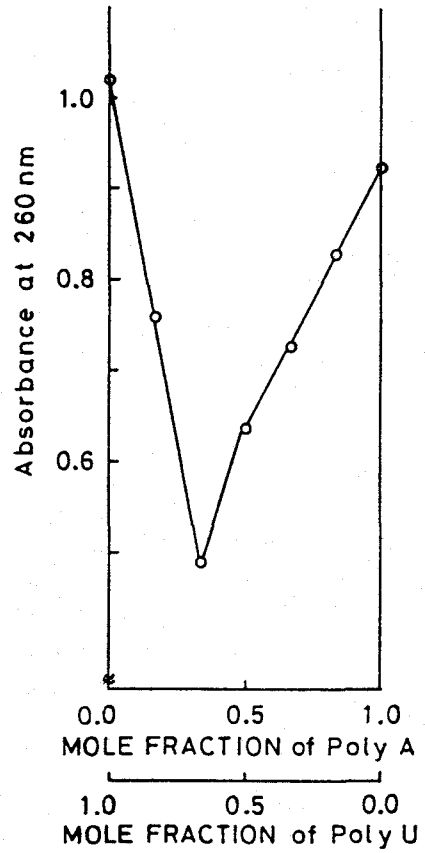


Figure II-3. Continuous Variation Curve of Poly U and Poly A.

Absorbance at 260 nm in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly U] = 1.0 x 10^{-4} mol/L, and [Poly A] = 9.9 x 10^{-5} mol/L.

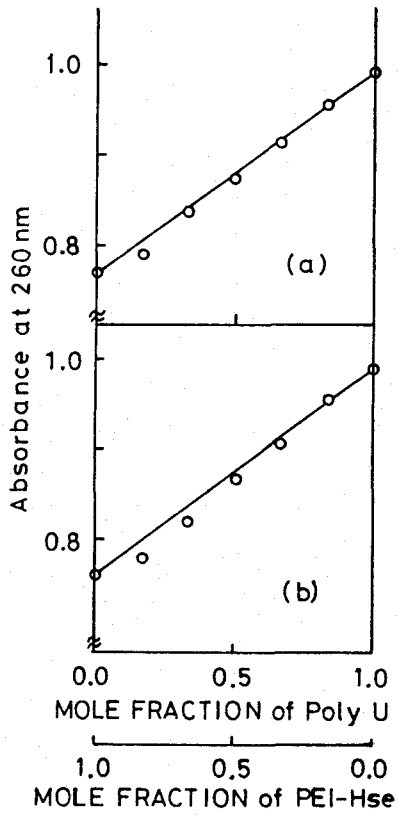


Figure II-4. Continuous Variation Curve of PEI-Hse-Thy and Poly U.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PEI-Hse-Thy] = 9.8 x 10^{-5} mol/L, and [Poly U] = 1.0 x 10^{-4} mol/L.

Figure II-4 shows the mixing curves for PEI-Hse-Thy : Poly U at pH 7.0. The hypochromicity, however, could not be observed after 3 hours (Figure II-4a) and even after 3 days (Figure II-4b). From these facts, the formation of the PEI-Hse-Thy : Poly A complex was concluded to be caused by the complementary interaction between adenine and thymine.

Time dependence of absorbance was also observed in this case as shown in Figures II-2 (a) and (b). The hypochromicity was scarcely observed in 3 hours after mixing of the polymer solutions (Figure II-2a), but the absorbance decreased and became constant in 3 days (Figure II-2b).

The fact may be caused by self association of the nucleic acid bases in the polyethyleneimine derivatives, which dissociated slowly to

form an intermolecular polymer complex. On the other hand, the polymer complex formations were immediately observed for the systems of Poly A : Poly U and PEI-Hse-Ade : Poly U after mixing of the polymer solutions. These facts suggest that the dissociation of the self-association of thymine bases accompanied by conformational change in PEI-Hse-Thy should be necessary to form the polymer complex in aqueous solution. A similar result was reported for the polymer complex formation between the polymethacrylate derivatives of uracil and adenine. In this case, the self association of the uracil bases in the polymer inhibited the polymer complex formation[24].

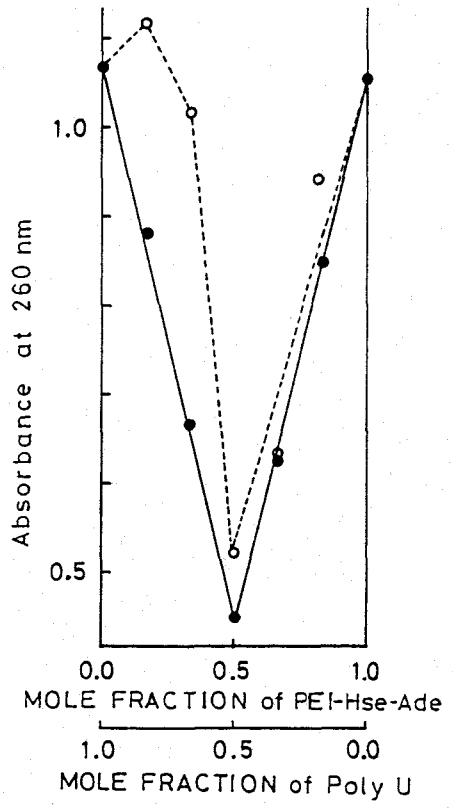


Figure II-5. Continuous Variation Curve of PEI-Hse-Ade and Poly U.
Absorbance at 260 nm after (a) (···○···) 3 hours and (b) (—●—) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly U] = 1.1 x 10^{-4} mol/L, and [PEI-Hse-Ade] = 8.3 x 10^{-5} mol/L.

Interaction of PEI-Hse-Ade and Poly U

Figure II-5 shows the mixing curve between PEI-Hse-Ade (5A) and Poly U in 3 hours (a) and 3 days (b) after mixing of the polymer solution. In this case, the formation of the polymer complex was observed even after 3 hours, as shown in Figure II-5a. The stoichiometry of the complex was 1:1 (thymine:adenine) and the maximum hypochromicity value was 49.6 % at this base ratio. This value was high as compared with the values of PEI-Hse-Thy : PEI-Hse-Ade and Poly A : Poly U systems. The formation of the polymer complex, however, was negligible at pH 2.2, where the adenine base existed in a protonated form. From these facts, it should be concluded that the polymer complex between PEI-Hse-Ade and Poly U was caused by the specific interaction between adenine and thymine, and the self association of PEI-Hse-Ade in aqueous solution was negligible.

II-4 CONCLUSION

Water soluble polyethyleneimine derivatives containing both the nucleic acid bases, thymine or adenine, and the homoserine unit were prepared. The polyethyleneimine derivatives, PEI-Hse-Thy and PEI-Hse-Ade formed the 1:1 polymer complex in aqueous solution. These polymers also formed the polymer complexes with polynucleotides, Poly A or Poly U, by the complementary interaction of the nucleic acid bases.

II-5 REFERENCES

1. K. Takemoto and Y. Inaki, "*Functional Monomers and Polymers*", K. Takemoto, Y. Inaki, and R.M. Ottenbrite, Eds, Marcel Dekker, p.149, 1987.
2. J. Pitha, *Adv. Polym. Sci.*, 50, 1 (1983).
3. K. Takemoto and Y. Inaki, *Adv. Polym. Sci.*, 41, 1 (1981).
4. C. G. Overberger and Y. Morishima, *J. Polym. Sci. Polym. Chem. Ed.*, 18, 1247, 1267 (1980).
5. A. G. Ludwick and C. G. Overberger, *J. Polym. Sci. Polym. Chem. Ed.*, 20, 2123, 2139 (1982).
6. K. A. Brandt and C. G. Overberger, *Nouv. J. Chim.*, 6, 673 (1982).
7. K. A. Brandt and C. G. Overberger, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 1981 (1985).
8. C. G. Overberger and S. Kikyotani, *J. Polym. Sci. Polym. Chem. Ed.*, 21, 525, 541 (1983).
9. C. G. Overberger and C. X. Lu, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 1321 (1985).
10. C. G. Overberger and C. C. Chen, *J. Polym. Sci. Polym. Chem. Ed.*, 24, 167 (1986).
11. N. M. Brahme and W. T. Smith, Jr., *J. Polym. Sci. Polym. Chem. Ed.*, 22, 813 (1984).
12. W. T. Smith, Jr. and N. M. Brahme, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 879 (1985).
13. A. G. Ludwick, K. S. Robinson, and J. McCloud, Jr., *J. Polym. Sci. Polym. Symp.*, 74, 55 (1986).
14. T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, 21, 11 (1989).

15. Sunamoto and K. Takemoto, to be published.
16. Y. Inaki, Y. Sakuma, Y. Suda, and K. Takemoto, *J. Polym. Sci. Polym. Chem. Ed.*, 20, 1917 (1982).
17. Y. Inaki, S. Sugita, T. Takahara, and K. Takemoto, *J. Polym. Sci. Polym. Chem. Ed.*, 24, 3201 (1986).
18. G.J. Thomas, Jr. and Y. Kyogoku, *J. Am. Chem. Soc.*, 89, 4170 (1967).
19. C. G. Overberger and Y. Inaki, *J. Polym. Sci. Polym. Chem. Ed.*, 17, 1739 (1979).
20. J. Pitha, P. M. Pitha, and E. Stuart, *Biochemistry*, 10, 4595 (1971).
21. C. G. Overberger, Y. Inaki, and Y. Nambu, *J. Polym. Sci. Polym. Chem. Ed.*, 17, 1759 (1979).
22. A. Ferro, T. Minaga, W. N. Piper, and E. Kun, *Biochim. Biophys. Acta*, 519, 299 (1978).
23. T. Sato, K. Kojima, T. Ihda, J. Sunamoto, and R. M. Ottenbrite, *J. Bioactive and Compatible Polym.*, 1, 448 (1986).
24. S. Fang, Y. Inaki, and K. Takemoto, *J. Polym. Sci. Polym. Chem. Ed.*, 22, 2455 (1984).

Chapter III SYNTHESIS AND INTERACTION STUDIES OF WATER SOLUBLE NUCLEIC ACIDANALOGS: POLYETHYLENEIMINE DERIVATIVES CONTAINING URACIL AND 5-FLUOROURACIL.

III-1 INTRODUCTION

Recently, polymer chemists have been exploring synthetic methods of preparing both natural and synthetic macromolecular analogs for biological use. Evaluation and modification of these materials by the chemist has led to the preparation of an array of polymeric agents with various application for biological systems.[1] Nucleic acids are the biopolymers, and their most essential function is based on the formation of specific base - base pairing through hydrogen bounding between purine and pyrimidine bases. Some synthetic polynucleotides are known to have biological activities. A synthetic double stranded polynucleotide complex of poly inosinic acid (Poly I) and poly cytidylic acid (Poly C) (Poly I - Poly C complex) is effective as an interferon inducer,[2-6] while it has a high level of toxicity.[7] Very recently, it was reported that the 1:1 complex of poly uridylic acid (Poly U) and poly adenylic acid (Poly A) (Poly U - Poly A complex) was also effective as an interferon associated enzyme inducer, which had a low level of toxicity.[8] Therefore, a polymer complex of polynucleotides with the synthetic nucleic acid analogs is expected to have enhanced bioactivity.[8]

The chemistry of synthetic nucleic acid analogs has recently received much attention and a number of synthetic polymers containing nucleic acid bases have been prepared and their properties have been studied.[9-13] These synthetic nucleic acid analogs were almost insoluble in water at neutral pH values, because most of them consisted of a hydrophobic polymer backbone with pendant nucleic acid bases. The interactions between the synthetic nucleic acid analogs, and with polynucleotides were studied. However, these studies were limited to organic solvents or to water - organic mixed solvents. It is, therefore, very important to prepare the water soluble synthetic nucleic acid analogs for biomedical field applications.

Previously, water soluble polyethyleneimine derivatives

containing cytosine, hypoxanthine, thymine, and adenine were prepared, and the interactions of these polymers and with polynucleotides in aqueous solution were studied.[14,15] In the present study, water soluble polyethyleneimine derivatives containing uracil and 5-fluorouracil are prepared and their interactions with Poly A in neutral aqueous solution are investigated.

As 5-fluorouracil is a famous anticancer agent, it is interesting to study the interaction of the polymer containing 5-fluorouracil unit with polynucleotides. The bioactivity of the polymer complex of nucleic acid analogs with polynucleotides will be reported later.[16]

III-2 EXPERIMENTAL

Materials

(±)- α -N-[3-(uracil-1-yl)propionyl]amino- γ -butyrolactone (4U; **Ura-Hse-L**)

To a solution of (±)- α -amino- γ -butyrolactone hydrobromide (910 mg; 5.0 mmol) in N, N-dimethylformamide (20 mL), pentachlorophenyl 3-(uracil-1-yl)- propionate [13,17] (3U) (2.2 g; 5.0 mmol), triethylamine (0.7 mL; 5.0 mmol), and imidazole (340 mg; 5.0 mmol) were added and stirred for one day at 50° C. After the reaction, the solvent was evaporated under reduced pressure, and acetone (150 mL) was added to the oily residue and stirred for 4 h to give a light brown precipitate. The precipitate was washed with water (50 mL) and dried under reduced pressure. The yield was 1.2 g (90 %); mp 236-238° C. IR (KBr, cm^{-1}) : 1770 (lactone), 1650, 1530 (amide), 1680, and 1620 (uracil). Thin layer chromatography (TLC): Rf. 0.54 (benzene : ethanol = 3:1). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.50 (t, 2H), 3.90 (t, 2H), 4.40 (m, 4H), 4.70 (m, 1H), 5.60 (d, 1H), 7.55 (d, 1H), and 8.45 (d, 1H).

ANAL. Calcd for $C_{11}H_{13}N_3O_5$: C, 49.44; H, 4.90; N, 15.72. Found: C, 49.00; H, 5.01; N, 15.53.

(±)- α -N-[3-(5-fluorouracil-1-yl)propionyl]amino- γ -butyrolactone (4FU; **5FU-Hse-L**)

To a solution of 3-(5-fluorouracil-1-yl)propionate [18] (2FU) (1.2 g;

6.0 mmol) in N, N-dimethylformamide (30 mL), pentachlorophenyl trichloroacetate (4.1 g; 10.0 mmol) and a catalytic amount of triethylamine were added. The mixture was stirred at 80° C overnight. The precipitate was filtered and washed thoroughly with diethyl ether to give 2.5 g of pentachlorophenyl 3-(5-fluorouracil-1-yl)- propionate (3FU) in 93% yield.

5FU-Hse-L (4FU) was prepared from pentachlorophenyl 3-(5-fluorouracil- 1-yl)propionate (3FU) (2.3 g; 5.0 mmol) and (±)-α -amino- γ -butyrolactone hydrobromide (910 mg; 5.0 mmol) according to the procedure described for Ura-Hse-L (4U) (yield 89 %; 1.3 g; mp 218-221° C). IR (KBr, cm^{-1}): 1770 (lactone), 1650, 1530 (amide), 1700, and 1680 (5-fluorouracil). TLC: Rf. 0.44 (benzene : ethanol = 3:1). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 3.80 (t, 2H), 4.50 (m, 4H), 4.70 (s, 1H), 8.00 (d, 1H), and 8.50 (d, 1H).

ANAL. Calcd for $C_{11}H_{12}N_3O_5F$: C, 46.16; H, 4.23; N, 14.68. Found: C, 46.52; H, 4.11; N, 14.50.

Poly N- [*{2-[3-(uracil-1-yl)propionyl]amino-4-hydroxy}butanoyl*] *ethyleneimine* (5U; PEI-Hse-Ura)

To a suspension of Ura-Hse-L (4U) (400 mg; 1.5 mmol) in water (5 mL), 1 N NaOH aqueous solution (1.5 mL; 1.5 mmol) was added. The mixture was stirred at 40° C for 1 h to afford a clear light brown solution. After the the solvent was evaporated under reduced pressure, the resulting residue was washed with acetone (20 mL), then with diethyl ether (30 mL) to give the sodium salt of the uracil derivative (Ura-Hse-Na) in 98 % yield (430 mg, powder). To a suspension of Ura-Hse-Na (430 mg; 1.5 mmol) in dry N, N - dimethylformamide (10 mL), pentachlorophenyl trichloroacetate (910 mg; 2.2 mmol) and a catalytic amount of triethylamine were added. The mixture was stirred at 0° C for 2 h and then at 50° C until the evolution of gas ceased to afford a clear light brown solution. After the the solvent was evaporated under reduced pressure, the residue was washed with diethyl ether (30 mL) to give of pentachlorophenyl ester of uracil derivative (Ura-Hse-PCP) as a powder (yield 92 %; 720 mg). TLC: Rf. 0.82 (benzene : ethanol = 3:1).

To a solution of polyethyleneimine [14] (70 mg; 1.5 mmol, M_{wt}: 22,000) in N, N-dimethylformamide (10 mL), Ura-Hse-PCP (720 mg; 1.5

mmol) and imidazole (90 mg; 1.5 mmol) were added and stirred at 60° C for 48 h. After the reaction, the solution was concentrated under reduced pressure and poured into an excess of acetone to precipitate the polymer. The polymer obtained was purified by reprecipitation from N, N-dimethylformamide into excess acetone. The polymer was dissolved in a small amount of water and freeze-dried to give a light brown powder: (360 mg, 78%); mp 210-215° C. IR (KBr, cm^{-1}): 1650 and 1550 (amide). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.50 (t, 2H), 3.70 (m, 8H), 4.30 (m, 3H), 5.65 (d, 1H), 7.50 (d, 1H), and 8.60 (s, 1H).

Poly N- [{2-[3-(5-fluorouracil-1-yl)propionyl]amino-4-hydroxy} butanoyl] ethyleneimine (5FU; PEI-Hse-5FU)

This polymer was prepared from 5FU-Hse-L (4FU) (1.0 g; 3.6 mmol) and polyethyleneimine (160 mg; 3.6 mmol) according to the procedure described for PEI-Hse-Ura (5U) (yield 68%; 820 mg; mp 166-172° C). IR (KBr, cm^{-1}): 1650 and 1550 (amide). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 3.70 (m, 8H), 4.30 (m, 3H), 8.00 (d, 1H), and 8.60 (s, 1H).

Contents of Nucleic Acid Bases in the Polymer

The polyethyleneimine derivatives (500 mg) were hydrolyzed in 6 N hydrochloric acid (10 mL) at 80° C for 48 h, to yield polyethyleneimine hydrochloride and the carboxyethyl derivatives of the nucleic acid bases. A quantitative calculation was made by using the corresponding carboxyethyl derivatives as standard samples.[19]

The nucleic acid base contents (unit mol%) of the polymers are tabulated in Table III-I.

Interaction between Synthetic Polymers and Polynucleotides

Interaction of the synthetic polymers and polynucleotides were estimated from the hypochromicity values in the UV spectra.[20] The UV spectra were measured with a JASCO UV-660 spectrometer equipped with a temperature controller at 20° C. Poly A (sodium salt)($S^{\circ}_{20,w}$: 6-12), Poly C (sodium salt) ($S^{\circ}_{20,w}$: 6-12) and Poly U (sodium salt) ($S^{\circ}_{20,w}$: 6-12) were obtained from Yamasa Shoyu Co. Ltd.. PEI-Hse-Ade (5A) was prepared by the procedure in the previous paper.[15] PEI-Hse-Ura (5U), PEI-Hse-5FU (5FU),

PEI-Hse-Ade (5A) and polynucleotides were dissolved in Kolthoff buffer (pH 7.0) (1/10 M KH_2PO_4 - 1/20 M $Na_2B_4O_7 \cdot 10H_2O$). These solutions stocked for 2 days at 20° C, and then mixed to give a polymer mixture of 10^{-4} M total concentration of nucleic acid base units in aqueous solution.

III-3 RESULTS AND DISCUSSION

Preparation of the Nucleic Acid Analogs

Natural and synthetic polynucleotides are known to form polymer complexes by specific base - base interactions between nucleic acid bases. The synthetic nucleic acid analogs such as polymethacrylamide, polyethyleneimine and poly-L-lysine derivatives containing nucleic acid bases were also found to form polymer complexes with polynucleotides by specific base - base interactions.[12] Since the solubilities of these nucleic acid analogs in water were low, the specific interactions were studied in organic solvents or water - organic mixed solvents, such as dimethyl sulfoxide, ethylene glycol, and water - propylene glycol.[12,21,22]

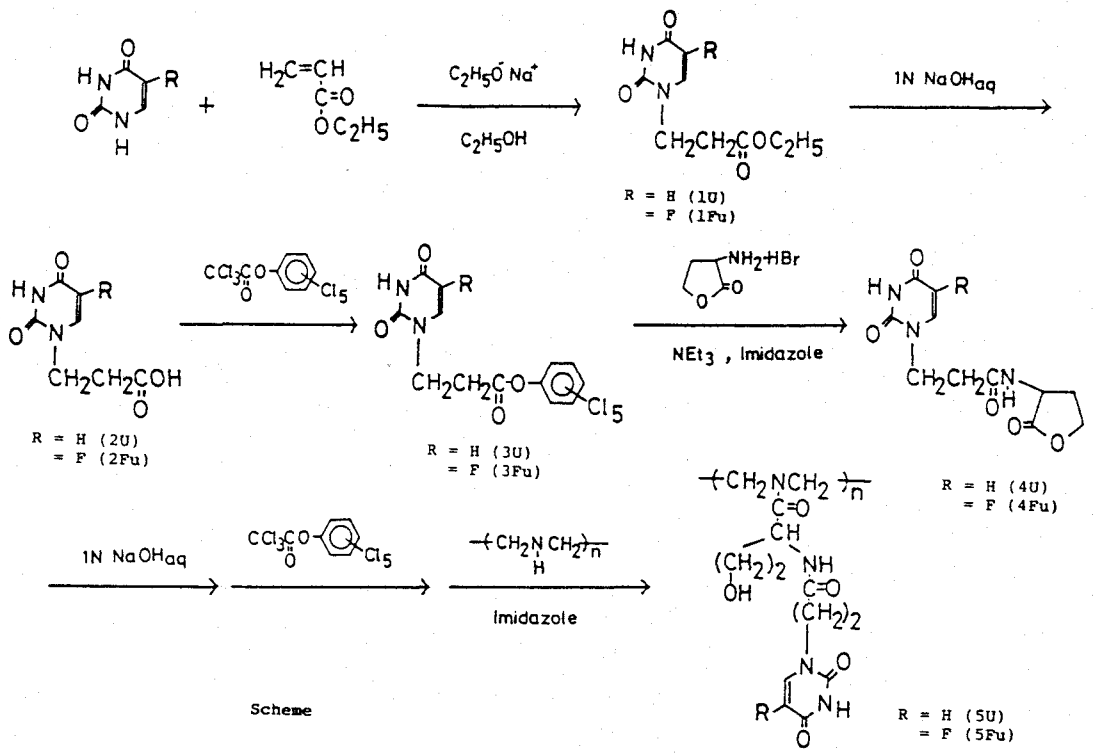
However, complex formation of polynucleotides and its application to biomedical field have been studied in aqueous solution, because organic solvent causes denaturation of polynucleotides. Therefore, it is necessary to prepare a water soluble polymer complex of nucleic acid analogs with polynucleotides for application of the polymer complex to biomedical fields.[1] The water soluble polyethyleneimine derivatives of nucleic acid bases make it possible to study the interaction with polynucleotides in neutral aqueous solution.

Various kinds of synthetic nucleic acid analogs have been prepared by the polymerization of the corresponding monomers or by grafting of the nucleic acid base derivatives on the polymers.[9-13] The most convenient method of preparing the nucleic acid analog is the grafting of the nucleic acid base derivatives onto functional polymers. By this method, the nucleic acid analogs have been prepared such as polyacrylate, polylysine, and polyethyleneimine derivatives containing nucleic acid bases.[13] In order to prepare the water soluble polymers containing high content of nucleic acid bases, hydrophilic units should be introduced to the side chains.

Overberger and Inaki prepared polyethyleneimine derivatives containing nucleic acid bases, in which amino acids were used as the spacer.[23] One of these derivatives was the polyethyleneimine having nucleic acid base and serine as a spacer, which was soluble in water. For the preparation of the serine derivative, however, protection of the functional groups was necessary.

In the previous study, (±)- α -amino- γ -butyrolactone hydrobromide was used as a starting compound.[14,15] This lactone has a stable lactone ring with the hydroxyl and carbonyl groups protected, and gave homoserine unit by ring opening reaction. By using this lactone, polyethyleneimines containing both nucleic acid base and homoserine units were successfully prepared in high yield.

Shown in Scheme III-1 is the preparations of the water soluble nucleic acid analogs containing uracil and 5-fluorouracil. The butyrolactone derivatives of uracil and 5-fluorouracil were obtained as stable solid in high yield.



Scheme

Homoserine Derivatives of Nucleic Acid Bases

Starting with uracil or 5-fluorouracil, the carboxyethyl derivatives of the nucleic acid bases were prepared by a Michael type addition reaction of ethyl acrylate followed by hydrolysis. The carboxyethyl derivatives of uracil or 5-fluorouracil were reacted with (±)- α -amino- γ -butyrolactone hydrobromide to give γ -butyrolactone derivatives of uracil or 5-fluorouracil. For the coupling reactions, pentachlorophenyl ester derivatives were used with imidazole as a catalyst.[24]

Grafting onto Polyethyleneimine

The grafting of nucleic acid base derivatives with a hydroxyl group onto polyethyleneimine polymer backbone was also carried out by the activated ester method. Since the reactivity of the γ -lactone is low, the direct reaction of the lactone derivative with polyethyleneimine was not effective. Therefore, the lactone derivatives were hydrolyzed to the 3-hydroxybutyric acid derivatives, followed by condensation with polyethyleneimine using the activated ester method. The grafting reaction was carried out in N, N-dimethylformamide, where a small amount of 4-pyrrolidinopyridine was added as an effective catalyst. Nucleic acid base contents of the polymers were determined by UV spectroscopy of hydrolyzed samples. A quantitative calculations were made by using the corresponding carboxyethyl derivatives as standards. The nucleic acid base units (unit mol%) on the polymer are tabulated in Table Ⅲ-Ⅰ.

Table I The unit mol% of nucleic acids for PEI-Hse-Ura and PEI-Hse-5FU.

	PEI-Hse-Ura	PEI-Hse-5FU
unit mol%	94	91

Interaction of Synthetic Polymers with Polynucleotides

The formation and stoichiometry of the polymer complex between nucleic acid analogs are affected by several factors. One of the factors is the proparty of the polymer backbone: its flexibility, steric

regularity, electric charge, branching, and molecular weight. These factors can reflect the compatibility and penetration ability of the polymer and the stability of the polymer complex. A polynucleotide has a flexible, sterically regular and negatively charged polymer backbone, whereas that of the synthetic analogs are probably less flexible, sterically inhomogeneous and neutral. Other important factors for the specific polymer complex are temperature and solvent conditions for the interaction . Further, the pH and concentration of salt are important for the complex formation in an aqueous solution.[25,26] In the present study, the formation of the polymer complexes were investigated for the PEI-Hse-Ura - PEI-Hse-Ade, and PEI-Hse-5FU - PEI-Hse-Ade systems, and for the polyethyleneimine derivatives - polynucleotides (Poly A, Poly C) systems.

Interaction of PEI-Hse-Ura and PEI-Hse-Ade

Shown in Figure III-1 are the mixing curves for PEI-Hse-Ura (5U) and PEI-Hse-Ade (5A) at pH 7.0 in the Kolthoff buffer solution. As shown in Figure III-1b, PEI-Hse-Ura with PEI-Hse-Ade have the highest hypochromicity value when the base unit ratio is about 1:1 (uracil : adenine), suggesting that the formation of a stable 1:1 polymer complex due to complementary nucleic acid base interactions. The hypochromicity value obtained (15.2 %) was higher than that for the PEI-Ade - PEI-Thy system which had not spacer group.[22]

Time dependence of hypochromicity value was observed for the PEI-Hse-Ura - PEI-Hse-Ade system as shown in Figure III-1. The hypochromicity was barely observable 3 hours after mixing the polymer solutions (Figure III-1a), but the absorbance decreased (hypochromicity increased) and became constant after 3 days (Figure III-1b). On the other hand, the polymer complex formation was immediately observed for Poly A - Poly U (see next section; Figure III-3) and PEI-Hse-Ade - Poly U[15] after mixing of the polymer solutions. These facts suggest that the conformational change in PEI-Hse-Ura may be necessary to form the polymer complex in aqueous solution. This may be caused by the self association of uracil bases in the polyethyleneimine derivatives, which dissociated slowly to form an intermolecular polymer complex. Similar results were reported for the polymer complex formation between

polymethacrylate derivatives of uracil and adenine, where the self association of the uracil bases in the polymer inhibited the polymer complex formation.[24,27,28] Therefore, the conformational change of the synthetic polymers may be important for the formation of a stable polymer complex.

Interaction of PEI-Hse-Ura with Poly A

Shown in Figure III-1 are the mixing curves for PEI-Hse-Ura with Poly A at pH 7.0. From the curve in Figure III-1b, the maximum hypochromicity value obtained was 54.4 %. The overall stoichiometry of the complex based on nucleic acid base units was 1:1 (uracil : adenine).

As a control experiment, the interaction between Poly A and Poly U was studied under the condition used here. As shown in Figure III-2, the complex formation was observed immediately after mixing of the polymer solutions, and the maximum hypochromicity value was 40.3 %. The hypochromicity value for the PEI-Hse-Ura - Poly A system was higher than the Poly U - Poly A system, PEI-Hse-Ade - Poly U system,[15] and any other nucleic acid analog - polynucleotide system.[9,10,21]

Hypochromicity in UV spectra has widely used to indicate the interaction of nucleic acid derivatives. The hypochromicity is known to be caused by the interaction between chromophors such as nucleic acid bases.[29] Therefore, the hypochromicity observed in Figure III-1b indicates the interaction between uracil and adenine chromophors in PEI-Hse-Ura and Poly A, respectively. To determine whether the interaction between the bases is due to the complementary nucleic acid base interaction, the interaction of PEI-Hse-Ura with Poly U was measured under the same condition. Figure III-1 shows the mixing curves for PEI-Hse-Ura - Poly U at pH 7.0. The hypochromicity, however, could not be observed after 3 hours (Figure III-1a) nor even after 3 days (Figure III-1b). This result indicates that the uracil bases of PEI-Hse-Ura do not interact with the uracil bases nor the phosphate units of Poly A. Based on these facts the formation of the PEI-Hse-Ura - Poly A complex in Figure III-1b may be concluded to be caused by the complementary base - base interaction between adenine and uracil.

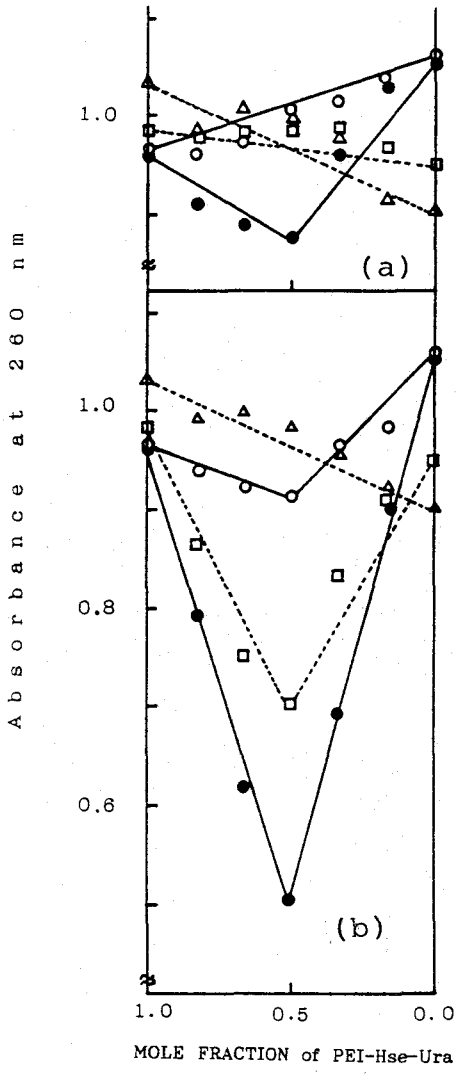


Figure Ⅲ-1. Continuous Variation Curve for PEI-Hse-Ura and PEI-Hse-Ade (—○—), Poly A (—●—), Poly U (···△···), Poly C (···□···).

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C.

{[PEI-Hse-Ura] = 1.1 x 10^{-4} mol/L, and [PEI-Hse-Ade] = 8.5 x 10^{-5} mol/L.}

{[PEI-Hse-Ura] = 1.1 x 10^{-4} mol/L, and [Poly A] = 1.2 x 10^{-4} mol/L.}

{[PEI-Hse-Ura] = 1.3 x 10^{-4} mol/L, and [Poly U] = 1.1 x 10^{-4} mol/L.}

{[PEI-Hse-Ura] = 1.2 x 10^{-4} mol/L, and [Poly C] = 1.4 x 10^{-4} mol/L.}

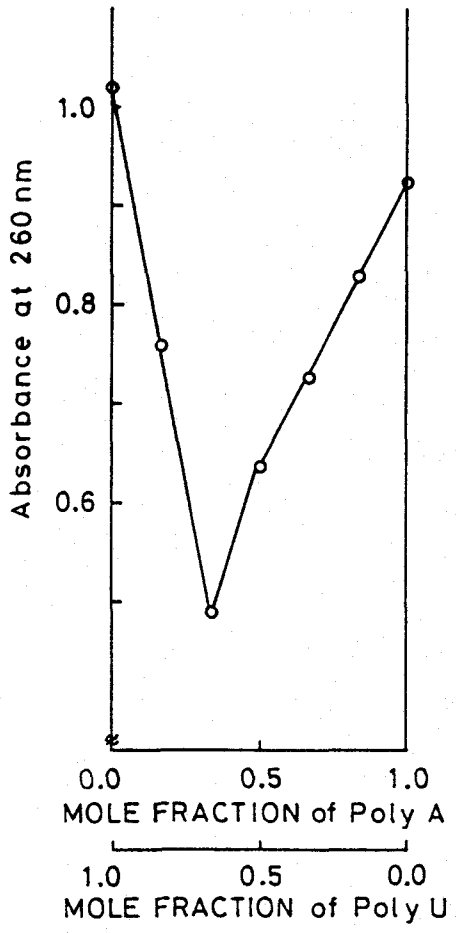


Figure Ⅲ-2. Continuous Variation Curve for Poly U and Poly A.

Absorbance at 260 nm in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C.
[Poly U] = 1.1 x 10^{-4} mol/L, and [Poly A] = 1.0 x 10^{-4} mol/L.

Interaction of PEI-Hse-Ura with Poly C

It is known that the complementary base of uracil is not cytosine but adenine.[30] However, as shown in Figure III-1b, the hypochromic effect was also observed for the interaction of PEI- Hse-Ura and Poly C 3 days after mixing the polymers. The overall stoichiometry of this system was 1:1 (uracil : cytosine). The maximum hypochromicity value (20.1 %), however, was smaller than that of the PEI-Hse-Ura - Poly A system (Figure III-1b).

As a control experiment, the interaction between Poly U and Poly C was also studied under the conditions used. As shown in Figure III-3a, the complex formation was observed immediately after mixing the polymer solutions, and the same curve was obtained after 3 days (Figure III-3b). The maximum hypochromicity (5.5 %) was observed at base unit ratio of 1:1 (uracil : cytosine). The maximum hypochromicity value of the Poly U - Poly C system (Figure III-3b), however, was smaller than that for the PEI-Hse-Ura - Poly C system (Figure III-1b). Therefore, the interaction between PEI-Hse-Ura and Poly C (Figure III-1b) was concluded to be caused by the interaction between uracil and cytosine bases.

Interaction of PEI-Hse-5FU and PEI-Hse-Ade

Halogenated uracil derivatives are known to have biomedical activity. In particular, 5-fluorouracil derivatives are famous antitumor and anticancer agents. The halogenation of uracil base causes change of acidity of the pyrimidine, which may relate to the interaction with the adenine derivatives. It is, therefore, interesting to study the effect of the 5-fluorouracil unit on the polymer complex formation with polynucleotides.

Shown in Figure III-4 are the mixing curve between PEI-Hse-Ade (5A) and PEI-Hse-5FU (5FU) which has the 5-fluorouracil unit. After 3 days (Figure III-7b) the highest hypochromicity value was measured for base unit ratio of about 1:1 (5-fluorouracil : adenine), suggesting that the formation of the 1:1 polymer complex due to the interaction between the nucleic acid bases. The hypochromicity value of 12.3 %, however, was smaller than that of the PEI-Hse-Ura - PEI-Hse-Ade system (15.2 %; Figure III-1b), but was higher than that of the PEI-Hse-Thy - PEI-Hse-Ade system(10.4 %).[15] In Figure III-4, the time dependence of hypochromicity value is shown. Based on

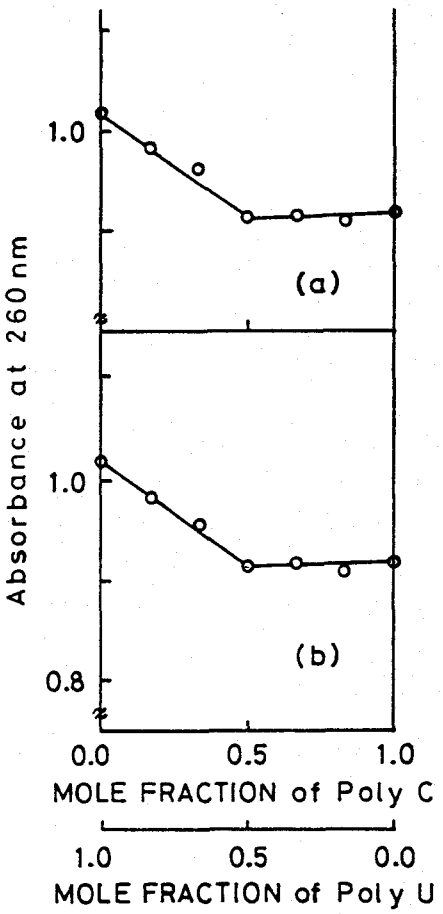


Figure Ⅲ-3. Continuous Variation Curve for Poly U and Poly C.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly U] = 1.1 x 10^{-4} mol/L, and [Poly C] = 1.3 x 10^{-4} mol/L.

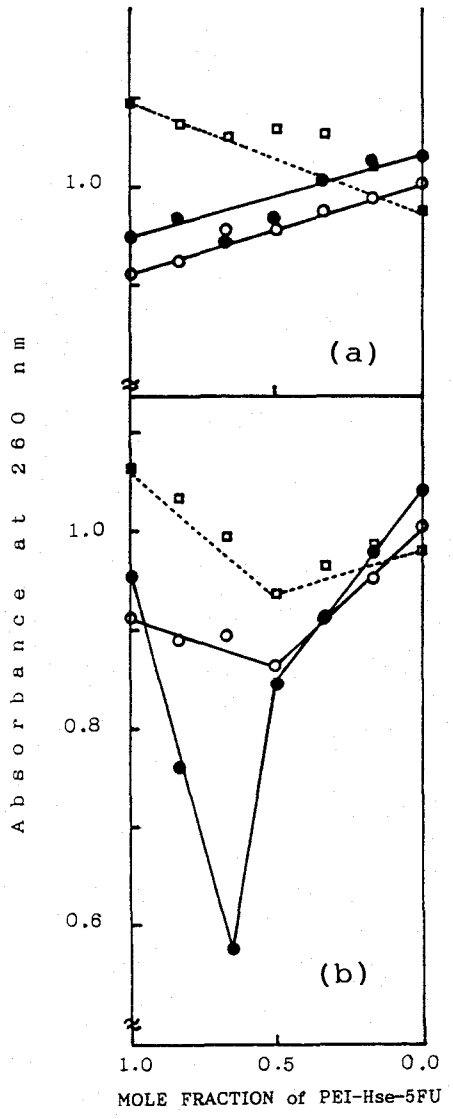


Figure Ⅲ-4. Continuous Variation Curve for PEI-Hse-5FU and PEI-Hse-Ade (—○—),Poly A (—●—), Poly C (···□···).

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C.
{[PEI-Hse-5FU] = 9.0 x 10^{-5} mol/L, and [PEI-Hse-Ade] = 8.5 x 10^{-5} mol/L.}
{[PEI-Hse-5FU] = 9.6 x 10^{-5} mol/L, and [Poly A] = 1.2 x 10^{-4} mol/L.}
{[PEI-Hse-5FU] = 1.0 x 10^{-4} mol/L, and [Poly C] = 1.4 x 10^{-4} mol/L.}

the results for the interaction between the polyethyleneimine derivatives, the expected enhancement of the interaction of 5-fluorouracil was not observed.

Interaction of PEI-Hse-5FU with Poly A

Shown in Figure III-4 are the mixing curves for PEI-Hse-5FU with Poly A at pH 7.0. The maximum hypochromicity value was obtained for the base unit ratio of 2:1 (5-fluorouracil : adenine). The maximum hypochromicity value of the PEI-Hse-5FU - Poly A system was 40.3 %, which was smaller than the PEI-Hse-Ura - Poly A system (54.4%; Figure III-1b), equal to the Poly U - Poly A system (40.3%; Figure III-2), and higher than the PEI-Hse-Thy - Poly A system (38.5%).[15] These facts suggest that the incorporation of the 5-fluorouracil base results in a decrease in the stability of the polymer complex.

A remarkable effect of 5-fluorouracil was observed in the stoichiometry of the polymer complex. The stoichiometry of the polymer complex between PEI-Hse-5FU and Poly A was 2:1 (5-fluorouracil : adenine, Figure III-4b), which is the same as the Poly A - Poly U polymer complex. On the other hand, the stoichiometry of the PEI-Hse-Ura - Poly A polymer complex was 1:1 (Figure III-1b). The reason of the formation of the 2:1 complex of Poly A with Poly U is due to the tendency of Poly A to form a single strand.[25] The 2:1 polymer complex of PEI-Hse-5FU - Poly A, therefore, may be formed the same way as the Poly A - Poly U complex.

The halogenation of uracil base in Poly U is reported to affect on the stoichiometry and stability of the polymer complex with Poly A; a triplestranded complex is formed in preference to doublestranded complex in the case of the Poly A - poly-5-bromouridylic acid.[31,32] In the case of the polymethacrylate type nucleic acid analogs, the stability of the polymer complex was increased by halogenation on uracil base.[33] The effect of halogenation on uracil may be related to acidity of the uracil derivatives resulting in change of the pKa values of the ^{3}N position in pyrimidine ring: 9.25 (Uridine), 8.50 (5-iodouridine), 8.20 (5-bromouridine), 8.20 (5-chlorouridine), and 7.75 (5-fluorouridine).[34] These pKa values indicate that the most strong hydrogen bonding interaction should be occurred between the 5-fluorouracil derivative and the adenine derivative.

From the results of the interactions in the present study, however, the hypochromicity value of PEI-Hse-Ura was higher than that of PEI-Hse-5FU for the complex formation with PEI-Hse-Ade or Poly A. The reason of this fact may be that pyrimidine bases need both the proton donating power of N^3H and the proton accepting power of the carbonyl group for the interaction with purine bases. Although 5-fluorouracil has higher proton donation power of N^3H, has lower proton accepting power of the carbonyl group compared with uracil base. These compensating factors may cause lower activity of PEI-Hse-5FU than PEI-Hse-Ura for the interaction with the adenine derivatives.

Interaction of PEI-Hse-5FU with Poly C

Shown in Figure III-4 are the mixing curve for PEI-Hse-5FU and Poly C, where the nucleic acid bases are not a complementary base pair.[30] The maximum hypochromicity value of 4.0% was observed for the base ratio of 1:1 (5-fluorouracil : cytosine). This value is the same as the Poly U - Poly C system.

Selectivity of The Base - Base Interaction

The maximum hypochromicity values and stoichiometries of the polymer complexes are summarized in Table III-II. In this table, the value of selectivity (S) was calculated by the following equation:

$$S = H_{(A)} / H_{(C)}$$

where $H_{(A)}$, and $H_{(C)}$ are the maximum hypochromicity values of the uracil derivatives with Poly A and with Poly C, respectively. The selectivity value means the selectivity of the uracil derivatives to the complementary Poly A based on Poly C. PEI-Hse-Ura has the highest hypochromicity value for Poly A, but the selectivity value (S: 2.7) is low. On the other hand, PEI-Hse-Thy[15] has the lowest hypochromicity value for Poly A, but the selectivity value is high. The selectivity value of PEI-Hse-5FU is high, while the hypochromicity value is the same with that of PEI-Hse-Ura.

The data in table II, therefore, indicates that the higher the hypochromicity value, the lower the selectivity for the interaction with the complementary base.

Table II The maximum hypochromicity values. Stoichiometry and the values of selectivity(S) for nucleic acid analogs - polynucleotides systems.

	Poly U	PEI-Hse-Ura	PEI-Hse-5FU	PEI-Hse-Thy[15]
Poly A	40.3%[a] / 2:1	54.4% / 1:1	40.3% / 2:1	38.4% / 2:1
Poly C	5.5% / 1:1	20.1% / 1:1	4.0% / 1:1	3.0% / 1:1
S	7.3	2.7	10.1	12.8

a: The maximum hypochromicity values are above the stoichiometry (uracil derivative : adenine or cytosine) ratios.

III-4 CONCLUSION

Water soluble nucleic acid analogs containing uracil (PEI-Hse-Ura) and 5-fluorouracil (PEI-Hse-5FU) were prepared. PEI-Hse-Ura was found to form polymer complexes both with Poly A and with Poly C. PEI-Hse-5FU has highly basic 5-fluorouracil base, but formed the polymer complex only with Poly A. The results indicated that the complex formation ability of the 5-fluorouracil base is not higher than the uracil base.

III-5 REFERENCES

1. R. M. Ottenbrite, "*Anionic Polymeric Drugs* ",L. G. Donaruma, R. M. Ottenbrite, O. Vogl, Eds., John Wiley & Sons, Inc., p 1, (1980).
2. J. H. Park, M. A. Gallin, A. Billiau, and S. Baron, *Arch. Ophthal.*, 81, 840 (1969).
3. H. B. Levy, *J. Bioact. Compat. Polym.*, 1, 348 (1986).
4. A. K. Field, A. A. Tytell, G. P.Lampson, and M. R. Hillman, *Proc. Nat. Acad. Sci. U.S.A.*, 58, 1004 (1967).
5. M. M. Nemes, A. A. Tytell, G. P. Lampson, A. K. Field, and M. R. Hillman, *Proc. Soc. Exp. Biol. Med.*, 132, 776 (1969).

6. V. DeVita, G. Canellos, P. Carbone, H. Levy, and H. Gralnick, *Proc. Am. Assoc. Cancer Res.*, 11, 21 (1970).
7. M. Absher and W. R. Stinebring, *Nature*, 223, 715 (1969).
8. T. Sato, K. Kojima, T. Ihda, J. Sunamoto, and R. M. Ottenbrite, *J. Bioact. Compat. Polym.*, 1, 448 (1986).
9. K. Takemoto and Y. Inaki, *Adv. Polym. Sci.*, 41, 1 (1981).
10. J. Pitha, *Adv. Polym. Sci.*, 50, 1 (1983).
11. A. G. Ludwick, K. S. Robinson, and J. McCloud, Jr., *J. Polym. Sci., Polym. Symp.*, 74, 55 (1986).
12. Y. Inaki and K. Takemoto, "*Current Topics in Polymer Science, Volume I*", R. M. Ottenbrite, L. A. Utracki and S. Inoue, Eds., Hanser Publisher, p 79, (1987).
13. K. Takemoto and Y. Inaki, "*Functional Monomers and Polymers*", K. Takemoto, Y. Inaki, and R. M. Ottenbrite, Eds, Marcel Dekker, Inc., p 149, (1987).
14. T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, 21, 11 (1989).
15. T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, 20, 1059 (1988).
16. J. Sunamoto and K. Takemoto, to be published.
17. Y. Sakuma, Y. Inaki, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, 22, 2061 (1984).
18. H. Kubota, N. Yoshino, and Y. Ogiwara, *J. Polym. Sci., Polym. Lett. Ed.*, 21, 367 (1983).
19. T. Ishikawa, Y. Inaki, and K. Takemoto, *Polym. Bull.*, 1, 85 (1978).
20. G. J. Thomas, Jr. and Y. Kyogoku, *J. Am. Chem. Soc.*, 89, 4170 (1967).
21. J. Pitha, P. M. Pitha, and E. Stuart, *Biochemistry*, 10, 4595 (1971); *Biopolymer*, 9, 965 (1970).
22. C. G. Overberger, Y. Inaki, and Y. Nambu, *J. Polym. Sci., Polym. Chem. Ed.*, 17, 1759 (1979).
23. C. G. Overberger and Y. Inaki, *J. Polym. Sci., Polym. Chem. Ed.*, 17, 1739 (1979).
24. Y. Inaki, Y. Sakuma, Y. Suda, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, 20, 1917 (1982).
25. D. Thiele, W. Guschlbauer, and A. Faver, *Biochim. Biophys. Acta*, 272, 22 (1972).
26. K. Fujioka, Y. Baba, A. Kagemoto, and R. Fujishiro, *Polym. J.*, 12, 843 (1980).

27. S. Fang, Y. Inaki, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, 22, 2455 (1984).
28. Y. Inaki, S. Sugita, T. Takahara, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, 24, 3201 (1986).
29. P. O. P. Ts'o, Ed., "*Basic Principles of Nucleic Acid Chemistry, Vols. 1*" and 2, Academic Press, New York (1974).
30. Z. G. Kudritskaya and V. I. Danilov, *J. Theor. Biol.*, 59, 303 (1976).
31. S. Higuchi, *Biopolymer*, 23, 493 (1984).
32. S. Higuchi, *Biopolymer*, 23, 831 (1984).
33. S. B. Fang, Y. Y. Jiang, Y. Inaki, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, in press.
34. M. Gueron and R. G. Shulman, *Ann. Rev. Biochem.*, 37, 571 (1968).

Chapter Ⅳ INTERACTION AND CONFORMATIONAL STUDIES ON WATER SOLUBLE POLYETHYLENEIMINE DERIVATIVES CONTAINING NUCLEIC ACID BASES INTRODUCTION

Ⅳ-1 INTRODUCTION

Recently, the relationship between conformation of the polymers and their functions have received much attention. It is well known that a number of natural polymers, such as polypeptides, polynucleotides, and polysaccharides, play important functions in biological system by various highly ordered conformation. In the case of enzyme, for example, the sterical structure of the active site influence the selectivity and activity of the enzyme. Nucleic acid also have an ordered form such as A, B, Z type double stranded helix or superhelicity, which closely relate to their function. The conformational change of nucleic acids, which can be observed by CD spectra, plays an important role in replication or transcliption of genetic code.

The chemistry of nucleic acid model polymer has recently received much attention and a number of synthetic polymers containing nucleic acid bases have been prepared and their properties have been studied.[1-13] These synthetic nucleic acid analogs, however, hardly soluble in neutral aqueous solution. It is, therefore, very important to prepare the water soluble synthetic nucleic acid analogs in order to apply the analogs to simulate the nucleic acid and biological field. For this purpose, previously, water soluble polyethyleneimine derivatives containing cytosine, hypoxanthine, thymine, uracil, 5-fluorouracil and adenine were prepared, and interactions of these polymers and with polynucleotides in aqueous solution were studied.[14-16] It is very important to study the relationship between the base - base interaction observed by UV spectra and conformation change of the polymers, which can be observed by CD spectra.

The present paper deals with the conformation change of the polynucleotides which was caused by the interaction with water soluble polyethyleneimine derivatives containing nucleic acid bases in neutral aqueous solution. The results were discussed with the

hypochromocity observed in UV spectra.

Ⅳ-2 EXPERIMENTAL

Materials

Polyethyleneimine derivatives containing nucleic acid bases (PEI-Hse-Thy, PEI-Hse-Ura, PEI-Hse-5FU, and PEI-Hse-Ade) were prepared according to the method reported earlier.[14-16]

Interaction of the polymers was estimated by hypochromicity values in UV spectra as reported previously[17].

The UV spectra were measured with an JASCO UV-660 spectrometer equipped with a temperature controller at 20° C and the circular dichroism (CD) spectra were measured with a JASCO CD J-40 spectrometer at room temperature. Polyadenylic acid (Poly A) (sodium salt) ($S^{\circ}_{20,w}$: 6-12), Polycytidylic acid (Poly C) (sodium salt) ($S^{\circ}_{20,w}$: 6-12), and Polyuridylic acid (Poly U) (sodium salt) ($S^{\circ}_{20,w}$: 6-12) were obtained from Yamasa Shoyu Co. Ltd. PEI-Hse-Ura (5U), PEI-Hse-Thy (5T), PEI-Hse-5FU (5FU), PEI-Hse-Ade (5A) and polynucleotides were dissolved in Kolthoff buffer (pH 7.0) (1/10 M KH_2PO_4 - 1/20 M $Na_2B_4O_7 \cdot 10H_2O$). These solutions stocked for 2 days at 20° C were mixed to give a polymer mixture of 10^{-4} mol/L total concentration of nucleic acid base units in aqueous solution.

Ⅳ-3 RESULTS AND DISCUSSION

Conformation and Interaction of the polymers

Natural and synthetic polynucleotides are known to form a polymer complex by specific base - base interaction between nucleic acid bases in neutral aqueous solutions. The synthetic nucleic acid analogs such as polyethyleneimine and polylysine derivatives containing nucleic acid bases were also found to form polymer complexes with polynucleotides by the specific base - base interactions, which were detected by UV spectra[2,3] and more stable than synthetic polynucleotides. The complex formation of the nucleic acid analogs with polynucleotides in water is very important

for the investigation of bioactivity of the polymers[18] For this purpose, we reported to prepare the water soluble polyethyleneimine derivatives containing nucleic acid bases. This nucleic acid analogs are easily soluble in water entire range of pH values. The interactions in water were thus studied by UV spectra between the polyethyleneimine derivatives, between the polymers and polynucleotides.

The formation of stable complexes were observed in polyethyleneimine derivatives - polynucleotides systems. In the case of PEI-Hse-Thy or PEI-Hse-5FU - Poly A system, maximum hypochromicity value was observed about 40 % in 2:1 (uracil : adenine) similar as Poly U - Poly A system. On the other hand, in the case of PEI-Hse-Ura - Poly A system, stoichiometry of maximum hypochromicity (52 %) was 1:1 and PEI-Hse-Ade - Poly U system, maximum hypochromicity (58 %) was observed at 1:1 base ratio. Thus, it is important and interesting to study conformational properties of the polymers during the formation of polymer complexes by CD spectra, which is compared with the data of UV spectra.

Interaction of PEI-Hse-Ura with Poly A

Figure Ⅳ-1 shows the CD spectra of PEI-Hse-Ura with Poly A system at pH 7.0. In this figure, with increasing of the molar ratio of Poly A, positive band at 262 and 220 nm increased and a negative band at 245 nm decreased. These bands were assigned to Poly A, because PEI-Hse-Ura is optically inactive. The intensity at 262 nm was plotted against mixing ratio of the polymers in Figures Ⅳ-2B with the result of the UV mixing curve of this system (Figure Ⅳ-2A).

In UV spectra, the hypochromicity which is caused by nucleic acid base interaction was scarcely observed in 3 hours, but increased and became constant in 3 days after mixing of the polymer solutions. The time dependence of hypochromicity in UV spectra was caused by conformational change of PEI-Hse-Ura. The rate of complex formation for polyethyleneimine derivatives was slow compared with that of polynucleotide systems. The absorbance of a mixed solution of the polymers decreased slowly and became constant after 3 days.

This was caused by self association of the nucleic acid bases in the polyethyleneimine derivatives, which dissociated slowly to form the

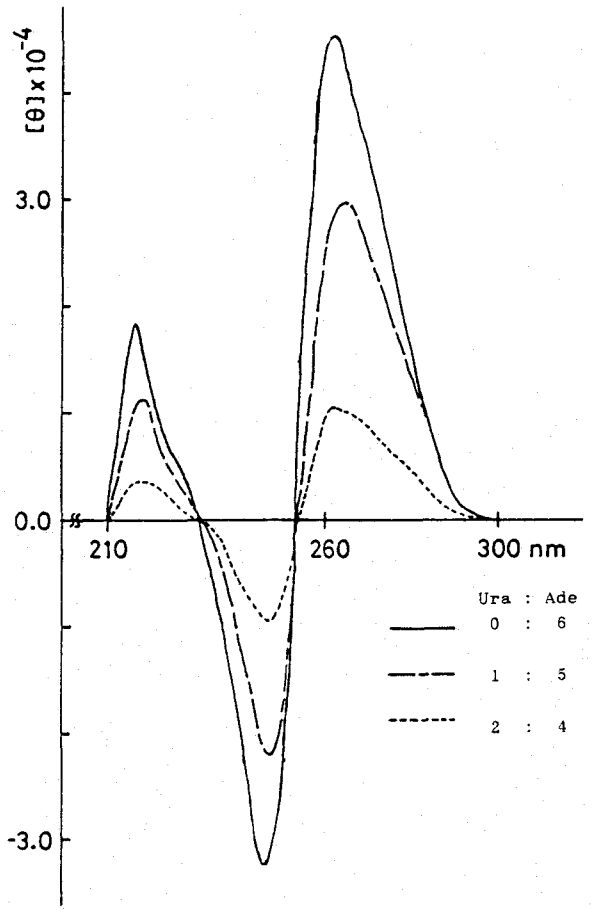


Figure Ⅳ-1. CD spectra of PEI-Hse-Ura and Poly A after 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PEI-Hse-Ura] = 1.1 x 10^{-4} mol/L and [Poly A] = 1.2 x 10^{-4} mol/L.

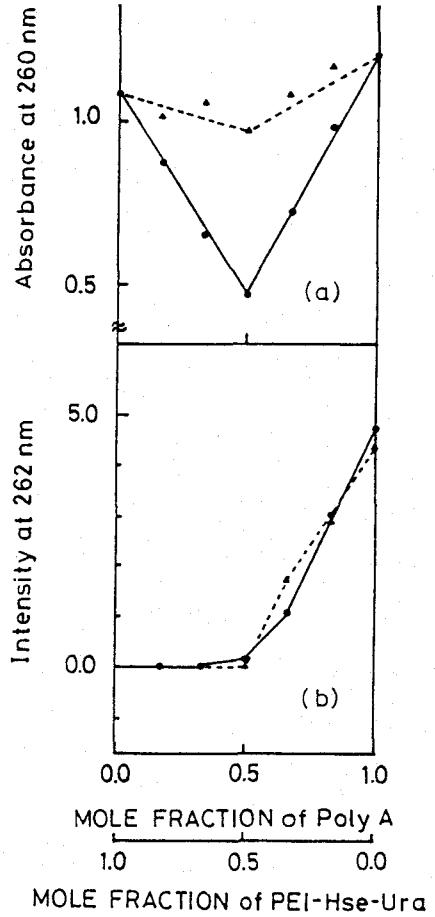


Figure Ⅳ-2. Continuous Variation Curve for PEI-Hse-Ura and Poly A .
(A) UV absorbance at 260 nm (B) CD intencity at 262 nm after 3 hours (···▲···) and 3 days (—●—) in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PEI-Hse-Ura] = 1.1 x 10^{-4} mol/L and [Poly A] = 1.2 x 10^{-4} mol/L.

intermolecular polymer complex.

On the other hand, significant decrease of CD spectra was observed in 3 hours after mixing of the polymer solutions and time dependence were hardly observed (Figure Ⅳ-2B). It is suggesting that the conformational of Poly A was little change with increasing of hypochromicity on UV spectra. Poly A is well known to form single stranded structure caused by stacking of adenine bases in neutral aqueous solution. Decrease of intensity in CD spectra observed in Figure Ⅳ-2B means conformational change of Poly A. Time dependence of CD spectra (Figure Ⅳ-2B), however, do not correspond to that of UV spectra (Figure Ⅳ-2A): change of the CD spectra was

fast and change of UV spectra was slow.

From the highest hypochromicity value in the mixing curve (Figure Ⅳ-2A), the stoichiometry of the complex was obtained as 1:1.

The same stoichiometry of the complex was obtained from the hypochromicity in CD spectra (Figure Ⅳ-2B).

From these facts, interaction between PEI-Hse-Ura and Poly A was considered as shown in Figure Ⅳ-3:

Ⅰ) Before mixing, poly A forms single stranded structure by base stacking and PEI-Hse-Ura exists in random coiled conformation.

Ⅱ) Mixing of the polymer solutions immediately causes conformational change of Poly A as shown by decrease of intensity of CD spectra after 3 hr (Figure Ⅳ-2B). In this step as shown in Figure Ⅳ-2A, the hydrogen bonding interaction between adenine bases in Poly A and uracil bases in PEI-Hse-Ura is weak, where a part of uracil bases in PEI-Hse-Ura interact with adenine bases in Poly A and other uracil bases form intramolecular interaction.

Ⅲ) During 3 days, most of the uracil and adenine bases of the

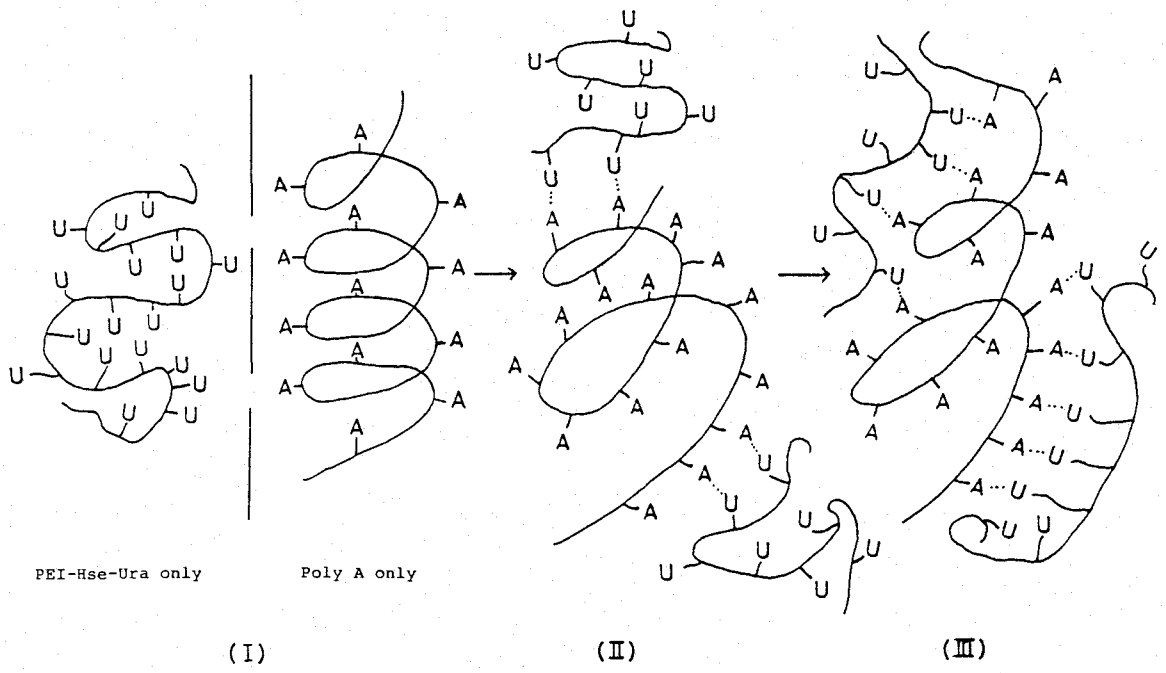


Figure Ⅳ-3. (Ⅰ) Before mixing (Ⅱ) 3 hours after mixing of polymer solutions (Ⅲ) 3 days after mixing of polymer solutions.

polymers form the base pairs and the hypochromicity value of UV spectra became the highest. The conformation of Poly A, however, scarcely changed during 3 days.

Interaction of PEI-Hse-Thy with Poly A

Figures Ⅳ-4A and Ⅳ-4B show the hypochromicities of UV and intensities of CD spectra at 262 nm for the PEI-Hse-Ura - Poly A system against mixing ratio of the polymers, respectively. These figures were essentially the same as those of the PEI-Hse-Ura - Poly A system.

A remarkable effect of thymine was observed in the stoichiometry of the polymer complex in UV spectra(Ⅳ-4A).

The stoichiometry of the polymer complex between PEI-Hse-Thy and Poly A was 2:1 (thymine : adenine, Figure Ⅳ-4A), which is the same as the Poly A - Poly U polymer complex (Figure Ⅳ-9A).

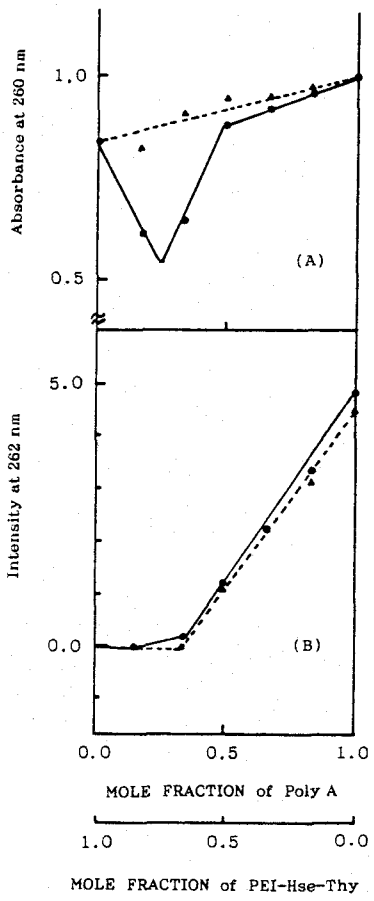


Figure Ⅳ-4. Continuous Variation Curve for PEI-Hse-Thy and Poly A .
(A) UV absorbance at 260 nm (B) CD intencity at 262 nm after 3 hours (···▲···) and 3 days (—●—)in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C.
[PEI-Hse-Thy] = 1.1 x 10^{-4} mol/L and [Poly A] = 1.1 x 10^{-4} mol/L.

On the other hand, the stoichiometry of the PEI-Hse-Ura - Poly A polymer complex was 1:1 (Figure Ⅳ-2A). The reason of the formation of the 2:1 complex of Poly A with Poly U is due to the tendency of Poly A to form a single strand.[19] The 2:1 polymer complex of PEI-Hse-Thy - Poly A, therefore, may be formed the same way as the Poly A - Poly U complex. In CD spectra (Ⅳ-4B), time dependence was scarcely observed different from UV spectra. But after 3days, the intensities of CD spectra at 262 and 245 nm increased (hyperchromicity) for the high base ratio of thymine. It may be caused by a little conformational change of Poly A due to formation of

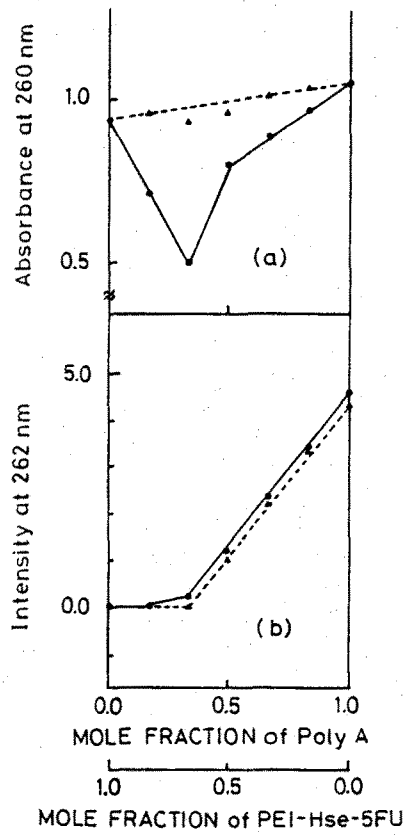


Figure Ⅳ-5. Continuous Variation Curve for PEI-Hse-5FU and Poly A .
(A) UV absorbance at 260 nm (B) CD intencity at 262 nm after 3 hours (···▲···) and 3 days (—●—)in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C.
[PEI-Hse-5FU] = 9.6 x 10^{-5} mol/L and [Poly A] = 1.2 x 10^{-4} mol/L.

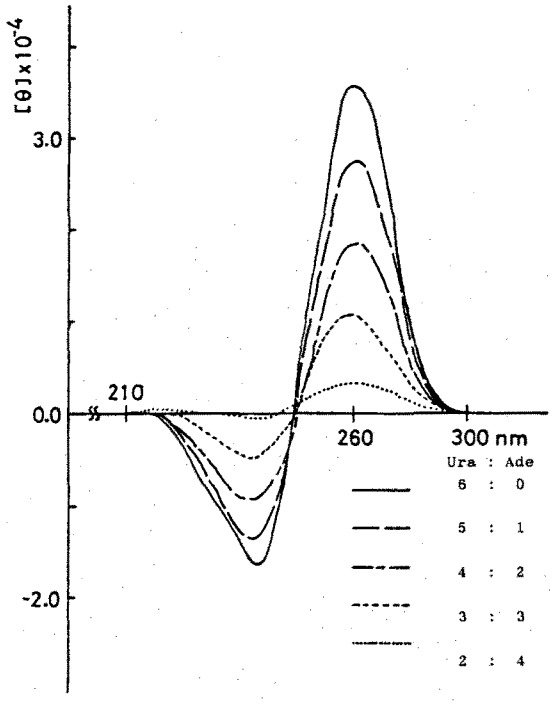


Figure Ⅳ-6. CD spectra of PEI-Hse-Ade and Poly U after 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly U] = 1.1 x 10^{-4} mol/L and [PEI-Hse-Ade] = 8.3 x 10^{-5} mol/L.

stable complex with PEI-Hse-Thy. The stoichiometry of maximum hypochromicity in CD spectra was the same as that in UV spectra.

Interaction of PEI-Hse-5FU with Poly A

Figures Ⅳ-5A, Ⅳ-5B show the UV mixing curves for the PEI-Hse-5FU with Poly A at pH 7.0 and CD intensities at 262 nm against mixing ratio of the polymers, respectively. The maximum hypochromicity value of UV spectra was 40.3 %, which was smaller than that of the PEI-Hse-Ura - Poly A system (54.4%; Figure Ⅳ-2) and higher than that of the PEI-Hse-Thy - Poly A system (38.5%). The hypochromicity of the CD spectra (Figure Ⅳ-5B) was also smaller than that of the PEI-Hse-Ura - Poly A system (Figure Ⅳ-2B) and higher than that of the PEI-Hse-Thy - Poly A system (Figure Ⅳ-4B).

And in this system, the stoichiometry of maximum hypochromicity in CD spectra was the same as that in UV spectra similar of PEI-Hse-Thy - Poly A system.

Interaction of PEI-Hse-Ade and Poly U

In the case of PEI-Hse-Ade - Poly U system, interaction was studied by CD and UV spectra. Figure Ⅳ-6 shows the CD spectra of this system. In this figure, a positive band at 270 nm and a negative band at 245 nm were assigned to Poly U. The shift of wave-length (λ_{max}) was scarcely observed by complex formation. Figures Ⅳ-7A, Ⅳ-7B show the UV mixing curves for the PEI-Hse-Ade (5A) and Poly U at pH 7.0 and CD intensities at 270 nm. In UV spectra, the hypochromicity was observed immediately after mixing of polymer solutions, different from the case of other polyethyleneimine derivatives - Poly A system. It may be caused by a weak intramolecular interaction of PEI-Hse-Ade compared with that of PEI-Hse-Ura, PEI-Hse-Thy, and PEI-Hse-5FU. Weak intramolecular interaction in PEI-Hse-Ade was supported by negligible temperature dependence of absorbance of UV spectra.

Figure Ⅳ-7B shows that the CD spectra changed immediately by mixing of the polymers, which was essentially the same as PEI-Hse-Ura, PEI-Hse-Thy, or PEI-Hse-5FU with Poly A systems. These facts suggest that the conformation of Poly U changed immediately by mixing of the polymer and was kept for 3 days.

As shown in Figures Ⅳ-7A and Ⅳ-7B, the polymer ratio for the maximum hypochromicity in UV spectra was not the same as that in CD spectra. The reason of this unusual fact might be that the conformational change of Poly U was small by formation of 1:1 complex, but the significant conformational change of Poly U occurred by formation of the 2:1 (adenine:uracil).

Interaction of Poly U and Poly A

As a control experiment, interaction and conformation of Poly U - Poly A system were studied. A number of studies of this system were reported in literatures, but time dependence of conformational change was scarcely known. Figure Ⅳ-8A shows CD spectra of this system in 3 hours after mixing of polymer solutions, and Figure Ⅳ-8B shows that after 3 days. The CD spectra (intensity and wavelength)

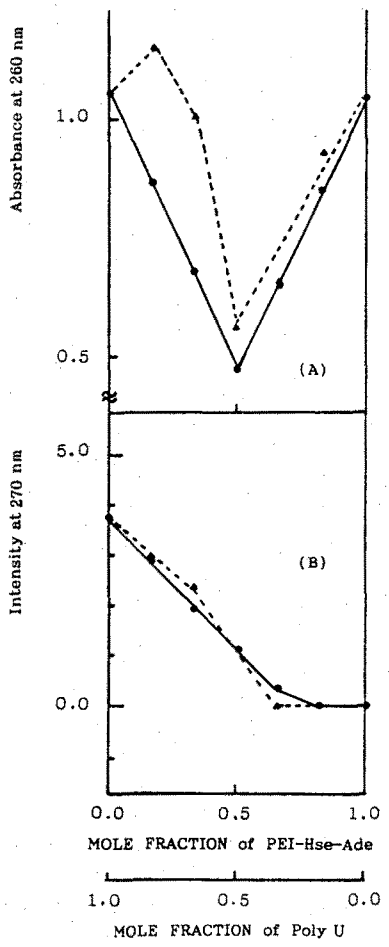


Figure Ⅳ-7. Continuous Variation Curve for PEI-Hse-Ade and Poly U .
(A) UV absorbance at 260 nm (B) CD intencity at 262 nm after 3 hours (···▲···) and 3 days (—●—)in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly U] = 1.1 x 10^{-4} mol/L and [PEI-Hse-Ade] = 8.3 x 10^{-5} mol/L.

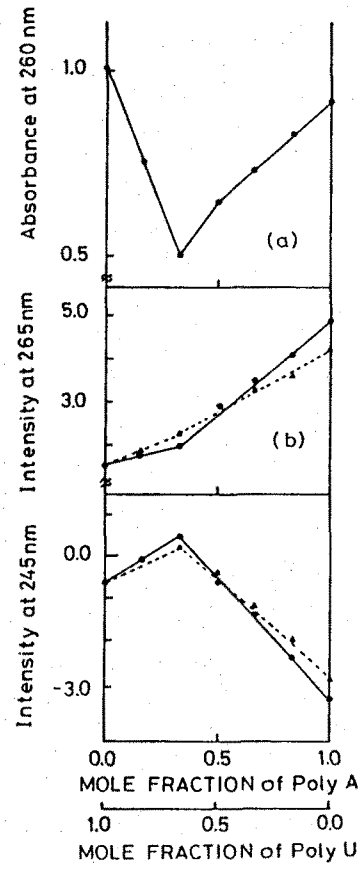


Figure Ⅳ-9. Continuous Variation Curve for Poly A and Poly U .
(A) UV absorbance at 260 nm (B) CD intencity at 262 nm (C) CD intencity at 245 nm after 3 hours (···▲···) and 3 days (—●—) in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly U] = 1.1 x 10^{-4} mol/L and [Poly A] = 1.0 x 10^{-4} mol/L.

drastically changed with time, which was different from polyethyleneimine derivatives - polynucleotides system. Figure Ⅳ-9A shows the mixing curve by UV spectra, and Figures Ⅳ-9B and Ⅳ-9C show intensities of CD spectra at 265 nm and 245 nm, respectively. As previously reported for the Poly U - Poly A system, the hypochromicity in UV spectra was immediately observed, and time dependence of the hypochromicity was scarcely observed (Figure Ⅳ-9A). As shown in Figures Ⅳ-9B and Ⅳ-9C, time dependence of CD spectra was clearly observed. From these facts, it was suggested that the uracil and adenine bases immediately form the base pair by mixing, and became a stable complex with

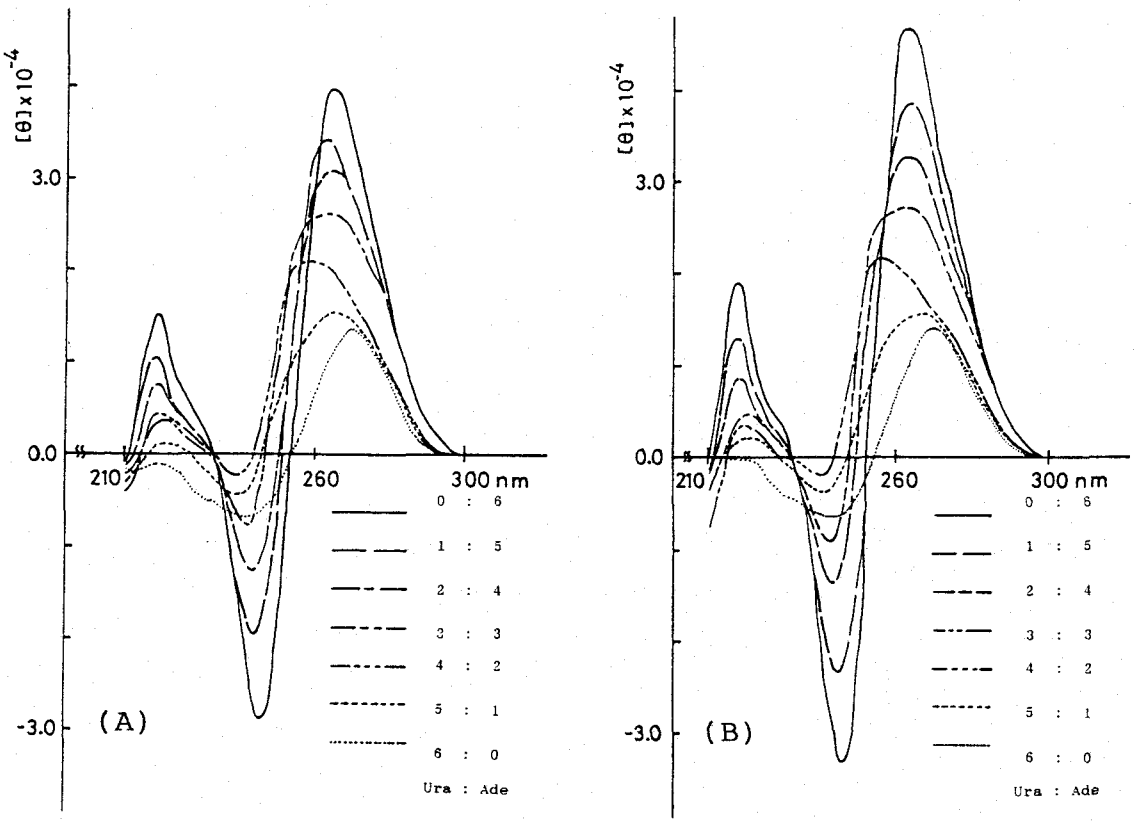


Figure Ⅳ-8. CD spectra of Poly A and Poly U 3 hours (A) and 3 days (B) after mixing of polymer solutions in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [Poly U] = 1.1 x 10^{-4} mol/L and [Poly A] = 1.0 x 10^{-4} mol/L.

conformational change in 3 days.

Ⅳ-4 CONCLUSION

The interactions between water soluble polyethyleneimine derivatives and polynucleotides were studied by CD spectra. In all cases, the conformational change by formation of complex was observed. The interactions between nucleic acid bases in polymers were slow as shown by UV spectra, but the conformational change of the polynucleotide was fast as shown by CD spectra. The result, however, is not true for the case of Poly U - Poly A system:UV change was fast and CD change was slow. The stoichiometry of the complex obtained by UV spectra was the same as that by CD spectra for all the polyethyleneimine derivatives - polynucleotides systems

except for the PEI-Hse-Ade - Poly U system.

Ⅳ-5 REFERENCES

1. K. Takemoto and Y. Inaki, " *Functional Monomers and Polymers* ", K. Takemoto, Y. Inaki, and R.M. Ottenbrite, Eds, Marcel Dekker , p.149, 1987.
2. J. Pitha, *Adv. Polym. Sci.*, 50, 1 (1983).
3. K. Takemoto and Y. Inaki, *Adv. Polym. Sci.*, 41, 1 (1981).
4. C. G. Overberger and Y. Morishima, *J. Polym. Sci. Polym. Chem. Ed.*, 18, 1247, 1267 (1980).
5. A. G. Ludwick and C. G. Overberger, *J. Polym. Sci. Polym. Chem. Ed.*, 20, 2123, 2139 (1982).
6. K. A. Brandt and C. G. Overberger, *Nouv. J. Chim.*, 6, 673 (1982).
7. K. A. Brandt and C. G. Overberger, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 1981 (1985).
8. C. G. Overberger and S. Kikyotani, *J. Polym. Sci. Polym. Chem. Ed.*, 21, 525, 541 (1983).
9. C. G. Overberger and C. X. Lu, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 1321 (1985).
10. C. G. Overberger and C. C. Chen, *J. Polym. Sci. Polym. Chem. Ed.*, 24, 167 (1986).
11. N. M. Brahme and W. T. Smith, Jr., *J. Polym. Sci. Polym. Chem. Ed.*, 22, 813 (1984).
12. W. T. Smith, Jr. and N. M. Brahme, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 879 (1985).
13. A. G. Ludwick, K. S. Robinson, and J. McCloud, Jr., *J. Polym. Sci. Polym. Symp.*, 74, 55 (1986).
14. T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, 21, 11 (1989).
15. T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, 20, 1059 (1988).
16. T. Wada, Y. Inaki, and K. Takemoto, *J. Bioact. Compat. Polym.*, 4, No.2 (April) (1989).
17. G. J. Thomas, Jr. and Y. Kyogoku, *J. Am. Chem. Soc.*, 89, 4170 (1967).
18. T. Sato, K. Kojima, T. Ihda, J. Sunamoto, and R. M. Ottenbrite, *J. Bioactive and Compatible Polym.*, 1, 448 (1986).
19. D. Thiele, W. Guschlbauer, and A. Faver, *Biochim. Biophys. Acta*, 272, 22 (1972).

Chapter V SYNTHESIS AND INTERACTION STUDIES ON WATER SOLUBLE POLY-L-LYSINE DERIVATIVES CONTAINING THYMINE AND HYPOXANTHINE.

V-1 INTRODUCTION

Nucleic acids play an important role in realizing the replication and transcription of genetic codes for the protein synthesis. The essential function of nucleic acids is based on the higher structures and the specific base - base pairing through hydrogen bounding between purine and pyrimidine bases. DNA has a double-stranded structure consisting of two polynucleotide chains twisted about each other in a double helix. The structures of nucleic acids and synthetic polynucleotides has been investigated in detail by circular dichroism (CD) and optical rotatory dispersion and it was shown that those structures are closely related to the interaction between complementary nucleic acid bases.

The chemistry of nucleic acid model polymer has recently received much attention and a number of synthetic polymers containing nucleic acid bases have been prepared and their properties have been studied.[1-12] It is very interesting to study the relationship between the interaction and the conformation for the polymer complex between nucleic acids and these analogs .These synthetic nucleic acid analogs, however, hardly soluble in water at neutral pH values. It is, therefore, very important to prepare the water soluble synthetic nucleic acid analogs in order to study the interaction and the conformation of the analogs.

For this purpose, previously, water soluble polyethyleneimine derivatives containing various nucleic acid bases were prepared, and interactions of these polymers with polynucleotides in aqueous solution were studied.[21,22,23] It was also studied that the conformation of the polynucleotides was changed by the interaction with the nucleic acid analogs. The nucleic acid analogs of polyethyleneimines, however, are optically inactive, therefore it was difficult to study the conformational change of the analogs caused by the interaction with polynucleotides. In the present study, water soluble and optically active poly-L-lysine derivatives containing

thymine and hypoxanthine are prepared, and the conformation of the polymer complexes with polynucleotides are studied. Poly-L-lysine derivatives containing nucleic acid bases have already prepared and their conformation in solution have been studied to be a α-helical structure. However, it was very difficult to study the conformation of the polymer in aqueous solution at neutral pH because of insolubility of the polymer. For the water soluble poly-L-lysine derivatives in the present paper, the conformational of the polymer is able to be studied in neutral aqueous solution by CD spectra. The results of the CD spectra are discussed with the results of the UV spectra for the poly-L-lysine derivatives and polynucleotide systems.

V-2 EXPERIMENTAL

Materials

Poly-L-lysine hydrobromide (PLL · HBr)

Poly- ε -N-carboxybenzoyl-L-lysine (Poly-Cbz-L-lysine) was prepared by NCA method in previous paper.[25] Degree of polymerization was determined ca. 40 by viscosity measurement in N,N-dimethylformamide. The polymer was deprotected by 25 % hydrobromic acid acetic acid solution to poly-L-lysine hydrobromide. The deprotection was found to be complete from NMR, UV and IR spectra.[21]

Poly- ε -N- [{ *2-* [*3-(thymin-1-yl)propionyl*] *amino-4-hydroxy* } *butanoyl*] *lysine* (5T; PLL-Hse-Thy)

To a suspension of ($\pm$)- α-N-[3-(thymin-1-yl)propionyl]amino-γ-butyrolactone[22] (4T; Thy-Hse-L)(390 mg; 1.5 mmol) in water (5 mL), 1 N NaOH aqueous solution (1.5 mL; 1.5 mmol) was added. The mixture was stirred at 40° C for 1 h to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The resulting residue was washed with acetone (20 mL), then with diethyl ether (30 mL) to give a sodium salt of thymine derivative (Thy-Hse-Na) in 98 % yield(430 mg, powder). TLC: Rf. 0.00 (benzene : ethanol=3:1), (ethanol), (n-butanol : acetic

acid : water =4:5:3)

To a suspension of Thy-Hse-Na (430 mg; 1.5 mmol) in 10 mL of dry N,N-dimethylformamide, pentachlorophenyl trichloroacetate (910 mg; 2.2 mmol) and a catalytic amount of triethylamine were added. The mixture was stirred at 0° C for 2 h and then at 50° C until evolution of gas ceased to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The residue was washed with diethyl ether (30 mL) to give a powder of pentachlorophenyl ester of thymine derivative (Thy-Hse-PCP)(yield 92 %; 720 mg). TLC: Rf. 0.82 (benzene : ethanol = 3:1).

To a solution of poly-L-lysine hydrobromide (PLL · HBr) (320 mg; 1.5 mmol) in N,N-dimethylformamide (10 mL), Thy-Hse-PCP (720 mg; 1.5 mmol) triethylamine (0.5 mL, 3.0 mmol) and a catalytic amount of imidazole were added and stirred at 60° C for 48 h. After the reaction, the solution was concentrated under reduced pressure and poured into excess acetone to precipitate the polymer. The obtained polymer was purified by reprecipitation from N,N-dimethylformamide into excess acetone. The polymer was dissolved again in a small amount of water and freeze-dried to give a light brown powder. (250 mg, 62 %); mp 180-182° C. IR (KBr, cm^{-1}): 1650 and 1550 (amide). TLC: Rf. 0.00 (benzene : ethanol = 3:1), (ethanol). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.50 (t, 2H), 3.70 (m, 8H), 4.30 (m, 3H), 5.65 (d, 1H), 7.50 (d, 1H), and 8.60 (s, 1H).

Poly- ε -N- [· { 2- [3-(hypoxanthyl-9-yl)propionyl] amino -4-hydroxy} butanoyl] -L-lysine (5H; PLL-Hse-Hyp)

This polymer was prepared from (±)- α -N-[3-(hypoxanthyl-9-yl)propionyl]amino- γ -butyrolactone[21] (4H; Hyp-Hse-L) (1.4 g; 5.0 mmol) and poly-L-lysine hydrobromide (PLL · HBr) (1.1 g; 5.0 mmol) according to a similar procedure described for PLL-Hse-Thy (5T) (yield 65%; 1.4 g; mp 166-172° C). IR (KBr, cm^{-1}): 1650 and 1550 (amide). ^{1}H-NMR (in dimethyl sulfoxide-d_6 at 25° C, ppm): 2.60 (t, 2H), 3.70(m, 8H), 4.30 (m, 3H), 7.80 (s, 1H), 8.00 (s, 1H), and 8.60 (s, 1H).

Hydrolysis of The Polymer

The poly-L-lysine derivatives were hydrolyzed in 6 N hydrochloric acid at 80° C for 48 h, into poly-L-lysine hydrochloride

and the carboxyethyl derivatives of nucleic acid bases. Quantitative calculation was made using the corresponding carboxyethyl derivatives as standard samples.[26] The nucleic acid base content in the polymers is tabulated in Table V-I.

Interactions Between The Polymers

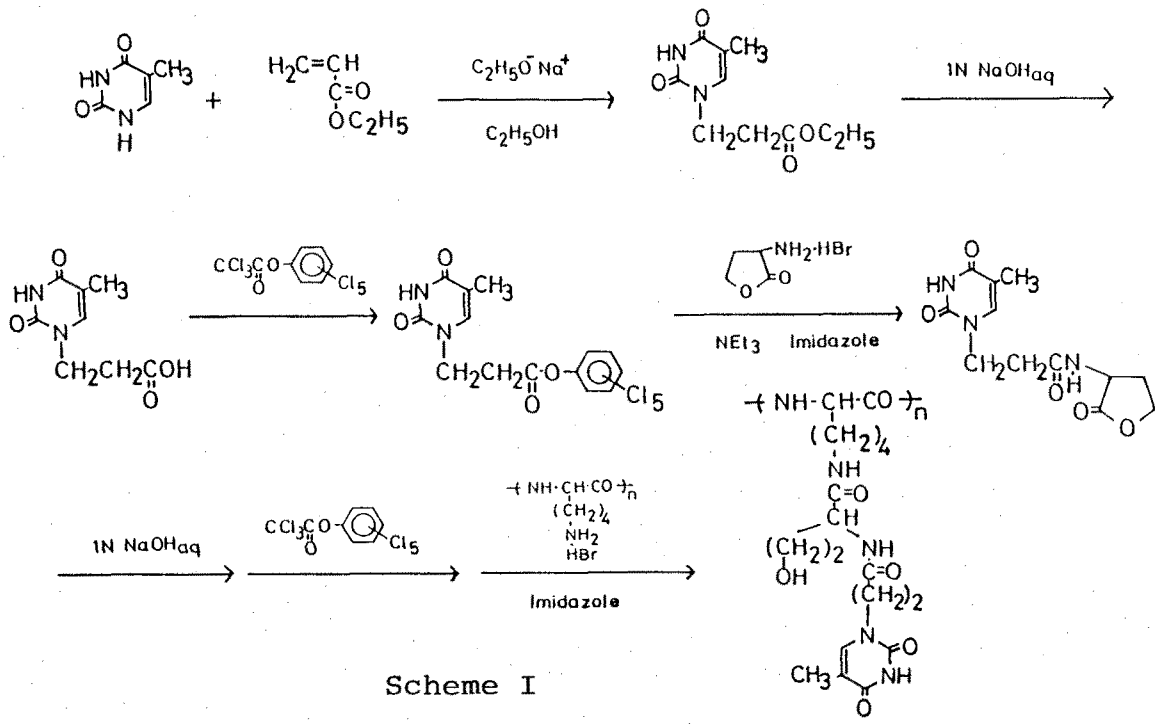
Interactions of the polymers were determined from hypochromicity values in UV spectra as reported previously.[27] The UV spectra were measured with a JASCO UV-660 spectrometer equipped with a temperature controller at 20° C and the circular dichroism (CD) spectra were measured with a JASCO CD J-40 spectrometer at room temperature. Poly A (sodium salt) ($S^{\circ}_{20,w}$: 6-12), Poly C (sodium salt) ($S^{\circ}_{20,w}$: 6-12) were obtained from Yamasa Shoyu Co. Ltd. PLL-Hse-Thy(5T), PLL-Hse-Hyp(5H) and polynucleotides were dissolved in Kolthoff buffer (pH 7.0) (1/10 M KH_2PO_4 - 1/20 M $Na_2B_4O_7 \cdot 10H_2O$). These solutions stocked for 2 days at 20° C, were mixed to give a polymer mixture of 10^{-4} M total concentration of nucleic acid base units in aqueous solution.

V-3 RESULTS AND DISUCUSSION

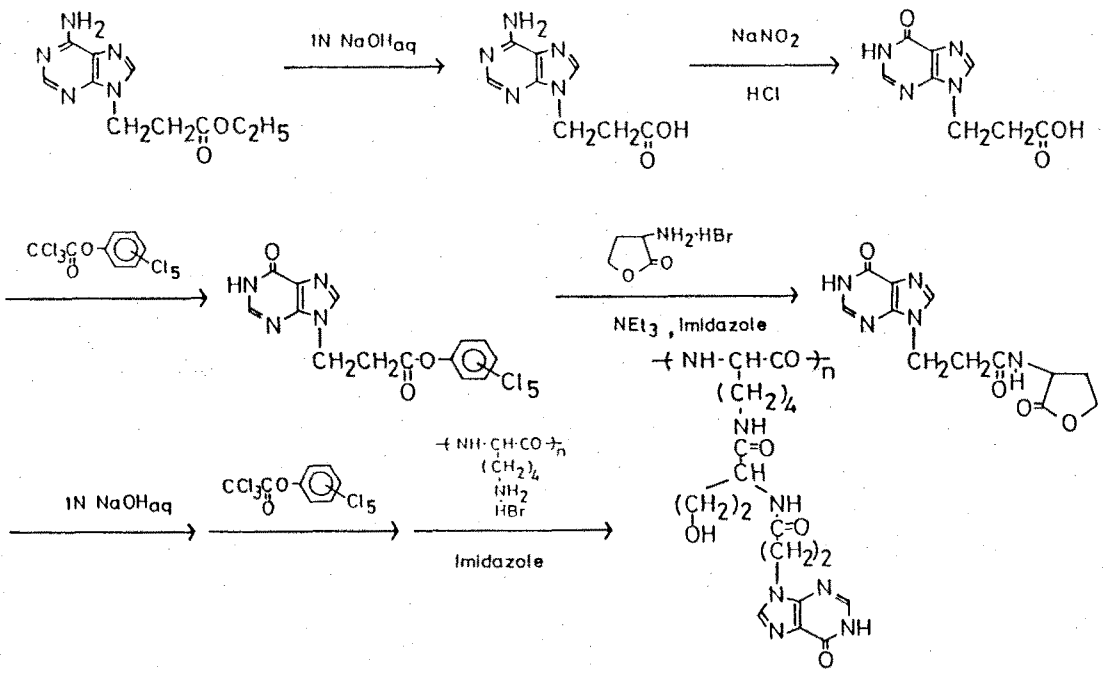
Grafting onto Poly-L-lysine

In the previous papers, the preparations were reported for the water soluble polyethyleneimine derivatives containing nucleic acid bases. These polyethyleneimine derivatives have both nucleic acid bases and hydroxyl groups as pendant groups. The preparations were also reported for the poly-L-lysine derivatives of nucleic acid base. Combination of these methods gave the water soluble poly-L-lysine derivatives which contained nucleic acid bases and hydroxyl groups as pendant groups. (Scheme V-1 and Scheme V-2).

The grafting of nucleic acid base derivatives having hydroxyl group onto poly-L-lysine polymer backbone was carried out by the activated ester method. Since the reactivity of the γ-lactone is low, the direct reaction of the lactone derivative with poly-L-lysine was hardly occurred. Therefore the lactone derivatives were hydrolyzed to the 3-hydroxybutylic acid derivative, followed by the condensation with poly-L-lysine using the activated ester method. The grafting reaction was carried out in N, N-dimethylformamide,



Scheme I



Scheme II

where a small amount of 4-pyroridino pyridine was an effective catalyst.

Nucleic acid base content of the polymer was determined by UV spectroscopy on their hydrolyzed samples. The quantitative calculation was made by using the corresponding carboxyethyl derivatives as standard samples, and was tabulated in Table V-I.

Table I

	PLL-Hse-Thy	PLL-Hse-Hyp
Content (unit Mol%)	73	72

Conformation of The Poly-L-lysine Derivatives

Poly-L-lysine is known to exist in random coiled, α -helical, and β -sheet conformations, depending on the temperature, pH, and solvent used. The side chain of the polymer has a significant effect on the conformation of the main chain. To determine how the thymine - homoserine and the hypoxanthine - homoserine substituents affect the conformation of the polymer, their CD spectra were measured in aqueous solution.

The CD spectra of PLL-Hse-Thy in aqueous solution at various pH are shown in Figure V-1. The molar ellipticity at 222 nm ($[\theta]_{222}$) is known to be related to the helix content of poly-L-lysine.

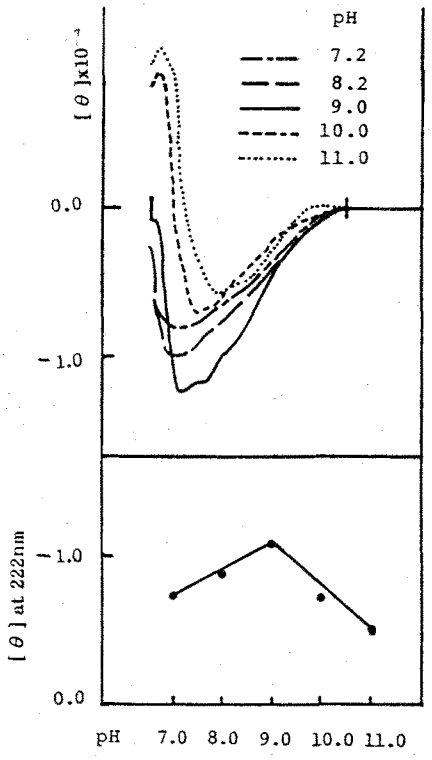


Figure V-1. CD spectra of PLL-Hse-Thy in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C (A) and intensity at 222 nm (B). [PLL-Hse-Thy] = 9.5 x 10^{-5} mol/L

Figure V-1B shows the plots of the $[\theta]_{222}$ values against pH of the system. The figure shows that PLL-Hse-Thy is in a helical structure, where the helix content is about 40% at pH 7.0. The helix content of PLL-Hse-Thy was lower than that of PLL-Thy[26] lacking of the homoserine unit, because of the hydrophilic homoserine unit. The helix content increased with increase of pH value according to neutralization of free amino units in PLL-Hse-Thy. After the highest helix content at pH=9.0 (about 50% helix content), the value decreased with increase of pH value because the thymine base became a anionic form above pKa = 10. The result indicates that PLL-Hse-Thy exists both in helical and in random coiled conformations. The similar result was obtained for PLL-Hse-Hyp.

Interaction of The Polymers

The formation of the polymer complexes were observed for the polyethyleneimine derivatives - polynucleotides (Poly A, Poly C, Poly U and Poly I) systems by specific base - base interactions with nucleic acid bases. The conformation of the polynucleotide was observed to change by complex formation with the polyethyleneimine derivatives. As the polyethyleneimine derivatives, however, are optically inactive, the study of the conformation of these polymer was impossible. Previously, the conformation of poly-L-lysine derivatives of nucleic acid bases was studied.[26] However, it was difficult to measure the conformation of the polymer complex in neutral aqueous solution because of insolubility of the polymer. The water soluble poly-L-lysine derivatives of thymine or hypoxanthine in this study make it possible to measure the conformation of the polymer complex with polynucleotide by CD spectra.

Interaction of PLL-Hse-Thy with Poly A

The poly-L-lysine derivatives containing both the nucleic acid bases and the homoserine units are soluble in water, therefore it is possible to study the interactions of the polymers with nucleic acids and polynucleotides in aqueous solution. Figure V-2 shows the mixing curves of UV spectra for the PLL-Hse-Thy with Poly A at pH 7.0. The maximum hypochromicity value was obtained as 12.3 % (Figure V-2b). The overall stoichiometry of the complex based on nucleic acid base units, therefore, was 2:1 (thymine : adenine). The

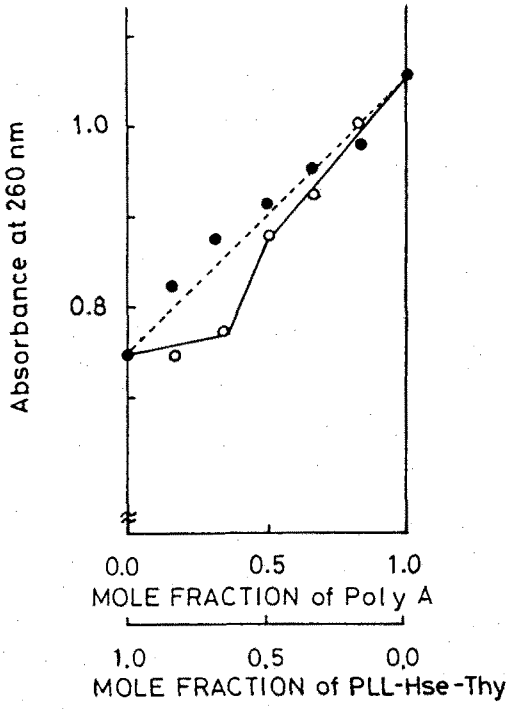


Figure V-2. Continuous Variation Curve of PLL-Hse-Thy and Poly A.

Absorbance at 260 nm after 3 hours (··· ●···) and 3 days (—○—) in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PLL-Hse-Thy] = 9.5 x 10^{-5} mol/L, and [Poly A] = 9.6 x 10^{-5} mol/L.

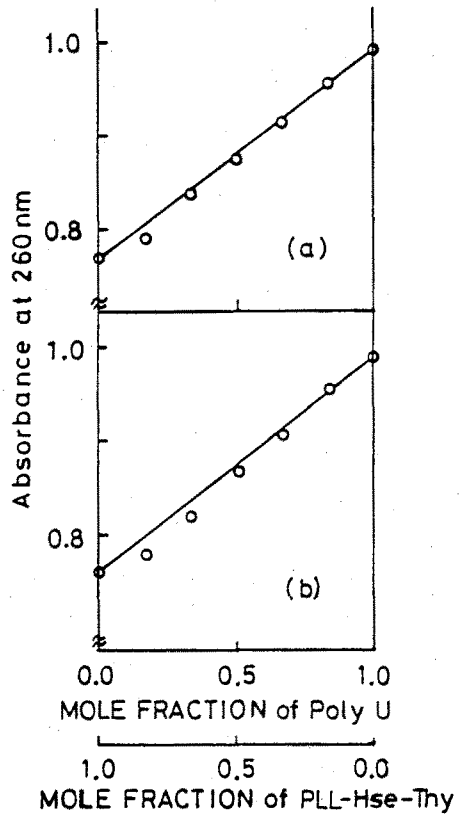


Figure V-3. Continuous Variation Curve of PLL-Hse-Thy and Poly U.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PLL-Hse-Thy] = 9.5 x 10^{-5} mol/L, and [Poly U] = 1.2 x 10^{-4} mol/L.

maximum hypochromicity value was lower than that of the PEI-Hse-Thy : Poly A system.[22] The stoichiometry of the polymer complex Poly U : Poly A was same as that of the PLL-Hse-Thy : Poly A complex. The formation of the Poly U - Poly A complex was observed immediately after mixing of the polymer solutions, and the maximum hypochromicity (40.3 %) was observed at the base ratio of 2:1 (uracil:adenine). The reason of the formation of the 2:1 complex is known to be ability of single strand formation for Poly A. The 2:1 polymer complex of PLL-Hse-Thy : Poly A, therefore, may be formed by the same reason for the Poly U : Poly A complex.

Time dependence of absorbance was observed in this case as shown in Figures V-2 (a) and (b). The hypochromicity was scarcely observed in 3 hours after mixing of the polymer solutions

(Figure V-2a), but the absorbance decreased and then became constant in 3 days (Figure V-2b). The fact may be caused by self association of the nucleic acid bases in the poly-L-lysine derivatives, which dissociated slowly to form an intermolecular polymer complex. The dissociation of the self - association of thymine bases accompanied by conformational change in PLL-Hse-Thy should be necessary to form the polymer complex in aqueous solution. In the case of interactions between polyethyleneimine derivatives and polynucleotides, conformational change of polyethyleneimine derivatives were necessary to form the complexes, while conformational change of polynucleotides was scarcely observed. A similar result was reported for the polymer complex formation between the polymethacrylate derivatives of uracil and adenine. In this case, the self association of the uracil bases in the polymer inhibited the polymer complex formation.[28]

To make sure that such complex formation is due to the complementary nucleic acid base interaction, the interaction of PLL-Hse-Thy with Poly U was measured under the same condition. Figure V-3 shows the mixing curves for PLL-Hse-Thy : Poly U at pH 7.0. The hypochromicity, however, could not be observed after 3 hours (Figure V-3a) and even after 3 days (Figure V-3b). From these facts, the formation of the PLL-Hse-Thy : Poly A complex was concluded to be caused by the complementary interaction between adenine and thymine.

The formation of the polymer complex between PLL-Hse-Thy and Poly A was also studied by the CD spectra as shown in Figure V-4. PEI-Hse-Thy shows a negative band at about 220 nm suggesting the formation of α-helical structure. Intensities of the positive band at 260 nm due to stacking interaction of Poly A was plotted against the ratio of the polymers in Figure V-4B. In this Figure, the maximum hypochromicity in CD spectra was observed at 2:1 base ratio (Thymine : Adenine). The stoichiometry of the polymer complex obtained by the CD spectra coincided with the value obtained by the hypochromicity in UV spectra. It was suggested that the conformational change of polynucleotides and polyethyleneimine derivatives were necessary to form the complex. The measurement of the conformational change of the PLL-Hse-Thy, unfortunately, was difficult because of overlapping of the negative band at 222 nm of

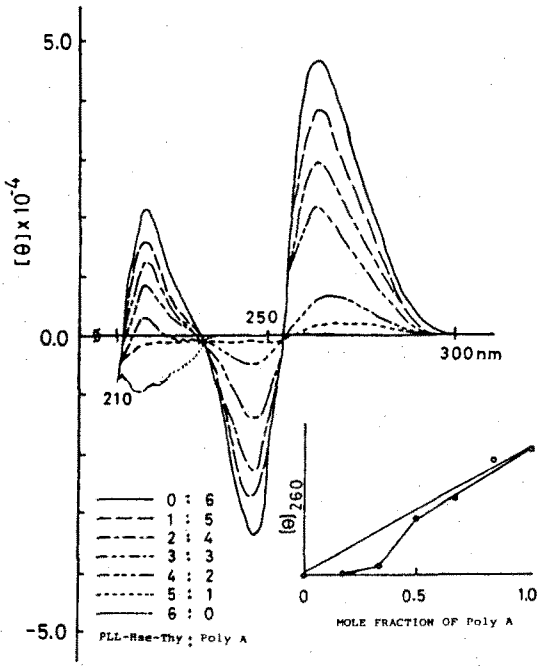


Figure V-4. CD spectra of PLL-Hse-Thy and Poly A after 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20 ° C. [PLL-Hse-Thy] = 9.5 x 10^{-5} mol/L, and [Poly A] = 9.6 x 10^{-5} mol/L.

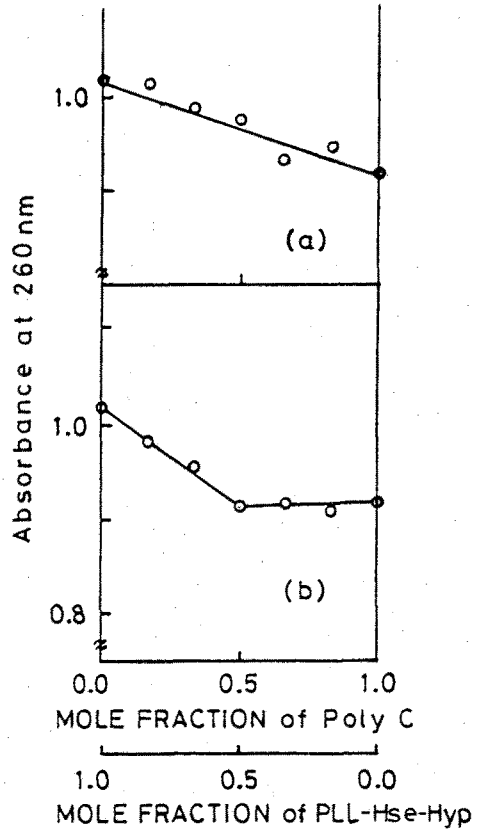


Figure V-5. Continuous Variation Curve of PLL-Hse-Hyp and Poly C.

Absorbance at 260 nm after (a) 3 hours and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20° C. [PLL-Hse-Hyp] = 9.5 x 10^{-5} mol/L, and [Poly C] = 9.6 x 10^{-5} mol/L.

PLL-Hse-Thy and the positive band of Poly A.

Interactions of PLL-Hse-Hyp with Poly C

Interactions between PLL-Hse-Hyp(5H) and Poly C which contains the complementary nucleic acid base can be observed by UV spectra in aqueous solution at pH 7.0, as shown in Figure V-5. The overall stoichiometry of the complex based on the nucleic acid base units was approximately 1:1 (hypoxanthine : cytosine) under the conditions used. The maximum hypochromicity value (10 %) was smaller than that of the Poly I - Poly C (33%) system. The stoichiometry of Poly I - Poly C complex is reported to be 1:1 buffer solution,[29] which is the same as the PLL-Hse-Hyp - Poly C system.

The CD spectra of this system is essentially the same as that of PLL-Hse-Thy - Poly A system (Figure V-4). PLL-Hse-Hyp had a negative band at 220 nm suggesting that PLL-Hse-Hyp had also α-helical structure. Figure V-6 shows the relation between the intensity of CD spectra at 260 nm and the ratio of the polymers. The stoichiometry of the polymer complex obtained by the CD spectra coincided with the value obtained by the hypochromicity in UV spectra.

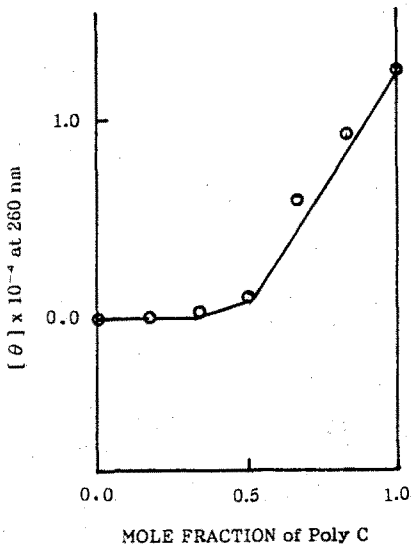


Figure V-6. Intensity of CD spectra at 260 nm in PLL-Hse-Hyp - Poly C system. [PLL-Hse-Hyp] = 9.5 x 10^{-5} mol/L, and [Poly C] = 9.6 x 10^{-5} mol/L.

V-4 REFERENCES

1. Y. Inaki and K. Takemoto, "*Current Topics in Polymer Science, Volume I*", R. M. Ottenbrite, L. A. Utracki and S. Inoue, Eds., Hanser Publisher, p 79, (1987).
2. J. Pitha, *Adv. Polym. Sci.*, 50, 1 (1983).
3. K. Takemoto and Y. Inaki, *Adv. Poly. Sci.*, 41, 1 (1981).
4. C. G. Overberger and Y. Morishima, *J. Polym. Sci. Polym. Chem. Ed.*, 18, 1247, 1267 (1980).
5. A. G. Ludwick and C. G. Overberger, *J. Polym. Sci. Polym. Chem. Ed.*, 20, 2123, 2139 (1982).
6. K. A. Brandt and C. G. Overberger, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 1981 (1985).
7. C. G. Overberger and S. Kikyotani, *J. Polym. Sci. Polym. Chem. Ed.*, 21, 525, 541 (1983).
8. C. G. Overberger and C. X. Lu, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 1321 (1985).
9. C. G. Overberger and C. C. Chen, *J. Polym. Sci. Polym. Chem. Ed.*, 24, 167 (1986).

10. N. M. Brahme and W. T. Smith Jr., *J. Polym. Sci. Polym. Chem. Ed.*, 22, 813 (1984).
11. W. T. Smith, Jr. and N. M. Brahme, *J. Polym. Sci. Polym. Chem. Ed.*, 23, 879 (1985).
12. A. G. Ludwick, K. S. Robinson, and J. McCloud, Jr., *J. Polym. Sci. Polym. Symp.*, 74, 55 (1986).
13. J. H. Park, M. A. Gallin, A. Billiau, and S. Baron, *Arch. Ophthal.*, 81, 840 (1969).
14. H. B. Levy, *J. Bioactive and Compatible Polymers*, 1, 348 (1986).
15. A. K. Field, A. A. Tytell, G. P.Lampson, and M. R. Hillman, *Proc. Nat. Acad. Sci. U.S.A.*, 58, 1004 (1967).
16. M. M. Nemes, A. A. Tytell, G. P. Lampson, A. K. Field, and M. R. Hillman, *Proc. Soc. Exp. Biol. Med.*, 132, 776 (1969).
17. V. DeVita, G. Canellos, P. Carbone, H. Levy, and H. Gralnick, *Proc. Am. Assoc. Cancer Res.*, 11, 21 (1970).
18. M. Absher and W. R. Stinebring, *Nature*, 223, 715 (1969).
19. T. Sato, K. Kojima, T. Ihda, J. Sunamoto, and R. M. Ottenbrite, *J. Bioactive and Compatible Polymer*, 1, 448 (1986).
20. R. M. Ottenbrite, "*Anionic Polymeric Drugs* ",L. G. Donaruma, R. M. Ottenbrite, O. Vogl, Eds., John Wiley & Sons, Inc., p 1, (1980).
21. T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, 21, 11 (1989).
22. T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, 20, 1059 (1988).
23. T. Wada, Y. Inaki, and K. Takemoto, *J. Bioactive and Compatible Polymer*, 4, Aprile (1989) in Press.
24. J. Sunamoto and K. Takemoto, to be published.
25. C. E. Seeney and H. J. Harwood, *J. Macromol. Sci. -Chem.*, A9, 779 (1975).
26. T. Ishikawa, Y. Inaki, and K. Takemoto, *Polym. Bull.*, 1, 85 (1978).
27. G. J. Thomas, Jr. and Y. Kyogoku, *J. Am. Chem. Soc.*, 89, 4170 (1967).
28. S. Fang, Y. Inaki, and K. Takemoto, *J. Polym. Sci. Polym. Chem. Ed.*, 22, 2455 (1984).
29. D. Thiele, W. Guschlbauer, and A Faver, *Biochim. Biophys. Acta*, 272, 22 (1972).
30. K. Fujioka, Y. Baba, A. Kagemoto, and R. Fujishiro, *Polym. J.*, 1^{2}, 843 (1980).

CONCLUSION

The purpose of this study is to prepare the water soluble nucleic acid analogs and to investigate the interaction between nucleic acid analogs and with polynucleotides in aqueous solution.

In chapter I, the synthesis and the interaction of the water soluble polyethyleneimine derivatives containing hypoxanthine and cytosine were studied. Polyethyleneimine derivatives containing cytosine and hypoxanthine bases formed stable polymer complex similar as Poly C - Poly I complex in neutral aqueous solution. And these nucleic acid analogs also formed stable polymer complex with polynucleotides by complementary bases - base interaction.

In chapter II, the synthesis and the interaction of the water soluble polyethyleneimine derivatives containing adenine and thymine were investigated. Polyethyleneimine derivatives containing adenine and thymine bases formed stable polymer complex, too. And these polymers also formed stable polymer complex with polynucleotides by complementary base - base interaction in aqueous solution.

In chapter III, the synthesis and the interaction of the water soluble polyethyleneimine derivatives containing uracil and 5-fluorouracil were studied. Polyethyleneimine derivatives containing uracil and 5-fluorouracil bases formed stable polymer complexes with polyethyleneimine derivative containing adenine base. And the substituent effect at 5-position of pyrimidine base on base - base interaction were made in comparison of polyethyleneimine derivatives containing uracil, thymine, and 5-fluorouracil. The polymer containing uracil base form very stable polymer complexes with both Poly A and Poly C, but selectivity of interaction was low. On the other hand, the polymer containing 5-fluorouracil, which has highly basic 5-fluorouracil base, formed the polymer complex only with Poly A. The results indicated that the complex formation ability of the 5-fluorouracil base is not higher than the uracil base.

In chapter IV, interaction and conformation studies of water soluble polyethyleneimine derivatives containing nucleic acid bases were investigated. The interactions between water soluble polyethyleneimine derivatives and polynucleotides were studied by

CD spectra. In all cases, the conformational change by formation of complex was observed. The interactions between nucleic acid bases in polymers were slow, but the conformational change of the polynucleotide was fast. The result, however, is not true for the case of Poly U - Poly A system : UV change was fast and CD change was slow. The stoichiometry of the complex obtained by UV spectra was the same as that by CD spectra for all the polyethyleneimine derivatives - polynucleotides systems.

In chapter V, the synthesis and the interaction of the water soluble poly-L-lysine derivatives containing thymine and hypoxanthine were studied. The water soluble poly-L-lysine derivatives containing nucleic acid bases have α-helical structure in ca. pH 9.0 aqueous solutions. And form polymer complexes with polynucleotides by complementary base - base interaction.

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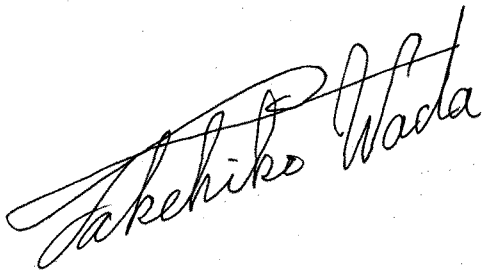

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