



Title	2' 置換ポリヌクレオチドの合成と性質に関する研究
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論文目録

氏名

垣内信子

博士論文題名

2' 置換ポリヌクレオチドの合成と性質に関する研究

1. Polynucleotides. XL. Synthesis and properties of poly(2'-azido-2'-deoxyadenylic acid).

M. Ikehara, T. Fukui and N. Kakiuchi.

Nucleic Acids Res., 3 2089, (1976)

ポリヌクレオチド 40. ポリ(2'-アジド-2'-デオキシアデニル酸)の合成と性質

2. Polynucleotides. XLVI. Synthesis and properties of poly(2'-amino-2'-deoxyadenylic acid).

M. Ikehara, T. Fukui and N. Kakiuchi.

Nucleic Acids Res., 4 989 (1977)

ポリヌクレオチド 46. ポリ(2'-アミノ-2'-デオキシアデニル酸)の合成と性質

3. Polynucleotides. XLV. Synthesis and properties of poly(2'-azido-2'-deoxyinosinic acid).

T. Fukui, N. Kakiuchi and M. Ikehara.

Nucleic Acids Res., 4 2629 (1977)

ポリヌクレオチド 45. ポリ(2'-アジド-2'-デオキシイノシン酸)の合成と性質

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

M. Ikehara, T. Fukui and N. Kakiuchi

Nucleic Acids Res., 4 4249 (1977)

ポリヌクレオチド 50. ポリ(2'-クロロ-2'-デオキシアデニル酸)及びポリ(2'-ブロモ-2'-デオキシアデニル酸)の合成と性質

5. Polynucleotides. LII. Synthesis and properties of poly(2'-fluoro-2'-deoxyadenylic acid).

M. Ikehara, T. Fukui and N. Kakiuchi

Nucleic Acids Res., 5 1877 (1978)

ポリヌクレオチド 52. ポリ(2'-フルオロ-2'-デオキシアデニル酸)の合成と性質

6. Interferon inducing activity of a 2'-modified double-stranded complex, poly(2'-azido-2'-deoxyinosinic acid). poly(cytidylic acid).

E. De Clercq, T. Fukui, N. Kakiuchi and M. Ikehara.

J. Pharm. Dyn., 1 62 (1978)

2'置換ニ重鎖コンプレックス、ポリ(2'-アジド-2'-デオキシイノシン酸)-ポリ(シチジル酸)のインターフェロン誘発活性

7. Polynucleotides. LVI. Synthesis and properties of poly(2'-fluoro-2'-deoxyinosinic acid).

M. Ikehara, N. Kakiuchi and T. Fukui

Nucleic Acids Res., 5 3315 (1978)

ポリヌクレオチド 56. ポリ(2'-フルオロ-2'-デオキシイノシン酸)の合成と性質

8. Interferon induction by a 2'-modified double-helical RNA poly(2'-azido-2'-deoxyinosinic acid) poly(cytidylic acid).

E. De Clercq, P. F. Torrence, B. D. Stoller, J. Hobbs, T. Fukui

N. Kakiuchi and M. Ikehara

Eur. J. Biochem., 88 341 (1978)

2'置換ニ重鎖RNAポリ(2'-アジド-2'-デオキシイノシン酸) ポリ(シチジル酸)によるインターフェロン誘発

9. Influence of various 2- and 2'-substituted polyadenylic acids on murine leukemia virus reverse transcriptase.

E. De Clercq, T. Fukui, N. Kakiuchi, M. Ikehara, M. Hattori, and W. Pflleiderer

Cancer Lett., in press

2-及び2'-置換ポリアデニル酸類のネズミ白血病ウイルスの逆転写酵素に与える影響

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1. Biologic activities of poly(2-azaadenylic acid) and poly(2-azainosinic acid)

E. De Clercq, G. F. Huang, P. F. Torrence, T. Fukui, N. Kakiuchi and M. Ikehara

Nucleic Acids Res., 4 3643 (1977)

ポリ(2-アザアデニル酸)及びポリ(2-アザイノシン酸)の生物活性

2. Polynucleotides. XLIV. Synthesis and properties of poly(2-azaadenylic acid) and poly(2-azainosinic acid)

T. Fukui, N. Kakiuchi and M. Ikehara

Biochim. Biophys. Acta., 520 441 (1978)

ポリヌクレオチド 44 ポリ(2-アザアデニル酸)及びポリ(2-アザイノシン酸)の合成と性質

論文内容の要旨

博士論文題名

Z'-置換ポリスチレンの合成と性質に
関する研究

学位申請者

垣内 信子

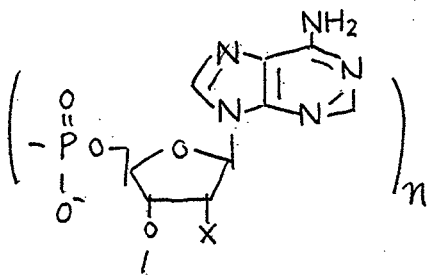


緒 論

核酸は生体を構成する重要な成分の一つである。

天然の核酸には大別して DNA と RNA の二種類が存在する。これら二者は互いに生体内での存在部位も、担っている役割も異なっている。DNA と RNA の化学構造上の相異点は、糖部 2' 位に水酸基が置換しているか否かということである。従って、2' 位置換基の核酸の構造全体に及ぼす影響を考察するのは興味あることである。

今回演者は、2' 位にアゾ基、アミノ基、及びハロゲンを導入して 2' 置換ポリヌクレオチドを合成し、その物理化学的性質、並びに生物活性に関する研究を行った。



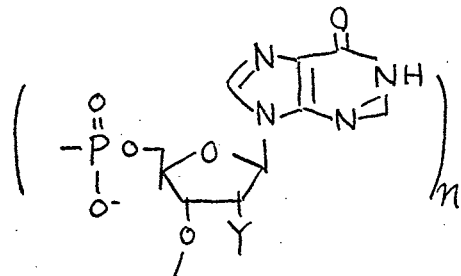
$X = N_3 : \text{poly(Az)}$

$NH_2 : \text{poly(Aa)}$

$F : \text{poly(At)}$

$Cl : \text{poly(Acl)}$

$Br : \text{poly(Abr)}$



$Y = N_3 : \text{poly(Iz)}$

$F : \text{poly(It)}$

$Cl : \text{poly(Icl)}$

本 論

第 1 章 ポリ(2'-アジド-2'-デオキシアデリンスラレオチド)の 合成と性質

アジド基は電気陰性度は水酸基に似ており、その大きさは水酸基より大きい。池原らにより 8, 2'-O-サイクロアデニシンを本発原料とし、2'-アジド-2'-デオキシアデニシン (Az) を合成するルートが開発された。¹⁾ Az は Fig 1 のようにジリン酸化⁴⁾を行い、polynucleotide phosphorylase を用いて、ポリ

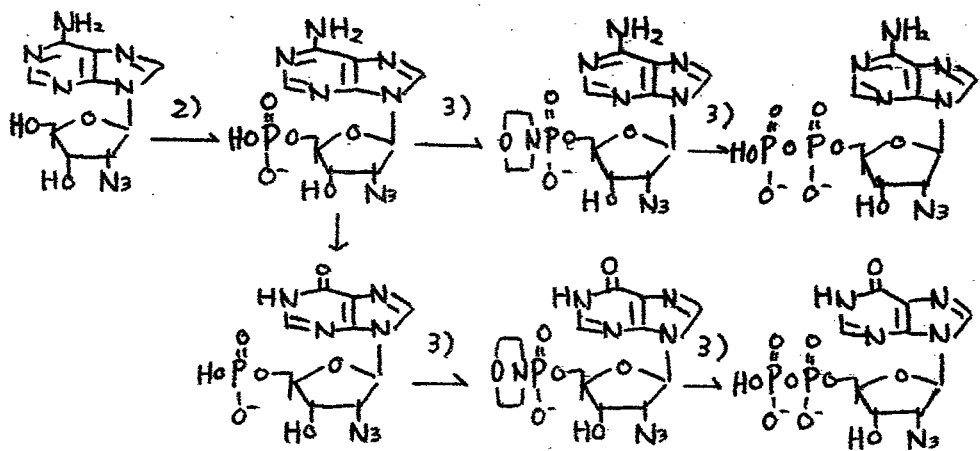
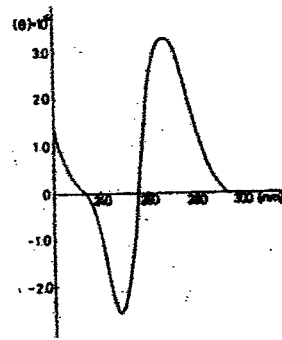
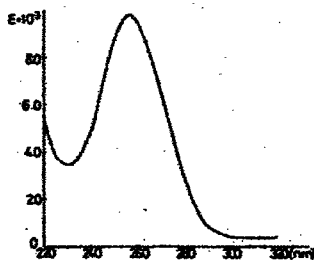


Fig 1

ヌクレオチドとした。分離精製は Sephadex G-50 ゲル
カラムで行い、void volume に溶出される poly(Az), poly(Iz) を得た。

poly(Az) は、0.15 M Na イオン濃度 pH 7.0 室温に於て
Fig 2 のような UV, CD スペクトルを示す。これは poly(A)
の UV, CD スペクトルによく似ている。UV スペクトルは λ_{max}
257 nm 2'。分子吸光係数 (ϵ) は 97802' モイマー⁻¹ の

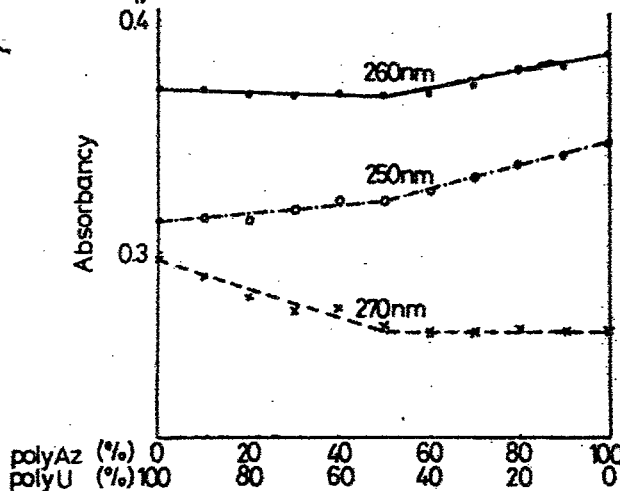


UV absorption spectrum of poly (Az) at pH 7.0. CD spectrum of poly (Az) at pH 7.0

Fig 2

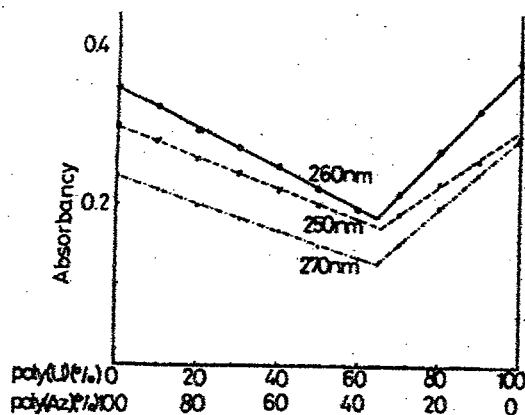
hypochromicity は 35 % であった。これは poly(A) の hypochromicity と同じであり、poly(Az) は poly(A) と同程度の stacking をしていることが判る。一方 CD スペクトルの場合は poly(A) の 60 % 程度であった。又、poly(Az) は poly(U) と 0.04 M Na⁺ イオン存在下 mixing すると 30 分後の測定では 1:1, 0.15 M

Fig3 a



Mixing curves of poly (Az) and poly (U) at 0.04M Na⁺.

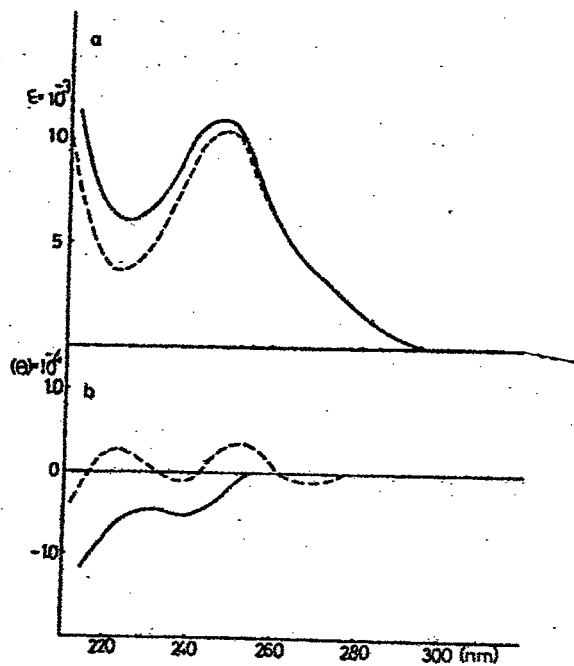
Fig 3 b



Mixing curves of poly (Az) and poly (U) at Na^+ concentration of 0.15M.

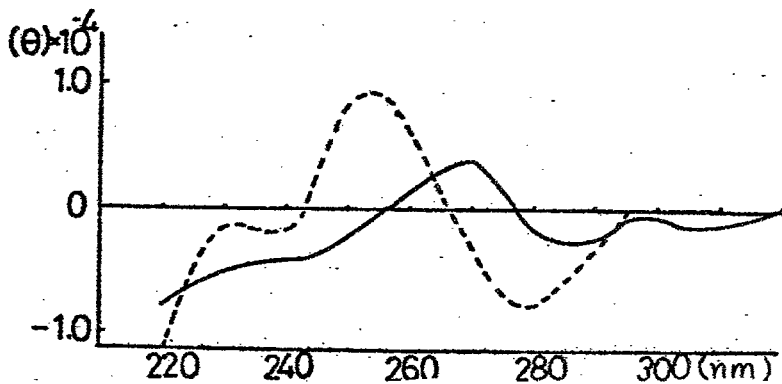
Naイオン濃度, over night の測定では 1:2 の complex の形成が観測された。これらの complex の melting temperature (T_m) は、0.04M Naイオン濃度で 46°C 、0.15M Naイオン濃度で 65°C であり、poly(A)・poly(U) complex の場合が 51° 、 62° であるので、ほぼ同程度の熱的安定性を有している。

poly(Iz) の 0.15M Naイオン濃度, pH 7.0, 室温 での UV, CD スペクトルを Fig 4 に示す。poly(I) では 225, 255 nm 付近に正の cotton 効果が見られるのに対して、poly(Iz) ではほとんど正の cotton 効果は見られない。0.95M Naイオン濃度では、poly(I) の CD スペクトルに似ているが、peak が長波長側にシフトしており、cotton 効果の amplitude も小さい。poly(I) に比べてより flexible な構造をとっているものと思われる。0.15M Naイオン存在下。



U.V. and C.D. spectra of poly(Iz) and poly(I) in neutral solution containing 0.15M Na⁺.
— poly(Iz), --- poly(I)

Fig 4



UV spectra of poly(Iz) and poly(I) in neutral solution containing 0.95M Na : — poly(Iz) --- Poly(I)

Fig 5

PH 7.0 2 poly (Iz) と poly (C) の mixing を行くと, poly (Iz)
50% のところに屈曲点がある。 poly (Iz) · poly (C) = 1 : 1
complex の形成が観察された。 この poly (Iz) · poly (C)
5

complexの T_m を種々の塩濃度で測定し、この値を $\log_{10}[Na]$ に対してプロットするとFig7のようになり、直線関係が成立

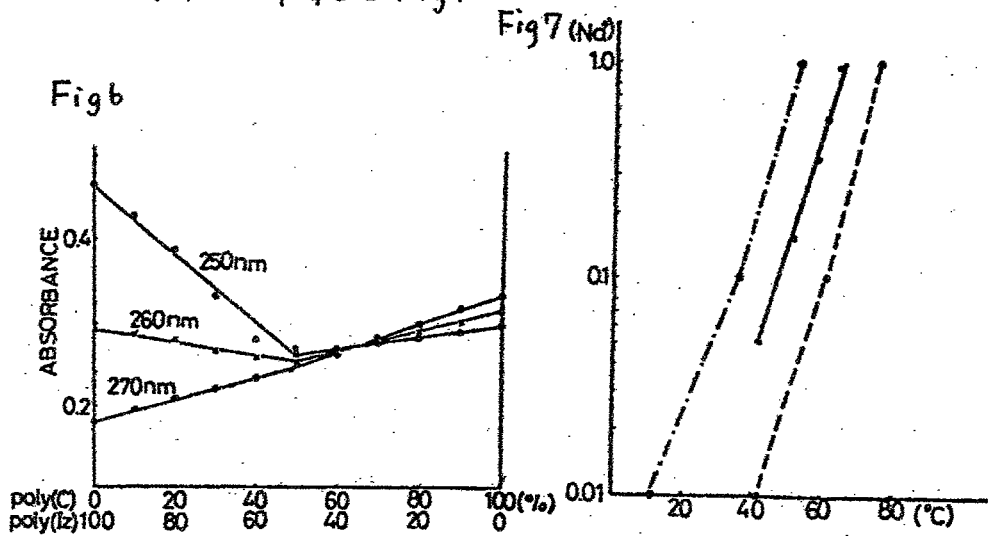


Fig 6 Mixing experiment of poly(Iz) and poly(C)

Fig 7 Relationship of Na^+ concentration and T_m of poly(Iz)·poly(C) (—), Poly(I)·poly(C) (----) and poly(dI)·poly(C) (- · - · -).

する。いずれの Na イオン濃度においても、poly(I)·poly(C) poly(dI)·poly(C)の中間の値である。このようにpoly(Iz)はpoly(C)とのcomplexの安定性において、ribo体とdeoxy体の中間の性質を有していることが判った。このように、2位のアジド基は、poly(Az)においては水酸基と同じ効果を有しているのに対して、poly(Iz)では若干の違いがあり、分子全体に影響を与えていることが判った。

第2章 ポリ(2'-アミ-2'-デオキシアデニル酸)の合成と性質
アミ基の電気陰性度は水酸基と大きく異なっているが、分子量は

とく似ている。poly(Aa)の合成の基質となるAaDPはAaDP
 のリボ糖置換により合成し、polynucleotide phospho-
 ryaseによる重合反応、分離精製の後、13%の収率で
 poly(Aa)を得た。0.15 M Na⁺イオン存在下 25°C 2⁻UV

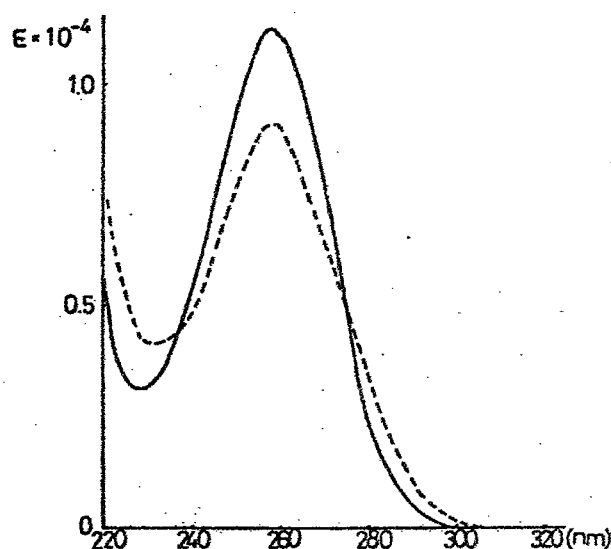


Fig 8 UV absorption spectra of poly(Aa), — at pH 7.0, --- at pH 6.3.

スペクトルは Fig 8 のようになる。pH 7.0 で λ_{max} 258 nm
 分の吸光係数 11200 であった。ヌクレオシドの hypo-
 chromicity は 25% 2⁻。poly(A) には比べて著しく小さい。pH 7
 以下で吸光度の急激な減少を生じる。pH 6.3 では λ_{max} は変化
 しないが hypochromicity は 40% となる。このとき CD スペク
 トルは Fig 9 のようになる。pH 7.0 では AaDP とよく似た CD スペ
 クトルを示す。この条件では random coil 状と思われるが、
 pH が弱酸性になると特異な CD スペクトルを示す。このとき

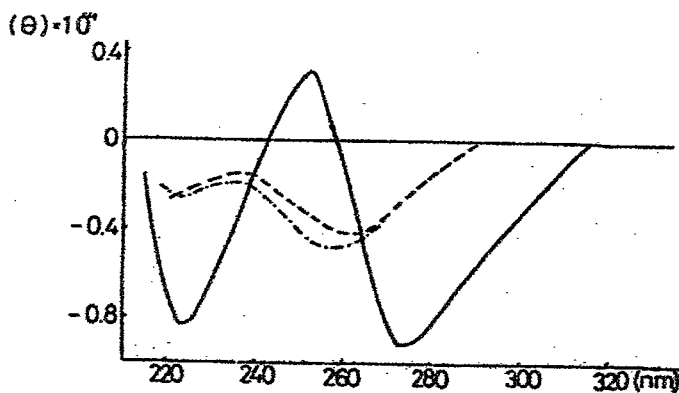


Fig 9-a CD spectra of poly(Aa) taken at 20°(—), 46°(---) and 66°(-.-.-).

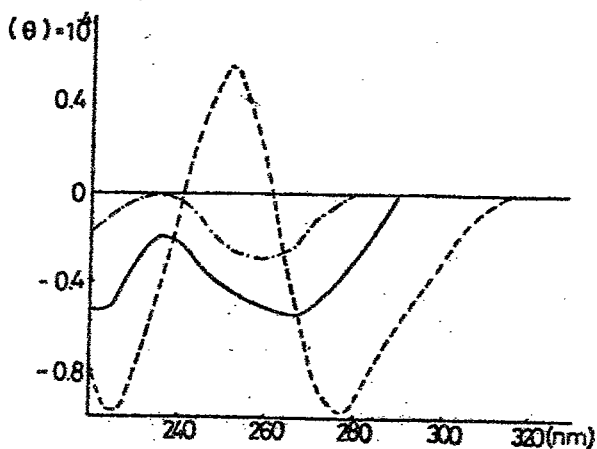


Fig 9-b CD spectra of AaDP and poly(Aa), —poly(Aa) at pH 7.0, ---poly(Aa) at pH 5.7, -.-.- AaDP at pH 7.0.

何らかの ordered structure をとっているものと思われる。この温度の上昇に従って崩壊し、もとの AaDP に似た CD スペクトルにもどる。この ordered structure の T_m 値と $\log_{10}[Na^+]$ の関係は Fig 10 のようになり、Na イオン濃度はこの ordered structure の熱的安定性に対して大きな影響を与えないことが判る。

以上のことから弱酸性における poly(Aa) の ordered structure は poly(A) における塩基部にプロトン化

いわゆる "acid form" ではなく、2'位アミ基がプロトネートすることにより stacking が強化されたことによるものである

と考えられる。このように

2'-NH₂ は電気陰性度

が水酸基と大きく異

なために、分子全体

が大きく異った構造

をとるようになる。この

様に 2'位の置換基の

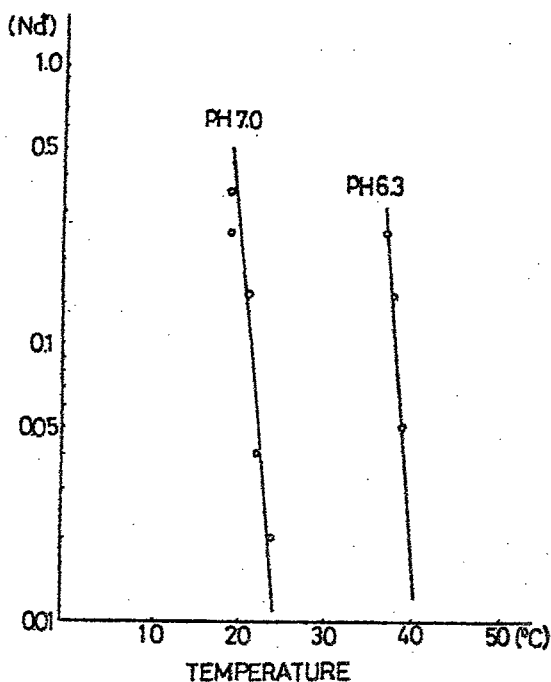
電気陰性度はポリ

ヌクレオチドの con-

formation に重大

な影響のあることが ^{Fig. 10} Dependence of T_m of Poly(Aa) on Na⁺ concentration

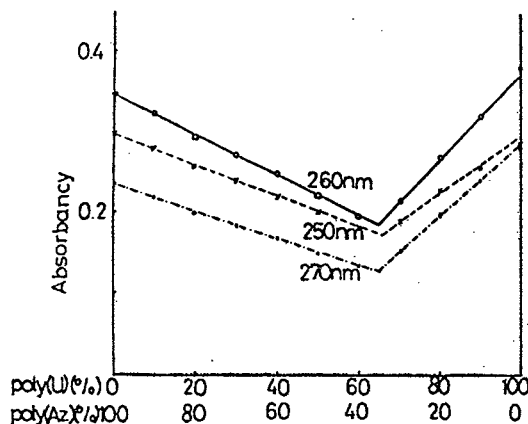
が判った。



第3章 ポリ(2'-ヒドロキシ-2'-デオキシプリンヌクレオチド)の合成と性質

第1章、第2章において、大きさから水酸基と類似しているアミ基を有する poly(Aa) II. 特に self ordered structure 形成において、poly(A) と全く異った性質を示し、より電気陰性度の近いアミト基を有する poly(Az) が、poly(A) と似た

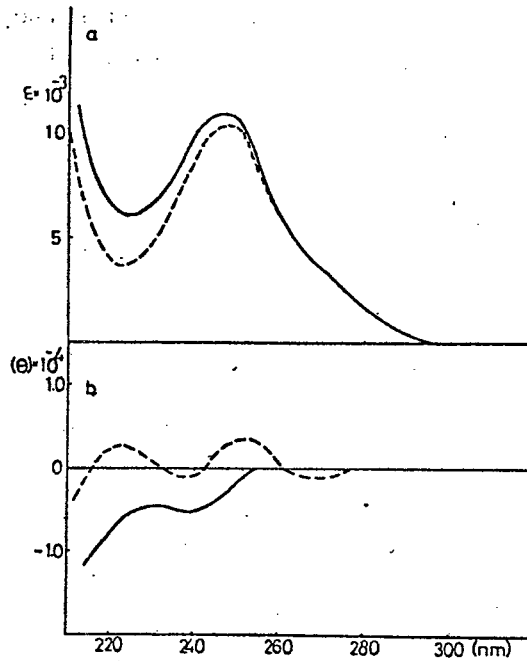
Fig 3 b



Mixing curves of poly (Az) and poly (U) at Na^+ concentration of 0.15M.

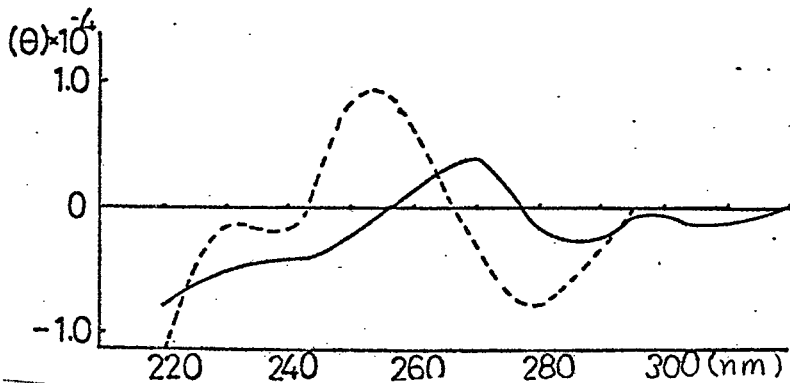
Naイオン濃度, over night の測定では 1:2 の complex の形成が観測された。これらの complex の melting temperature (T_m) は、0.04M Naイオン濃度で 46°C , 0.15M Naイオン濃度で 65°C であり、poly(A)-poly(U) complex の場合が 51° , 62° であるので、ほぼ同程度の熱的安定性を有している。

poly(Iz) の 0.15M Naイオン濃度, pH 7.0, 室温での UV, CD スペクトルを Fig 4 に示す。poly(I) では 225, 255 nm 付近に正の cotton 効果が見られるのに対して、poly(Iz) ではほとんど正の Cotton 効果は見られない。0.95M Naイオン濃度では、poly(I) の CD スペクトルに似ているが、peak が長波長側にシフトしており、cotton 効果の amplitude も小さい。poly(I) に比べてより flexible な構造をとっているものと思われる。0.15M Naイオン存在下。



U.V. and C.D. spectra of poly(Iz) and poly(I) in neutral solution containing 0.15M Na⁺.
 — poly(Iz), --- poly(I)

Fig 4



UV spectra of poly(Iz) and poly(I) in neutral solution containing 0.95M Na : — poly(Iz) --- Poly(I)

Fig 5

PH 7.0 2" poly (Iz) と poly (C) の mixing を行くと, poly (Iz)
 50% のところに屈曲点がある。 poly (Iz) · poly (C) = 1:1
 complex の形成が観察された。 この poly (Iz) · poly (C)

について調べた。これから三者と poly(U) とを 0.04 M Na イオン存在下 pH 7.0 で mixing を行い、30 分後に UV スペクトルを測定すると 1:1 の complex 形成がみとめられた。又、0.15 M Na イオン存在下に mixing して over night 後に UV スペクトルを測定すると、いずれも poly(Ax)・poly(U) 1:2 complex が形成していることが判った。Fig 12 は poly(Af) の例である。これから complex の T_m 値は Table II のようになった。このようにいずれの場合にも complex の

Table II

A-U	0.04M[Na ⁺]30min.	0.15M[Na ⁺]1day
A-U	51 (2-1)	62 (3-1)
Af-U	49 (2-1)	64 (3-1)
Ac1-U	46 (2-1)	56 (3-1)
Abr-U	45 (2-1)	53 (3-1)

熱的安定性は $Af-U > Ac1-U > Abr-U$ となり、2'置換基の大きさが大きくなり、電気陰性度が減少するほど熱的安定性の減少がみられる。これは、poly(Ax)の conformation がハロゲンが大きくなるに従って complex 形成に不利になる傾向があるためと思われる。

poly(I_f), poly(I_{ce})の 0.15 M Na イオン濃度, pH 7.0 20°C にあける UV スペクトルは Fig 13 のようになる。いずれも poly(I) に類似のスペクトルを示す。このとき CD スペクトルは Fig 14 のようになり、poly(I_f) とは poly(I) とは全く

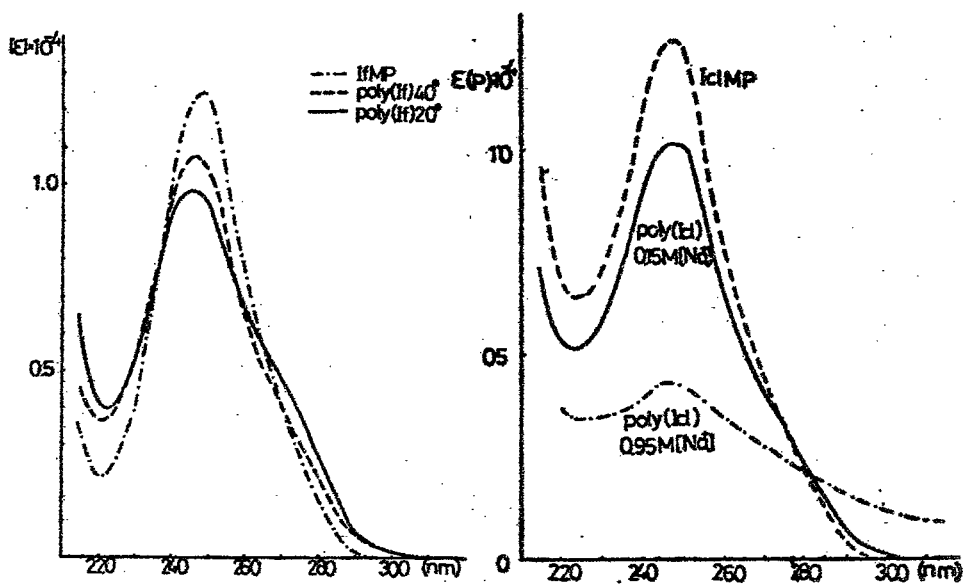


Fig3 a UV spectra of poly(If)

b UV spectra of poly(IcI)

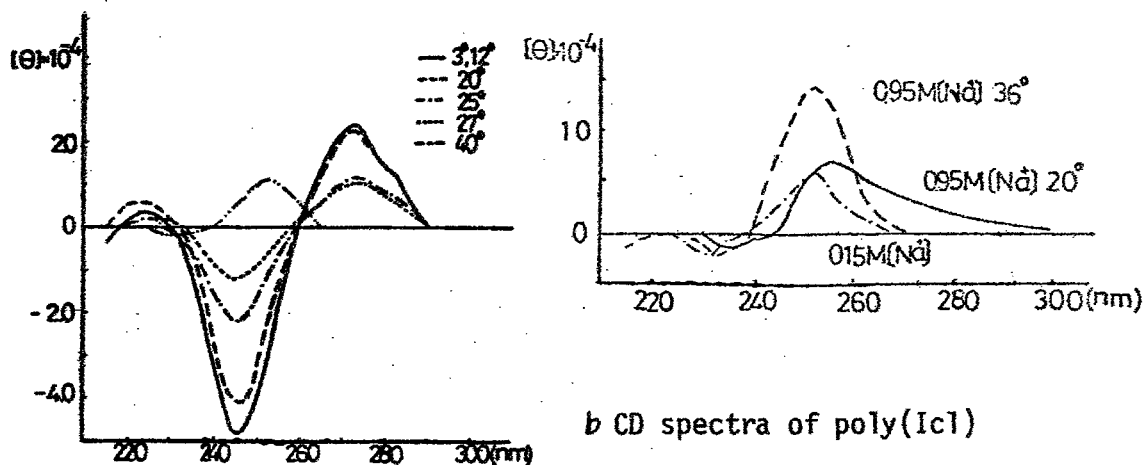


Fig4 a CD spectra of poly(If)

b CD spectra of poly(IcI)

異なった特徴的な CD スペクトルを示す。この条件下、
poly(If) は何らかの ordered structure を形成してい
るものと思われる。これは温度の上昇に従って崩壊し、 T_m
である 27°C に至ると CD スペクトルは逆転し、poly(I) の

random coil 状にあるときの CD スペクトルと似たパターンを示している。一方 poly(Ic) は同じ条件下で random coil 状態にあると思われる CD スペクトルを示し、poly(Ic) は poly(I_f) にみられる様な ordered structure は形成しないことがわかる。又、poly(I_f), poly(Ic) は poly(c) と中性条件下 1:1 の complex を形成する。この complex

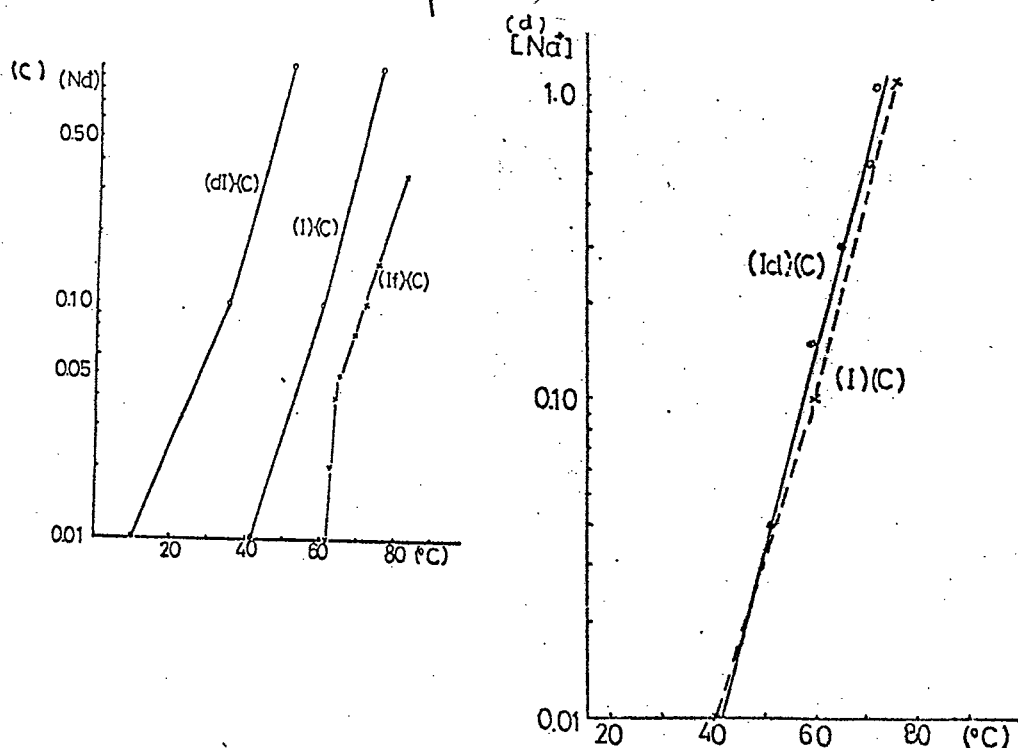


Fig. 14(c)(d) Relationship between T_m of various double-stranded complexes and Na^+ concentration.

の熱的安定性を Na イオンを変化させて調べた結果が Fig. 14(c)(d) である。両者ともに T_m 値と $\log_{10} [Na^+]$ の間に直線関係が成立する。poly(I_f) · poly(c) は poly(I) · poly(c) complex に比べ、いあれの Na イオン濃度にある。20

T_m 値が $15 \sim 20^\circ \text{C}$ 高いが、 $\text{poly}(\text{Ic}) \cdot \text{poly}(\text{C})$ はほとんど同じである。

前記の $\text{poly}(\text{Ax})$ の場合と同様 $\text{poly}(\text{Ix})$ においても、2'置換基の電気陰性度が大きくなり、その大きさが減少する程、self-ordered structure が形成しやすく、又 $\text{poly}(\text{C})$ との complex の熱的安定性も高いことがわかる。

第4章 ポリ2'置換ヌクレオチドの生物活性

1) インターフェロン誘導活性

インターフェロンは細胞がある特定の物質 (interferon inducer) の誘発をうけて作り出す分量 $2 \sim 3$ 万程度の糖タンパク質で細胞にウイルス抵抗性等の生理作用をおこさせる作用がある。二本鎖 RNA は inducer として重要なもので、特に $\text{poly}(\text{I}) \cdot \text{poly}(\text{C})$ complex は高い活性を有することが知られている。今回前章までに合成した $\text{poly}(\text{Az})$, $\text{poly}(\text{Iz})$, $\text{poly}(\text{It})$, $\text{poly}(\text{At})$ について interferon 誘発活性を調べた。Table III, IV に結果を示す。 $\text{poly}(\text{Az})$ を含む complex はほとんど活性を示さない。しかし、 $\text{poly}(\text{Iz}) \cdot \text{poly}(\text{C})$ は human の系で $\text{poly}(\text{I}) \cdot \text{poly}(\text{C})$ とほとんど同じ活性を有している。 $\text{poly}(\text{At})$ を含む complex は活性は示すが $\text{poly}(\text{A})$ を含む complex より値は小さい。 - 又 $\text{poly}(\text{I}) \cdot \text{poly}(\text{C})$

は. rabbit と human の系で poly(I)·poly(C) とほとんど同じ
 活性を示し mouse の系ではむしろ高い値を示している. この結果は,
 interferon 誘発活性には 2' 位水酸基が必須であるとした
 従来の定説には一致せず, interferon 誘発には二本鎖
 RNA 分子全体の構造が重要であると思われる.

Table III

Interferon inducing activity of various 2'-azido analogues of (A)n-(U)n and (I)n-(C)n

System	polynucleotide	interferon titer ^{AK}		
		0.1 µg/ml.	1 µg/ml.	10 µg/ml.
Primary rabbit kidney cells "superinduced" with cycloheximide and actinomycin D	(A)n-(U)n ⁺	log ₁₀ (units/ml)		
	(A)n-(rT)n			2.9
	(A)n-(Uz)n			3.4
	(Az)n-(U)n			<1.0
	(Az)n-(rT)n			<1.0
	(Az)n-(Uz)n			<1.0
	(I)n-(C)n			3.8
	(I)n-(br ⁵ C)n			3.9
	(Iz)n-(C)p			3.3
	(Iz)n-(br ⁵ C)n			< 1.0
	(I)n-(Cz) ⁻			< 1.0
Human skin fibroblast cells "primed" with interferon and "superinduced" with cycloheximide and actinomycin D	(I)n-(C)n	3.0	3.7	3.9
	(I)n-(brC)n	3.5	3.9	4.2
	(Iz)n-(C)n	3.0	3.7	4.1
	(Iz)n-(brC)n	< 1.3	< 1.3	< 1.3
L-929 cells 'primed' with interferon	(I)n-(C)n	1.5	2.3	2.3
	(I)n-(brC)n	1.5	1.8	2.0
	(Iz)n-(C)n	< 0.5	0.8	1.0
	(Iz)n-(brC)n	< 0.5	< 0.5	< 0.5
Intact rabbits	(I)n-(C)n	-	3.8	4.7
	(I)n-(brC)n	-	3.5	4.7
	(Iz)n-(C)n	-	< 1.0	1.7

Table IV

Interferon inducing activity of poly(dIf)-derived complexes in different systems

Interferon titer(log 10 units/ml) obtained at polynucleotide concentration of

Polynucleotide	0.1 $\mu\text{g/ml}$.	1 $\mu\text{g/ml}$.	10 $\mu\text{g/ml}$.
1. Primary rabbit kidney cells "superinduced" with cyclohexamide and actinomycin D.			
poly(I)-poly(C) ₅	2.5	3.8	4.2
poly(I)-poly(br ⁵ C)	2.2	2.2	3.0
poly(If)-poly(C) ₅	3.9	4.1	4.3
poly(If)-poly(br ⁵ C)	2.7	3.0	3.5
poly(A)-poly(U)	1.7	3.2	3.4
poly(A)-poly(rT) ₃	3.5	4.2	3.9
poly(A)-poly(br ⁵ U)			< 1.0
poly(Af)-poly(U)	< 1.0	< 1.0	< 1.0
poly(Af)-poly(rT) ₃	< 1.0	< 1.0	1.1
poly(Af)-poly(br ⁵ U)			< 1.0
2. Human skin fibroblast cells "primed with interferon and superinduced" with cyclohexamide and Actinomycin D.			
poly(I)-poly(C) ₅	4.1	4.1	4.1
poly(I)-poly(br ⁵ C)	4.3	4.1	4.1
poly(If)-poly(C) ₅	4.1	4.2	4.3
poly(If)-poly(br ⁵ C)	3.8	3.4	3.5
poly(A)-poly(U)	4.1	4.3	4.2
poly(A)-poly(rT) ₃	4.1	4.1	4.0
poly(A)-poly(br ⁵ U)			< 1.0
poly(Af)-poly(U)	1.9	2.9	2.9
poly(Af)-poly(rT) ₃	< 1.0	2.5	2.0
poly(Af)-poly(br ⁵ U)			< 1.0
3. Mouse L-929 cells pretreated with DEAE-dextran			
poly(I)-poly(C) ₅	2.11	3.65	3.68
poly(I)-poly(br ⁵ C)	< 0.5	2.29	3.66
poly(If)-poly(C) ₅	< 0.5	< 0.5	< 0.5
poly(If)-poly(br ⁵ C)	< 0.5	< 0.5	< 0.5
4. Mouse L-929 cells primed with interferon			
poly(I)-poly(C) ₅	1.32	1.67	2.00
poly(I)-poly(br ⁵ C)	0.84	1.03	1.32
poly(If)-poly(C) ₅	1.68	2.00	2.36
poly(If)-poly(br ⁵ C)	< 0.5	< 0.5	< 0.5
5. Intact rabbits			
poly(I)-poly(C)	at 1 h.	2.2	2.5
	at 2 h.	4.5	4.7
	at 4 h.	3.3	3.3
	at 7 h.	2.2	1.8
poly(If)-poly(C)	at 1 h.	< 1.0	1.7
	at 2 h.	3.5	3.7
	at 4 h.	3.0	2.9
	at 7 h.	2.2	2.2

2) リバーストランスクリプターゼに対する活性

RNA腫瘍ウィルスの gene を合成する reverse transcriptase は、RNA を Template にし、³ oligonucleotide primer に dNTP を付加重合する酵素である。

今回 poly(Az), poly(Af) について、murine leukemia virus の reverse transcriptase に対する活性を調べた。Fig 15 が template primer 非存在下の結果で、

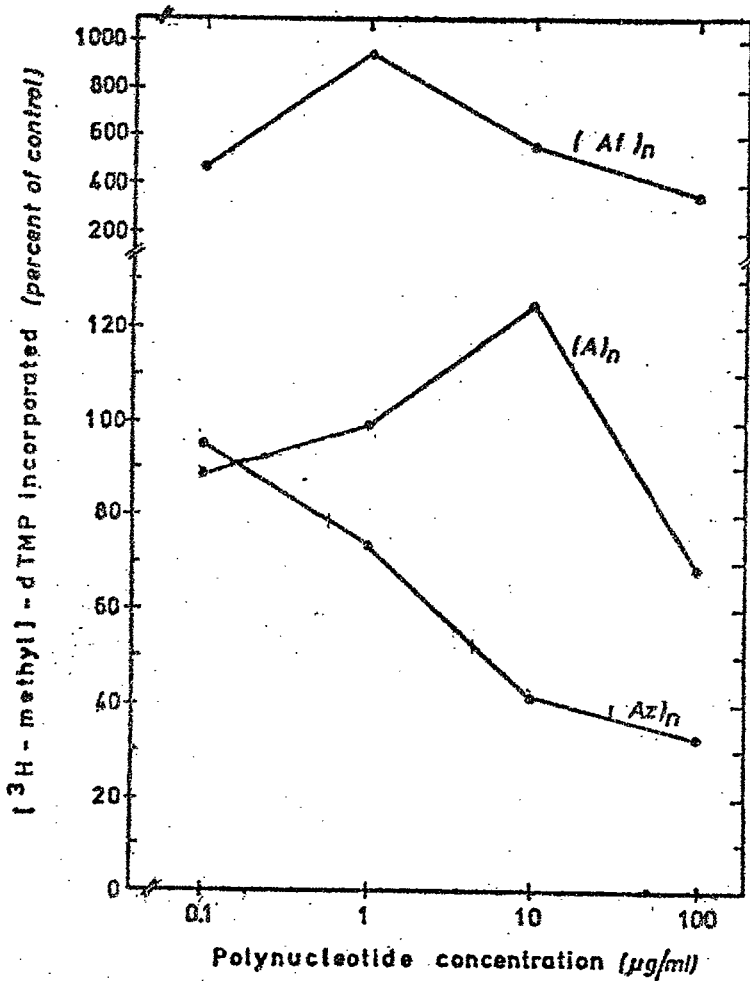


Fig 15 Effect of poly(Az), poly(Af) on MuLV reverse transcriptase in the absence of poly(A). oligo(dT) as template-primer

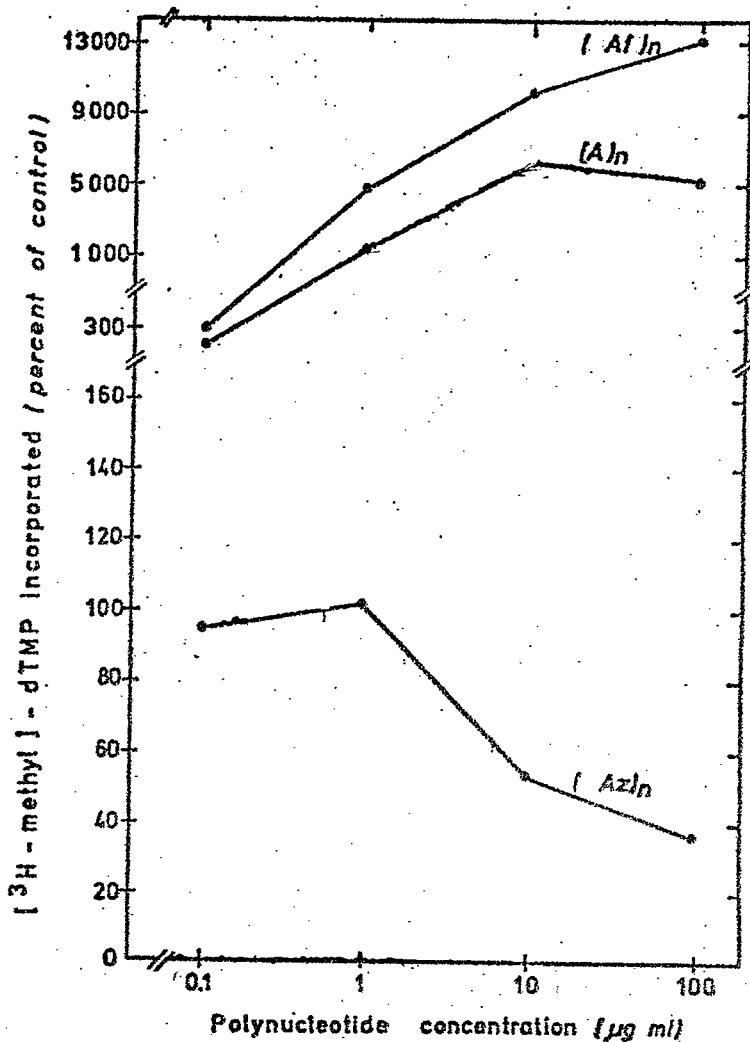


Fig 16 Effect of poly(Az) and poly(Af) on MuLV reverse transcriptase in the presence of oligo(dT) as primer

poly(Af)は、 $[^3\text{H}]\text{-dTMP}$ のとりこみを促進している。

Fig 16 2"は、primer存在下での template 作用を調べているが poly(Af)は poly(A)より高い template 活性を有しているが poly(Az)は template 活性がみとめられない。

Fig 17 2"は、template primer存在下での inhibition

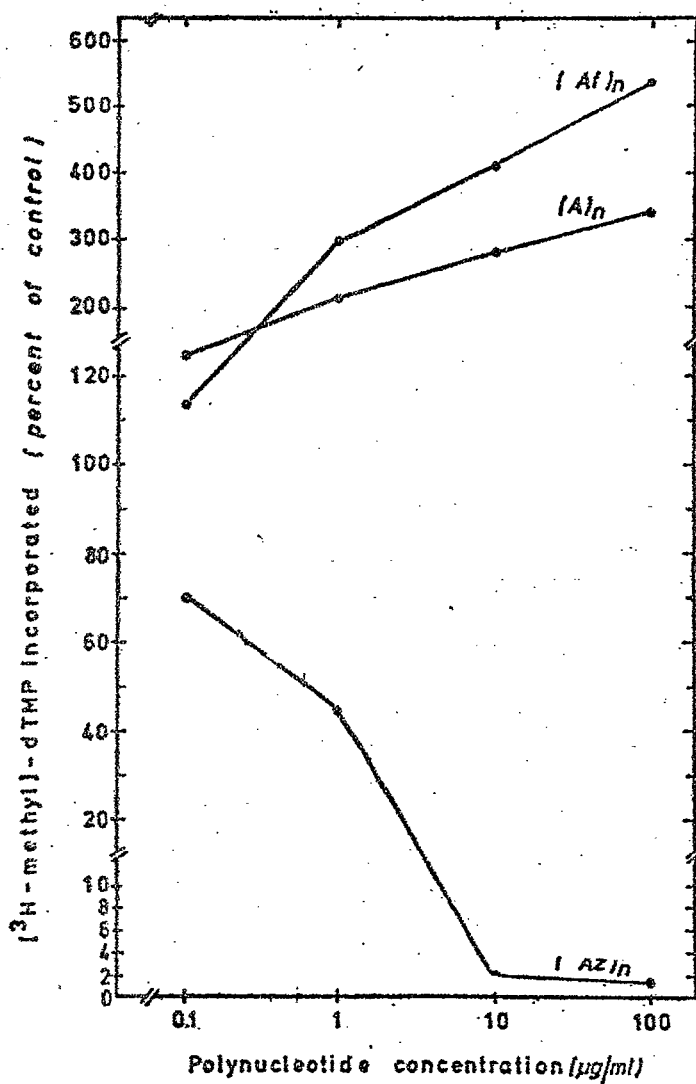


Fig 17 Effect of poly(Az) and poly(Af) on MuLV reverse transcriptase in the presence of poly(A) oligo(dT) as template-primer

効果と調へた結果である。poly(Az)が顕著な阻害作用を有しているのかわかる。このように poly(Af)は reverse Transcriptase に高い template 活性を有し。一方、poly(Az)は阻害作用を有している。

結 論

1) アデニン, イノシンの2'位をアジトで置換した $\text{poly}(\text{Az})$, $\text{poly}(\text{Iz})$ では, $\text{poly}(\text{Az})$ の UV スペクトル, CD スペクトル, 及び $\text{poly}(\text{U})$ との complex の熱的安定性が $\text{poly}(\text{A})$ とよく似ているのに対して, $\text{poly}(\text{Iz})$ は $\text{poly}(\text{I})$ における self-ordered structure をとり難く, $\text{poly}(\text{C})$ との complex の熱的安定性は, $\text{poly}(\text{I}) \cdot \text{poly}(\text{C})$ と, $\text{poly}(\text{dI}) \cdot \text{poly}(\text{C})$ の中間に位置している。

2) 2'位にアジ1基を導入した $\text{poly}(\text{Aa})$ は, 弱酸性にふる。 $\text{poly}(\text{A})$ では観察されなかった self-ordered structure を形成する。これは2'-アジ1基のフットネートにより, 塩基間の stacking が強化されたものと思われる。中性条件では stacking の程度は $\text{poly}(\text{A})$ より小さく, フットネートされない状態での2'-アジ1基は stacking を妨げる。

3) アデニンの2'位にハロゲンを導入した, $\text{poly}(\text{At})$, $\text{poly}(\text{Acl})$, $\text{poly}(\text{Abr})$ では, ハロゲンの電気陰性度の増大による減少に伴って self-structure の

stacking の強化, $\text{poly}(U)$ との complex の熱的安定性の増大が観察された。

4) イノシンの 2' 位にハロゲンを導入した $\text{poly}(If)$, $\text{poly}(Ice)$ では, $\text{poly}(If)$ が特異な self-ordered structure を形成し, $\text{poly}(C)$ と非常に安定な complex を形成するのに対し, $\text{poly}(Ice)$ では, そのような self-ordered structure は形成せず, $\text{poly}(C)$ との complex の安定性は, $\text{poly}(I) \cdot \text{poly}(C)$ とほとんど同じである。

5) $\text{poly}(Iz) \cdot \text{poly}(C)$, $\text{poly}(If) \cdot \text{poly}(C)$ では, $\text{poly}(I) \cdot \text{poly}(C)$ と同じ程度, 又はそれ以上の interferon 誘導活性を有している。これは 2' 置換ポリヌクレオチドとしてはじめての例であり、従来の説を訂正した。

又, $\text{poly}(At)$ は $\text{poly}(A)$ より高い RNA 腫瘍ウイルスの reverse transcriptase の template 活性をもっていることが判った。

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2'-置換ポリヌクレオチドの 合成と性質に関する研究

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2'-置換ポリヌクレオチドの 合成と性質に関する研究

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略語表

Az	2'-azido-2'-deoxyadenosine
AzMP	2'-azido-2'-deoxyadenosine-5'-monophosphate
AzDP	2'-azido-2'-deoxyadenosine-5'-diphosphate
Poly(Az),	(Az)n poly(2'-azido-2'-deoxyadenylic acid)
Iz	2'-azido-2'-deoxyinosine
IzMP	2'-azido-2'-deoxyinosine-5'-monophosphate
IzDP	2'-azido-2'-deoxyinosine-5'-diphosphate
Poly(Iz),	(Iz)n poly(2'-azido-2'-deoxyinosinic acid)
Aa	2'-amino-2'-deoxyadenosine
AaDP	2'-amino-2'-deoxyadenosine-5'-diphosphate
Poly(Aa)	poly(2'-amino-2'-deoxyadenylic acid)
Af	2'-fluoro-2'-deoxyadenosine
AfMP	2'-fluoro-2'-deoxyadenosine-5'-monophosphate
AfDP	2'-fluoro-2'-deoxyadenosine-5'-diphosphate
Poly(Af),	(Af)n poly(2'-fluoro-2'-deoxyadenylic acid)
Ac1	2'-chloro-2'-deoxyadenosine
Ac1MP	2'-chloro-2'-deoxyadenosine-5'-monophosphate
Ac1DP	2'-chloro-2'-deoxyadenosine-5'-diphosphate
Poly(Ac1)	poly(2'-chloro-2'-deoxyadenylic acid)
Abr	2'-bromo-2'-deoxyadenosine
AbrMP	2'-bromo-2'-deoxyadenosine-5'-monophosphate
AbrDP	2'-bromo-2'-deoxyadenosine-5'-diphosphate
Poly(Abr)	poly(2'-bromo-2'-deoxyadenylic acid)
Ix	2'-deoxy-2'-halogenoinosine
Poly(Ix)	poly(2'-halogeno-2'-deoxyinosinic acid)
If	2'-fluoro-2'-deoxyinosine
IfMP	2'-fluoro-2'-deoxyinosine-5'-monophosphate
IfDP	2'-fluoro-2'-deoxyinosine-5'-diphosphate
Poly(If), Poly(If)	poly(2'-fluoro-2'-deoxyinosinic acid)
Ic1	2'-chloro-2'-deoxyinosine
Ic1MP	2'-chloro-2'-deoxyinosine-5'-monophosphate
Ic1DP	2'-chloro-2'-deoxyinosine-5'-diphosphate
Poly(Ic1)	poly(2'-chloro-2'-deoxyinosinic acid)
Poly(A)	poly(adenylic acid)
Poly(C)	poly(citidylic acid)
Poly(I)	poly(inosinic acid)
Poly(U)	poly(uridylic acid)
PNPase	polynucleotide phosphorylase
Pi	inorganic phosphate
Tm	midpoint of thermal melting

Poly(dA)	poly(2'-deoxyadenylic acid)
poly(dI)	poly(2'-deoxyinosinic acid)
Poly(Cz)	poly(2'-azido-2'-deoxycitidylic acid)
Poly(Uz)	poly(2'-azido-2'-deoxyuridylic acid)
Poly(Ca)	poly(2'-amino-2'-deoxycitidylic acid)
Poly(Ua)	poly(2'-amino-2'-deoxyuridylic acid)
Poly(Cf)	poly(2'-fluoro-2'-deoxycitidylic acid)
Poly(Uf)	poly(2'-fluoro-2'-deoxyuridylic acid)
Poly(Ccl)	poly(2'-fluoro-2'-deoxycitidylic acid)
Poly(Ucl)	poly(2'-fluoro-2'-deoxyuridylic acid)
Poly(Um)	poly(2'-O-methyl-uridylic acid)
Poly(Ue)	poly(2'-O-ethyl-uridylic acid)
Poly(Am)	poly(2'-O-methyl-adenylic acid)
Poly(Ae)	poly(2'-O-ethyl-adenylic acid)
Poly(Aac)	poly(2'-O-acetyl-adenylic acid)
Poly(Im)	poly(2'-O-methyl-inosinic acid)
Poly(n ² A)	poly(2-azaadenylic acid)
Poly(c ³ A)	poly(3-deazaadenylic acid)
Poly(c ⁷ A)	poly(7-deazaadenylic acid)

緒 論

核酸は生体を構成する重要な成分の一つである。天然の核酸には大別してDNAとRNAの二種類が存在する。これら二者は互いに生体内での存在部位も、担っている役割も大きく異なっている。

DNAとRNAの化学構造上の違いは、糖が *deoxyribose*, *ribose* かということである。従って糖部 2' 位に水酸基が存在するか否かということとは物理化学的、又生物学的に重要な意味を持っている。

2' 位に水酸基の代りに種々の置換基を導入した 2' 置換ポリヌクレオチドは、これらDNA, RNAの構造と機能を考察する上で、興味あるモデル化合物である。

そこで、今回著者は、2' 位にアジド基, アミ基, 各種ハロゲンの置換したポリヌクレオチドを合成し、その物理化学的性質、及び生物活性に関する研究を行った。

第一章 ポリ(2'-アジド-2'-デオキシ/プリンヌクレオチド) の合成と性質^{57) 59)}

アジド基は、その電気陰性度は3.2で、水素の2.0、水酸基の3.5の間に位置し、水酸基に近い。分る量は水酸基にくらべて大きく、立体構造は直鎖状である。アジド基を2'位に導入することにより、水酸基と電気陰性度は似ているが立体構造にどのような影響を与えるかを考察できる。

ピリミジン系においては、2'置換ポリヌクレオチドとしてポリ(2'-デオキシ-2'-アジドウリシル酸)(poly(Uz))¹²⁾、ポリ(2'-デオキシ-2'-アジドシチシル酸)(poly(Cz))²⁾が報告されている。poly(Uz)ではpoly(U)より安定なself-structureをとることができ、又、poly(Uz)・poly(A) complexはpoly(A)・poly(U) complexと同程度の安定性を有することと報告している。一方poly(Cz)は弱酸性条件におけるself-structureやpoly(I)・poly(Cz) complexのいずれにおいても、天然のpoly(C)より不安定であることが示されている。

しかしプリン系においてはヌクレオチドの合成の困難さのため2'置換ポリヌクレオチド合成の例はなかった。最近池原らにより8,2'-O-cycloadenosineを出発物質にして、arabinosyl adenine^{4) 27)}誘導体を経て、2'置換アデノシンを合成するルートが開発された。

polynucleotide phosphorylase (PNPase) は Grunberg-Manago らにより発見され³⁾ 生体内の RNA を無機リン酸存在下ヌクレオシド 5'-ジホスフェートに分解すること知られている。この逆反応を利用して多くのポリヌクレオチドが合成された。

そこで、この酵素を用いて、2'-アジド-2'-デオキシアデニル酸^{a)} 5'-モノホスフェート^{b)} (AzMP) は対応するヌクレオシドを山崎らの方法に従って triethyl phosphate 中、 POCl_3 を用いて 5' 位をリン酸化し、活性炭

第 1 節、ポリ (2'-アジド-2'-デオキシアデニル酸) の合成

2'-アジド-2'-デオキシアデニン^{a)} 5'-モノホスフェート^{b)} (AzMP) は対応するヌクレオシドを山崎らの方法に従って triethyl phosphate 中、 POCl_3 を用いて 5' 位をリン酸化し、活性炭

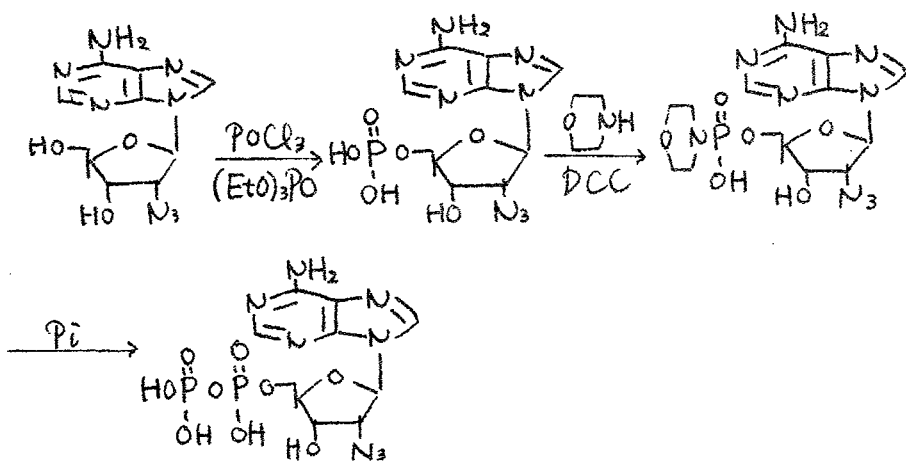


Fig 1

カラムで脱塩後、Dowex 1x2 (formate) カラムを用いて精製した。

60~80%の収率で AzMP を得た。このカラムクロマトグラフィー

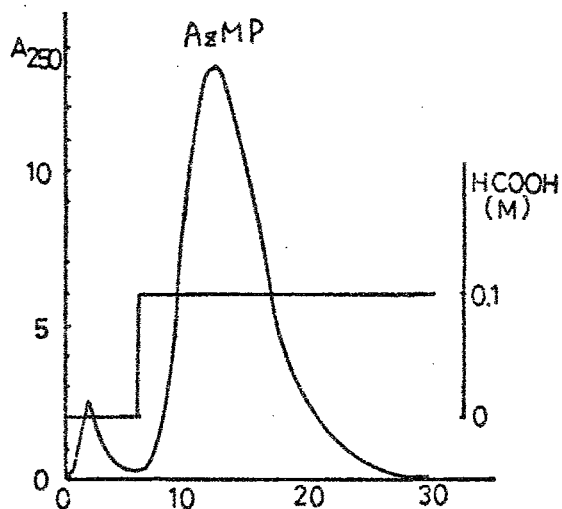


Fig 2 Dowex 1x2 (formate) カラム
クロマトグラフィーによる AzMP の分離

の溶出パターンを Fig 2 にあげた。

このものは crude

snake venom 5'

nucleotidase で処理

することにより、完全に

脱リン酸化をうけ、

もとのヌクレオシドに戻る

ことより 5'リン酸である

ことを確認した。次に AzMP を Moffatt-Khorana^{b)} の

方法により morpholidate を経由して、5'-diphosphate

とした。活性炭カラムで脱塩後 DEAE Sephadex A-25 (bicarbonate)

カラムクロマトグラフィーによって分離精製し、50~70%の収率で

目的の diphosphate 体を得た。このカラムクロマトグラフィーの一例

を Fig 3 に示した。AzMP 及び AzDP のクロマトグラムの性質を Table I に示す。

Table I

	R _m PA-A		R _f		
	PEP [*]	PH 7.5	PH 3.5	PPC [*]	C↓ G↓ M↓
AzMP		1.05	0.98	0.32	0.43 ~
AzDP		1.44	2.29	0.06	- 0.48

* 展開液の組成は実験の部参照

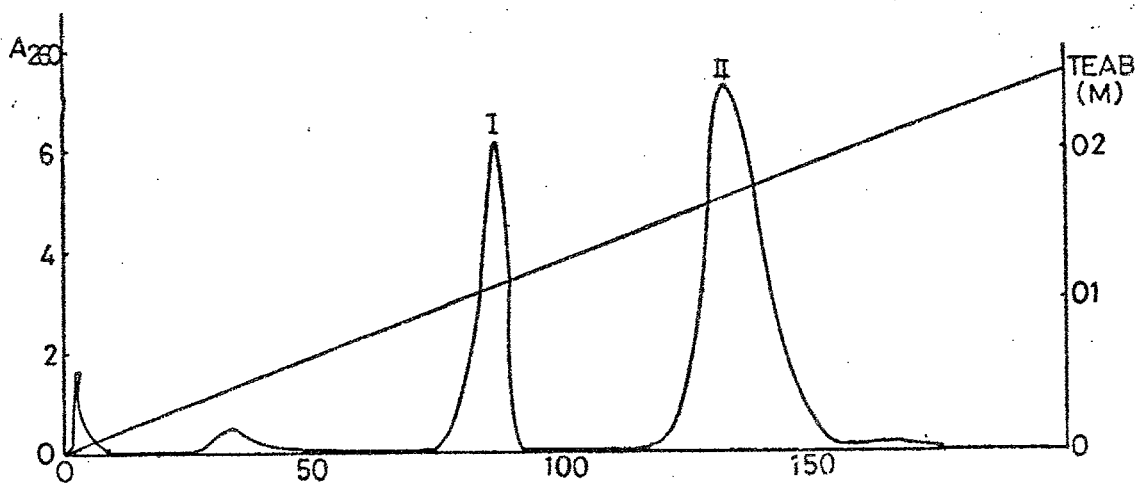


Fig 3 DEAE Sephadex A-25 (bicarbonate) に J3 AzDP の分離

peak I AzMP

peak II AzDP

次に重合反応を行った。AzDP 4 mM, $MgCl_2$ 2 mM, Tris HCl (pH 8.5) 100 mM, PNPase (E. coli) 2 units/ml 37°C で反応させた。時間をあつて遊離する無機リンの定量により反応の進行をしらべると Fig 4 のようになる。20 時間後に isoamyl alcohol - $CHCl_3$ (1:3) 混液で除蛋白に反応をとめ凍結乾燥の後、Sephadex G50 でゲル濾過を行った。poly(Az) は void volume 付近に

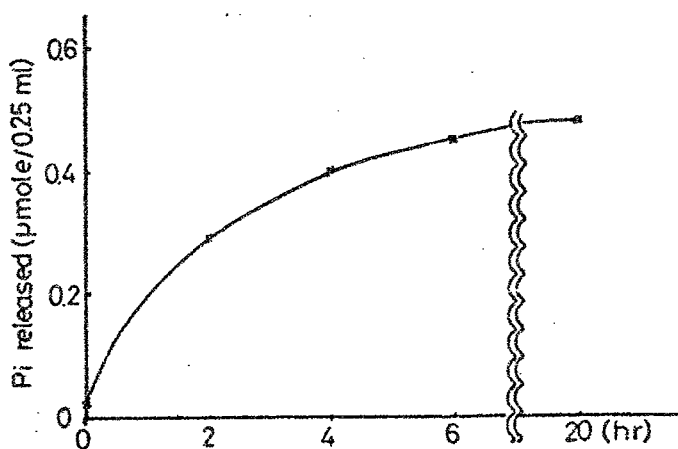


Fig 4 poly(Az) 合成の Time course

溶出される。収率は18%であった。このように A_2DP は2'位に水酸基がないにもかかわらず PLase の基質となることが判った。しかし、天然の基質である ADP の同じ条件で2時間で平衡に達し、単離収率50%程度で得られるのに対し、反応速度が速く、低収率である。

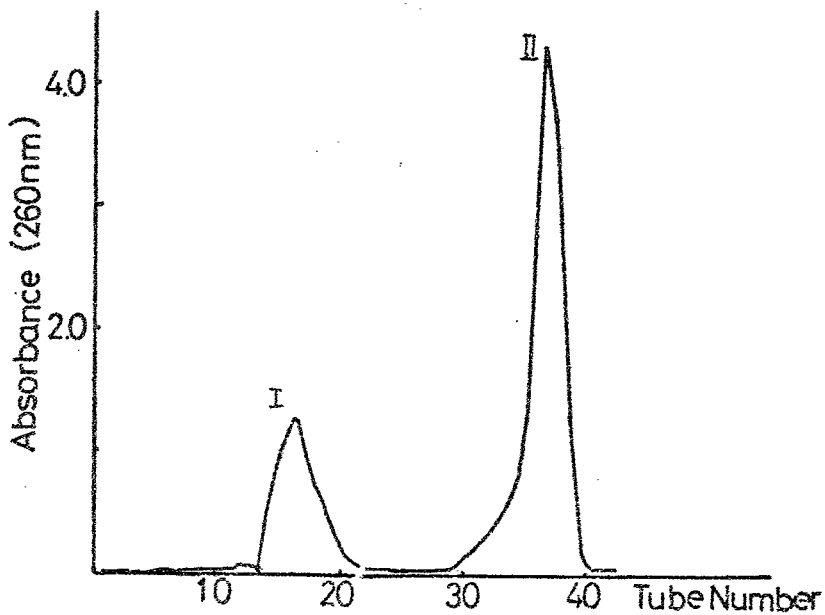


Fig 5 Sephadex G-50 gel filtration of poly(Az)
peak I; poly(Az), peak II; A_2DP and A_2MP

えられた poly(Az) は snake venom phosphodiesterase で完全水解され、 A_2MP と少量の A_2I になった。このヌクレオシドは A_2MP の酵素中に混在していると思われる。5' nucleotidase により脱リン酸化されて生じたものである。

第2節ポリ(2'-アジド-2'-デオキシアデニル酸)の性質

・ UVスเปクトル

0.15 M Naイオン存在下 pH 7.0 で UVスเปクトルを測定すると Fig 6a のようになる。 λ_{max} は 256 nm への定量あり

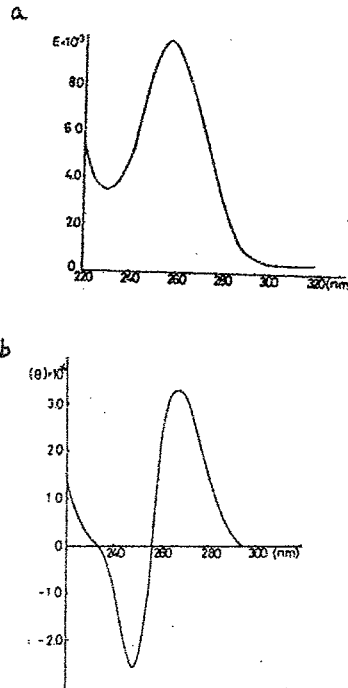


Fig.6 UV CD spectrum of poly (Az) in the presence of 0.15M Na⁺ at pH 7.0 and 16°.

求めた分子吸光係数 ($\epsilon(P)$) は 97802. hypochromicity は AzMP の 35% である。この UVスเปクトルは poly(A) とよく似ており、hypochromicity も同じである。hypochromicity は stacking 状態を反映するので、poly(Az) は poly(A) と同程度の stacking をしているものと考えられる。

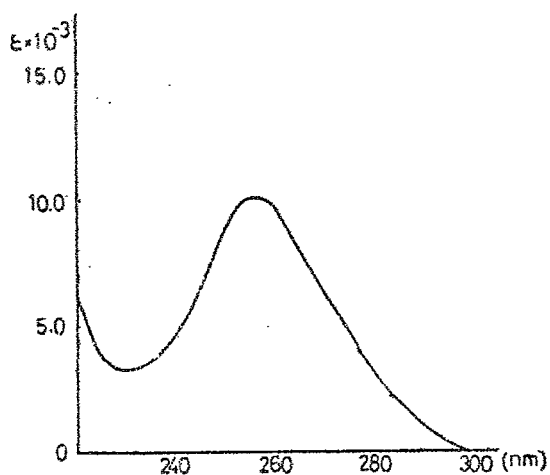


Fig. 7 a

UV absorption spectra of poly (A)

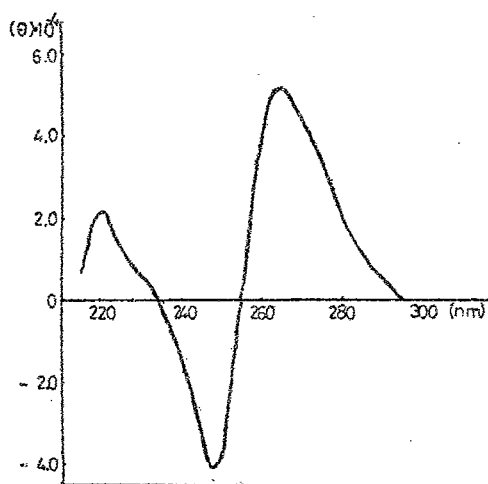


Fig. 7 b

CD Spectrum of Poly(A).

UV測定と同条件下、CDスペクトルを測定すると Fig. 7b のようになる。poly (A) とよく似たスペクトルパターンであるが、その正負の cotton 効果の分る比率^{1), 2)} ([θ]) は poly (A) の約 60% 程度しかない。

poly (U)^{1), 2)}, poly (C)²⁾ においても [θ] 値が poly (U), poly (C) より小さい。前記のごとく、hypochromicity の値より poly (A) の stacking の程度は poly (A) とほぼ同じである。
8

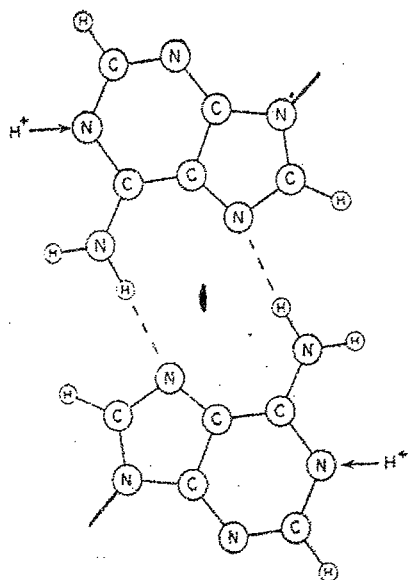
あると思われるので、この $[\theta]$ 値の減少は stacking の強度の減少ではなく、conformation の変化によるのではないかとと思われる。poly(dA) は hypochromicity はむしろ poly(A) より大きく、従って stacking の強度は poly(A) より大きいと思われるが、CD スペクトルの $[\theta]$ 値は小さくなっている。⁸⁾ これは poly(A) と poly(dA) の conformation に違いがあるためである。poly(A₂) の場合も若干 poly(A) と conformation が異なっていると思われる。

°PH transition と acid form の安定性

poly(A) を酸で滴定するとその UV 吸収は pH 6.0 付近で変化する。⁹⁾ この変曲点以下の pH にあって poly(A) は安定な二次構造¹⁰⁾ いわゆる "acid form" を形成しており、その構造は Fig 8 に示すようなものである。

Fig 8 "acid form" の構造

そこで poly(A₂) についても acid form 形成について調べた。0.15 M NaIT₂



存在下、室温において poly (Az) を酸滴定し、260nm

における吸光度を

調べると Fig 9 の

ようになる。このように

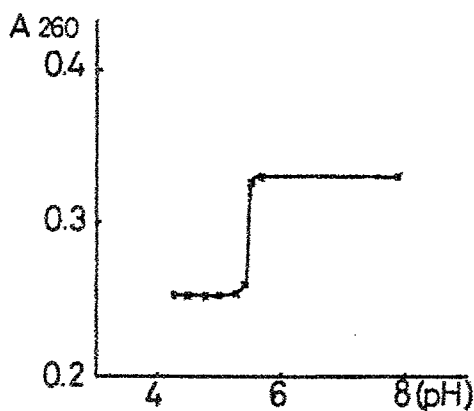
poly (Az) の pH

transition は

5.5 である。poly (A) Fig 9

に比べ 0.5 pH unit

小さくなる。という。



Acid titration curve of poly (Az).

次に 0.15 M Na イオン存在下で poly (Az) の temperature

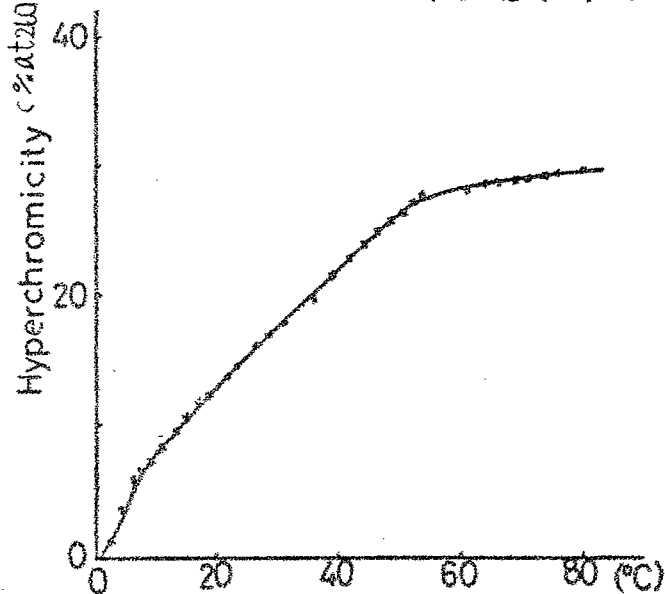


Fig 10 (a) poly (Az) の temperature

-absorbance profile (pH 7.0)

-absorbance

profile を測定

した。pH 7.0 以下

Fig 10 (a) のように

co-operative

な melting が見

られる。これは

pH 5.0 以下

Fig 10 (b) のように

33°C に T_m が観

察された。

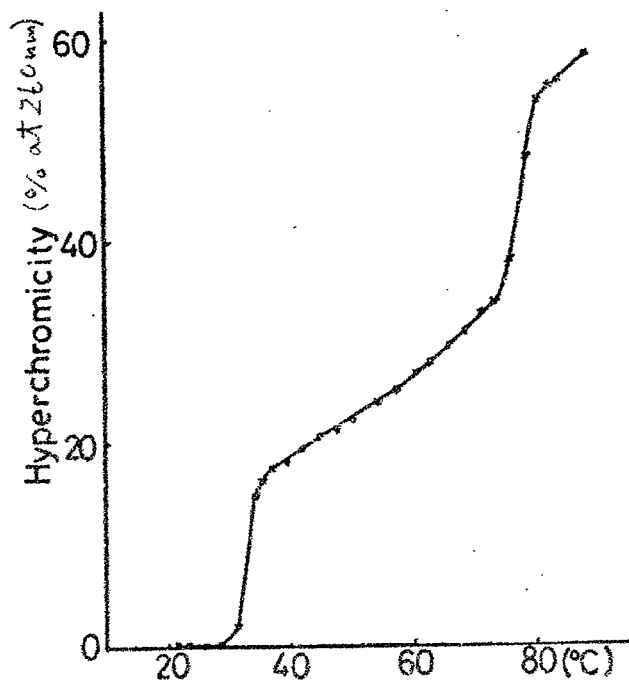


Fig 10(b) poly (Az) の temperature.- absorbance profile (PH 5.0)

80°C 付近の第2の transition については不明である。

poly(A) は pH 5.0 で T_m が 60°C であった。poly (Az) は poly(A) に比べて, acid form が形成し難く, 形成された acid form も熱的安定性が低いことがわかる。poly(dA) にも, acid form の不安定化の傾向がある⁵⁾。この点に於いても poly(Az) は poly(dA) に似ているといえる。

• poly(U) との complex 形成

poly(A) は poly(U) と中性条件下, 安定な 2本鎖又は⁽¹³⁾⁽¹⁴⁾⁽¹⁵⁾ 3本鎖 complex を形成することが知られている。そこで,

0.04M NaIオン濃度存在下、 $\text{pH} 7.02$ poly(Az)と poly(U)と mixingを行い、30分後に 250nm 260nm

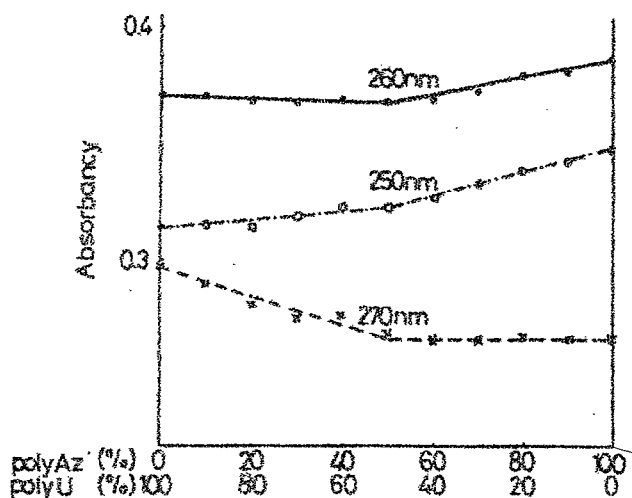


Fig 11 Mixing curves of poly (Az) and poly (U) at 0.04M Na⁺.

270nmにおける吸光度を測定するとFig 11のように、いずれの波長においても poly (Az) 50%のところで屈曲するから、poly (Az)・poly (U)=1:1の2本鎖 complexが形成されていることが判った。又、0.15M NaIオン存在下、mixingを行い一日後の測定ではFig 12のように poly (Az) 33%のところで屈曲し、この条件で poly (Az)・poly (U)は1:2の3本鎖 complexを形成したことが判る。このときCDスペクトルは mixing前後でFig 13のように変化した。このような性質は poly (A)・poly (U) complexとよく似ており、poly (Az)は poly (A)とよく似た complex 形成能を

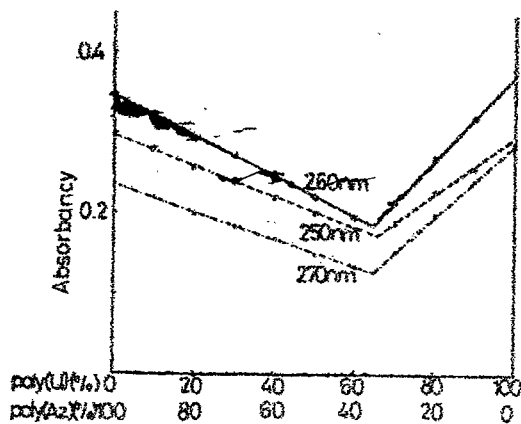


Fig 12 Mixing curves of poly (Az) and poly (U) at Na^+ concentration of 0.15M.

もっていること判る。又、形成した complex は Fig 14 の 535 Watson-Crick¹⁶⁾ 型の二本鎖、及 Watson-Crick と Hoogsteen¹⁴⁾ 型の三本鎖を形成しているものと思われる。

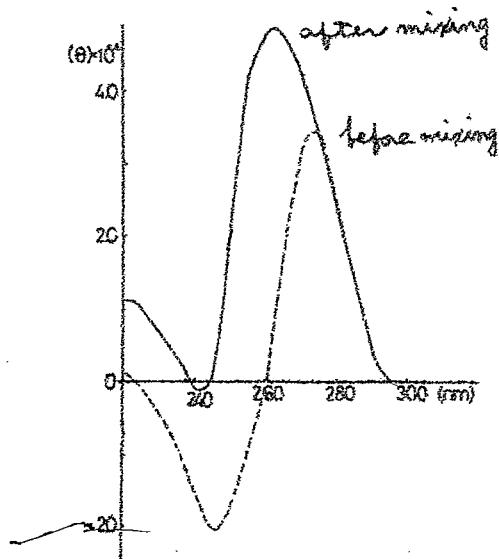


Fig 13 CD curves of poly (Az) plus poly (U) before and after mixing at 0.15 M Na^+ concentration.

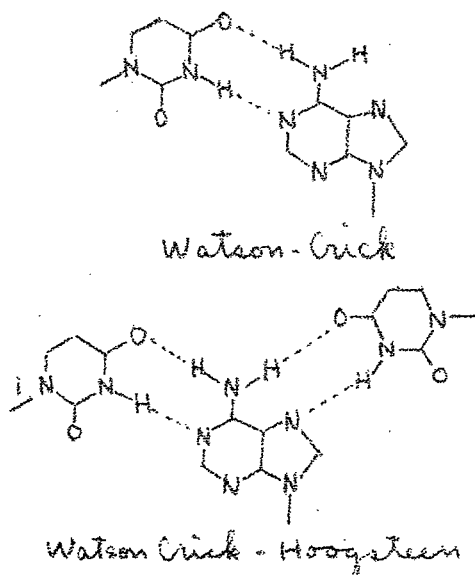
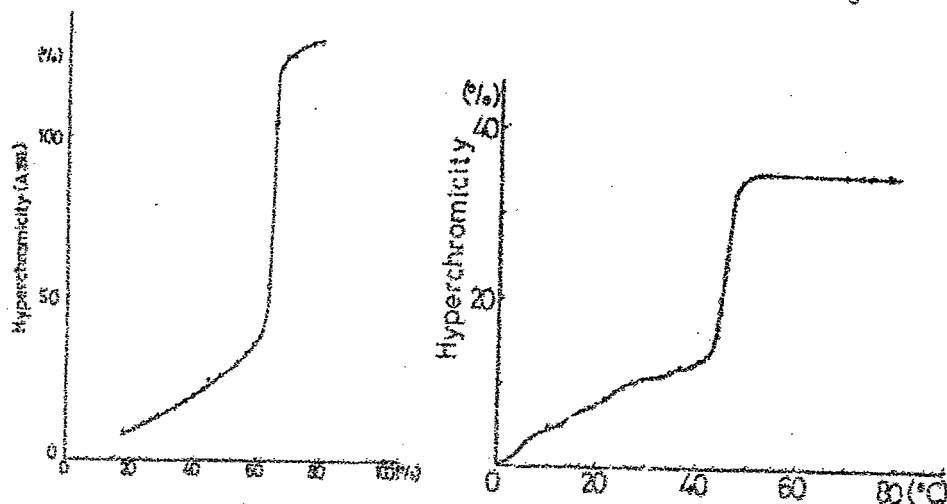


Fig 14: (A)-(U) complexの構造

次にこれら complex の熱的安定性について調べた。Fig 15(a)



(a) Temperature-absorption profile of poly(Az).poly(U) in the presence of 0.15M Na⁺.

(b) Temperature-absorption profile of poly(Az).poly(U) in the presence of 0.04M Na⁺.

Fig 15

Temperature absorption profile である。0.15M Na⁺イオン濃度で65℃、0.04M Na⁺イオン濃度で46℃のT_m値であった。同条件で poly(A).poly(U) complex では各々62℃、51℃であるので、poly(Az).poly(U) complex はそれらとほぼ同程度の安定性を有している。

このように poly(Az) は poly(A) とたいへんよく似た UV スペクトル、CD スペクトル、poly(U) との complex 形成能力を有しており、又 complex の熱的安定性も類似している。poly(Az) の2'位のアジド基は、complex 形成により水酸基とほぼ同様な効果を有していることが判った。このことは、アジド基の電気陰性度が水酸基のそれと似ているためと考えられ、

RNAの conformation の決定にIJ, 2'置換基が大きく関与していることを示している。

第3節 ポリ(2'-アジド-2'-デオキシイリシン酸)の合成

2'-アジド-2'-デオキシイリシン-5'-モノホスフェート (I₂MP) は A₂MP と酢酸酸性中 NaNO₂ と反応させて合成した。反応条件は、pH3.5

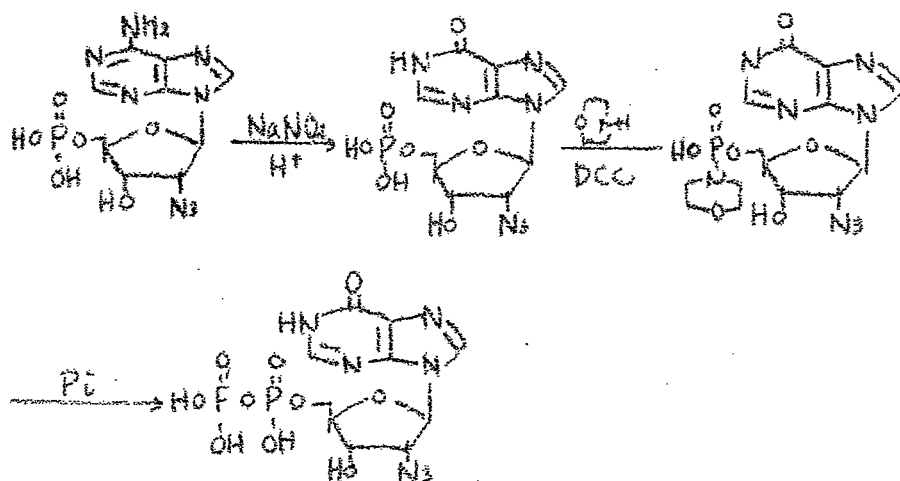


Fig 16

の電気泳動で原料のスポットが消失し、よりR_m値の大きいスポットが現れたことより確認した。活性炭カラムで脱塩の後、Dowex 1x2 (formate) のカラムクロマトグラフィーを行って 80~97% の収率で I₂MPE 得た。次に I₂MPE を第1節で述べたと同様にして、5'-diphosphate 体 (I₂DP) に変えた。精製は DEAE Sephadex A-25 (Amersham Pharmacia) で行い、60~70% の収率で I₂DPE 得た。

これらのクロマトグラフ上の諸性質は Table 2 の通りになる。

Table 2

	PEP (R_{mpA-A})		PPC (R_f)		
	PH 7.5	PH 3.5	G↓	F↓	M↓
I ₂ MP	0.98	2.00	0.13	0.67	0.73
I ₂ DP	1.19	—	0.03	0.22	0.49

重合反応は、I₂DP 4 mM, MnCl₂ 2 mM, TrisHCl (pH 8.5) 100 mM, PNPase (E. coli) 2.4 units/ml, 37°C の条件で行った。反応の進行と遊離の無機リンの定量による調べると

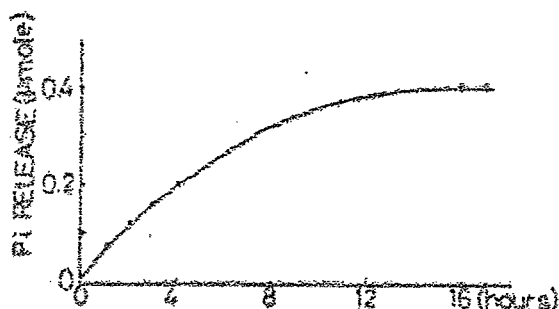


Fig 17 Time course of polymerization of 2'-azido-2'-deoxyinosine 5'-diphosphate

Fig 17 のようになる。16時間付近で反応は plateau に達している。IDP の重合反応の場合、2時間で plateau に達し、遊離する無機リンも 0.6 ~ 0.7 nmole / 0.25 ml であるので、I₂DP は IDP より反応の基質として劣るといえる。17時間後に isooamyl-alcohol : CHCl₃ = 1:3 混液で除蛋白して反応を止め、MnCl₂ を除くために 0.01 M TrisHCl (pH 8.5) に対して透析し、Sephadex

G50 2' グリッド 図を行った。poly(I₂)は void volume 付近に溶出され、14%の収率が得ることができた。得られた poly(I₂)は snake venom phosphodiesterase により完全に水解され、IMP と trace 量の I₂ になった。

第4節 ポリ(2'-アクト-2'-デオキシリボリン酸)の性質 ・UVスペクトルとCDスペクトル

0.15 M Naイオン濃度、pH 7.0、室温で UVスペクトルを測定すると Fig 18 (a) のようになる。λ_{max} 247 nm で、リン酸の定数と求めた 分子吸光度は 10950 である。IMP 5% の

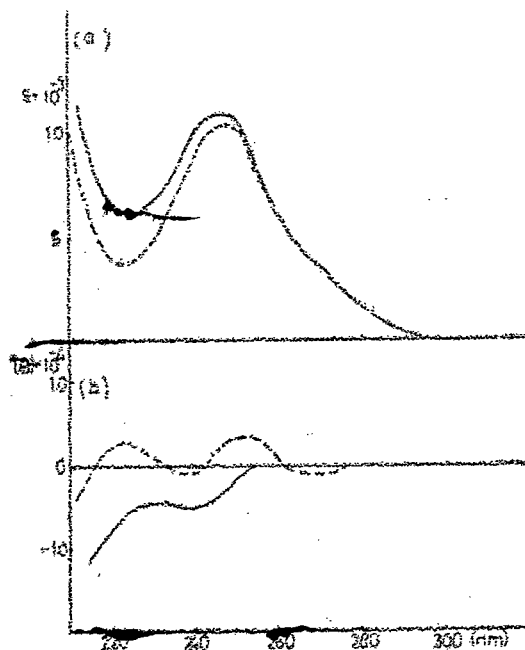


Fig 18 U.V. and C.D. spectra of poly(I₂) and poly(I) in neutral solution containing 0.15M Na⁺.
— poly(I₂), --- poly(I)

hypochromicity は11%であった。UVスペクトルパターン
 の hypochromicity も poly(I) とほぼ一致している。次に
 同条件で CD スペクトルを測定すると Fig 18-(b) のようになる。
 poly(I) では 255nm 225nm 付近に正の Cotton 効果
 が存在するのに対して、poly(I₂) では正の Cotton 効果
 が観察されない。0.95 M Na⁺ イオン濃度では 285nm 付近

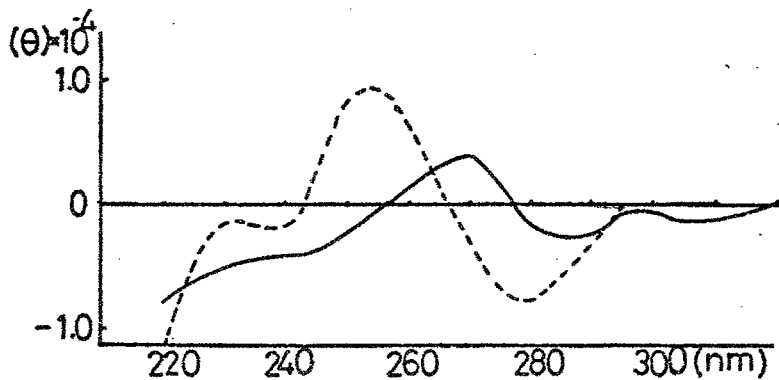


Fig 19 CD spectra of poly(I₂) and poly(I) at 0.95 M Na⁺
 — poly(I₂) - - - poly(I)

に負、270nm 付近に正の Cotton 効果があらわれ、poly(I₂)
 はこの条件で何らかの ordered structure をとっている。
 poly(I) は比較的高塩濃度における本鎖又は4本鎖をとっ
 ているとされている⁽¹⁷⁾⁽¹⁸⁾。このとき CD スペクトルは Fig 19 のように 280nm⁽¹⁹⁾
 付近に強い負の CD バンドが存在するのが特徴である。高塩
 濃度における poly(I₂) はこれに比べてスペクトルパターンは異な
 るが、正負の Cotton 効果が長波長側に shift して、[θ] 値
 もたゞはんいさい。このことから、poly(I₂) は低イオン濃度では

ordered structure とらず、高塩濃度では類似の ordered structure ととり得るが、その構造は poly(I) に比べて、より flexible なものであることが推察できる。この様に poly(Iz) は rigid な ordered structure ととり難いことが判った。

• self-ordered structure の熱的安定性

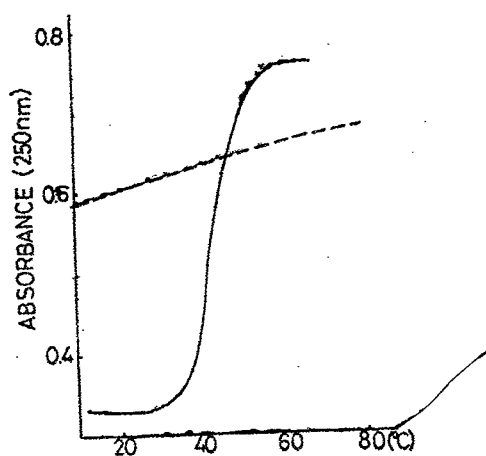


Fig. 20 T_m of poly(Iz)
 — 0.95M Na⁺
 ---- 0.15M Na⁺

Fig. 20 は、NaI オン濃度が 0.15M と 0.95M のときの 250nm の吸光度の温度変化である。0.15M では co-operative な melting は観測されない。0.95M では 43°C に T_m が観測される。同じ条件で poly(I) の T_m 値は 42°C であるので

ほぼ同程度の熱的安定性を有している。poly(Us) においても self-ordered structure の形成がみられるが、この場合は poly(U) のそれより高い T_m 値を有していると報告されている¹⁾。これ、poly(Iz) ではそのような安定化はみられない。

• poly(C) との complex 形成

中性条件で poly(I) は poly(C) と 1:1 complex を形成
 することから知られている。そこで、0.15M NaI 存在下、pH7.0
 で poly(I₂) と poly(C) の mixing を行くと Fig 20 のようになる。

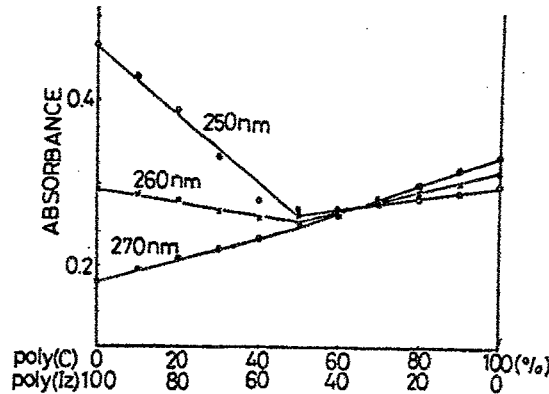


Fig 20 Mixing experiment of poly(I₂) and poly(C)

poly(I₂) 50% のところには屈曲点があり、poly(I₂)・poly(C) 1:1
 の complex の形成

が観察された。

このとき CD スペクトル

は mixing

前後で Fig 21 の

ように変化した complex

形成を示唆して

いる。この complex

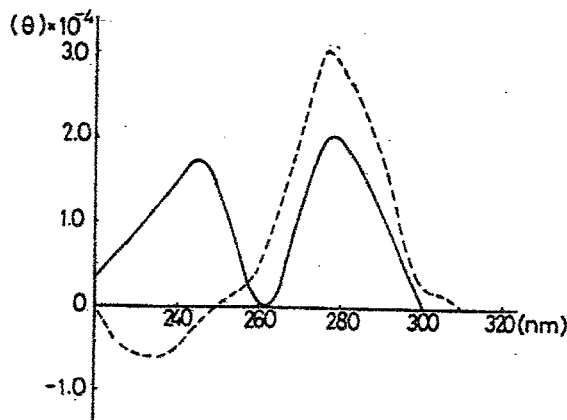


Fig 21 CD spectra of poly(I₂) and poly(C) (1:1)
 before and after mixing. ----- before mixing,
 ——— after mixing
 20

の CD スペクトルは $\text{poly(I)} \cdot \text{poly(C)}$ complex のそれによく似ている。

° $\text{poly(I)} \cdot \text{poly(C)}$ complex の熱的安定性

$\text{poly(I}_2) \cdot \text{poly(C)}$ の 1:1 の melting を種々の塩濃度で観測すると、Fig 22 のようになる。この thermal denaturation curve より T_m 値を求めこれを $\log_{10} [\text{Na}^+]$ に対してプロットすると Fig 23 のようになり、直線関係が成立する。 $\text{poly(I)} \cdot \text{poly(C)}$ complex 及び $\text{poly(dI)} \cdot \text{poly(C)}$

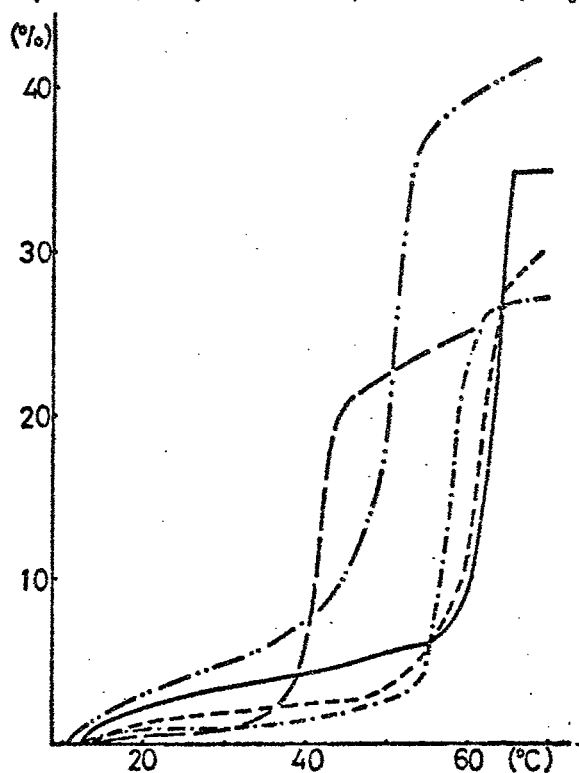


Fig 22 Temperature absorbance profile of $\text{poly(I}_2)\text{poly(C)}$ complexes at various Na^+ concentration
 --- 0.04 M, --- 0.15 M, --- 0.35 M, --- 0.55 M, --- 0.95 M,

complex)においても同様の直線関係が成立²¹⁾し、その傾きは
 ほぼ $\text{poly(Iz)} \cdot \text{poly(C)} \text{ complex}$ と同じであるのど、Naイオン
 による安定化の程度は同じである。しかし、いずれのNaイオン
 濃度においても、 $\text{poly(Iz)} \cdot \text{poly(C)} \text{ complex}$ は $\text{poly(I)} \cdot$
 $\text{poly(C)} \text{ complex}$ より低い T_m 値を有しているが、 $\text{poly(dI)} \cdot$
 $\text{poly(C)} \text{ complex}$ より安定である。このように、 poly(Iz)
 が poly(C) との complex の熱的安定性において、ribo体
 と deoxy 体の中間的な性質を有していることが判った。

第2節において述べた様に、 poly(Az) は self-structure
 形成においても、 poly(U) との complex 形成においても、
 poly(A) とよく似た性質であった。しかし、 poly(Iz) では
 poly(I) と若干性質の変化がみられる。 poly(Iz) は

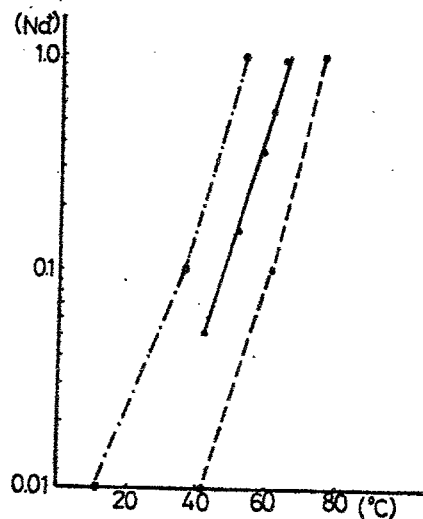


Fig23. Relationship of Na^+ concentration and T_m of
 $\text{poly(Iz)} \cdot \text{poly(C)}$ (—), $\text{Poly(I)} \cdot \text{poly(C)}$ (----)
 and $\text{poly(dI)} \cdot \text{poly(C)}$ (- - - - -).

2'位置換基の変化による影響を受けやすい傾向があるの
が判る。

第 二 章 ポリ(2'-アミノ-2'-デオキシアデニル酸) の合成と性質⁵⁷⁾

アミノ基は電気陰性度は2.92、水酸基と大きく異なっているが、含窒量はよく似ている。又 pK_a 値は水酸基より大きく、より容易にプロトン化すると考えられる。

このような性質を有するアミノ基を有するポリヌクレオチドとしては $\text{poly}(\text{Ua})$ ²⁾, $\text{poly}(\text{Ca})$ ²²⁾ が報告されている。前述のように Az の合成が可能となり、 Az より合成された AzDP を接触置換して AaDP がえられた。これを用いて $\text{poly}(\text{Aa})$ を合成し、その諸性質を調べた。

第 1 節 ポリ(2'-アミノ-2'-デオキシアデニル酸)の合成

2'-アミノ-2'-デオキシアデノシン 5'-ジホスフェート (AaDP) は AzDP を酸性水溶液中で 10% palladium charcoal で接触置換して合成した。反応室結は pH 3.5 の電気泳動で AzDP よりおくれる位置に 1 スポットがあらわれることにより確認した。分離精製は DEAE Sephadex A-25 (bicarbonate) で行った。80% の収率で AaDP を得た。そのプロトタイプ²⁾ の性質を Table 3 に示す。重合反応は AaDP 4 mM, MnCl_2 2 mM, TrisHCl (pH 8.5) 80 mM, PNPase (*M. luteus*) 5 units/ml を用いて 37°C で行った。時間をあて無機

Table 3

	PEP(R _{mp} A-A)		PPC(Rf)		
	PH 3.5	PH 7.5	C↓	G↓	M↓
AaDP	1.18	1.20	0.05	0.11	0.48

リンの定量により反応の進行を測定したのが Fig 24 である。

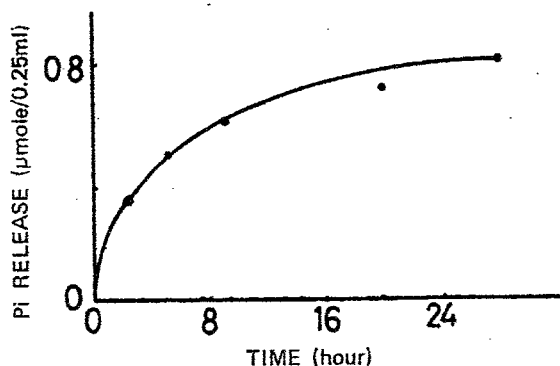


Fig 24 Time course of AaDP polymerization.

27時間後に isoamyl alcohol:CHCl₃ = 1:3 混液で除蛋白して反応をとめ、Mnイオンを除くために 0.01M EDTA, 0.01M TrisHCl (pH 7.0) で透析し、更に水に対して透析した。分離精製は Sephadex G-50 で100過を行った。

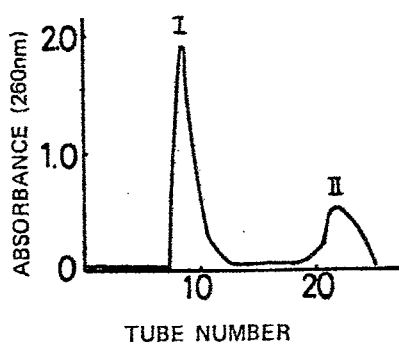


Fig 25 Sephadex G-50 column chromatography of poly (Aa).

peak I poly (Aa)
peak II AaDP

poly(Aa) は void volume 付近に溶出される。収率は 13% であつた。Fig 25 は溶出パターンである。

第2節 ポリ(2'-アジ1-2'-デオキシアデニル酸)の性質

・UV スペクトル

0.15 M Naイオン存在下 25°C で UV スペクトルは pH 7.0 時 Fig 26 のようになる。 λ_{max} 258 nm で無機リンの定量より

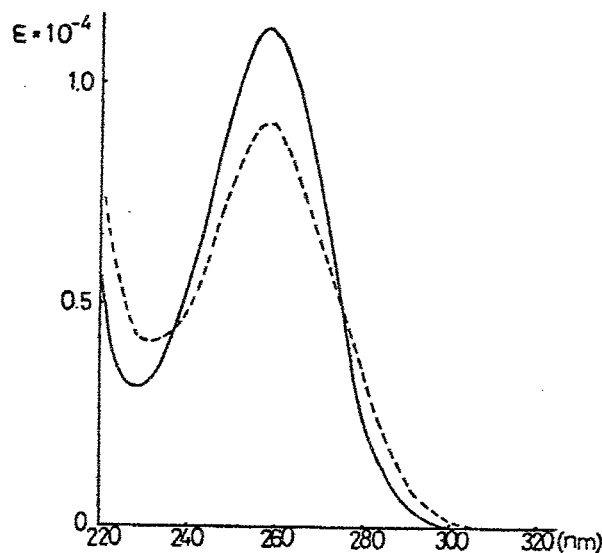


Fig 26. UV absorption spectra of poly(Aa), — at pH 7.0, --- at pH 6.3.

求めた分子吸光係数は 11200 であつた。Aa の λ_{max} 258.5 nm で分子吸光係数は 15000 であるので hypochromicity は 25% である。これは poly(A) の 35% に比べ、低い値である。poly(Aa) はこの条件では poly(A) ほど stack した

状態でないことがわかる。pH 6.3 に下げると λ_{max} は変化しないが $\epsilon(P)$ は 9.15 となり、pH 7.0 より 20%、Aa より 40% の hypochromicity が観測される。 λ_{max} の変化がないことより、N-1 位に protonate してある、いわゆる "acid form" をとっていると思われる。

・pH transition と多酸性における構造

0.15 M NaCl 存在下 15°C で酸滴定を行うと Fig 27 のようになる。258 nm の吸光度は pH 7.0 付近で sharp な transition を有している。しかし、transition pH の前後で λ_{max} の位置の変化はなく、塩基部には protonate が起っていないものと考えられる。他に protonation が起りやすい部位としては 2' 位 NH_2 があり、この程度の pKa 値を有するものと考えられる。2' NH_2 に protonation することにより、強く stack する構造をとるようになったのではないかと思われる。

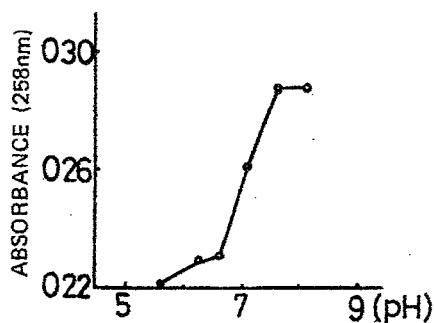


Fig 27 Acid titration of poly(Aa).

° CDスペクトル

0.15 M NaCl 存在下 24°C での poly(Aa) の CD スペクトルを Fig 28 に示した。pH 7.0 において poly(Aa) は AaDP とよく似た CD スペクトルパターンを示す。従って poly(Aa) は poly(A) のように強固な stacking により、rigid な構造をとっていることがわかる。これは UV スペクトルの λ_{max} における hypochromicity が poly(A) より顕著に小さい値をとるという事実とよく一致している。一方 pH 5.7 あるいは pH 6.3 では CD スペクトルは大きく変化した。274, 225 nm に大きな trough, 252 nm に peak を有するようになる。故に poly(Aa) はこのような弱酸性条件下で ordered structure を形成しているのかわかる。この CD スペクトルは poly(A) の

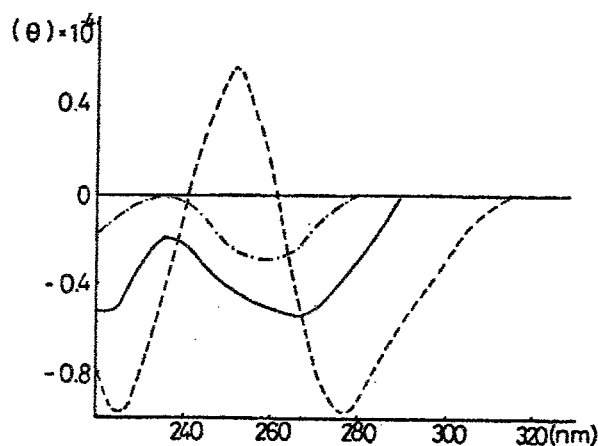


Fig 28 CD spectra of AaDP and poly(Aa), —poly(Aa) at pH 7.0.
 ---poly(Aa) at pH 5.7, - · - · - AaDP at pH 7.0.

acid form の CD スペクトルとは全く異なっているのが、この型の ordered structure ではないと思われる。この ordered structure は温度を上げるとくずれ、元の monomer に似た CD スペクトルに戻る。pH 6.3 の場合が Fig 29 である。20°C で形成していた ordered structure は 46°C に温度を上げることによって崩壊し、それ以上温度を上げても大きな変化はみられない。こういったことはクリミジンヌクレオチドでは観察されていない。

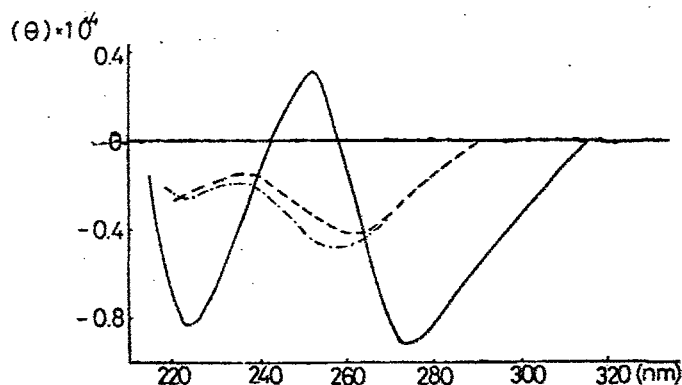


Fig 29 CD spectra of poly(Aa) taken at 20°(—), 46°(---) and 66°(-.-.-).

・ poly(Aa) の self-ordered structure の熱的安定性

poly(Aa) の弱酸性における ordered structure の熱的安定性について調べた。0.15M Naイオン存在下 pH 7.0, pH 6.3, pH 5.7 の各々の temperature-absorbance profile は Fig 30 のようになる。ここで与えられた T_m 値は

各々 22°C, 38°C, 54°C であつた。pH が下がると共に T_m 値が上昇する傾向がある。 T_m 値を Na イオン濃度に対してプロットすると Fig 31 のようになる。pH 6.3, pH 7.0 のいずれの場合にも、 T_m 値と $\log_{10} [Na^+]$ との間に直線

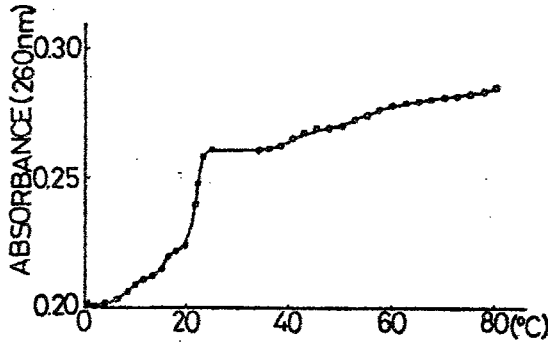


Fig 30(a) Temperature-absorption profile of poly(Aa) taken at 7.0.

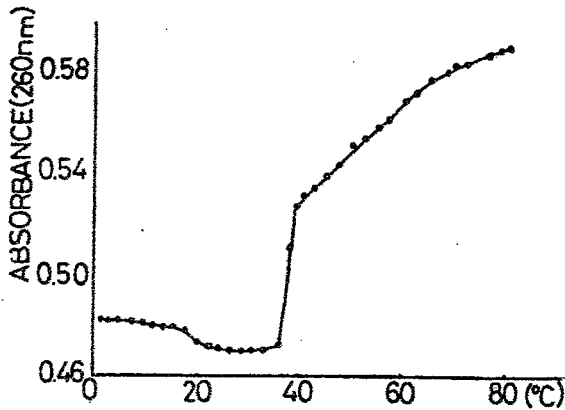


Fig 30(b) Temperature-absorption profile of poly(Aa) taken at pH 6.3.

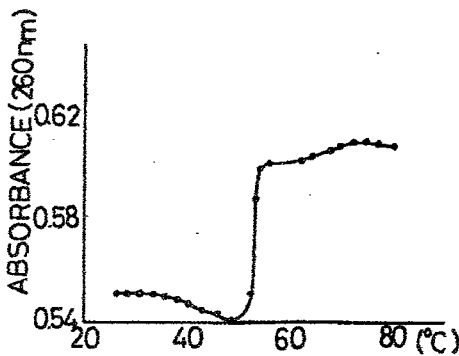


Fig 30(c) Temperature-absorption profile of poly(Aa) taken at pH 5.7.

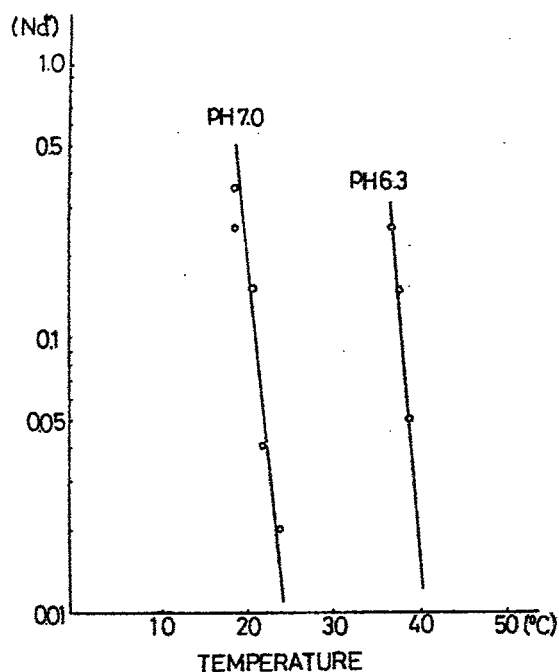


Fig 31 Depending of T_m of poly(Aa) on sodium ion concentration.

関係が成立する。そして塩濃度の上昇に伴って T_m 値が若干減少する傾向がある。poly(A) の acid form の安定性は塩濃度の上昇に伴い大きく減少²³⁾する。他の水素結合を含む complex では塩濃度の上昇に従って T_m 値は上昇する。このように、水素結合を含む complex では、塩濃度はその T_m 値に大きく影響を与えるが、poly(Aa) の ordered structure ではあまり変化がないので、塩基間の水素結合を含んでいないと思われる。poly(Aa) の弱酸性における ordered structure は前述のように 2 個のアミノ基の protonate により stacking が強化された

と考へらる。

・ poly(U) との complex 形成 とその熱的安定性

0.15 M NaCl 存在下で, poly(Aa) と poly(U) と mixing すると Fig 32 のように, poly(Aa) 33% のところに屈曲点が見られ, poly(Aa)・poly(U) = 1:2 の

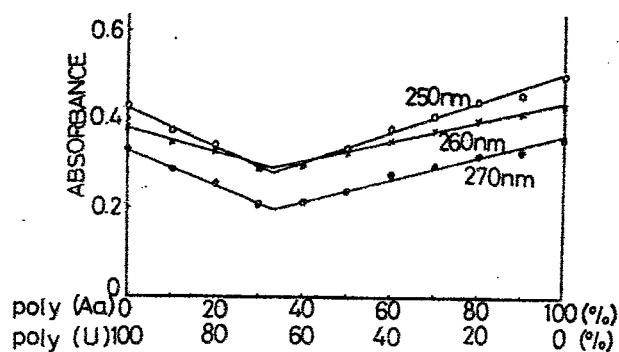


Fig 32 Mixing experiments of poly(Aa) with poly(U).

3重鎖 complex の形成が観察された。これは Fig 33

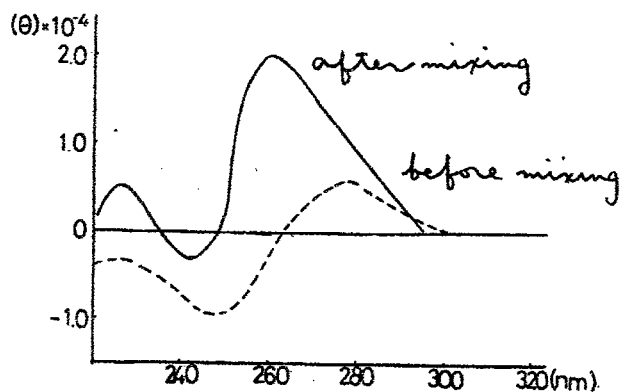


Fig 33 CD spectrum of poly(Aa)·2poly(U) complex.

のように mixing 前後で CD スペクトルが変化することに
 より確認できる。poly(Ua)³²⁾の場合、poly(A)と1:1
 complex を形成しないと報告されている。又 poly(Ca)
 は、poly(I)と complex を形成するが、その T_m 値は非常
 に低いものがある。poly(Aa)においても 0.04 M イオン存在下
 における mixing 実験では、明らかな屈曲点が見られず、
 この条件で complex 形成が定結していないことと思われる。0.15 M
 Na イオン濃度で形成した poly(Aa)-poly(U) complex の
 temperature-absorbance profile は Fig 34 のように
 なり、54°C を中心に co-operative な melting を示す。この
 値は poly(A)-poly(U) complex の同条件下の T_m 値より
 8°C 低くなっている。

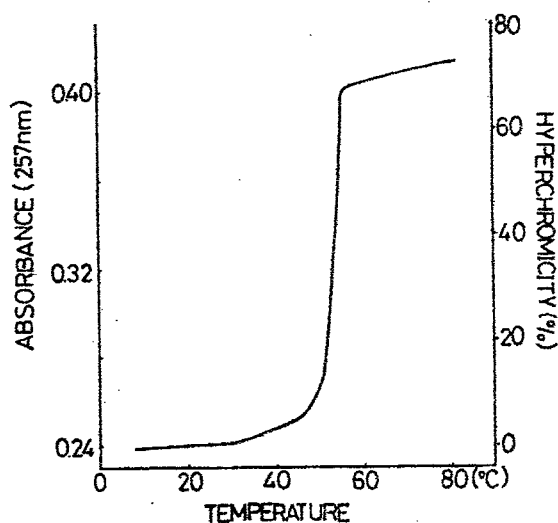


Fig 34 Temperature-absorption profile of poly(Aa)·2poly(U) complex.

poly(Aa) は poly(U) と complex を形成するがその熱的安定性は poly(A)-poly(U) complex より低い。poly(Ua) は poly(A) との complex 形成が観測できない。又、poly(Ca)-poly(I) complex は 0.10 M NaI 存在下 (pH 7.5) で 25°C と低い T_m 値と有する不安定なものである。このように 2'位アミ基の存在は complex 形成に不利であることが判った。

第三章 ポリ(2'-ハロゲン-2'-デオキシポリヌクレオチド) の合成と性質

第一章、第二章において、電気陰性度、そのsizeの全く異った、アジド基、及びアミノ基が2'位に置換したことにより生じる性質の変化について考察した。この結果 poly(A) アナログでは、size の水酸基と類似しているアミノ基を有する poly(Aa) は、特に self-structure の形成において poly(A) とは異った性質を示すことが明らかにになり、電気陰性度により水酸基に近いアジド基を有する poly(Az) は、置換基の size の違いにもみかわらず、poly(A) に似ていることが判った。又、poly(I) アナログでは、その基のもつ特異性により poly(A) アナログより影響を受けやすいことが判った。

ハロゲン類は、 $F(3.94) > Cl(3.00) > Br(2.68)$ の順に電気陰性度は小さくなり、その共有結合半径は逆に $0.64 < 0.99 < 1.11$ と大きくなる。これらを2'位に導入し、相互の性質を比較することにより、2'位置置換基の影響をより明らかにすることができると考えられる。

最近、池原等により 2'-ハロゲン-2'-デオキシアデノシンの合成法が開発²³⁾され、これらを 5'-diphosphate に導き、PKase で重合することにより対応するポリヌクレオチドが得られる。

第1章 ホリ(2'-ハロゲン-2'-デオキシアデニル酸)の合成

2'-ハロゲン-2'-デオキシアデニン 5'-モノホスフェート (AMP)⁵⁾
は第1章で述べたと同様に、小嶋らの方法に従って
triethyl phosphate 中 $POCl_3$ を用いて対応するヌクレ
オシド²³⁾の5'位をリン酸化した。Dowex 1×2 (formate)
カラムクロマトグラフィーにより精製を行い 60~80% の収率で
5'-phosphate 体を得た。これはすべて crude snake
venom 5'-nucleotidase により脱リン酸化され、もとの
ヌクレオシドに戻ることより、5'-phosphate 体であることを確
認した。しかし、脱リン酸化反応は天然の 5'-AMP より長時間
を要した ことより若干切れにくい傾向がある。

次にこれを Moffat-Khorana の方法により morpholidate
経由で 5'-diphosphate 体とした。DEAE Sephadex
A-25 (bicarbonate) カラムクロマトグラフィーで分離精製
を行った。収率は 30~60% 程度である。これらえられた
monophosphate 体、及び diphosphate 体のクロマト
グラフ上の諸性質は Table 4 のようになる。

次にこれらの重合反応を行った。基質 4mM, $MgCl_2$
2mM, TrisHCl (pH 8.5) 60~100mM, PNPase (E. coli)
4~4.5 units/ml で 37°C 24時間反応させた。除蛋白の
後 Sephadex G50 に 37°C で 2'-polynucleotide を

Table 4

	PEP(PAA)		PPC		
	pH 7.5	pH 3.5	C↓	G↓	M↓
AfMP	1.04	1.00	0.13	0.23	0.49
AfDP	1.35			0.13	0.44
AceMP	0.96	0.97	0.17	0.35	0.58
AceDP	1.25			0.08	0.54
AbrMP	0.96	0.95	0.28	0.40	0.54
AbrDP	1.27			0.08	0.50

単離した。Fig 35に poly(Abr) の gel filtration の
溶出パターンを示した。これに polynucleotide の収率は
poly(Af) 55%, poly(Ace) 25%, poly(Abr) 13% である。

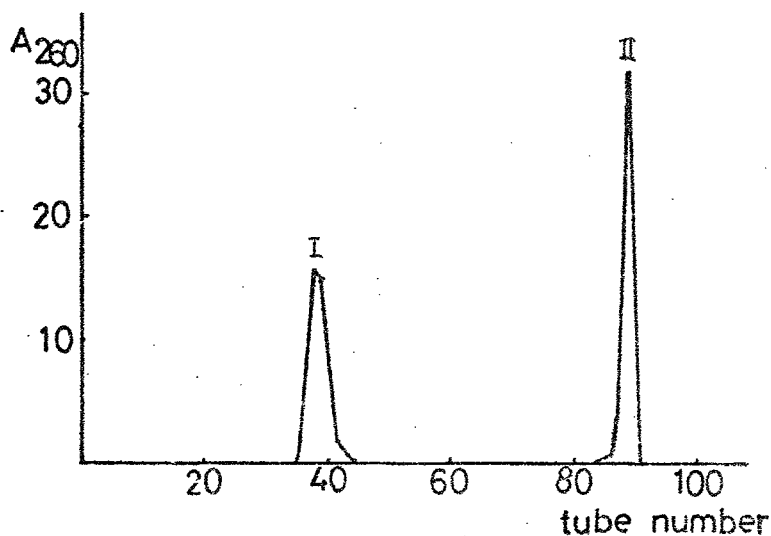


Fig.35 Sephadex G-50 gel filtration of poly(Abr).
peak I: poly(Abr), peak II: AbrDP.

前述のように PNPase は、2'-デオキシ体と重合反応の基質としてはいない。2'位ハロゲン基は水酸基と同様に認識されているのが判る。又、ハロゲンの原子半径の増大とともに収率の低下がみられる。2'位に大きな置換基が導入されることは PNPase の基質としては不利になる傾向がある。

又、 Mg^{++} イオンと同濃度の Mn^{++} イオンに変え、他は同じ条件で反応を行くと、リンの定量による反応の check では $poly(AT)$ では 14% 反応が進行していたが、 $poly(Ace)$, $poly(Abr)$ ではほとんど反応が進行していなかった。このことより、2'ハロゲン体の重合反応は Mg^{++} イオン依存性であることが判る。

第2節 ポリ(2'-ハロゲノ-2'-デオキシアデニル酸)の性質

○UV スペクトル

0.15 M Na イオン存在下 25°C で $poly(AT)$ は Fig 36 のような UV スペクトルを示した。pH 7.0 では $\lambda_{max} 255 nm$, pH 4.5 では $253 nm$ であった。 $poly(Ace)$ と $poly(Abr)$ はたゞ々 1 μm ほどあり、そのうち $poly(Ace)$ を Fig 37 に示した。

$poly(Ace)$, $poly(Abr)$ とともに pH 7.0 で $\lambda_{max} 257 nm$, pH 4.5 で $\lambda_{max} 255 nm$ であった。この三者の pH 7.0 におけるリンの

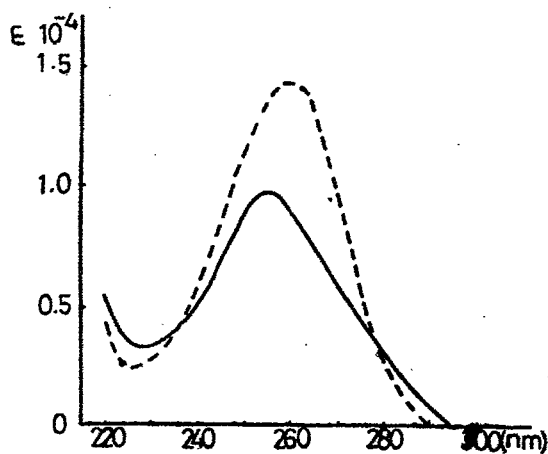


Fig 36 U. V. absorption spectra of poly(Af) and Af 5'-MP.
Poly(Af) —, AfMP ----.

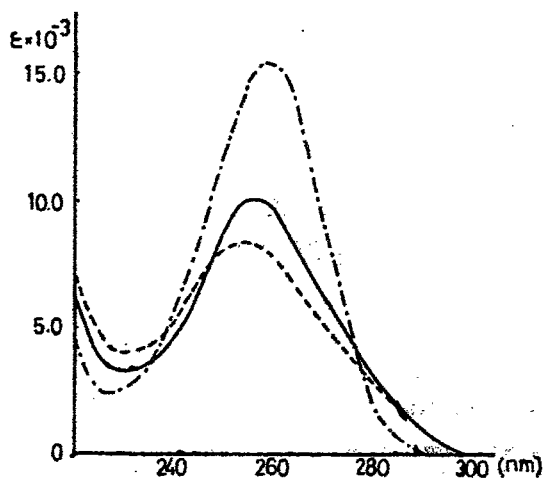


Fig. 37 UV absorption spectra of poly (AcI). — at pH 7.0.
..... at pH 4.5, ---- AcI-MP.

定量により求めた分子吸光係数 ϵ と hypochromicity ϵ
Table 5 に示した。このように hypochromicity の大さは
 $\text{poly}(+) > \text{poly}(\text{AT}) \geq \text{poly}(\text{Ace}) > \text{poly}(\text{Abr})$ となる。
 hypochromicity は stacking の程度を反映しており、

Table 5

	$\epsilon(P) \times 10^{-4}$ at $\lambda_{\max}$		hypochromicity(%)
	(a) polymer	(b) monomer	$[1-(a)/(b)] \times 100$
A	1.00	1.54	35
Af	0.97	1.43	32
Ac1	1.05	1.54	32
Abr	1.07	1.50	29

2'位のハロゲンのsizeが大きくなるにつれて、stackingの程度が小さくなっているのが判る。ヌクレオシドのNMRスペクトルより得た $J_{H1-H2'}$ の値は、Af 3.5 Hz, Ac1 5 Hz, Abr 7.5 Hz である。²⁵⁾ Altonaらは $S \rightleftharpoons N$ conformation 平衡において通常ヌクレオシド、ヌクレオチドでは、S conformerのハ-ロエンタジは $10 \times J_{H1-H2'}$ と与えられると報告している。²⁶⁾ これを2'-ハロゲンアティシンの場合にあてはめれば、Af < Ac < Abrの順でS conformerの比率が大きくなる。すなわち2'-endo型という確率が大きくなる。2'-endo型である、2'置換基はequatorial方向に存在することになり、2'置換基が大きくなれば、polynucleotideの場合のbase-base interactionが妨げられると思われ、これがhypochromicityの減少となってあらわれると思われる。

Alderfer²⁷⁾は、2'-O-フルビル体のシリーズで $\text{poly(dA)} > \text{poly(A)} > \text{poly(Am)} > \text{poly(Ae)}$ とhypochromicity₄₀

が減少し、2'置換基のsizeが大きくなるとstackingが妨げられると報告している事実とよく一致する。

・CDスペクトル

0.15 M Naイオン存在下、pH7.0, 25°CでCDスペクトルを測定するとFig 38のようになる。poly(Af), poly(Ace), poly(Abr)の三者はたいへんよく似ており、いずれもpoly(A)型のCDスペクトルを示す。しかし、正負のcotton効果のamplitudeは $\text{poly(Af)} > \text{poly(Ace)} > \text{poly(Abr)}$ の順で減少している。その値はTable 6に示す。hypochromicityの減少と分るに円率 $[\theta]$ の減少がよく対応しているの2、 $[\theta]$ 値の減少はstackingの減少によると考えられる。

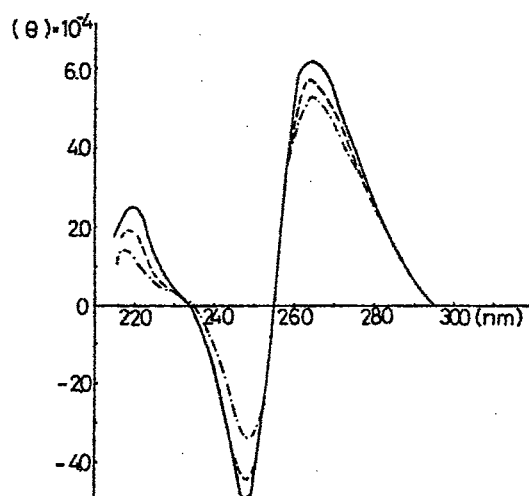
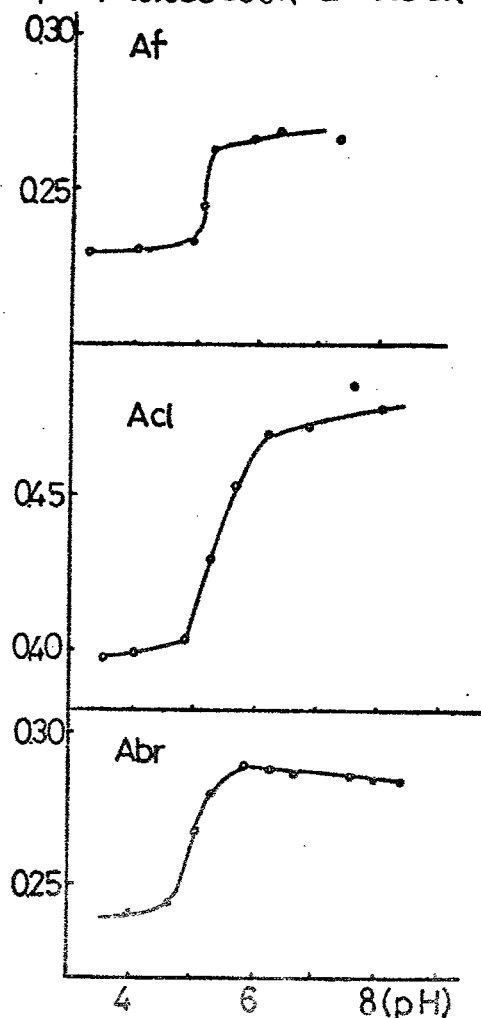


Fig 38 CD spectra of poly(Af), poly(Ace) and poly(Abr). Poly(Af) —, poly(Ace) ----, poly(Abr) -.-.-.

Table 6

polynucleotide	$[\theta] \times 10^{-4}$		
	peak1 (nm)	trough (nm)	peak2 (nm)
poly(A)	2.1 (220)	-4.1 (248)	5.2 (264)
poly(Af)	2.5 (220)	-4.9 (248)	6.3 (264)
poly(Acl)	1.9 (218)	-4.4 (248)	5.7 (264)
poly(Abr)	1.4 (217)	-3.4 (249)	5.3 (264)

・ pH transition と "acid form" の安定性



poly(Af), poly(Acl),
poly(Abr)の acid form 形成
について調べた。

0.15 M NaCl 存在下で 25°C
で酸滴定すると Fig 39 のように
なる。その pH transition
point の値と pH 4.5 で
形成した acid form の
 T_m 値を Table 7 に示した。

Fig 39

Acid titration curves of
poly(Ax) in the presence
0.15M NaCl at 25°C

Table 7

polynucleotide	pH t _{1/2}	T _m at pH 4.5
poly(A)	5.5	60
poly(Af)	5.2	37
poly(Acl)	5.5	63
poly(Abr)	5.0	56

stackingの強い polynucleotide は塩基部に protonate され、このことが知られており、poly(Af)の pH t_{1/2}、及び pH 4.5 の T_m が低い値を示すのはそのためと考えられる。poly(Abr) の場合には、protonate した後に形成する double-strand complex が不安定なものであるために、これら二つの値が小さくなっていくものと考えられる。又、poly(Acl) は poly(A) に近く似ている。

・ poly(U) との complex 形成とその安定性

0.15 M Na⁺ イオン存在下、pH 7.0 で poly(Af), poly(Abr) poly(Abr) と poly(U) と mixing すると Fig 40 のようになる。いずれの poly(Ax) も poly(U) と 1:2 の 3本鎖 complex を形成したことがわかる。

又、0.04 M Na⁺ イオン存在下、pH 7.0 で mixing を行い、30分後の測定では Fig 41 のように、1:1 の 2本鎖 complex の形成がみられる。しかし、この場合には、時間の経過とともに mixing 曲線は変化し、2本鎖、3本鎖のまざった複雑な

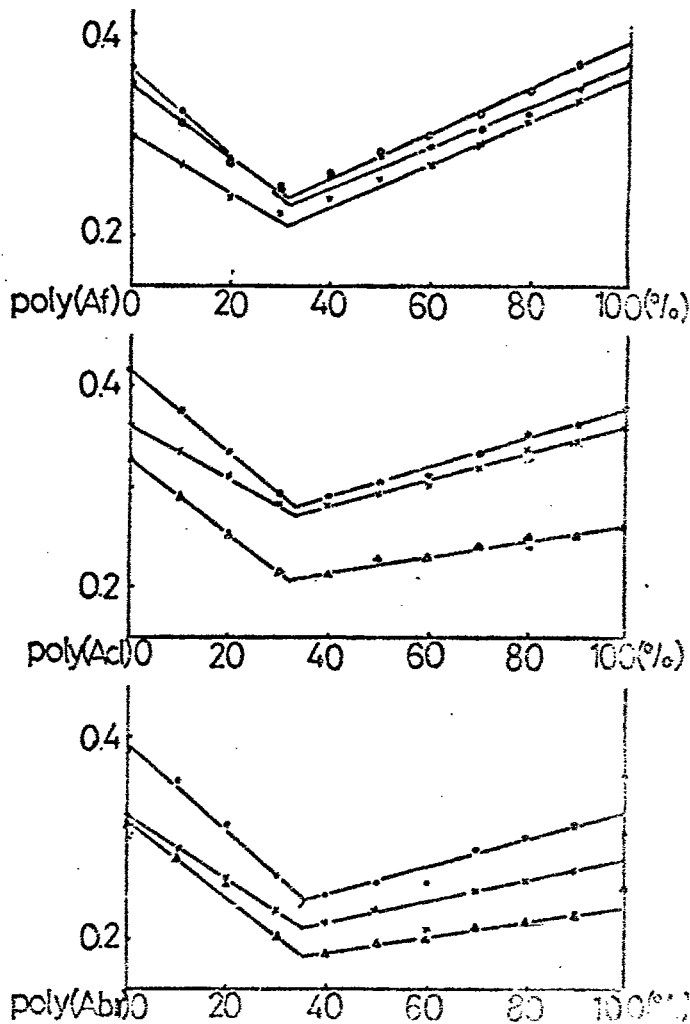


Fig 40

Mixing curves of
poly(Ax) with poly(U)
in the presence
0.15M Na⁺

250nm ————
255nm ————
260nm ————
270nm ————

ものとなり、poly(At)-poly(U)の系では1週間後には
完全に 1:2 complexに移行した。これらのcomplexの
形成は、poly(Ax)-poly(U)混合物のmixing前後の
CDの変化によっても確認した。Fig 42 は、poly(At)-
poly(U) 1:2 complex形成における、CDの正の値
の変化である。

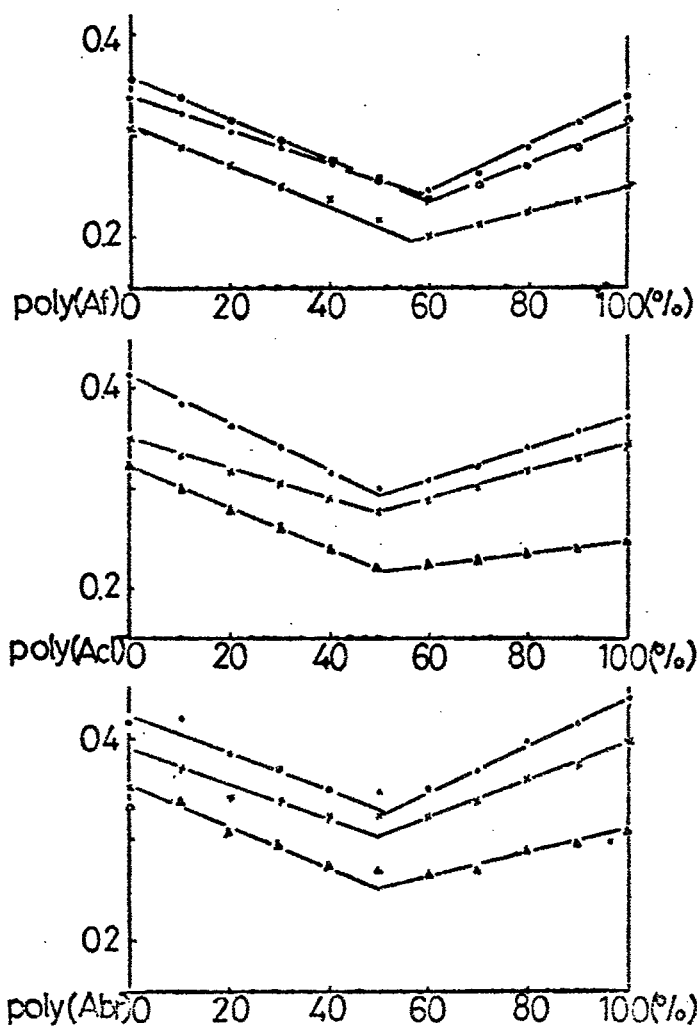


Fig41

Mixing curves of
poly(Ax) with poly(U)
in the presence
0.04M Na⁺

250nm —x—x—
255nm —o—o—
260nm —·—·—
270nm —▲—▲—

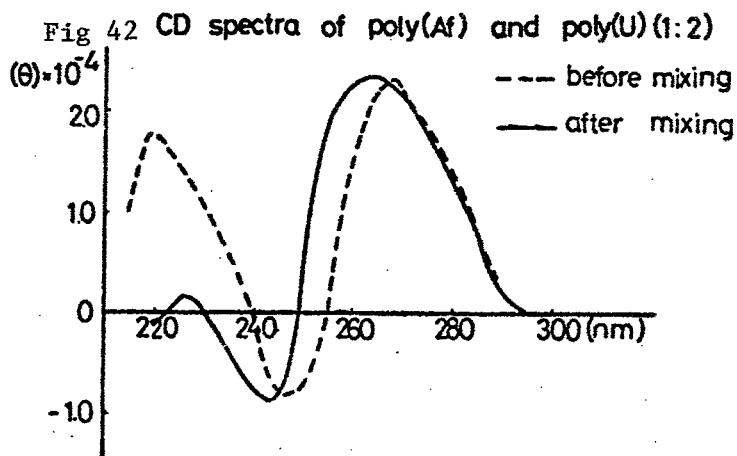


Table 8

complex	T _m	
	0.04M [Na ⁺] 30min.	0.15M[Na ⁺] 1day
poly(A).poly(U)	51°C(2→1)	62°C(3→1)
poly(Af).poly(U)	49	64
poly(Acl).poly(U)	46	56
poly(Abr).poly(U)	45	53

これらの complex の T_m 値は Table 8 のようになる。いずれの塩濃度によっても complex の熱的安定性は poly(Af)・poly(U) > poly(Acl)・poly(U) > poly(Abr)・poly(U) であつた。2' 置換基の大きいほど T_m 値の下る傾向があるのがわかる。これは、2' 位に大きな置換基を有すると、poly(U) との complex 形成に不利な conformation をとるためと思われる。

・ poly(I) との complex 形成とその安定性

0.15 M Na イオン濃度 pH 7.0 2' poly(Af), poly(Acl),

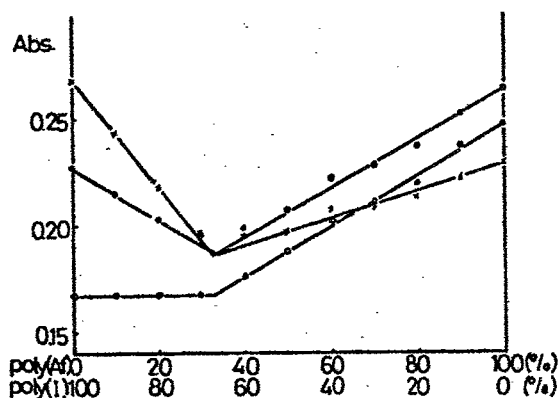


Fig43 Mixing curves of poly(Af) with poly(I) in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0) at 25°. At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

$\text{poly}(\text{Abr}) \pm \text{poly}(\text{I})$ と mixing すると、いずれも $\text{poly}(\text{I})$ の 67% のところで屈曲した mixing curve がえられ、 $\text{poly}(\text{Ax}) \cdot \text{poly}(\text{I})$ 1:2 の 3 本鎖 complex の形成が観察された。
 このことは、 $\text{poly}(\text{Ax}) \cdot \text{poly}(\text{I})$ と 1:2 に混合する前後の CD の変化でも確認できる。例として、 $\text{poly}(\text{At})$ の場合をあげたのが Fig 43, Fig 44 である。これら complex の T_m 値は Table 9 のようになり、三者でほとんど差がみられない。しかし、これに 10 mM の MgCl_2 を加えるとわずかに差を生じ、 $\text{poly}(\text{At}) \cdot \text{poly}(\text{I}) > \text{poly}(\text{Acl}) \cdot \text{poly}(\text{I}) > \text{poly}(\text{Abr}) \cdot \text{poly}(\text{I})$ となる。
 $\text{poly}(\text{Ax}) \cdot \text{poly}(\text{U})$ についても同様、2' 置換基が大きくなるに従って熱的安定性が低下している。

このように $\text{poly}(\text{Ax})$ は self-structure と、 $\text{poly}(\text{U})$ あらうは $\text{poly}(\text{I})$ との complex 形成も $\text{poly}(\text{A})$ と似た性質であるが、F, Cl, Br の順で stacking の程度が減少し、

Fig 44 CD spectra of $\text{poly}(\text{At})$ and $\text{poly}(\text{I})$ (1:2)

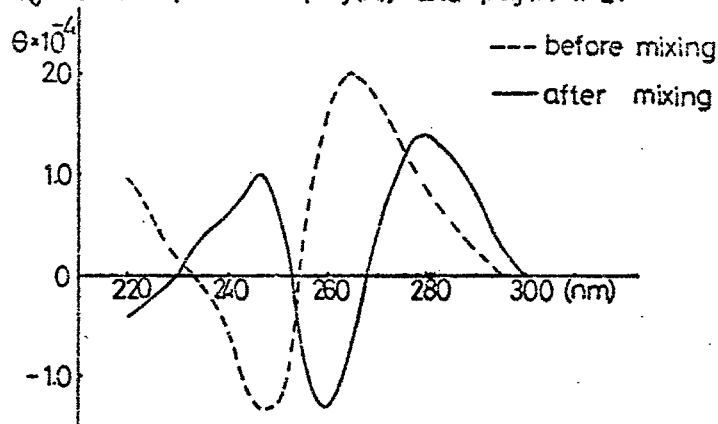


Table 9

complex	T _m (°C)	
	0.15M[Na ⁺]	0.15M[Na ⁺]+10mM MgCl
poly(A).poly(U)	42	54
poly(Af).poly(U)	36	51
poly(Acl).poly(U)	36	49
poly(Abr).poly(U)	35	47

complexの熱的安定性が低下するのが判った。

第4節 ポリ(2'-ハロゲン-2'-デオキシイリシン酸)の合成

2'-ハロゲン-2'-デオキシイリシン-5'-モノホスフェート(IxMP)は第1章で述べたと同様にして、対応するAxMPと酢酸酸性中NaNO₂と反応させて deamination し、Dowex 1×2(formate)カラムクロマトグラフィーにより70-80%の収率で得た。次にMoffat-Khoranaの方法により morpholidate 経由で5'-diphosphate 体と合成した。分離精製は Sephadex A-25(bicarbonate)カラムクロマトグラフィーで行い、60%程度の収率で IxDP とえた。これらのクロマトグラフィーの諸性質は Table 10 のようになる。

重合反応は IxDP 4mM, MgCl₂ 2mM, TrisHCl (pH8.5) 100mM の条件で poly(Ir) は PNPase(E. coli) 4.5 units/ml, poly(Ic) の場合 PNPase(M. luteus) 40 units/ml 用いた。ポリヌクレオチドは Sephadex G50

Table 10

	PEP (RmpA-A)		PPC(RF)		
	pH 7.5	pH 3.5	C	G	M
IfMP	1.04	2.50	0.07	0.34	0.32
IfDP	1.41		0.04	0.10	0.31
Ic1MP	1.10	2.70	0.12	0.31	0.45
Ic1DP	1.40		0.08	0.21	0.45

ゲルコロム2 void volume付近に溶出される。収率は
 poly(I_f) 12%, poly(I_c) 16%であった。poly(A_f), poly(A_c)
 のPNase 4.5-4 units/ml 用いて収率各々 55%, 25%
 であったのに比べ著しく低い。Fig 45 に poly(I_c) のゲル
 コロムの溶出パターンを示した。

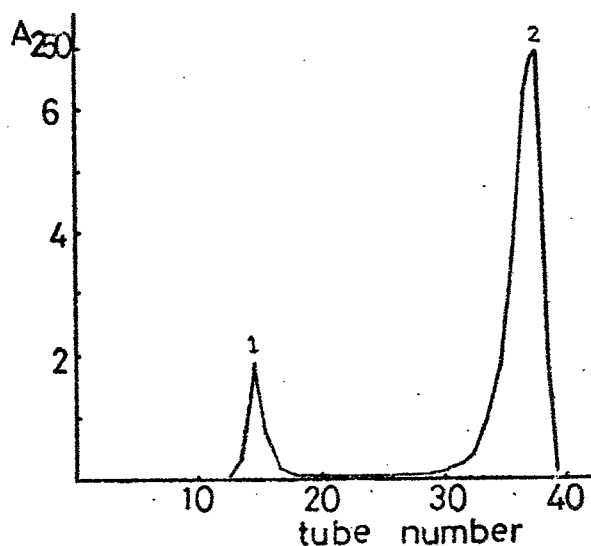


Fig.45 Sephadex G-50 gel filtration of poly(Ic1)
 peak1:poly(Ic1), peak2:Ic1DP

第4節 ポリ(2'-ハロゲン-2'-デオキシリボシン酸)の性質

・UVスペクトル

poly(If) を pH 7.0 で UV スペクトルを測定すると Fig. 46 のようになる。20°C においては、poly(If) の UV スペクトルに似ているが、275 nm 付近にふくらみがある。後述するようにこの条件で poly(If) の T_m 値は 27°C であるが、これより高い温度、例えば 40°C では、このふくらみは消失している。If MP の hypochromicity は 20°C において 20%、40°C で 12% であった。これは、0.75 M Na⁺ イオン存在下では、30°C においても、 T_m 値である 47°C 以上例えば 52°C においても、275 nm 付近にふくらみは見られない。

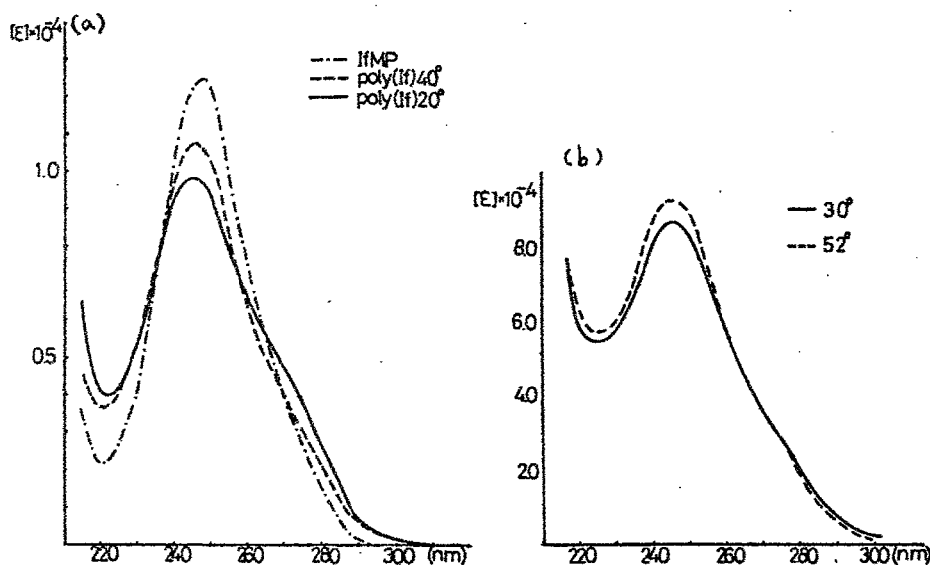


Fig. 46(a) UV spectrum of poly(If) and If MP at pH 7.0 in the presence of 0.15M Na⁺ ion.

Fig. 46(b) UV spectrum of poly(If) in the presence of 0.75M Na⁺.

従って、poly(Ic)は低イオン濃度と高イオン濃度では異なる ordered structure が存在していることが推定される。

- ⑤ poly(Ic)のUVスペクトルはFig 47のようになる。これはたゞ単に poly(I)に似ている。0.15 M Naイオン濃度ではIcMPより hypochromicity は20% である。0.95 M Naイオン濃度においては、より大きな hypochromicity が観察され、長波長側の background が高くなっている。これは aggregation によるものと思われる。

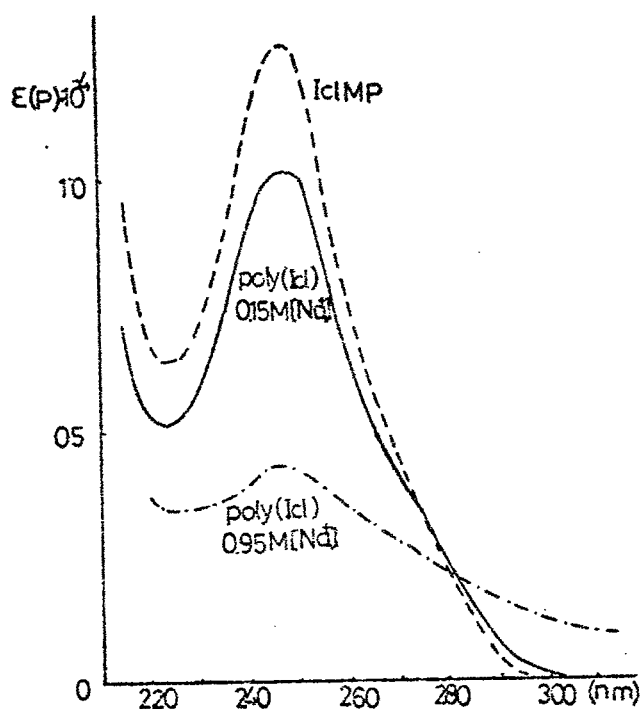


Fig 47 UV spectra of poly(Ic) and IcMP at pH7.0

・ self-ordered structure の熱的安定性.

poly (If) の 0.15 M Na イオン 濃度, 0.75 M Na イオン 濃度 における thermal melting curve を Fig 48 (a) に示す. 0.15 M のときは 27°C , 0.75 M では 47°C に各々 melting point が観察される. しかし, 0.15 M における ordered structure と 0.75 M における ordered structure は, 後述の CD スペクトルの結果より 全く異なったものである.

Na イオン 濃度 を変化させて, T_m の変化をみると, Fig 48(b) のようになる. poly (If) の curve は折れ曲っており, 高塩濃度になるほど $\log_{10} [\text{Na}^+]$ の変化に対して T_m 値の変化が大きくなる. これより 低塩濃度に

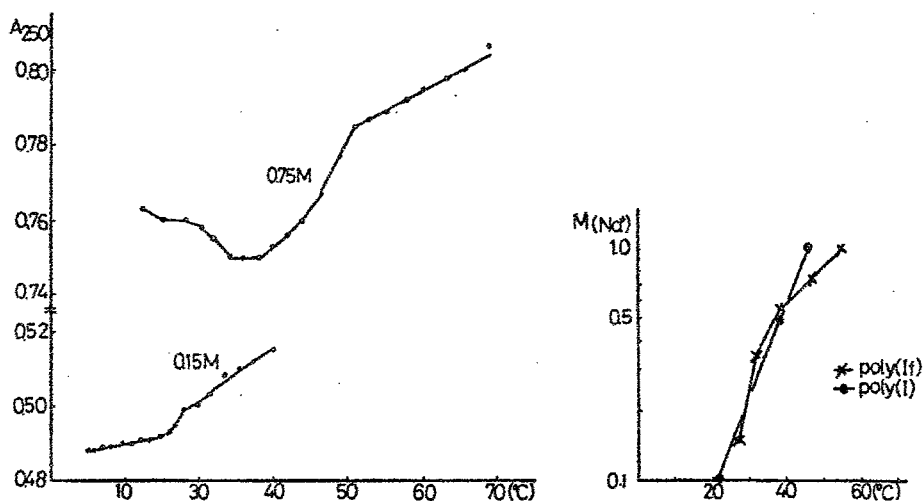
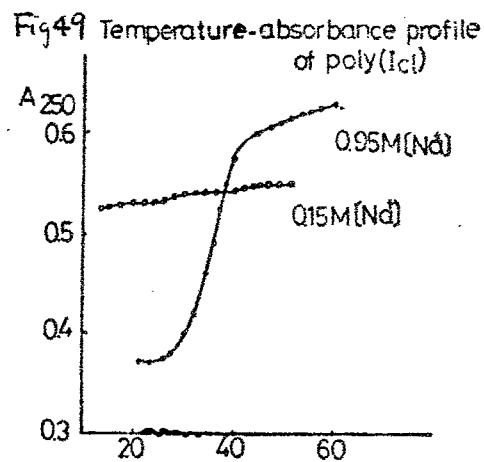


Fig 48(a) Thermal melting profiles of poly(If) at Na⁺ concentration of 0.15M and 0.75M.
Fig 48(b) Relationship of T_m of poly(If) and poly(I) to Na⁺ concentration.

おける ordered structure はあまり塩濃度の変化
 による安定性に変化はうけないが、高塩濃度における
 ordered structure 2 は大きく安定化をうける。

- お. poly(Icl) の 0.15 M Na⁺ イオン濃度, 0.95 M
 Na⁺ イオン濃度 2 の thermal melting curve を Fig
 49 に示す。0.15 M 2 は顕著な transition をみられない。
 0.95 M 2 は T_m が 36°C
 となる。



○ CD スペクトル

0.15 M Na⁺ イオン濃度, pH 7.0 2 poly(I⁺) の CD スペ
 クトルを測定したものが Fig 50 である。 T_m 値より低温
 2 は長波長側に正, 短波長側に強い負の Cotton 効果
 があらわれる。これは同条件下の poly(I)⁽¹⁹⁾ には観察
 されないものである。この条件で poly(I⁺) は何らかの
 ordered structure をとっている。しかし CD スペクトル
 のパターンより、この ordered structure は高塩濃度に

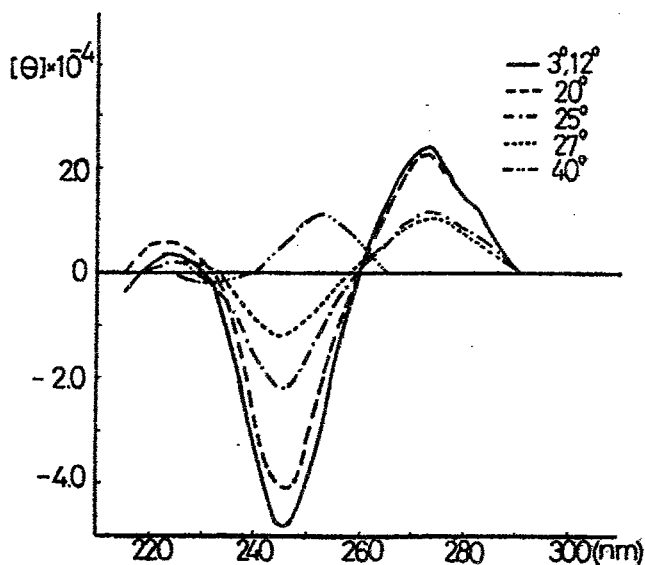


Fig50 CD spectra of poly(If) at 0.15M Na⁺ concentration.

おける poly(I) がとるような4本鎖 complex ではないと思われる。温度が上昇するにつれて、正負の cotton 効果の amplitude は減少し、T_m 以上では cotton 効果は逆転し、poly(I) が random coil 状態にあるときの CD スペクトルと似たスペクトルを示す。

より高い濃度においては、275 nm 付近に負の cotton 効果がみられる。Fig 51 は 0.75 M Na⁺ イオン濃度の例である。30°C において観察された CD スペクトルが 40°C では大きく変化し、poly(I) の4本鎖 complex の CD スペクトルに似たパターンを示す。T_m 以上では負の cotton 効果は消失しており、random coil になっているものと思われる。

Fig 51 CD spectra of poly(I_f) at 0.75M(Na⁺)

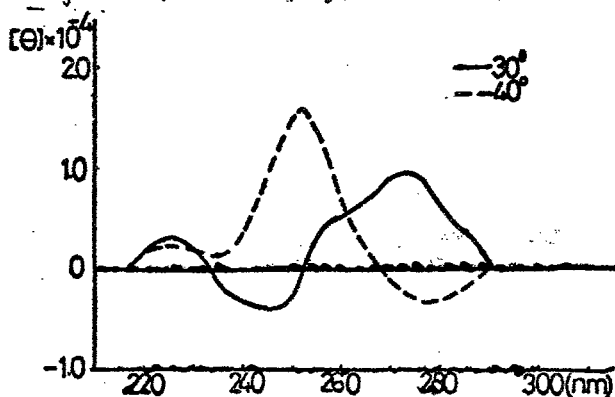
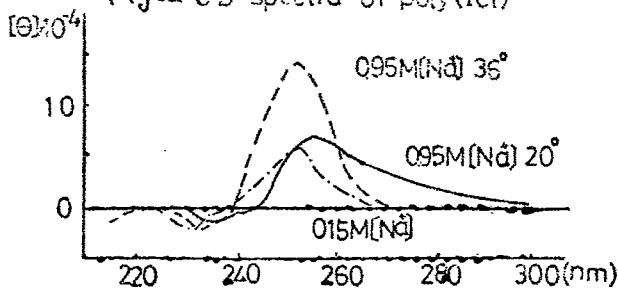


Fig 52 CD spectra of poly(I_c)



- 5 poly(I_c)のCDスペクトルはFig 52のようになる。

0.15M Naイオン濃度では、poly(I_c)はpoly(I_f)にみられるようなCDスペクトルは観察されず、random coil状態で存在しているものと思われる。又、高塩濃度においても、poly(I_f), poly(I)に観察される長波長側における負のcotton効果はみられない。何らかのaggregationによるものと思われる長波長側の正のcotton効果がみられる。温度を

上げるとこれは消失し、 0.15M Na イオン濃度におけるものとよく似た CD スペクトルを示す。これもやはり、random coil 状態を反映しているものと思われる。このように poly(Ice) は、 poly(I) 、 poly(If) にみられる高塩濃度における 4 本鎖の形成、あるいは poly(If) にみられる低塩濃度における self ordered structure の形成いづれも観測できなかった。従って、 poly(Ice) は self complex を形成し難いことがわかった。これは 2' 位 フロールの存在による self structure の不安定化によると考えられる。 poly(Ar) の場合と同様、2' 位 ハロゲンの size が大きくなり、電気陰性度が小さくなるほど self ordered structure がとり難くなることが判る。

・ poly(C) との complex 形成とその安定性

0.15M Na イオン濃度、 $\text{pH } 7.02$ poly(If) と poly(C) と mixing すると Fig 53 のように poly(If) 50% のところには屈曲点が生じ、この条件下、 poly(If) poly(C) 1:1 の complex が形成されることが判る。この CD スペクトルは Fig 54 のように、 $\text{poly(I)} \cdot \text{poly(C)}$ 1:1 complex の CD スペクトルとたいへんよく似ている。

次に poly(Ice) と同様に poly(C) と mixing すると、

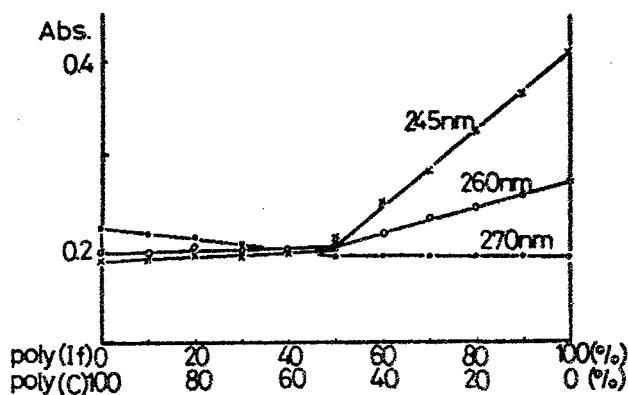


Fig 53. Mixing curves of poly(If) with poly(C)

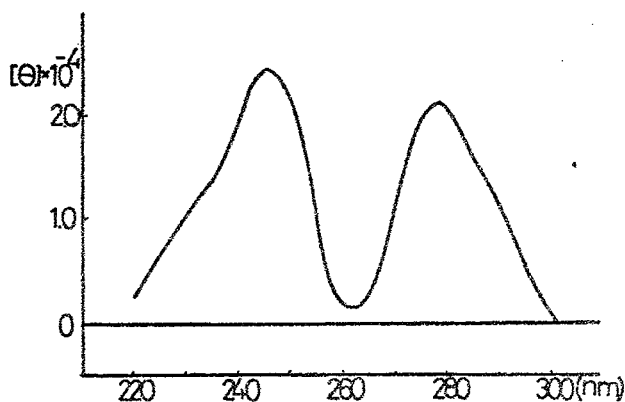
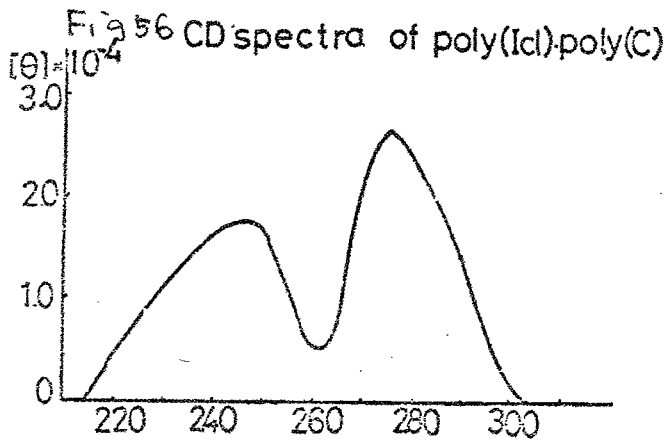
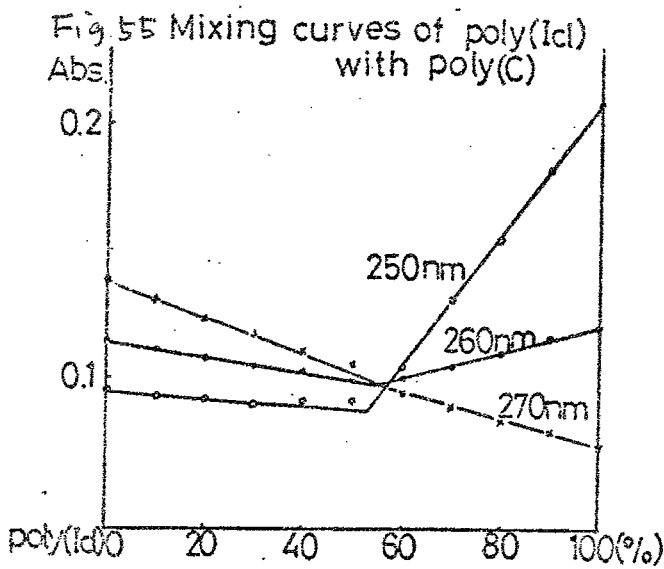


Fig. 54 CD spectrum of poly(If)·poly(C) complex.

Fig 55 のように poly (Ic) · poly (C) : 1:1 complex の形成がみられた。この complex の CD スペクトルも Fig 56 のように poly (I) · poly (C) 型である。

・ poly (Ix) · poly (C) の熱的安定性

poly (It) · poly (C) complex の種々の Na イオン濃度における thermal melting curve は Fig 57 のようになる。



得られた T_m 値を $\log_{10} [Na]$ に対してプロットすると、

Fig 58 のように、いずれの Na イオン濃度においても、poly(I)-poly(C) complex よりはるかに高い T_m 値を示す。このように 2' 位にフッ素が置換することにより、poly(C) との complex が著しく安定化されることが判った。

他の 2'-フッロ体では塩基により、その安定化の傾向は異なっている。poly(U) の場合、poly(U)-poly(A)

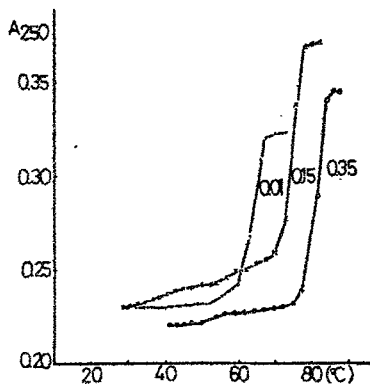


Fig. 57 Temperature absorbance profiles of poly(I)f-poly(C) at various Na^+ concentrations.

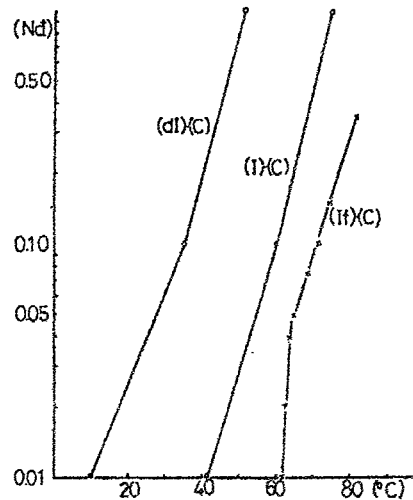


Fig. 58 Relationship between T_m of various double-stranded complexes and Na^+ concentration.

complex は poly(A)·poly(U) complex より、各塩濃度で常に 15 ~ 20°C 高い T_m 値を有し、かなり安定化している。しかし、poly(Cf) ²⁹⁾ と poly(Cf)·poly(I) は poly(I)·poly(C) complex より若干低い T_m 値である。又、前述の poly(Af)·poly(U) complex とは、poly(A)·poly(U) complex とほぼ同じ T_m 値を有している。今回 poly(If)·poly(C) が非常に安定な complex を形成することと考えると、2-7 ポリ体の塩基部にアミ基を有する component に含まれるとき影響は少ないが、塩基部に水素結合における proton acceptor の $\text{C}=\text{O}$ を有する component に含まれるとき、complex の強い安定化が起こる。

- 5. poly(Ice)·poly(C) の complex の熱的安定性

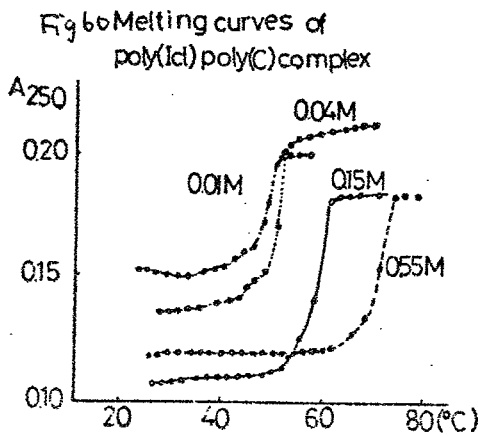
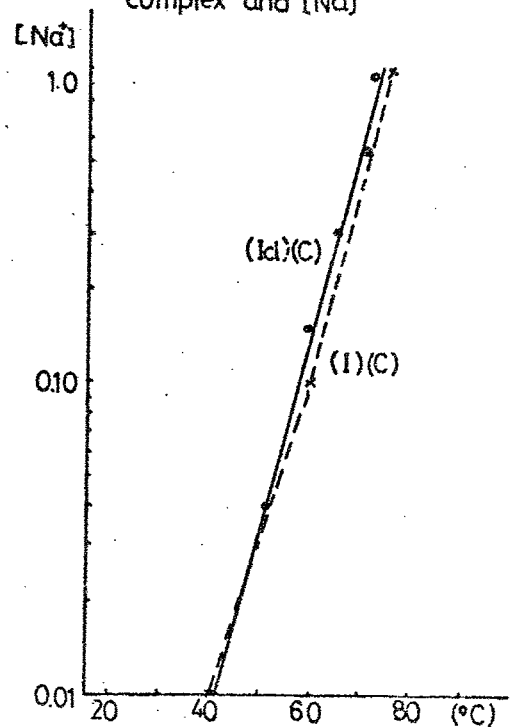


Fig 61 Relationship between T_m of (Icl)(C) complex and $[Na^+]$



について調べた。種々の Na^+ イオン濃度における poly(Icl)・poly(C) complex の thermal melting curve を Fig 60 に示した。この場合にも sharp な melting が観測され、得られた T_m 値を $\log_{10} [Na^+]$ に対してプロットすると Fig 61 のようになる。poly(Icl)・poly(C) complex の熱的安定性はほぼ poly(I)・poly(C) complex のそれと同じである。ペリリジン系においては³⁰⁾ poly(Ucl), poly(Ccl) は、各々相補的な poly(A) あるいは poly(I) と complex を形成し、その T_m 値は、対応する poly(A)・poly(U) 及び poly(C)・poly(I) より若干高いと報告されている。このように、2価に

Cl を置換しても, complex 形成能やその安定性には
大きな差はみられない. 2. $\text{poly}(\text{I}^{\text{I}})$ のそれと比較すると
 $\text{poly}(\text{I}^{\text{Ce}})$ 2-IT. complex の安定性が低下している.
 $\text{poly}(\text{A}^{\text{x}})$ の場合と同様に. $\text{poly}(\text{I}^{\text{x}})$ においても.
2' 位のハロゲンの電気陰性度が大きく. size の小さいほど
complex の安定性が高くなる.

第四章 2'置換ポリヌクレオチドの 生物活性⁶²⁾⁶⁴⁾⁶⁵⁾

第1節 インターフェロン誘導活性

二本鎖 RNA の興味ある生物活性のうちの一つにインターフェロン誘導活性があげられる。インターフェロンとは、細胞が特定の物質(インターフェロンインデューサー)の刺激を受け³¹⁾³²⁾産生する、分子量 2-3 万程度の糖蛋白質³¹⁾³²⁾で、細胞に作用して抗ウイルス作用と起させる働きがある。又、抗ガン作用、抗細胞増殖作用、生体の免疫能の促進、及びその阻害等の多くの生理作用を有し、生体防衛機構の解明、臨床応用への期待がもたれる興味ある物質である。

インターフェロンはウイルスの干渉現象から発見され³³⁾、インターフェロン誘導の活性を有するものはウイルス核酸であろうと考えられた³¹⁾³³⁾。実際にインターフェロン誘導能をもつ二本鎖 RNA が発見され³⁴⁾、合成二本鎖 RNA が³⁵⁾インターフェロンを誘導することが判った。その後、種々の合成 RNA のインターフェロン誘導能を検討した結果、poly(I)-poly(C)が最も高い誘導能力を有することがわかった³⁵⁾。しかし、poly(I)-poly(C)の臨床への応用に、その毒性、トランス等が問題となる。

そこで、多くのポリヌクレオチドアログについて、インターフェロン誘導活性が調べられ、インターフェロン誘導に必要な条件と³⁶⁾³⁷⁾は以下の通りである。

- i)二本鎖RNAが最も活性が高く、一本鎖、三本鎖はこれより活性が下る。³⁸⁾
- ii) 10⁶以上の/かき量を必要とする。³⁹⁾
- iii) T_m値、そしてRNaseに対する抵抗性が高い。
- iv) 塩基部の窒素とCHに変化させると活性を失う。⁴⁰⁾
- v) 両方の鎖の2'水酸基は不可欠である。

といったことがあげられてきた。

このうち v) については、これまでの種々の2'置換ポリヌクレオチドを/かき complex についての報告にもとづいてみる。

おもに、poly(A)・poly(U)型 complex については、⁴¹⁾ A・U⁴²⁾, ⁴³⁾ A・U⁴⁴⁾, ⁴⁵⁾ A・U⁴⁶⁾, ⁴⁷⁾ A・U⁴⁸⁾, ⁴⁹⁾ A・U⁵⁰⁾, ⁵¹⁾ A・U⁵²⁾, ⁵³⁾ A・U⁵⁴⁾, ⁵⁵⁾ A・U⁵⁶⁾, ⁵⁷⁾ A・U⁵⁸⁾, ⁵⁹⁾ A・U⁶⁰⁾ である。又、poly(I)・poly(C)系では、⁶¹⁾ I・dC, ⁶²⁾ I・C⁶³⁾, ⁶⁴⁾ I・C⁶⁵⁾, ⁶⁶⁾ I・C⁶⁷⁾, ⁶⁸⁾ I・C⁶⁹⁾ である。これらすべての complex は、いずれもインターフェロン誘導活性は低下しているか、全く消失している。

今回、前章までに/かき成した poly(A₂), poly(I₂), poly(AI), poly(II) について、それらの相補的二本鎖 complex についてインターフェロン誘導活性を調べた。Table 10は、

Table 10

Interferon inducing activity of various 2'-azido analogues of (A)n-(U)n and (I)n-(C)n

System	polynucleotide	interferon titer**		
		0.1 μ g/ml	1 μ g/ml	10 μ g/ml
Primary rabbit kidney cells * superinduced" with cycloheximide and actinomycin D	(A)n-(U)n*	$\log_{10}(\text{units/ml})$		
	(A)n-(rT)n			2.9
	(A)n-(Uz)n			3.4
	(Az)n-(U)n			< 1.0
	(Az)n-(rT)n			< 1.0
	(Az)n-(Uz)n			< 1.0
	(I)n-(C)n			3.8
	(I)n-(br5C)n			3.9
	(Iz)n-(C)n			3.3
	(Iz)n-(br2C)n			< 1.0
	(I)n-(Cz)n			< 1.0
Human skin fibroblast cells "primed" with interferon and "superinduced" with cycloheximide and actinomycin D	(I)n-(C)n	3.0	3.7	3.9
	(I)n-(brC)n	3.5	3.9	4.2
	(Iz)n-(C)n	3.0	3.7	4.1
	(Iz)n-(brC)n	< 1.3	< 1.3	< 1.3
L-929 cells 'primed' with interferon	(I)n-(C)n	1.5	2.3	2.3
	(I)n-(brC)n	1.5	1.8	2.0
	(Iz)n-(C)n	< 0.5	0.8	1.0
	(Iz)n-(brC)n	< 0.5	< 0.5	< 0.5
Intact rabbits	(I)n-(C)n	-	3.8	4.7
	(I)n-(brC)n	-	3.5	4.7
	(Iz)n-(C)n	-	< 1.0	1.7

* 表中 (A)n-(U)n は poly(A) · poly(U) complex を示す。以下同様

** VSD の生育を 50% 阻害する検体の希釈率をもつ interferon titer とする。

2'-アジド・ア+Uグ"について示した。poly(Az) を含む
complex は titer が 1 以下で、ほとんど活性を示さない。

しかし、poly(Iz) · poly(C) complex は Human の系で、

poly(I) · poly(C) complex とほとんど同じか又は高い活性
を示している。しかし他の系では若干低くなっている。又、

poly(I) · poly(brC) complex は poly(I) · poly(C) complex
より高い活性を有するのに対し、poly(Iz) · poly(brC) 2' は
poly(Iz) · poly(C) より活性が低い。

Table 11

Interferon inducing activity of poly(dIf)-derived complexes in different systems

Polynucleotide	Interferon titer(log 10 units/ml) obtained at polynucleotide concentration of		
	0.1 μ g/ml.	1 μ g/ml.	10 μ g/ml.

1. Primary rabbit kidney cells "superinduced" with cyclohexamide and actinomycin D.

poly(I)-poly(C) ₅	2.5	3.8	4.2
poly(I)-poly(br ⁵ C)	2.2	2.2	3.0
poly(If)-poly(C) ₅	3.9	4.1	4.3
poly(If)-poly(br ⁵ C)	2.7	3.0	3.5
poly(A)-poly(U)	1.7	3.2	3.4
poly(A)-poly(rT)	3.5	4.2	3.9
poly(A)-poly(br ⁵ U)	< 1.0	< 1.0	< 1.0
poly(Af)-poly(U)	< 1.0	< 1.0	< 1.0
poly(Af)-poly(rT)	< 1.0	< 1.0	1.1
poly(Af)-poly(br ⁵ U)			< 1.0

2. Human skin fibroblast cells "primed with interferon and superinduced" with cyclohexamide and Actinomycin D.

poly(I)-poly(C) ₅	4.1	4.1	4.1
poly(I)-poly(br ⁵ C)	4.3	4.1	4.1
poly(If)-poly(C) ₅	4.1	4.2	4.3
poly(If)-poly(br ⁵ C)	3.8	3.4	3.5
poly(A)-poly(U)	4.1	4.3	4.2
poly(A)-poly(rT)	4.1	4.1	4.0
poly(A)-poly(br ⁵ U)			< 1.0
poly(Af)-poly(U)	1.9	2.9	2.9
poly(Af)-poly(rT)	< 1.0	2.5	2.0
poly(Af)-poly(br ⁵ U)			< 1.0

3. Mouse L-929 cells pretreated with DEAE-dextran

poly(I)-poly(C) ₅	2.11	3.65	3.68
poly(I)-poly(br ⁵ C)	< 0.5	2.29	3.66
poly(If)-poly(C) ₅	< 0.5	< 0.5	< 0.5
poly(If)-poly(br ⁵ C)	< 0.5	< 0.5	< 0.5

4. Mouse L-929 cells primed with interferon

poly(I)-poly(C) ₅	1.32	1.67	2.00
poly(I)-poly(br ⁵ C)	0.84	1.03	1.32
poly(If)-poly(C) ₅	1.68	2.00	2.36
poly(If)-poly(br ⁵ C)	< 0.5	< 0.5	< 0.5

5. Intact rabbits

poly(I)-poly(C)	at 1 h.	2.2	2.5
	at 2 h.	4.5	4.7
	at 4 h.	3.3	3.3
	at 7 h.	2.2	1.8
poly(If)-poly(C)	at 1 h.	< 1.0	1.7
	at 2 h.	3.5	3.7
	at 4 h.	3.0	2.9
	at 7 h.	2.2	2.2

poly (A⁺), poly (I⁺) について調べた結果が Table 11 である。poly (A⁺) と含⁺ complex は 1 の系でその活性が著しく低い。2 の系で活性は示すが、対応する poly (A) と含⁺ complex より低い値を示している。一方 poly (I⁺) · poly (C) complex は、1, 2, 4 の系で poly (I) · poly (C) complex より高い活性を有している。特に 4 の系と著しい。

この結果は、インターフェロン誘導活性には、2' 水酸基は必須でないことを示すものであり、インターフェロンを誘導するレセプター部位による二重鎖 RNA の認識には二重鎖全体の構造が重要であることが判る。

又、Human の系で活性の高い poly (I⁺) · poly (C) complex, poly (I₂) · poly (C) complex は、臨床への応用という点で期待がもたれる。

第 2 節 リバーストランスクリプターゼに対する活性

RNA 腫瘍ウイルスの gene を合成するリバーストランスクリプターゼは Baltimore⁵⁰⁾, Temin⁵¹⁾ によって発見された。その機能は各のように RNA を template とし oligonucleotide を primer に deoxytriphosphate を 1-100 重合することである。

これは多くのポリヌクレオチドアナログの、この酵素に対する

果多量⁵³⁾の⁵⁴⁾に⁵⁵⁾ら⁵²⁾れ⁵³⁾て⁵⁴⁾い⁵⁵⁾る。そのうち $\text{poly}(\text{Am})$, $\text{poly}(\text{Ae})$, $\text{poly}(\text{u}^2\text{A})$, $\text{poly}(\text{C}^3\text{A})$, $\text{poly}(\text{C}^7\text{A})$ のこの酵素の polymerase 活性を阻害すること⁵²⁾が報告⁵³⁾され⁵⁴⁾て⁵⁵⁾い⁵⁶⁾る。

今回、 $\text{poly}(\text{Az})$, $\text{poly}(\text{Af})$ について Murine leukemia virus の reverse transcriptase に対する活性を調べた。

Fig 62 は template の $\text{poly}(\text{A})$, primer の $\text{oligo}(\text{dT})_{12-18}$

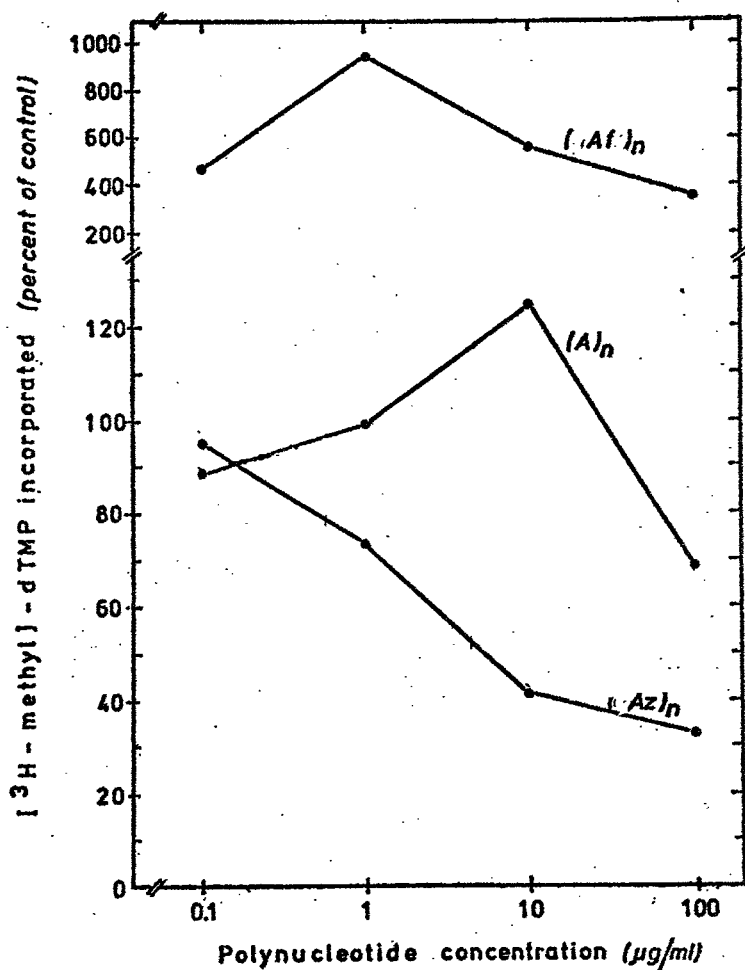


Fig 62 Effect of $\text{poly}(\text{Az})$ and $\text{poly}(\text{Af})$ on MuLV reverse-transcriptase in the absence of $\text{poly}(\text{A})$, $\text{oligo}(\text{dT})$ as template-primer

非存在下での $[^3\text{H}]$ dTMP のとりこみとみたものである。

poly (Af) はこの条件でもとりこみを示している。poly (A)

poly (Az) では低いとりこみ量である。Fig 63 は

primer oligo (dT) 存在下での各 polynucleotide の Template 活性とみたものである。poly (Af) は天然の

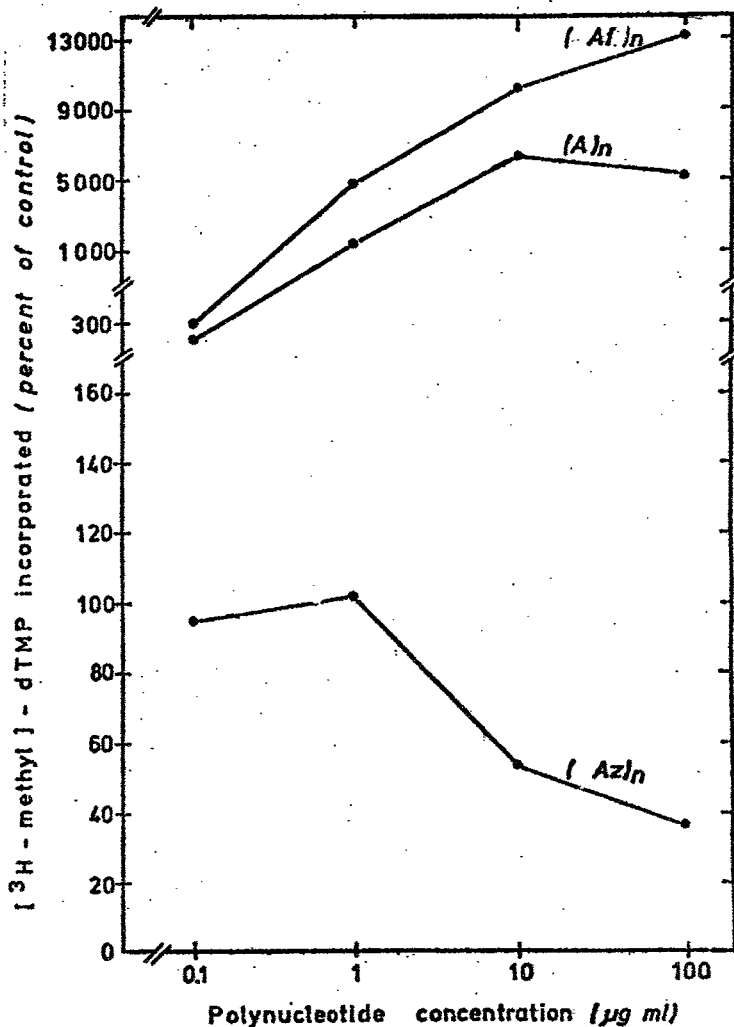


Fig 63 Effect of poly(Az) and poly(Af) on MuLV reverse transcriptase in the presence of oligo(dT) as primer

template 2 ある poly (A) より高い $[^3\text{H}]$ dTMP のとりこみと
 示してあり、より template 活性が大きいといえる。しかし
 poly (Az) はとりこみ量が低く、template 活性が
 ほとんどない。 Fig 64 17 template, primer 存在下での

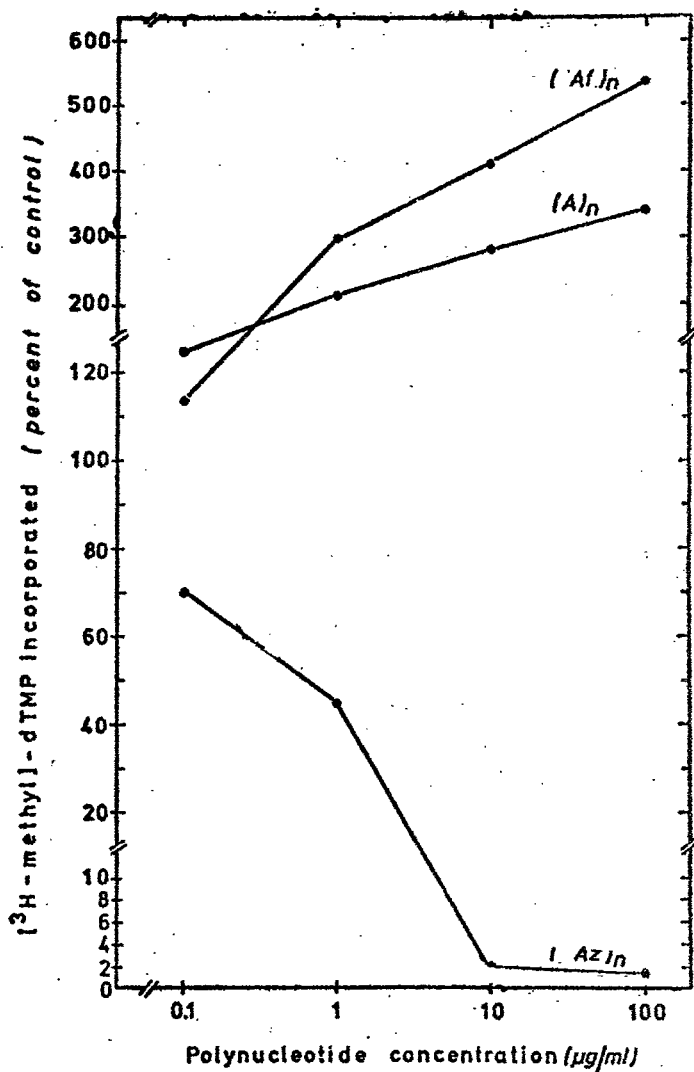


Fig 64 Effect of poly (Az) and poly (Af) on Mulv reverse transcriptase in the presence of poly (A) oligo (dT) as template-primer

阻害作用を調べた結果である。poly(A)⁺はpoly(A)⁻より高いとりこみ量を示し、poly(A)⁺は促進作用を有していることが判る。一方poly(A)⁻はtemplate存在下においても[3H]-dTTPのとりこみが低く、又poly(A)⁻量の増化に伴って低下している。poly(A)⁻は阻害作用を有している。

このように、poly(A)⁺はreverse transcriptaseに對して、poly(A)⁺より高いtemplate活性を有し、poly(A)⁻が強い阻害作用を有していることは、これらのpolynucleotideの構造と機能との関連に於いて興味深い。

結 語

1) アデニン, イノシンの2'位をアジドで置換した $\text{poly}(\text{Az})$ $\text{poly}(\text{Iz})$ では, $\text{poly}(\text{Az})$ が UVスペクトル, CDスペクトル及び $\text{poly}(\text{U})$ との complex の熱的安定性が $\text{poly}(\text{A})$ とよく似ているのに対して, $\text{poly}(\text{Iz})$ は $\text{poly}(\text{I})$ のとりうる self-ordered structure ととり難く, $\text{poly}(\text{C})$ との complex の熱的安定性は, $\text{poly}(\text{I}) \cdot \text{poly}(\text{C})$ $\text{poly}(\text{dI}) \cdot \text{poly}(\text{C})$ complex の中間である。

2) 2'位にアミノ基を導入した $\text{poly}(\text{Aa})$ は弱酸性により, $\text{poly}(\text{A})$ では観察されなかった self-ordered structure を形成する。これは2'位アミノ基の protonate により, 塩基間の stacking の強化によるものと思われる。中性条件では, stacking の程度は $\text{poly}(\text{A})$ より小さく, protonate されない状態での2'位アミノ基は stacking を妨げる。

3) アデニンの2'位にハロゲンを導入した, $\text{poly}(\text{At})$ $\text{poly}(\text{Acl})$, $\text{poly}(\text{Abr})$ では, ハロゲンの電気陰性度

の増大, size の減少に伴って self-structure の stacking の強化, poly(U) との complex の熱的安定性の増大が観察された。

4) イーシンの 2' 位にヒロゲンを導入した poly(I^H), poly(I^{ce}) 2' は, poly(I^H) が, かなり異なる self-ordered structure を形成し, poly(C) と非常に安定な complex を形成するのに対して, poly(I^{ce}) 2' は, self-ordered structure は形成し難く poly(C) との complex の熱的安定性は, poly(I), poly(C) とほとんど同じである。

5) poly(I^z)-poly(C), poly(I^H)-poly(C) complex は poly(I)-poly(C) complex と同程度。又はそれ以上の interferon 誘導活性を有している。これは 2' 置換ポリヌクレオチドとして、はじめての例であり、従来の説を訂正した。

又, poly(A^H) は poly(A) より高い RNA 腫瘍ウイルスの reverse transcriptase の template 活性を有していることが判った。

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実験の部

- ・UVスペクトルの分子吸光係数 $[\epsilon(P)]$, CDスペクトルの分子円率 $[\theta]$ は残基あたりの値を示し、無機リンの定量による残基濃度測定により求めた。
- ・UVスペクトル, CDスペクトルのサンプルは、標準条件として 0.05 M Na cacodylate (pH 7.0) - 0.10 M NaCl の系を用い、buffer solution 中で測定した。又、酸性条件として pH 5.7 以上は Na cacodylate buffer を用い、それ以下は Na acetate buffer を用いた。Na イオンの調整は NaCl 濃度を変化させることにより行った。
- ・UVスペクトルは日立 124 分光光度計、Hitachi Model 200-10 spectrophotometer, 日立 323 分光光度計を用い測定した。
CDスペクトルは JASCO - ORD/UV-spectrophotometer を用いた。
- ・CD サンプルは 10 mm のセル、又は 9 mm の mixing 用セルを用いて、0.5 OD ~ 1.0 OD になるように調整し測定した。

- 各測定用サンプリは *homocomplex* の場合 *buffer solution* 中 20~30 分室温放置後測定を行った。
heterocomplex は特に断らな…限り、一夜放置後測定した。
- T_m 測定は小松電子工業の分光光度計恒温装置 Model SPD-H124 と日立 124 分光光度計にとりつけて測定した。
温度は TAKARA thermister SPD-10 を用いて測定した。
- mixing 実験は、*Nacacodylate* 中 (pH 7.0) total 0.02 ~ 0.08 M 各 *polynucleotide* とそれと別の割合で混合し、UV スペクトルを測定し、行った。
- 電気泳動 (paper electrophoresis PEP) は 0.05M triethyl ammonium bicarbonate (pH 7.5) buffer 中、あるいは 0.2M morpholinium acetate (pH 3.5) buffer 中 35V/cm の条件で行った。相対移動度 (R_m) は adenosine-5'AMP に対する値で表わした。
- paper chromatography (PPC) は下降法で。
溶媒は次のものを用いた。

C↓ : isopropanol - 濃アニモニア水 - 水 (7:1:3)

G↓ : n-butanol - 酢酸 - 水 (5:2:3)

F↓ : ethanol - 1M ammonium acetate (7:3)

M↓ : n-propanol - 濃アニモニア水 - 水 (55:10:35)

- PEP, PPC には東洋口紙 No.51-A を用いた。
- poly (A), poly (C), poly (I), poly (U) は, ADP, CDP, IDP, UDP と PNPase により重合したものを用了。
- polynucleotide phosphorylase は, *M. luteus* は米国シグマ社より購入したものを用了。 *E. coli* PNPase は Grunberg-Manago の方法に従って *E. coli* B より精製した。各段階の精製度は次の通りである。

step	total unit	total protein (mg)	specific activity (unit/mg)
crude extract	6860	4700	1.46
I 硫酸分画	1910	530	3.6
Ca ₃ (PO ₄) ₂ 分画	1620	360	4.5
II 硫酸分画	1600	340	5.0
DEAE-cellulose	600	12	50

assay 条件 ; incubation mixture 0.25 ml

ADP 4 mM, TrisHCl (pH 8.5) 50 mM

MgCl₂ 2 mM, enzyme solution

これを 37°C 20 分 incubate し、allen 法⁵⁵⁾で

遊離される無機リンを定量した。この条件で

1 時間 1 μ mole の無機リンを遊離する酵素活性

を 1 unit と定義する。

また蛋白量は Lowry 法⁵⁶⁾により定量した。

- crude snake venom 中の nucleotidase は鹿児島県立衛生研究所より寄贈されたものである。酵素反応条件は

sample 約 3 μ l TrisHCl (pH 8.5) 100 mM

MgCl₂ 10 mM enzyme 6 μ g/ml

- 2. 37°C 4 時間 incubate し、反応液を PPC に apply し 2 調べた。

- snake venom phosphodiesterase は Worthington Biochem Co. より購入したものをを用い、反応条件は

sample 約 3 μ l TrisHCl (pH 8.5) 50~200 mM

enzyme 1.5 μ g/ml

- 2. 37°C 24 時間 反応させ、反応液を PPC apply し 2 調べた。

第 - 章の実験

2'-azido-2'-deoxyadenosine-5'-monophosphate の合成

氷冷下. triethyl phosphate 1 ml に POCl_3 0.067 ml (0.74 mmole) と加えて攪拌し. これに 2'-azido-2'-deoxyadenosine 107 mg (0.37 mmole) と加える. 1.5 時間攪拌の後. 氷水に於て反応をとめ. 活性炭カラムに apply し. 水洗し. ethanol: H_2O : C.NH_3 50:50:5 混液で溶出する. 溶出液を減圧留去し. 残渣を水にとり. Dowex 1×2 (formate) カラム (φ0.7×5 cm) に apply する. 水洗後. 0.1 N のギ酸で溶出する. 収量 3885 OD_{260} (0.26 mmole) 70%
UV: λ_{max} (at pH 7.0) 259.5 nm

2'-azido-2'-deoxyadenosine-5'-di-phosphate の合成

AEMP 3885 OD_{260} (0.26 mmole) (free) と t-butanol 2.5 ml, H_2O 2.5 ml, morpholine 0.1 ml (1.1 mmole) ととり. reflux する. DCC 235 mg (1.1 mmole) と t-butanol 5 ml にとり. 1 時間:

わたりて滴下する。reflux 3-5時間の後、DCC 120 mg (0.57 mmole), morpholine 0.05 ml (0.57 mmole) を加えて reflux 1時間する。反応の完結をPEPで確認し、冷却の後、析出したDCCureaを除き、溶媒を留去の後、水を加え、ether 3 ml 2-3回抽出する。水層を減圧留去し、残渣をpyridineで数回煮沸し、85% リニ酸 0.05 ml (0.73 mmole), tri-*n*-butyl amine 0.17 ml (0.73 mmole) をpyridineで数回煮沸する。これをmorpholidateと合してpyridine煮沸の後、pyridine 2 ml を加えて室温放置する。2日後、電気泳動でmorpholidateが消失したので、溶媒を留去し、水を約10 ml 加え、1N HCl で酸性にし、活性炭カラムにapplyする。水洗の後、ethanol : H₂O : cNH₃ (50 : 50 : 5) 混液で溶出する。溶出液を減圧留去の後、水にとかし、DEAE Sephadex A-25 (bicarbonate) カラム $\phi 2.0 \times 19$ cm にapplyする。0 M から 0.25 M triethyl ammonium bicarbonate (TEAB) buffer total 4 l の linear gradient で溶出する。main peak は272; 目的の diphosphate は 0.20 M 付近で溶出される。0.14 M で溶出

され3分の1は monophosphate 2-791 OD

AzDPの収量 2290 OD (59%)

UV: $\lambda_{\max}$ (at 259.5 nm) 259.5 nm

poly (2'-azido-2'-deoxyadenylic acid) の合成

AzDP 4 mM, $MgCl_2$ 2 mM, Tris HCl (pH 8.5) 100 mM, PNase (E. coli) 1.9 units/ml Σ total volume 7.3 ml 2-37°C incubate する。0, 2, 4, 6, 20 時間 に 0.25 ml ずつ sampling して無機リンの定量で、反応の進行を check する。20 時間後 isoamyl alcohol: $CHCl_3$ (1:3) 混液で除蛋白の後、凍結乾燥して濃縮し、Sephadex G 50 カラム $\phi 1.7 \times 85$ cm でゲルDした。poly (Az) は void volume に溶出され、51.3 OD 260 (18%) 得た。

2'-azido-2'-deoxyinosine-5'-monophosphate の合成

AzMP 1222 OD 260 (0.081 mmole) Σ 80% 酢酸 2 ml にとかし、 $NaNO_2$ 0.3 g (4.3 mmole) Σ 加える。一夜室温で放置する。pH 3.5 の電気泳動で反応の完結を確認、水でうすめる。活性炭カラムに apply する。

水洗の後. ethanol: H₂O: cNH₃ (50:50:5) 混液で
溶出する。溶媒留去後. Dowex 1×2 (formate) カラム
φ 0.7 × 5.8 cm に apply する。水洗の後. 0.3 N
HCOOH で溶出する。main peak は I 72° あった。

収量 958 mg (0.079 mmole) 97%

UV λ_{max} (pH 7.0) 249 nm

2'-azido-2'-deoxyinosine-5'-diphosphate の合成.

IzMP 958 mg (79 mmole) と t-butanol 2 ml
H₂O. 2 ml, morpholine 0.1 ml (1 mmole) にとり
reflux する。DCC 210 mg (1 mmole) と t-butanol
にとり、30 分にとけて室温下する。reflux 2 時間
後. 反応の完結を電気泳動で確かめ、3 時間後反応を
とめる。冷却後. 溶媒留去し、残渣を水にとり、不
溶物は口去し. ether 抽出 3 回行う。水層を留去し
pyridine 共沸 3 回行う。85% 11-酸 30 ml (0.44
mmole), tri-n-butyl amine 0.1 ml (0.44 mmole)
を pyridine 共沸 3 回行う。両者を混合して. pyridine
共沸、トルエン共沸の後. DMF 1 ml にとり、室温
放置 2 日後. 原料の消失を確かめ、水で DP 2- 反応を

とめる。活性炭カラムに apply し、水洗の後、exhanol
 $H_2O : CNH_3$ (50:50:5) 混液で溶出する。溶媒留去
し、残渣を水にとかし、DEAE Sephadex A-25 (bi-
carbonate) カラム $\phi 1.3 \times 8 \text{ cm}$ に apply する。0 から
0.25 M TEAB buffer total 3 l の linear
gradient で溶出する。main peak は 2 時間で目的
物は 0.16 M に溶出される。0.1 M に溶出されるのは
monophosphate で 60 OD 250

収量 656 OD (53 μmole) 68%

UV λ_{max} (pH 7.0) 249 nm

poly(2'-azido-2'-deoxyinosinic acid) の合成

IzDP 4 mM, $MnCl_2$ 2 mM, TrisHCl (pH 8.5) 80 mM
PUPase (E. coli) 2-4 unit/ml 全量 12.5 ml で
37°C incubate する。1, 2, 3, 4, 16, 17 時間後に
0.25 ml ずつ sampling し、遊離リンの定量によって
反応の進行を check する。17 時間後に isoamyl
alcohol: $CHCl_3$ (1:3) 混液で除蛋白し、凍結乾燥
し、Sephadex G50 カラム $\phi 1.7 \times 100 \text{ cm}$ でゲルに過す
る。poly (Iz) は void volume 付近に溶出される。
これを 0.01 M TrisHCl (pH 7.0) 0.01 M EDTA に透析し、

次に水に対して透析する。凍結乾燥の後 Sephadex G50 カラム $\phi 1.7 \times 110 \text{ cm}$ でゲルD過す。void volume に溶出された poly (I₂) は 75 OD₂₆₀ 14% で得た。

第二章の実験

2'-amino-2'-deoxyadenosine-5'-diphosphate の合成

A₂DP 747 OD₂₆₀ (49 μmole) を水 5 ml, 酢酸 1 ml にとく。palladium charcoal (10%) 20 mg を加え、室温 1 時間接触還元を行った。反応後、palladium charcoal を取り、口液を留め、水煮沸で酢酸を除き、DEAE Sephadex A-25 (bicarbonate) $\phi 1.7 \times 20 \text{ cm}$ カラムに apply する。0.15 M から 0.25 M TEAB buffer total 2 l の linear gradient で溶出する。main peak は 172. 0.20 M に溶出された。

収量 601 OD₂₆₀ (39 μmole) 80.4%

UV λ_{max} 258.5 nm

poly(2'-amino-2'-deoxyadenylic acid) の合成

AaDP 4mM, $MnCl_2$ 2mM, TrisHCl (pH 8.5) 80 mM, PNPase (*M. luteus*) 5 units/ml, 全量 8.75 ml で 37°C incubate した。2, 5, 9, 25, 28 時間後には 0.25 ml あつとり、無機リンの定量によって反応の進行を check した。28 時間後、isoamyl alcohol : $CHCl_3$ (1:3) 混液で除蛋白の後、0.01M TrisHCl (pH 7.0), 0.01M EDTA に透析し、蒸留水に對して透析する。凍結乾燥の後、Sephadex G50 カラム $\phi 1.7 \times 110$ cm でゲル口過す。目的の poly(Aa) は void volume 付近に溶出された。収量 42 mg (13%)

第三章の実験

2'-fluoro-2'-deoxyadenosine-5'-monophosphate の合成

At 40.3 mg (0.15 mmole) と $POCl_3$ 0.1 ml (1.1 mmole) triethyl phosphate 1 ml 氷冷下攪拌中の混液に加え、反応させる。2 時間後、電気泳動で原料の消失を確認。3 時間後に氷水に約 200 ml にあけ反応を止め、活性炭カラム

2' 脱塩する。次に Dowex 1x2 (formate) カラムクロマトグラフィーで分離精製する。peak は 172. 電気泳動で 1 スポットを呈する。

収量 1607 μ g (0.113 mmole) 76%

UV λ_{max} (PH 7.0) 259.5 nm

2'-fluoro-2'-deoxyadenosine-5'-diphosphate の合成

ATMP 1607 μ g (0.113 mmole) (free) と t-butanol 1.5 ml, H₂O 1.5 ml, morpholine 0.1 ml (1.1 mmole) に加え reflux 中。DCC 235 mg (1.1 mmole) と 2 ml の t-butanol に加えたものを滴下。1.5 時間後同量の morpholine と DCC を加え 2 時間 reflux し。冷却後水を加え。不溶物を口去し。ether 抽出 3 回後水層を蒸発乾固し。pyridine 中 3 回行う。80% 硝酸 40 ml (0.58 mmole) と tri-n-butylamine 0.14 ml (0.58 mmole) を pyridine 中 3 回行い両者を混合し pyridine 中 3 回、トルエン中 3 回。DMF 1 ml に加え 31°C 放置する。3 日後反応を止め。活性炭カラムで脱塩の後、DEAE Sephadex A-25 (bicarbonate) カラムクロマトグラフィーで分離精製した。

収量 707 μ g (0.05 mmole) 46%

UV λ_{max} (PH 7.0) 259.5 nm

poly(2'-fluoro-2'-deoxyadenylic acid) の合成

ATP 4 mM, $MgCl_2$ 2 mM, TrisHCl (pH 8.5) 100 mM,
E. coli PNPase 4-5 units/ml 全量 5 ml 2: 37° 24 時間
incubate した。除蛋白の後、凍結乾燥し、Sephadex
G50 カラムでゲル濾過し poly(AT) を得た。

収量 108 mg (0.011 mmole) 55%

2'-chloro-2'-deoxyadenosine-5'-mono-
phosphate の合成

Ace 32.1 mg (0.11 mmole) と $POCl_3$ 50 μ l (0.54 mmole)
triethyl phosphate 2 ml 氷冷下攪拌中の混液に加える。
反応させる。6 時間後に反応を止め、活性炭カラムで脱塩
の後、Dowex 1x2 (formate) カラムクロマトグラフィーによって
分離精製する。

収量 10.12 mg (61%)

UV λ_{max} (pH 7.5)

2'-chloro-2'-deoxyadenosine-5'-diphosphate
の合成

Ace MP 915 mg (61 μ mole) と morpholine 30 ml
(0.35 mmole) H_2O 1 ml t-butanol 1 ml に加える。

reflux 中. DCC 92mg (0.35 mmole) を *t*-butanol
1.5 ml にとかしたものを滴下. 2.5 時間 reflux させ
て. 反応をとめ. 冷却後 後処理を行う. 次に pyridine
2 ml を加え. こけに 80% H_3PO_4 18 μ l (0.26 mmole) tri-
n-butyl amine 62 μ l (0.26 mmole) を pyridine
共沸したものを合せ. さらに pyridine を加え. pyridine
1 ml にとかす. 室温 3 日 放置し. 反応をとめ 後処理し.
DEAE Sephadex A-25 (bicarbonate) カラムで
フーリー-2 分離精製した.

収量 330 OD (22 μ mol) 25%

poly (2'-chloro-2'-deoxyadenylic acid) の合成

Ace DP 4 mM, $MgCl_2$ 2.2 mM, Tris HCl (pH 8.5)
66 mM PNPase (E. coli) 3.9 units/ml 全量 4.5 ml
とし. 37°C 24 時間 反応させる. 除蛋白の後. Sephadex
G50 gel filtration を行う.

収量 45 OD 260 (4.4 μ mol) 25%

2'-bromo-2'-deoxyadenosine-5'-mono-
phosphate の合成

Abr 27.4 mg (83 μ moles) Σ POCl₃ 0.1 ml (1.1 mmole) triethyl phosphate 1 ml 氷冷下攪拌中の溶液に加え、攪拌下反応させる。4時間後、氷水にあり活性炭カラムで脱塩する。次にDowex 1 $\times$ 2 (formate) カラムクロマトグラフィーで分離精製する。

収量 790 α D260 (53 μ moles) 63%

2'-bromo-2'-deoxyadenosine-5'-diphosphate の合成

AbrMP 790 α D260 (53 μ moles) Σ morpholine 75 μ l (0.86 mmole), t-butanol 1 ml, H₂O 1 ml にとり加熱下、DCC 176 mg (0.86 mmole) Σ t-butanol 1.5 ml にとりかしたものと混ぜ、4時間還流し、室温放置した後、後処理する。pyridine 共沸し、80% 11-酸 30 μ l (0.4 mmole), tri-n-butyl amine 0.1 ml (0.4 mmole) Σ pyridine 共沸したもの Σ 混ぜさらに pyridine 共沸、トルエン共沸の後 DMF 1 ml 加えて室温放置する。3日後、反応をとめ、活性炭カラムで脱塩し、DEAE Sephadex A-25 カラムクロマトグラフィーで分離精製する。

収量 377 OD₂₆₀ (25 μ mole) 48%

poly (2'-bromo-2'-deoxyadenylic acid) の合成

AbrDP 4mM, MgCl₂ 2mM, TrisHCl (pH 8.5) 80 mM, PNPase (E. coli) 4.5 units/ml 全量 6 ml とし 37°C 24 時間反応した。除蛋白の後、Sephadex G50 gel filtration した。

収量 33 OD₂₆₀ (13%)

2'-fluoro-2'-deoxyinosine-5'-monophosphate の合成

AtMP 2000 OD₂₆₀ (0.14 mmole) を 30% 酢酸溶液 1.5 ml にとかし、NaNO₂ 0.3 g と 37°C 20 時間反応させる。pH 3.5 の電気泳動で反応の完結を確かめ、活性炭カラムで脱塩した。Dowex (1×2) カラムクロマトグラフィーで分離精製した。

収量 1290 OD₂₅₀ (0.11 mmole) 76%

2'-fluoro-2'-deoxyinosine-5'-diphosphateの合成

IfMP 1290 OD₂₅₀ (0.11 mmole) と t-butanol 1.5 ml morpholine 0.1 ml (1.1 mmole) H₂O 1.5 ml にとかし
OD 減下. DCC 235 mg (1.1 mmole) と t-butanol
2 ml にとかしたものと混ぜ. 3-5時間還流し. 冷却し
役処理する. pyridine 共沸し. 80% リニ酸 40 μl
(0.58 mmole) と tri-n-butylamine 0.14 ml
(0.58 mmole) と pyridine 共沸したものと混ぜ. トルエン
共沸し. DMF 1 ml にとかし 32°C で放置する. 2日
後. 反応をとめ. 脱塩の後 DEAE Sephadex A-25
カラムでグルコースで分離精製する.

収量 745 OD₂₆₀ (65 μmole) 59%

UV λ_{max} (pH 7.0) 248 nm

poly (2'-fluoro-2'-deoxyinosinic acid)の合成

IfDP 4 mM, MgCl₂ 2 mM, TrisHCl (pH 8.5) 100 mM,
PNPase (E. coli) 5.8 units/ml 全量 7.5 ml 37°C
24時間反応した. 除蛋白の後. Sephadex G50
gel filtration した.

収量 35 OD₂₅₀ (3.6 μmole) 12%
90

2'-chloro-2'-deoxyinosine-5'-monophosphate の合成

AceMP 2000 2260 (0.13 mmole) に 30% 酢酸溶液中 NaNO_2 0.3g と反応する。37°C 6時間、室温 20時間後、0.1g NaNO_2 を追加し、室温 4時間反応する。活性炭カラムで脱塩の後、Dowex 1×2 カラムクロマトグラフィーで分離精製した。

収量 1236 2250 (0.10 mmole) 78%

UV λ_{max} (pH 7.0) 248 nm

2'-chloro-2'-deoxyinosine-5'-diphosphate の合成

IceMP 1112 22 (0.091 mmole) に morpholine 80 μl (0.9 mmole) *t*-butanol 1 ml, H_2O 1 ml にとかれ、加熱下、DCC 186 mg (0.9 mmole) に *t*-butanol 1.5 ml にとかしたものと加え、4時間置流の後、冷却し、後処理する。pyridine 共沸し、85% 11-酸 0.09 ml (1 mmole), tri-*n*-butylamine 0.238 ml (1 mmole) に pyridine 共沸したものと加え、さらに pyridine 共沸トルエン共沸し、DMF 2 ml にとかして室温放置する。2日後、反応を止め、活性炭カラムで脱塩し、DEAE

Sephadex A-25 カラムクロマトグラフィーで分離精製
す。

収量 711 mg (57 μ mole) 63%

u v (PH 7.0) λ_{max} 258 nm

poly (2'-chloro-2'-deoxyinosinic acid) の合成

IClDP 4 mM, $MgCl_2$ 2 mM, Tris HCl (PH 8.5) 100
mM, PNPase (*M. luteus*) 40 units/ml 全量 5 ml
とし 37°C 24 時間反応す。除蛋白し。Sephadex
G50 gel filtration す。

収量 31 mg (3.1 μ mole) 16%

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des. XL.¹ Synthesis and properties of poly 2'-azido-2'-deoxyadenylic

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7'-azido-2'-deoxyadenylic acid (Poly Az) was synthesized by polyphosphorylation of 2'-azido-2'-deoxyadenosine diphosphate by polyphosphorylase. Poly (Az) has U.V. absorption similar to poly (A) and hypochromicity of 40% at neutral pH. CD curve also resembled to that of poly (A), but has smaller ellipticity. Titration of poly (Az) with HCl gave a transition at pH 5.5, but exact structure of the acid-form complex was not elucidated. Upon mixing with poly (U), poly (Az) forms 1:1 and 1:2 complexes having T_m 's somewhat higher than that of poly (A)·poly (U) complex in the same condition.

A number of polynucleotides containing analogs of pyrimidine and purine nucleosides have been reported.² Among these polynucleotides, ones which have 2'-substituted nucleosides are especially interesting because they have natural 3'-5'-phosphodiester linkages and are suitable for elucidating physical and biological properties of polynucleotides. However, up to the present they have been limited to only 2'-substituted pyrimidine nucleotides, i.e. 2'-halogeno-,³ azido-,⁴ methoxy-,⁵ and ethoxy-,⁶ compounds. We have found a new method for the synthesis of 2'-azido and 2'-amino-2'-deoxyadenosine and guanosine⁷ by way of purine cyclonucleosides,⁸ which are readily available from the naturally occurring nucleosides. In this communication we report the synthesis of poly 2'-azido-2'-deoxyadenosine (poly (Az)) and its physical properties, such as UV, C.D., T_m and hybridization with poly (U).

MATERIAL AND METHODS

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

2'-Azido-2'-deoxyadenosine (Ia) (63 mg, 0.21 mmole) was dissolved in a mixture of POCl_3 (0.5 ml) and triethylphosphate

Polynucleotides. XL.¹ Synthesis and properties of poly 2'-azido-2'-deoxyadenylic acid

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ABSTRACT

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

A number of polynucleotides containing analogs of pyrimidine and purine nucleosides have been reported.² Among these polynucleotides, ones which have 2'-substituted nucleosides are especially interesting because they have natural 3'-5'-phosphodiester linkages and are suitable for elucidating physical and biological properties of polynucleotides. However, up to the present they have been limited to only 2'-substituted pyrimidine nucleotides, i.e. 2'-halogeno-,³ azido-,⁴ methoxy-,⁵ and ethoxy-,⁶ compounds. We have found a new method for the synthesis of 2'-azido and 2'-amino-2'-deoxyadenosine and guanosine⁷ by way of purine cyclonucleosides,⁸ which are readily available from the naturally occurring nucleosides. In this communication we report the synthesis of poly 2'-azido-2'-deoxyadenosine (poly (Az)) and its physical properties, such as UV, C.D., T_m and hybridization with poly (U).

MATERIAL AND METHODS2'-Deoxy-2'-azidoadenosine 5'-diphosphate

2'-Azido-2'-deoxyadenosine (Ia) (63 mg, 0.21 mmole) was dissolved in a mixture of POCl₃ (0.5 ml) and triethylphosphate

(2 ml) with cooling to 0°. The solution was stirred for 2 hr at 0° and poured in ice-water.⁹ The aqueous solution was applied to a column of charcoal, which was washed with water and eluted with 50% EtOH containing 5% conc. ammonia. Eluents were evaporated in vacuo and the residue was dissolved in water. The aqueous solution was applied to a column (1.5 x 15 cm) of Dowex 1 x 2 (formate). After the water-wash the column was eluted with 0.1N formic acid to give 2'-azido-2'-deoxy AMP (2160 OD₂₆₀ units, 0.15 mmole) in a yield of 73%. UV: $\lambda_{\text{max}}^{\text{pH}2}$ 257 nm, $\lambda_{\text{max}}^{\text{pH}12}$ 259 nm.

2'-Azido-2'-deoxy AMP (0.15 mmole) was dissolved in a mixture of H₂O (1.5 ml), t-BuOH (1.5 ml) and morpholine (0.058 ml, 0.6 mmole). A solution of dicyclohexylcarbodiimide (124 mg 0.6 ml) in t-BuOH (2.25 ml) was added dropwise into the solution while refluxing.¹⁰ The refluxing was continued for 2 hr, the mixture was evaporated in vacuo, and the residue was equilibrated in a H₂O-ether (1:1) mixture. Insoluble material was filtered off and the aqueous layer was separated and evaporated. The residue was azeotropically dried with pyridine several times. To the residue, inorganic phosphate (0.04 ml, of 95%), which was previously dried by azeotropical evaporation with pyridine together with tri-n-butylamine (0.14 ml) and dissolved in pyridine (1 ml), was added. The reaction mixture was kept at 30° for 5 days. The reaction mixture was evaporated in vacuo, the residue dissolved in H₂O, and applied to a column of charcoal. The column was washed with H₂O and eluted with 50% EtOH containing 5% conc. ammonia. Eluents were concentrated and applied to a column (1.0 x 17.5 cm) of DEAE-Sephadex A 25. Elution was performed with 0.1-0.3 M triethylammonium bicarbonate buffer (total 2 l) in a linear gradient and 15 ml fractions were collected. Fractions No. 65-85 were pooled and evaporated. 2'-Azido-2'-deoxy ADP (760 OD₂₆₀ units, 35%) was obtained. UV: $\lambda_{\text{max}}^{\text{pH}2}$ 257 nm, $\lambda_{\text{max}}^{\text{pH}7}$ 259.5 nm, $\lambda_{\text{max}}^{\text{pH}12}$ 260 nm. Paper electrophoresis: R_{AMP} 1.44.

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

2'-Azido-2'-deoxy ADP (4 mM), polynucleotide phosphorylase (2.2 units /ml), Tris-HCl (pH 8.5) 100 mM and MgCl₂ or MnCl₂ 0.4 mM were adjusted to 0.25 ml with H₂O and incubated at 37°

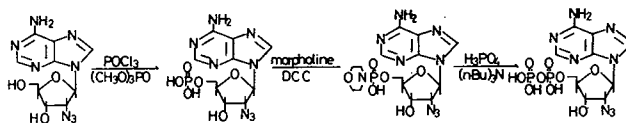
for 6 hrs. Pi release was 0.27 μmole (34%) or 0.33 μmole (41%) in the presence of Mg^{2+} or Mn^{2+} respectively.

A large scale incubation was performed in a total volume of 7.3 ml containing the same ingredients in same concentrations. After 20 hrs the mixture was deproteinised with isoamylalcohol- CHCl_3 (1:3) and the aqueous layer was evaporated. The residue was dissolved again in water and applied to a column of Sephadex G-50. Poly (Az) was eluted as a symmetrical peak in the void volume. The yield was 51.3 OD_{260} units (5.5 μmoles , 18%). AzDP (17.2 μmoles , 57%) was recovered. UV: $\lambda_{\text{max}}^{\text{pH } 7.0}$ 256 nm ($\epsilon = 8,900$). This sample was completely hydrolyzed with snake venom phosphodiesterase to give only 2'-azido-2'-deoxy A5'p. UV spectra were taken with a Hitachi 124 spectrophotometer in the presence of 0.1 M NaCl and 0.05 M Na cacodylate (pH 9.0) at 12°. CD spectra were taken with a JASCO ORD/UV-5 spectrometer equipped with a CD attachment in the presence of 0.1M NaCl and 0.05M Na cacodylate (pH 7.0) at 16°. Melting temperature was measured in the presence of 0.1M NaCl and 0.05M Na cacodylate at pH 7.0 with a Hitachi spectrophotometer equipped with a thermostated cell. The temperature inside the cell was measured with a thermocouple. Mixing curves were obtained by measuring the absorbance of mixture which contained 0.04 mM total concentration of poly (Az) and poly (U) in the ratios indicated in Fig 6. Salt concentration was 0.1 M NaCl and pH was adjusted to 7.0 with 0.05M Na cacodylate.

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

RESULTS AND DISCUSSION

As in the case with 2'-substituted pyrimidine nucleoside 5'-diphosphates⁴ the polymerization reaction of AzDp proceeds better in the presence of Mn^{++} than in the presence of Mg^{++} . The large scale preparation was thus performed with Mn^{++} ion and the time course is presented in Fig 1. The inorganic phosphate liberated was more than 50% after 20 hrs incubation, but the isolated yield of poly (Az) was only 18%. Probably



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

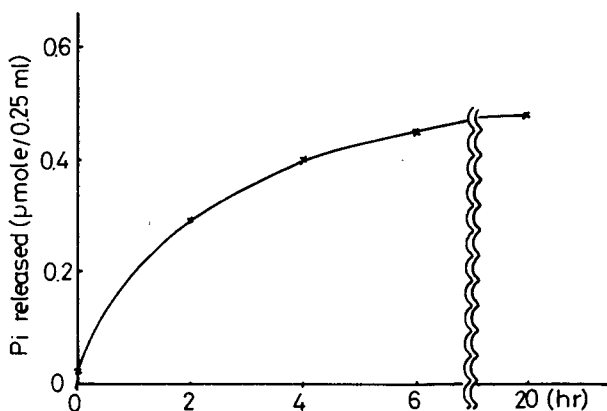


Fig. 1. Time course of polymerization of AzDP

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

UV Absorption of Poly (Az)

UV absorption of poly (Az) is shown in Fig 2. At pH 7.0 in the presence of 0.15M Na^+ , it shows λ_{max} at 256 nm. A blue shift of 4 nm of the λ_{max} compared to that of monomer was observed. Hypochromicity at neutrality was calculated as 40% assuming ϵ of the monomer, 2'-azido-2'-deoxyadenosine as 14,800.⁷ This hypochromicity is larger than that of poly (A) (37%) under the same conditions.¹¹ It is also larger than those of poly (2'-O-methyl A)¹² and poly (2'-O-ethyl A).¹³ Since poly (dA) has hypochromicity of 41%,¹⁴ the effect of 2'-substitution on hypochromicity follows the order, $\text{H} > \text{N}_3 > \text{OH} > \text{OMe} > \text{OEt}$. This cannot be explained solely in terms of steric distortion by 2'-groups of the vertical stacking

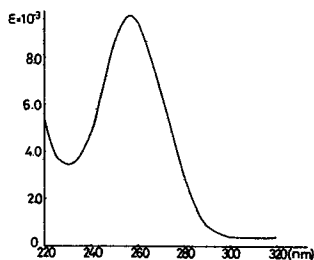


Fig. 2. UV absorption spectrum of poly (Az) at pH 7.0.

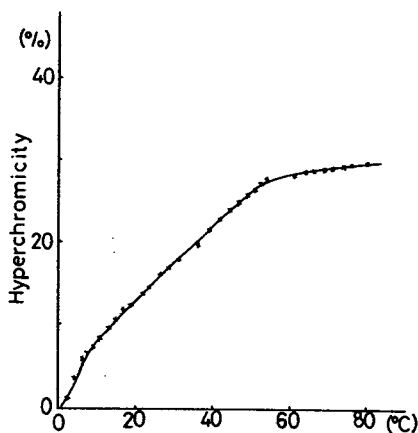


Fig. 3. Temperature-absorption profile of poly (Az) at pH 7.0.

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

Temperature-absorbance profile

The temperature-absorbance profile of poly (Az) in neutral conditions is shown in Fig 3. The UV absorption increased gradually on heating from 0° to 80° without showing any steep increase indicative of cooperative melting. As was observed for poly (A)¹¹ and poly (m²A),¹⁵ this curve suggests a flexible structure of poly (Az), which is only stabilized by the vertical stacking. This phenomena is in sharp contrast with that observed for poly (Uz).⁴ In the latter case a steep rise of the temperature-absorbance profile was observed and the T_m was reported to be 12° in the presence of 0.01M MgCl₂. This different influence of the azido group when introduced to the 2'-position of polynucleotides of purine and pyrimidine may be interpreted as indicating a difference in conformations of both types of polynucleotides stabilized by

different stacking interactions.

CD spectra of poly (Az)

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

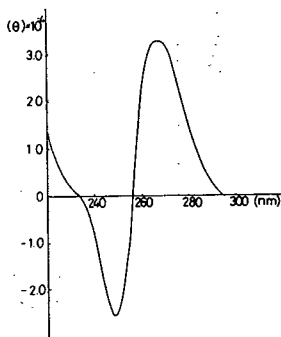


Fig. 4. CD spectrum of poly (Az) in the presence of 0.15 M Na^+ at pH 7.0 and 16° .

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

As was discussed previously¹⁷, the magnitude of rotatory strengths reflects both the stacking tendency and direction of adjacent bases. It may be deduced that in the molecule of poly (Az), adjacent bases stack rather strongly, but their arrangement is not similar to the case of poly (A). This unusual stacking conformation might be ascribed to steric distortion by azido groups.

Acid titration of poly (Az)

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

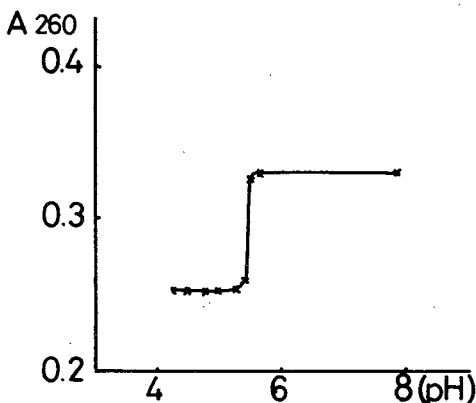


Fig 5. Acid titration curve of poly (Az).

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

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

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

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

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

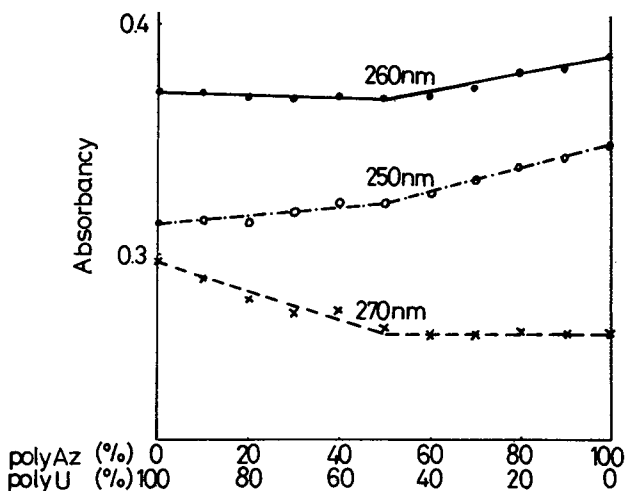


Fig. 6. Mixing curves of poly (Az) and poly (U) at 0.04M Na^+ .

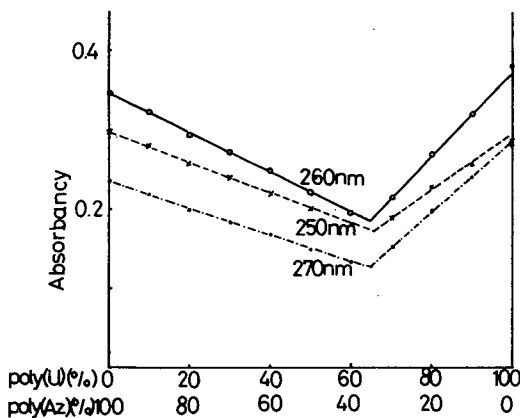


Fig. 7. Mixing curves of poly (Az) and poly (U) at Na^+ concentration of 0.15M.

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

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

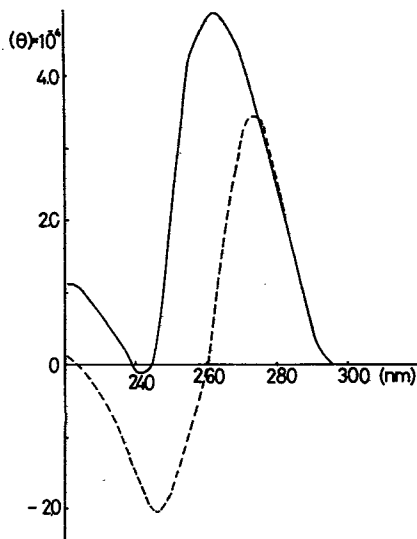


Fig.8. CD curves of poly (Az) plus poly (U) before and after mixing at 0.15 M Na^+ concentration.

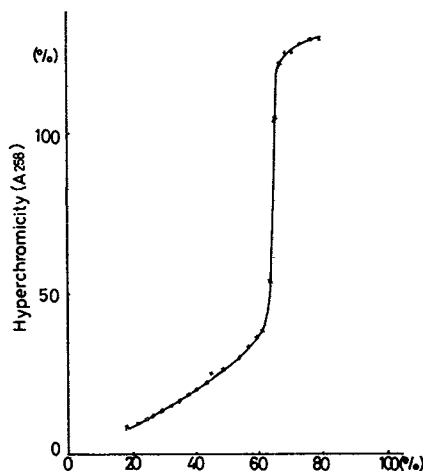


Fig.9. Temperature-absorption profile of poly (Az). poly (U) in the presence of 0.15M Na^+ .

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

When the absorbance of poly (Az)·poly (U) complex was measured at 0.04 M Na^+ concentration and pH 7.0, it rose gradually from 20° to 43° and then steeply until 48°. The curve tapered off after 50°. The hyperchromicity reached around 40%. This melting curve gave a T_m value 46° for the poly (Az)·poly (U) complex. As it was reported previously the poly (A)·poly (U) complex has a T_m of 47° in the same salt concentration¹⁹, this value of poly (Az)·poly (U) complex seems to be reasonable. In this comparison it may be deduced that the effect of an azido group for the stability of a double strand complex is same as an OH group.

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

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

This T_m value of poly (Az) · 2poly (U) is somewhat higher than that observed in poly(A) · 2poly(U) (60°).¹⁹ Since the T_m of poly (dA) · 2poly (U) was reported to be 46°, a comparable effect of 2'-substitution by OH or N_3 for stabilization of the complex can be noted. It is known that the methyl or ethyl group or the 2'-OH of poly (A) reduced the thermal stability of heteroduplexes.¹³ Therefore, not only the size of the 2'-substituent, but also its hydrophilicity must be taken into account for the stabilization of the complex.

Concluding Remarks

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

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

When poly (Az) form a complex with poly (U), it forms a 1:2 complex in 0.15M Na^+ solution. This complex has a T_m higher than that of poly (A) · 2poly (U). The stabilising effect of the azido group seems to be invariable by complexing. This may be because 2'-substitutions are working as a hydrogen-

bond acceptor by virtue of its polarizable nature.

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

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- 3 Burdon, R.H. (1971) in *Progress in Nucleic Acid Research and Molecular Biology*. Vol. II. pp. 33-79. Academic Press. New York

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Polynucleotides. XLVI. ¹ Synthesis and properties of poly (2'-amino-2'-deoxyadenylic acid)

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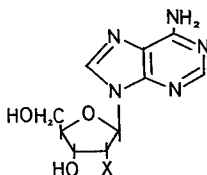
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ABSTRACT

Poly (2'-amino-2'-deoxyadenylic acid) [poly (Aa)] was prepared from chemically synthesized 2'-amino-2'-deoxy-ADP by the catalysis of polynucleotide phosphorylase. Poly (Aa) showed a similar UV absorption spectra to poly(A), but quite different CD spectra at pH 7.0 and 5.7. At the former pH it showed a single negative Cotton band and at the latter a curve with a large splitting of bands. Acid titration of poly(Aa) suggested protonated form below pH 7.0. Temperature absorption profiles and their dependency on sodium ion concentration suggested an ordered structure for poly (Aa) which is stabilized by stacking of bases and intrastrand interaction between 2'-amino and internucleotidic phosphate groups. Poly (Aa) forms a 1:2 complex with poly (U) at neutrality and its T_m was 45° in the presence of 0.15M sodium ion.

INTRODUCTION

Recently a versatile method for synthesizing 2'-azido- and 2'-amino-2'-deoxyadenosine (Chart, X=N₃ and NH₂) was developed.² Using this approach poly (2'-azido-2'-deoxyadenylic acid) [poly(Az)] has been synthesized³ and its properties have been elucidated. It was found that poly(Az) showed



quite similar characteristics to poly(A) in UV and CD spectroscopy, melting temperature (T_m) and complexing with poly(U), in contrast to the previous observation^{4,5} that introduction of an azido group in place of 2'-OH of poly (U) enhanced its thermal stability significantly.

We now wish to report on the synthesis and properties of poly (2'-amino-2'-deoxyadenylic acid) [poly(Aa)]. Interesting features such as the fact that poly(Aa) formed a protonated ordered structure at pHs lower than 7.0 and a triple stranded complex with poly (U) in contrast to its pyrimidine counterpart poly (Ua),^{6,7} are described.

Materials and Methods

2'-amino-2'-deoxyadenosine 5'-diphosphate

2'-Azido-2'-deoxyadenosine 5'-diphosphate³ (747 OD₂₆₀ units, 49.5 umoles) was dissolved in a mixture of water (5 ml) and acetic acid (1 ml). To the solution palladium charcoal (10%, 20 mg) was added and the mixture was stirred under an hydrogen atmosphere for 1 hr at room temperature. The catalyst was removed by filtration and the filtrate was evaporated in vacuo. Traces of acetic acid were removed by azeotropic distillation with water several times. The product showed one spot at R_f 1.07 on a paper electrophoretogram performed at pH 7.0. AaDP thus obtained was applied to a column (1.7 x 20 cm) of DEAE-Sephadex A-25 (bicarbonate form) and eluted with triethylammonium bicarbonate (0.15-0.25M, 1l. each) in a linear gradient. Fractions of 15 ml were collected every 1l min. AaDP was eluted at the buffer concentration 0.2 M. The yield was 601 OD₂₆₀ units (80 %). Paper electrophoresis: R_{pA} 1.07 (at pH 7.5), R_{pA} 1.00 (at pH 3.5). Hydrolysis of this sample with alkaline phosphatase gave exclusively 2'-amino-2'-deoxyadenosine and inorganic phosphate.

Poly (2'-amino-2'-deoxyadenylic acid) [poly (Aa)]

A solution (5 ml) containing 2'-amino-2'-deoxyadenosine 5'-DP 4 mM, MnCl₂ 2 mM, polynucleotide phosphorylase obtained from Micrococcus luteus⁸ by a method described by Klee and Singer⁹ 45 units/ml and TrisHCl (pH 7.5) 80 mM was incubated at 37° for 20 hrs. The time course of the polymerization is shown in Fig.1.

The viscosity of the solution increased significantly during the incubation. The incubation mixture was extracted with a mixture of isoamylalcohol-CHCl₃ (1:3, vol/vol) for

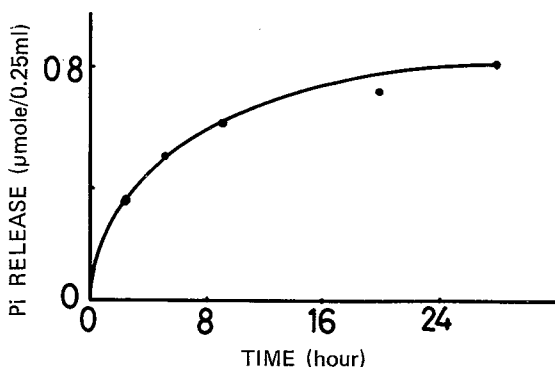


Fig. 1. Time course of AaDP polymerization.

deproteinization and dialyzed against water containing 0.01M Tris-HCl buffer (pH 7.0). The dialyzed solution was lyophilized, the residue dissolved in a small amount of water, and applied to a Sephadex G-50 column. As shown in Fig. 2, poly(Aa) was eluted in the void volume. Yield was 32 OD₂₆₀ units (15 % regardless of hypochromicity).

Physical measurements

UV spectra were taken with a Hitachi 124 spectrophotometer and CD spectra were taken with a JASCO ORD/UV-5 spectrophotometer equipped with a CD attachment. Calibration was performed with d-10-camphorsulfonic acid. T_m's were measured with a Hitachi EPS-3T spectrophotometer equipped with a Komatsu thermostated cell. Temperature inside the cell was measured by a Cu-Constantan thermocouple.

RESULTS AND DISCUSSION

UV spectrum of poly(Aa)

The UV spectrum of poly(Aa) was recorded in the presence

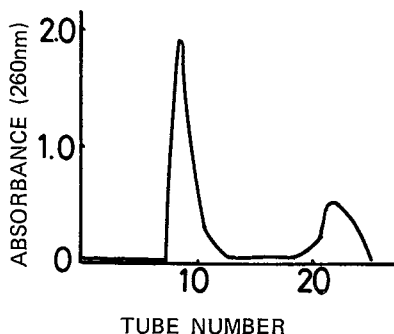


Fig. 2. Sephadex G-50 column chromatography of poly (Aa).

of 0.1M NaCl and 0.05M sodium cacodylate (pH 7.0 and 6.3) at 25°. As shown in Fig 3, poly(Aa) showed a spectrum at neutrality having a single λ max at 258 nm and ϵ equal to 11,200. Although the maximum wavelength was almost identical to that of poly(A)¹⁰, ϵ was somewhat smaller. Since the ϵ value of 2'-amino-2'-deoxyadenosine was reported to be 15,000 at 258.5 nm², hypochromicity of poly(Aa) was calculated to be 25%. This value is significantly smaller than that of poly(A) which was reported to be 39% at 260 nm.¹⁰ This unusually small hypochromicity and negligible hypsochromicity may be due to a structure of poly (Aa) with a lower degree of stacking of adenine bases in the polynucleotide array. At pH 6.3 poly(Aa) showed much lower ϵ value presumably due to an acid form described below.

Acid titration of poly (Aa)

When poly (Aa) was titrated with 0.1N hydrochloric acid in the presence of 0.15 M NaCl at 15°, the UV absorption increased sharply at pH 6.7 and reached a plateau at pH 7.6 (see Fig. 4). The midpoint of the transition was at pH 7.0. The λ max 258 nm of the UV spectra did not change throughout

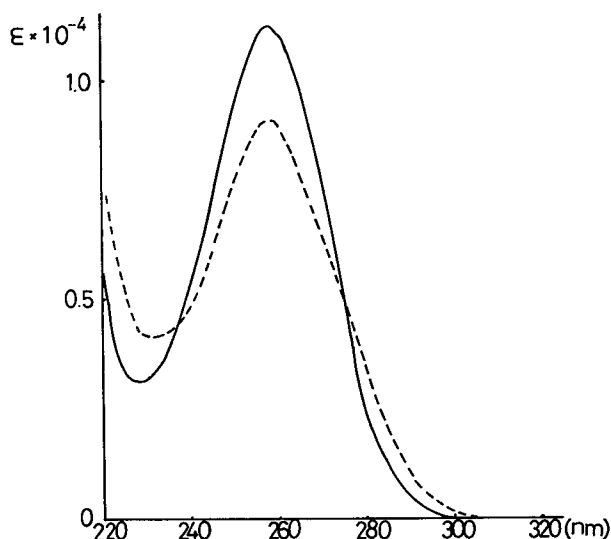


Fig. 3. UV absorption spectra of poly(Aa), — at pH 7.0, --- at pH 6.3.

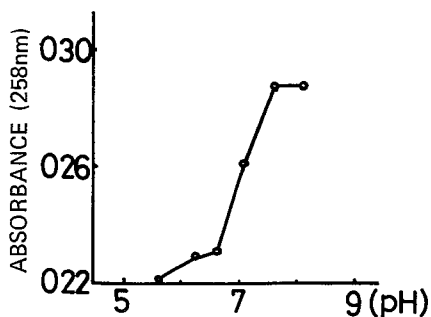


Fig. 4. Acid titration of poly(Aa).

the titration and below pH 5.5 a precipitation occurred, which disturbed the UV measurements. These phenomena imply that poly (Aa) must be completely protonated at the 2'-NH₂ group at around pH 6.7 and stacking of bases may be increased, despite the fact that no protonation at the N¹-atom of the adenine ring as reported in the case of poly(A), occurred.¹¹

CD spectrum of poly(Aa)

As shown in Fig. 5, the CD spectrum of poly (Aa) taken at pH 7.0 and 24° showed a simple curve resembling that of monomer AaDP. If it was taken at pH 5.7 two troughs at 280 and 235 nm and a peak at 252 nm appeared. This fact may suggest that poly (Aa) exists as a random coil structure at pH 7.0 and at pH 5.7 it transformed to an ordered structure upon protonation at the 2'-NH₂ group. This type

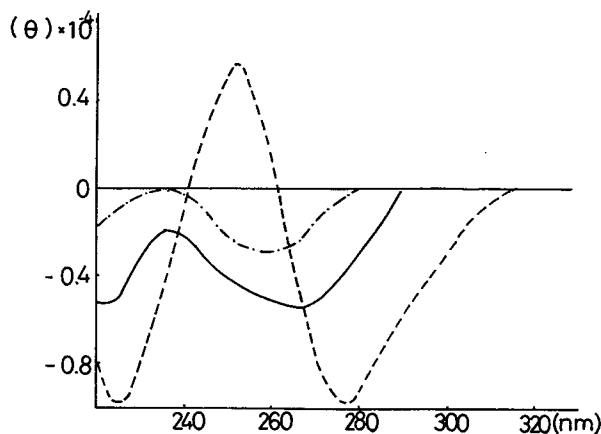


Fig. 5. CD spectra of AaDP and poly(Aa), —poly(Aa) at pH 7.0. ---poly(Aa) at pH 5.7, - · - · - AaDP at pH 7.0.

of structure has not been reported previously with pyrimidine 2'-NH₂ polynucleotides.^{6,7}

To examine the thermal stability of this ordered structure of poly(Aa), CD spectra were recorded at 20°, 46° and 60° in the presence of 0.15M sodium ion at pH 6.3. As shown in Fig 6, the curve at 20° closely resembled that shown in Fig. 5, which had two troughs at 274 and 225 nm and a peak at 252 nm. Raising the temperature to 46° this spectrum changed to one resembling the monomer spectrum and this is also the case for the 60° spectrum. Therefore, the 2'-NH₂ protonated acid form of poly (Aa) at 20° decomposed by thermal perturbation and a random structure might be formed above 46°.

Temperature-absorption profiles of poly (Aa)

The temperature absorption profiles of poly(Aa) at various pH's were recorded in Fig. 7. At pH 7.0 in the

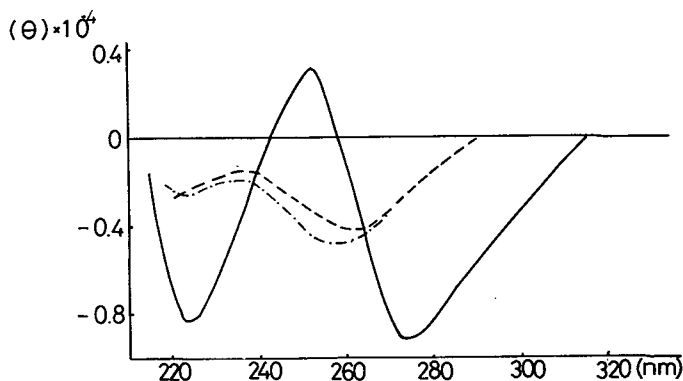


Fig. 6. CD spectra of poly(Aa) taken at 20°(—), 46°(---) and 66°(-.-.-).

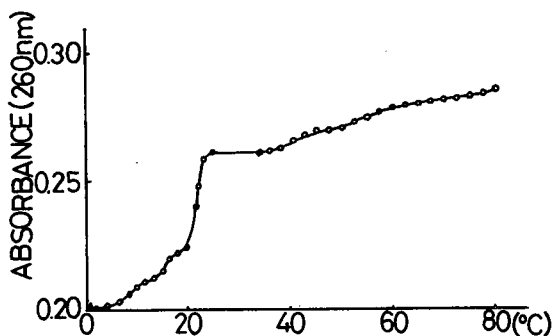


Fig. 7. Temperature-absorption profile of poly(Aa) taken at 7.0.

presence of 0.1M NaCl and 0.05M sodium cacodylate, the absorption steeply increased at 20° and reached a plateau at 24°, then it gradually increased up to 80°. This provides a T_m of 22° at least for the first melting step. When the pH goes down to 6.3 (Fig. 8), poly(Aa) showed a similar type of curve, though the transition point moved to 38°. At pH 5.7 the curve (Fig. 9) showed a clear transition point at 53-55° and about an 80% increase in absorption. This may account for the complete melting of poly (Aa) acid form. At pH 5.0 the polymer precipitated presumably due to double protonation both at the 2'-amino and N¹ of the adenine ring.

These facts imply that by the protonation of poly (Aa) initially at the 2'-amino group with decreasing pH a partially protonated structure was formed and this structure changed to a "fully protonated" one at pH 5.7. Since pK of

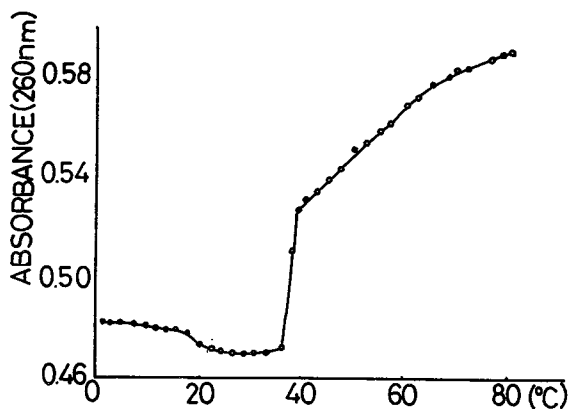


Fig. 8. Temperature-absorption profile of poly(Aa) taken at pH 6.3.

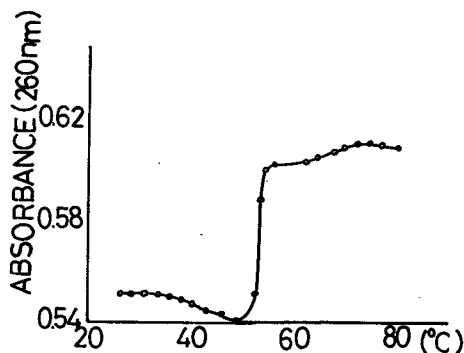


Fig. 9. Temperature-absorption profile of poly(Aa) taken at pH 5.7.

the adenine base is around 3.5^{12} and the λ max 258 of poly (Aa) does not show any change in this pH area, the protonation at the adenine base could be excluded.

Dependency of T_m on cationic concentration

As shown in Fig. 10 the T_m value at pH 7.0 was then measured at various cationic strengths. At 0.04, 0.15 and 0.35 M sodium ion concentration T_m 's were slightly lowered, 28° , 22° and 20° , respectively. This phenomenon is in contrast to the fact that in other polynucleotides T_m increased with increasing cationic concentration.¹³

It may be deduced from this experiment that in the poly (Aa) acid form the ordered structure is stabilized by intramolecular interaction between protonated 2'-amino group and internucleotidic phosphate dissociation. This situation could be observed in a Corey-Pauling-Koltun model of poly (Aa), though the exact structure must await X-ray diffraction study.

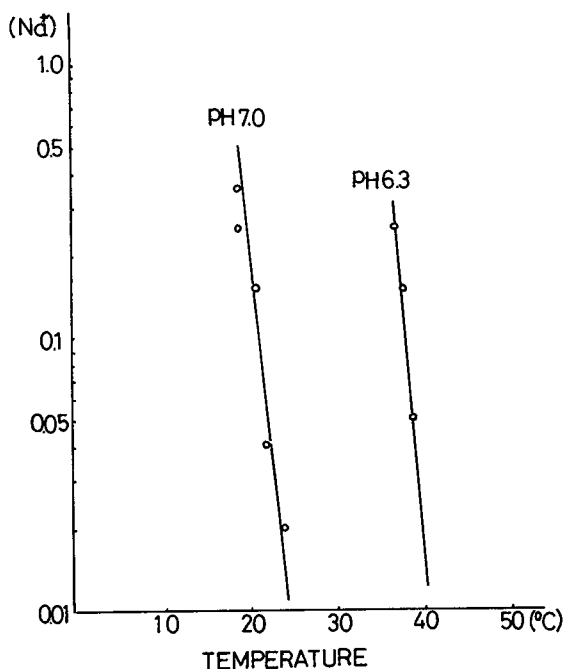


Fig. 10. Depending of T_m of poly(Aa) on sodium ion concentration.

Complex formation of poly(Aa) with poly (U)

A mixing experiment of poly (Aa) with poly(U) was performed at 0.15M sodium ion concentration and pH 7.0. Polynucleotides of 0.04M concentration each were mixed in various ratios, heated to 80° for 2 min and annealed at 20° for 15 hrs. As shown in Fig. 11, inflexion points appeared at a ratio of 1:2 for poly (Aa) vs poly (U) at wavelengths of 250, 260 and 270 nm. This indicated that a three-stranded complex poly (Aa)·2poly(U) was formed in these conditions as in the case of poly (A)·2poly(U).¹⁴ Separate experiments at 0.04M sodium ion concentration gave fluctuating curves and no definite results could be obtained.

Formation of this type of complex was further supported by the CD spectrum of poly (Aa) 2 poly (U) as shown in Fig. 12

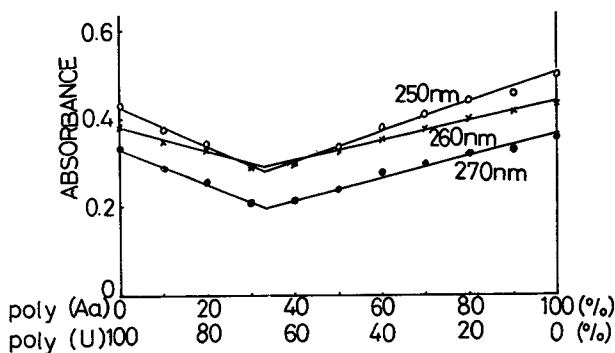


Fig. 11. Mixing experiments of poly(Aa) with poly(U).

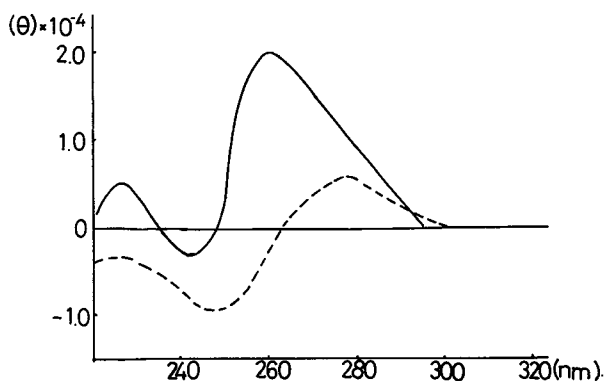


Fig. 12. CD spectrum of poly(Aa)·2poly(U) complex.

(solid line). The CD spectrum definitely differed from the calculated sum of the CD curves of the component nucleotides (dotted line).

T_m of poly (Aa)·2 poly(U)

T_m of poly (Aa)·2poly(U) complex was measured in the presence of 0.15M sodium ion at pH 7.0. As shown in Fig. 13, UV absorption steeply increased from 52° and reached a plateau at 55°. From this curve a T_m of 54° was obtained. This value is somewhat lower than that of poly (A)·2poly(U)¹⁵, which was reported to be 60° in the same conditions. This melting curve suggested a simultaneous dissociation of the two poly (U) strands from the poly(A)·2poly(U) complex.

DISCUSSION

From the experiments described above the following points may be emphasized. Polymerization of 2'-amino-2'-deoxyADP proceeds rather slowly relative to that of poly(A) and the yield of poly (Aa) was 15%. The UV spectrum of poly (Aa) closely resembled that of poly (A), though its hypochromicity is smaller. This may imply that the introduction of the 2'-NH₂ group instead of OH of poly (A) inhibits to some extent the stacking of bases.

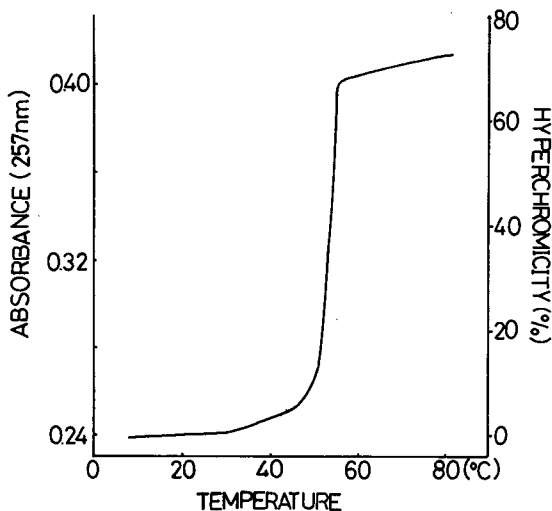


Fig. 13. Temperature-absorption profile of poly(Aa)·2poly(U) complex.

Acid titration showed that poly (Aa) must be protonated at pH lower than 7.0. Since the amino group in the carbohydrate moiety has a pK value around 7-8 and that of adenine is 3.5-4, this protonation must occur on the 2'-NH₂ group. From the thermal stability measurement and examination of dependency of T_m on cationic strength, it may be deduced that this protonated form of poly (Aa) would be stabilized by an intrastrand interaction of 2'-NH₃⁺ and phosphate. Therefore, the introduction of an amino group instead of a OH to the 2'-position of adenosine in the polynucleotide array led to an unusual structure, which has not been reported previously. In the previous reports of polynucleotides containing 2'-aminopyrimidinenucleotides^{6,7} this type of stable structure has not been detected, presumably due to the relatively weak nature of stacking interactions in pyrimidines.

Poly (Aa) forms a 1:2 complex with poly (U) as in the case of poly (A)-poly (U) interaction. Its sharp melting process and T_m value close to that of the poly (A)·2 poly (U) case suggested a well-ordered three-stranded helical structure probably stabilized by Watson-Crick-Hoogsteen type hydrogen bonding. Again this is in sharp contrast with the fact that poly (Ua) did not form any complexes with poly (A)⁷.

From these studies, together with results obtained with poly (Az)³, it may be deduced that the effect of 2'-substituents in purine and pyrimidine polynucleotides is widely different in nature and magnitude.

ACKNOWLEDGEMENT

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Polynucleotides. XLV¹⁾ Synthesis and properties of poly(2'-azido-2'-deoxyinosinic acid)

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ABSTRACT

Poly (2'-azido-2'-deoxyinosinic acid), [poly (Iz)], was synthesized from 2'-azido-2'-deoxyinosine diphosphate by the action of polynucleotide phosphorylase. Poly (Iz) has UV absorption properties similar to poly (I) and hypochromicity of 11% at 0.15M Na⁺ and neutrality. In solutions of high Na⁺ ion concentration, poly (Iz) forms a multi-stranded complex and its T_m at 1.0M Na⁺ ion concentration was 43°. Upon mixing with poly (C), poly (Iz) forms a 1:1 complex having a T_m lower than that of poly (I)·poly (C) complex in the same conditions. The effect of substitution at the 2'-position of the poly (I) strand was discussed in relation to the interferon-inducing activity.

INTRODUCTION

A number of polynucleotides containing analogues of pyrimidine and purine nucleotides have been reported.²⁾ The need to understand the way in which a substituent at the 2'-position of the ribose ring influences the structure and function of the polynucleotides has led to the synthesis of a variety of compounds of this type. Pyrimidine polynucleotides which contain 2'-halogeno³⁾, -azido^{4,5)}, -amino⁵⁾, -methoxy⁶⁾ and -ethoxy⁷⁾ substituents have been reported⁸⁾. We have found a new method for the synthesis of 2'-N-substituted nucleosides⁹⁾ by way of purine cyclonucleosides, which are readily available from the naturally occurring nucleosides. We have previously reported the synthesis and properties of poly (2'-azido-2'-deoxyadenylic acid)¹⁰⁾ as the first purine polynucleotide having 2'-N-substituents. We found that it possessed unique stacking features between adenine bases in the neutral form and in the case of complexing with poly(U). We have also synthesized poly(2'-amino-2'-deoxyadenylic acid) and studied its physical properties.¹¹⁾

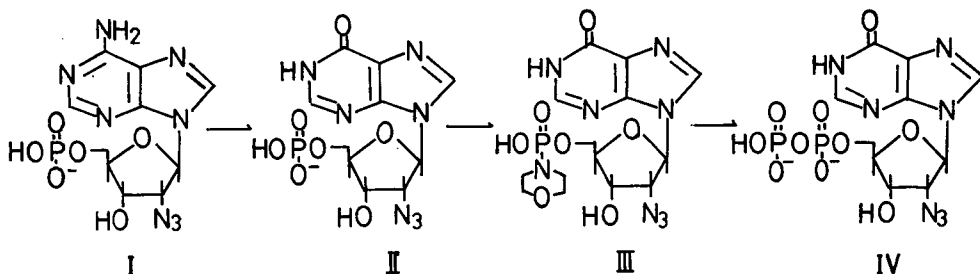
In this paper we describe the synthesis of poly(2'-azido-2'-deoxyinosinic acid) [poly(Iz)] catalyzed by *E.coli* polynucleotide phosphorylase together with its physical properties including UV, CD and T_m in neutral media and the results obtained on mixing with poly(C). It has been found that the introduction of the azido group at the 2'-position of 2'-deoxyinosinic acid in the polynucleotide chain led to the significant increase of the interferon inducing ability of the complex with poly(C).¹²⁾ Therefore, it seemed of interest to obtain information on the structure-function relationships of such an interferon inducer analogous to poly(I)·poly(C).

Materials and Methods

Synthesis of poly(2'-azido-2'-deoxyinosinic acid)

2'-Azido-2'-deoxyinosine 5'-phosphate (II)

2'-Azido-2'-deoxyadenosine 5'-phosphate¹⁰⁾ (I, 5000 OD₂₆₀ units, 0.33 mmol) and sodium nitrite (500 mg) was dissolved in water (5 ml). Acetic acid (4 ml) was added to the solution which was kept at 37° overnight.¹³⁾ The solution was absorbed on charcoal, which was washed thoroughly with water. Elution with 50% ethanol containing 5% conc. ammonia and evaporation of the eluents gave crude 2'-azido-2'-deoxyinosine 5'-phosphate. The residue was dissolved in water and applied to a column (12x20 cm) of Dowex 1x2 (formate form). Washing with 0.175N HCOOH and elution with 0.3N HCOOH gave 2'-azido-2'-deoxyinosine 5'-phosphate (II) (3640 OD₂₅₀ units, 0.29 mmole, 87%). UV: $\lambda_{\max}^{\text{pH } 7.0}$ 249 nm. Paper electrophoresis: R_{AMP} 1.0.



2'-Azido-2'-deoxyinosine 5'-diphosphate (IV)

2'-Azido-2'-deoxyinosine 5'-phosphate (3640 OD₂₅₀ units, 0.29 mmole) was dissolved in water (3 ml) and t-butanol (3 ml). After adding morpholine (0.14 ml, 1.2 mmoles) to the mixture, a t-butanol (4.5 ml) solution of DCC (252 mg, 1.2 mmoles) was added dropwise under reflux.¹⁴⁾ After refluxing for 4 hr, the mixture was evaporated in vacuo and the residue was equilibrated in an H₂O-ether (1:1) mixture. Insoluble material was filtered off and the aqueous layer was separated and evaporated. The residue was azeotropically dried with pyridine several times to give 2'-azido-2'-deoxyinosine 5'-phosphoromorpholidate (III) as a hard syrup. To the residue inorganic phosphate (85% aqueous solution 0.07 ml, 1.03 mmole), which was previously dried by evaporation with pyridine together with tri-n-butylamine (0.24 ml, 1.0 mmole) and dissolved in pyridine (2 ml), was added. The reaction mixture was kept at 30° for 2 days. The reaction mixture was evaporated in vacuo, the residue dissolved in water, and applied to a column of charcoal. The column was washed with water and eluted with 50% ethanol containing 5% conc. ammonia. Eluents were concentrated and applied to a column (1.7x20 cm) of DEAE-Sephadex A25 (bicarbonate form). Elution was performed with 0-0.25M triethylammonium bicarbonate buffer (pH 7.5, total 4 l) in a linear gradient and 20 ml fractions were collected. Fractions No 161-200 were pooled and evaporated. 2'-Azido-2'-deoxyIDP (1575 OD₂₅₀ units, 43%) was obtained as an amorphous powder. Paper electrophoresis: R_{AMP} 1.22. Ratio of base: labile phosphate: total phosphate, 1.0:1.1:2.1.

Polymerization of 2'-azido-2'-deoxyinosine 5'-diphosphate

The polymerization mixture (12.5 ml) contained Tris-HCl (pH 8.5, 80 mM), MnCl₂ (2 mM), 2'-azido-2'-deoxyIDP (4 mM), and 2.4 units of polynucleotide phosphorylase per milliliter of solution. Incubation was performed at 37°. Progress of the reaction was followed by inorganic phosphate analyses¹⁵⁾ on aliquots (0.25 ml) removed at various time intervals. (Fig 1) After 17 hrs the mixture was deproteinised with isoamylalcohol-CHCl₃ (1:3 v/v). The organic phases were

combined and extracted with water. The resulting aqueous solutions were combined and lyophilized to dryness. The residue was dissolved in water and applied to a Sephadex G50 column (1.7x110 cm) which was eluted with water. The polymer was eluted in the void volume. The appropriate fractions containing polynucleotide were combined and dialyzed against 5 l of 0.01M EDTA-0.01M Tris-HCl (pH 7.0) and then against 5 l of water. The resulting aqueous solution was lyophilized. Usually IzDP (50 μ moles) was polymerized as described above. After purification, the yield of poly(Iz) was 75 OD₂₅₀ units (7.04 μ moles, 14%). UV: λ max 247 nm (ϵ 10,950) at 18° in 0.10M NaCl-0.05M Na cacodylate (pH 7.0).

Physical Measurements

UV spectra were taken with a Hitachi 124 or 200 spectrophotometer in the same conditions described above. The extinction coefficient of poly(Iz) was determined by inorganic phosphate analysis after digestion with acid as described by Howard et al.¹⁶⁾ An average value for the three Pi determinations gave 10,950 at λ max for poly (Iz). CD spectra were taken with a JASCO ORD-UV-5 spectrometer equipped with a CD attachment in the presence of 0.1M NaCl and 0.05M Na cacodylate (pH 7.0). Melting temperature was measured with a Hitachi spectrometer equipped with a thermostated cell. The temperature inside the cell was measured with Sibaura thermister Model MGB-III type 218. Mixing curves were obtained by measuring the absorbance of mixtures which contained 0.04mM total concentration of poly(Iz) and poly(C) in the ratios indicated in Fig. 5. Salt concentration was 0.1M NaCl and pH was adjusted to 7.0 with 0.05M Na cacodylate.

RESULTS AND DISCUSSION

IzDP was a substrate for polynucleotide phosphorylase from E.coli on incubation at pH 8.5 in the presence of Mn²⁺ ions. Fig.1 shows the time course. As in the case of AzDP,¹⁰⁾ IzDP was a very poor substrate. The organic phosphate liberated was 40% after 17 hrs incubation, but the isolated yield of poly(Iz) was only 14%.

UV and CD spectra of poly(Iz)

UV absorption of poly(Iz) is shown in Fig 2a. Poly(Iz)

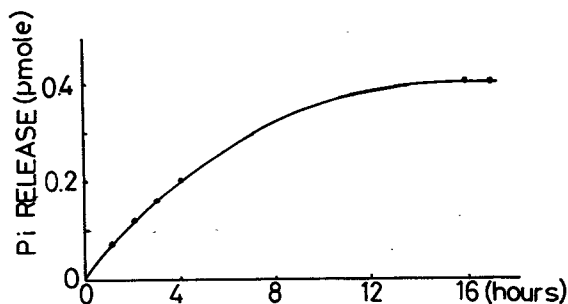


Fig. 1 Time course of polymerization of 2'-azido-2'-deoxyinosine 5'-diphosphate

showed $\lambda_{\max}$ 247 nm (ϵ 10,950) as compared with $\lambda_{\max}$ 249 nm (ϵ 12,500) for IzMP. A blue shift of 2 nm of the $\lambda_{\max}$ compared to that of the monomer was observed. The magnitude of this shift was the same as that observed for poly(I). Hypochromicity at neutrality in the presence of 0.15M Na^+ was calculated as 11% assuming ϵ of the monomer equal to 12,500.

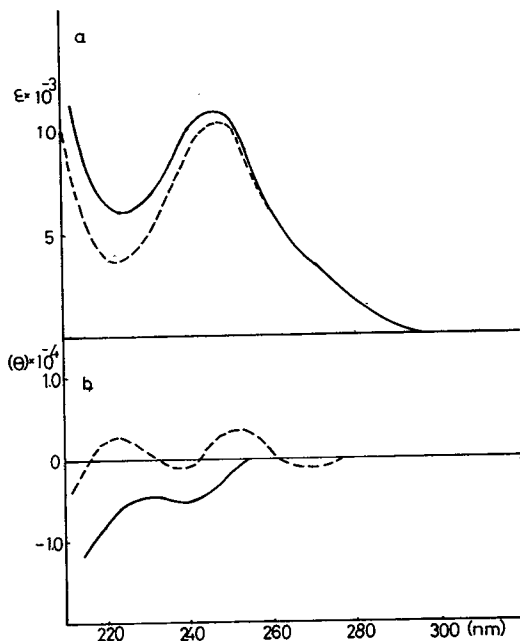


Fig 2 U.V. and C.D. spectra of poly(Iz) and poly(I) in neutral solution containing 0.15M Na^+ .
— poly(Iz), --- poly(I)

This hypochromicity is smaller than that of poly(I) (15%) under the same conditions.

The CD spectra of poly(Iz) and poly(I) at 8° in 0.10M MgCl-0.05M Na cacodylate (pH 7.0) are shown in Fig. 2b. The CD spectrum of poly(Iz) did not show any positive band in the UV region (210-320 nm). A trough appeared at around 240 nm ($[\theta] = -5,300$). This suggests a stacked random coil structure which was less stable than poly(I) for poly(Iz) in the neutral solution in the presence of 0.15M Na⁺.

According to Rich¹⁷⁾ poly(I) associates under appropriate environmental conditions to form a three-stranded helical complex with three hydrogen bonds involved in stabilization of each hypoxanthine triplet. Recently Arnott et al, presented a quadruplet structure for this complex.¹⁸⁾

As shown in Fig.3, the optical density of poly(Iz) was decreased by changing the solvent from 0.05M to 0.95M Na⁺ ion concentration. On going from 0.05M to 0.95M, the optical density at 248 nm was decreased by 19%. This fact suggests that, while poly(Iz) exists as a random coil structure in the 0.05M Na⁺ solution, on increasing the salt concentration to 0.95M Na⁺, the polymer associated to an ordered structure as was found in the case of poly(I).

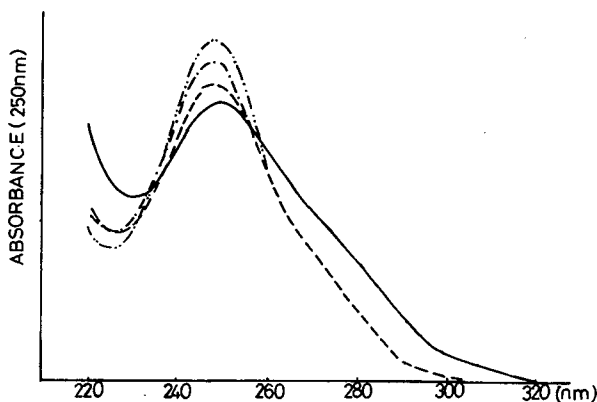
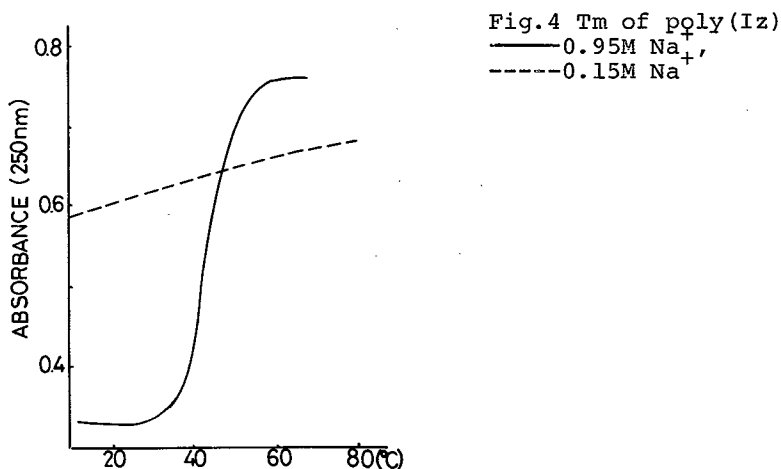


Fig.3 U.V. absorption of poly(Iz) at various Na⁺ ion concentration. — 0.95M, --- 0.51M, -.-.- 0.35M
..... 0.05M



Temperature-absorbance profile

The temperature-absorbance profile of poly(Iz) in neutral conditions is shown in Fig. 4. While the UV absorption at 0.15M Na⁺ ion increased gradually on heating from 0° to 80° without showing any steep increase indicative of cooperative melting, the curve taken with 0.95M Na⁺ ion showed a sharp transition at 43°. The T_m (43°) for poly(Iz) is the same as the T_m (43°) of poly(I). The melting profile for poly(Iz) is somewhat less cooperative and its hyperchromicity on melting is smaller than that of poly(I) (18%). These facts suggest that the thermal stability of the multi-stranded complex of poly(Iz) is almost the same as the poly(I) quadruplex. Therefore, we concluded that the stability of the three(or four)-stranded complex of polymer is not affected by substitution of the hydroxyl group by the azido group at the 2'-carbon of inosinic acid in the polynucleotide chain.

Hybridization experiments of poly(Iz) with poly(C)

It is well known that poly(I) forms a double-stranded complex upon mixing with poly(C). Poly(dI) also shows similar complex formation with poly(C) or poly(dC). We examined the complex of poly(Iz) with poly(C) by the continuous variation method. Poly(Iz) and poly(C) at 0.04 mM base concentrations were mixed in various ratios as indicated

in Fig.5 in the presence of 0.15M Na^+ at pH 7.0.

The curves at 250 nm, 260 nm and 270 nm clearly showed formation of a 1:1 complex, $\text{poly(Iz)} \cdot \text{poly(C)}$, as in the case of $\text{poly(I)} \cdot \text{poly(C)}$.

As shown in Fig. 6, this complex formation by poly(Iz) and poly(C) was also supported by measurements of CD before and after the mixing of two components. The CD curve before

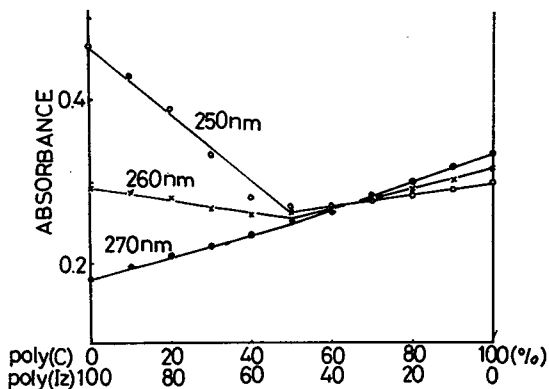


Fig.5 Mixing experiment of poly(Iz) and poly(C)

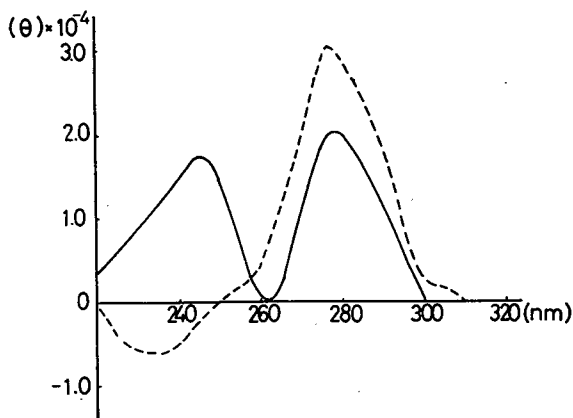


Fig. 6 CD spectra of poly(Iz) and poly(C) (1:1) before and after mixing. ---- before mixing, — after mixing

the mixing showed a peak at 277 and a trough at 235 nm. After the mixing the curve changed to a completely different one, which had two peaks at 277 nm and 245 nm, and a trough at 262 nm. The amplitude of the long wavelength CD band decreased as compared to that before mixing, and its shorter wavelength CD band was reversed in sign with an increase in magnitude. These changes indicate complex formation in the mixture.

Thermal stability of the poly(Iz)·poly(C) complex

The temperature-absorption profile at various ionic strengths are recorded. The T_m was 41° at $0.15M Na^+$ ion concentration, 51° at $0.15M Na^+$ ion concentration, 58° at $0.35M Na^+$ ion concentration, 61° at $0.55M Na^+$ ion concentration and 64° at $0.95M Na^+$ ion concentration. The T_m of poly(Iz)·poly(C) complex was 11° lower than that the T_m of poly(I)·poly(C) reported to be 62.5° at $0.15M Na^+$ ion concentration. These T_m 's showed a linear relationship with the ionic concentration as shown in Fig.7.

It has become evident that the presence, absence, or modification of the 2'-hydroxyl group of polynucleotides

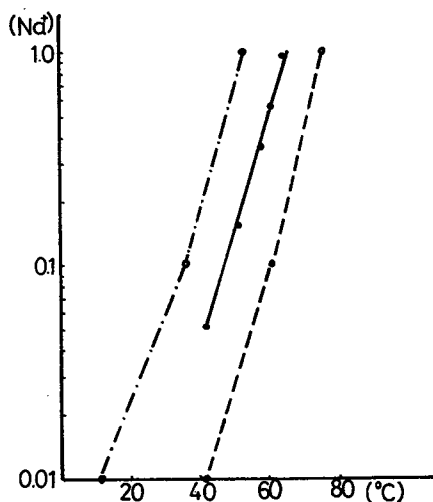


Fig.7. Relationship of Na^+ concentration and T_m of poly(Iz)·poly(C) (—), Poly(I)·poly(C) (----) and poly(dI)·poly(C) (- - - -).

results in significant differences in the conformation and relative stabilities of the ordered structures of such polynucleotides. Generally, double-stranded homopolymer pairs seem to follow a trend that the ribose duplexes have a higher T_m than the deoxyribose duplexes as well as the hybrid duplexes.¹⁹⁾ We have reported previously¹⁰⁾ that the poly (Az) forms a 1:2 complex with poly(U) in 0.15M Na^+ solution and this complex has a T_m higher than that of poly(A)·2poly(U) or poly(dA)·2poly(U).²⁰⁾ In the case of the poly(Iz)·poly(C) duplex, as described above, this complex has T_m 's lower than those of the ribose duplex, poly(I)·poly(C), but has T_m 's higher than those of the hybrid helix, poly(dI)·poly(C), at Na^+ ion concentrations between 0.05 and 0.96M. These results suggest that, not only the size of the 2'-substituent, but also its interaction with solvent molecules must be taken into account for the stabilization of the complex.

In conclusion it might be emphasized that the introduction of the azido group to the 2'-position of purine nucleotides in polymer chains caused rather small changes in the physical properties as compared to ribopolynucleotides. The enhancement of interferon-inducing activity by the 2'-azido group may be ascribed to resistance of poly(Iz) to enzymatic degradation.

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

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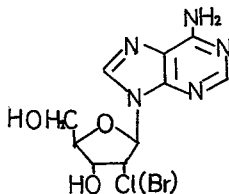
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ABSTRACT

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

INTRODUCTION

Recently we have developed a versatile method for synthesizing 2'-azido^{2,3}, 2'-amino^{2,3}, 2'-chloro³ and 2'-bromo⁴-2'-deoxyadenosine starting from 8,2'-O-cycloadenosine.⁵ Using these 2'-substituted 2'-deoxyadenosines as starting materials, poly (2'-azido-2'-deoxyadenylic acid) [poly (Az)]⁶, poly (2'-amino-2'-deoxyadenylic acid) [poly (Aa)]⁷ and poly (2'-azido-2'-deoxyinosinic acid) [poly (Iz)]⁸ were synthesized.



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

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

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

MATERIAL AND METHODS

General Procedure

UV absorption spectra were taken with a buffer containing 0.1M NaCl and 0.05M Na Cacodylate (pH 7.0) at 24-26° with a Hitachi Model 200-10 spectrophotometer. The concentrations of nucleotides were determined by phosphate analysis and are presented as per residue values. Hypochromicity was obtained by measuring UV absorption at $\lambda_{\max}$ before and after the digestion of polynucleotides. CD spectra were taken with a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment using 10 mm path-length cell. The concentration of nucleotides was 0.5-1.0 OD₂₆₀. The solution contained 0.1M NaCl and 0.05M Na Cacodylate (pH 7.0) and was measured at 24-26°. Mixing curves and T_m's were measured with a Hitachi 124 spectro-photometer equipped with a Komatsu thermostated cell SPD-H-124. The temperature inside the cell was measured with a Cu-constantan thermocouple. Solutions containing 0.04M or 0.15 M NaCl, 0.05M Na Cacodylate (pH 7.0) and each component polynucleotide were heated once at 60° for 10 min after mixing and measured at 30 min (in case of 0.09M Na⁺) to 10 hrs (in case of 0.15M Na⁺) after cooling to 24-26°.

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

Paper-chromatography (PPC) was performed in solvent systems : A, isopropanol-conc. ammonia-water (7:1:2); B, n-butanol-acetic acid-water (5:2:3); C, sat. (NH₄)₂SO₄-water-isopropanol (79:19:2); D, n-propanol-conc. ammonia-water (55:10:35), by the descending technique. Paper electrophoresis was performed in 0.05M triethylammonium bicarbonate buffer (pH 7.5) at

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

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

2'-Chloro-2'-deoxyadenosine (32.1 mg, 0.11 mmole) was dissolved in a mixture of $POCl_3$ (50 μ l, 0.54 mmole) and triethyl phosphate (2 ml) at 0°. The reaction mixture was stirred at 0° for 6 hrs. The mixture was poured in ice-water and absorbed on a column of charcoal. The column was washed thoroughly with water and eluted with 50% EtOH containing 5% ammonia. Eluents were evaporated in vacuo and the residue was dissolved in water (20 ml), and applied to a column of Dowex 1x2 (formate form). After a water-wash, the column was eluted with 0.1N $HCOOH$. The yield of Acl 5'-MP was 1012 OD₂₆₀ (61%). UV: $\lambda_{max}^{H_2O}$ 259 nm. PPC: Rf (A) 0.17, Rf (B) 0.35. PEP: R_{PA-A} 0.96. This sample was hydrolyzed completely with snake venom 5'-nucleotidase to give Acl and inorganic phosphate.

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

2'-Bromo-2'-deoxyadenosine (27.4 mg, 83 μ moles) was treated with $POCl_3$ (0.1 ml, 1.1 mmole) in triethyl phosphate (1 ml) as described above. Yield of Abr 5'-MP was 790 OD₂₆₀ (63%). UV: $\lambda_{max}^{H_2O}$ 259 nm. PPC: Rf (B) 0.28, Rf (C) 0.54. PEP: R_{PA-A} 0.96. This sample was hydrolyzed completely with snake venom 5'-nucleotidase to give Abr and inorganic phosphate.

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

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

the addition of water, the solvent was removed by evaporation in vacuo, and the residue was dissolved in water. The aqueous solution was brought to ca. pH 5 and applied to a charcoal column. The nucleotidic material was eluted with 50% EtOH containing 5% conc. NH_4OH and evaporated in vacuo. The residue was taken up in water and applied to a column (1.7x15 cm) of DEAE-Sephadex A-25(bicarbonate form). Elution was carried out with 0-0.25 triethylammonium bicarbonate buffer (2 l+2 l) in a linear gradient. The yield of Acl 5'-DP was 330 OD₂₆₀ (35%). PPC: Rf (B) 0.08, Rf (D) 0.54. PEP: $R_{\text{pA-A}}$ 1.25.

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

Abr 5'-MP (790 OD₂₆₀) was treated with morpholine (75 μl) dissolved in t-BuOH (1 ml) and water (1 ml) and DCC (176 mg, 0.86 mmole) in t-BuOH (1.5 ml) as described above. After the appropriate work up, the residue was allowed to react with 80% H_3PO_4 (30 μl , 0.4 mmole) and (n-Bu)₃N (0.1 ml, 0.4 mmole) in DMF (1 ml). After 3 days at room temperature the reaction mixture was applied to a column of DEAE-Sephadex A-25 as described above. The yield of Abr 5'-Dp was 377 OD₂₆₀ (48%). PPC: Rf (B) 0.05, Rf (C) 0.50. PEP: $R_{\text{pA-A}}$ 1.27.

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

A solution (4.5 ml) containing Acl 5'-DP (4 mM), MgCl_2 (2.2 mM), Tris-HCl (pH 8.5, 66mM) and *E. coli* polynucleotide phosphorylase¹⁰ (3.9 units/1 ml) was incubated at 37° for 24 hrs. The mixture was deproteinized with isoamyl alcohol-chloroform (1:3, vol/vol)mixture and the water-layer was lyophilized.

The residue was dissolved in water and filtered through a column (2.6 x 80 cm=425 ml) of Sephadex G-50 gel. The flow rate was 5 ml/20 min/fraction. The polynucleotide was eluted in the void volume and the yield was 45 OD₂₆₀ (25 %, ignoring hypochromicity). The fact suggests that the poly (Acl), thus obtained, has a chain length greater than 50 nucleotide units.

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

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

through a column of Sephadex G-50 as described above. The yield was 33 OD₂₆₀ (13%, ignoring hypochromicity).

Enzymatic digestion of polynucleotides

i) Polynucleotides (ca. 2 OD₂₆₀) were incubated with ribonuclease M¹¹ (2 mg/ml) 2 ul in water 50 ul containing 1M NH₄OAc (pH 7.5) 2 ul at 37° for 150 min. While poly (A) was hydrolyzed completely in these conditions, poly (Acl) and poly (Abr) were resistant to hydrolysis. This fact confirms that nucleosides in poly(Acl) and poly(Abr) were substituted at their 2'-positions.

ii) Polynucleotides (ca. 2 OD₂₆₀) were incubated with snake venom phosphodiesterase (5 mg/ml) 1 ul in water 50 ul containing 1M Tris-HCl (pH 8.5) 3 ul at 37° for 30 min. While poly(A) was completely hydrolyzed after 30 min, poly (Acl) and poly(Abr) were only hydrolyzed to extents of 8% and 9% to give Acl 5'-MP and Abr 5'-MP, which were identified directly with authentic samples, after 2 hrs incubation. This fact suggests that large electronegative substituents inhibit hydrolysis catalyzed by snake venom phosphodiesterase to some extent.

RESULTS AND DISCUSSION

UV absorption properties

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

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

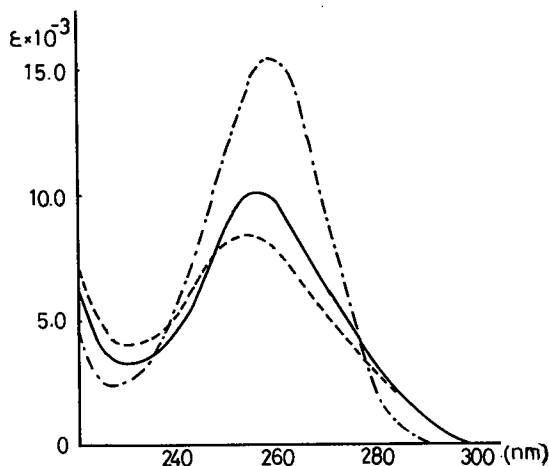


Fig. 1. UV absorption spectra of poly (Acl). — at pH 7.0. at pH 4.5, -.- Acl 5'-MP.

CD spectra

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

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

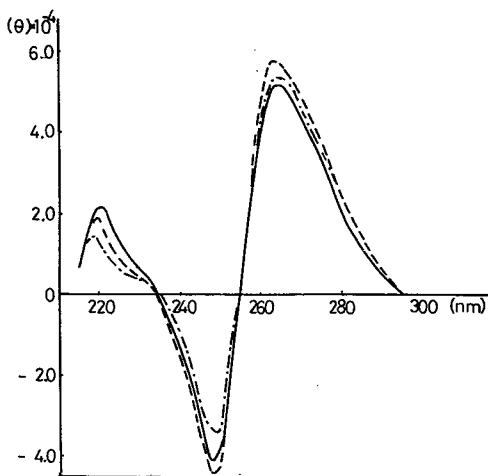


Fig. 2. CD Spectrum of Poly (Acl), Poly (Abr) and Poly (A).

—— Poly (A), ---- Poly (Acl), -.-.- Poly (Abr).

Table I Molecular Ellipticity of Polynucleotides

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

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

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

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

Table II Acid Titration of Polynucleotides

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

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

Formation of Complexes with Poly (U)

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

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

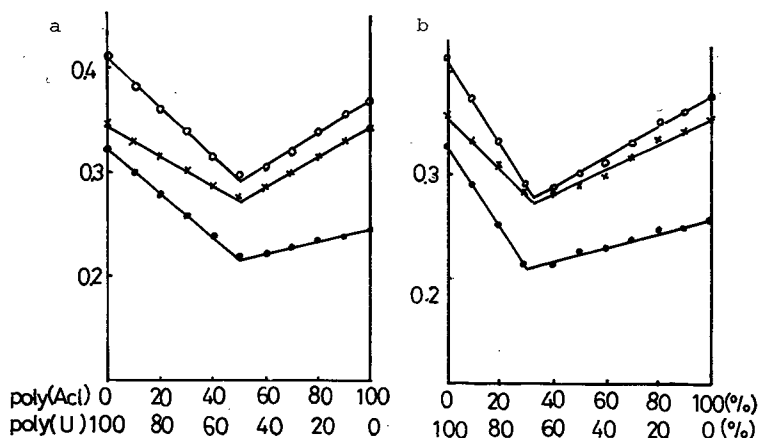


Fig. 3. Complex Formation of Poly (Acl) with Poly (U).

complex described below.

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

Thermal Transition of Complexes

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

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

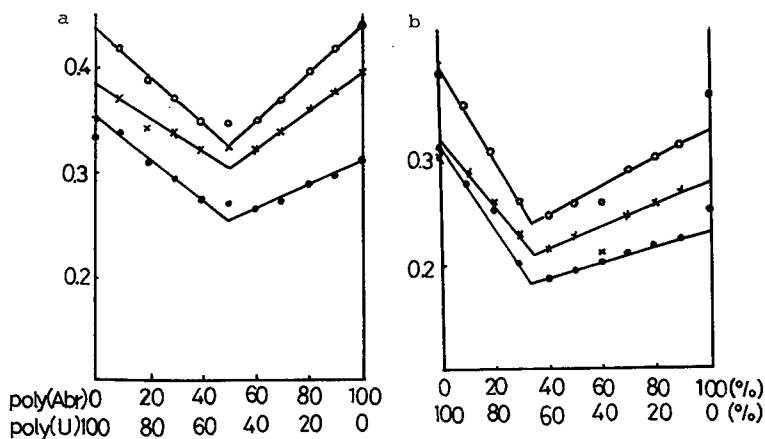


Fig. 4. Complex Formation of Poly (Abr) with Poly (U).

Table III. Thermal Transition Points of Complexes.

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

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

CONCLUSIONS

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

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

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

Poly (A) is known to form the so-called acid structure at pHs below 4.5.¹⁴ The transition pHs (5.5-6.0) to form the acid structure of the polynucleotides discussed here are in almost the same range and it may be deduced that 2'-halogeno or azido substituents do not significantly affect the pK values of these polynucleotides. However, as regards their stability these substituents have large effects. For 2'-halogeno compounds the decrease in thermal stability relative to poly (A) is in the range of 20-25° and for poly (Az) it is more than 40°. This destabilizing effect may be due to size and polarity of these substituents.

In the case of the complexes formed between poly (U) and these poly (A) analogs, again the 2'-substituents did not inhibit the formation of double- or triple-stranded complexes, though the thermal stability was affected. The Cl, Br and N₃ substituents at the 2'-position significantly lowered the thermal stabilities of the double helical complexes, poly (Acl)·poly (U), poly (Abr)·poly (U) and poly (Az)·poly (U), to the extent of 5-6°. This tendency was also observed in the case of the triple-stranded complexes, poly (Acl)·2poly (U) and poly (Abr)·2poly (U). However, in the case of poly (Az)·2 poly (U), the T_m was increased 3° relative to poly (A)·2poly (U). It seems reasonable to assume that these substituents in the 2'-position may affect the thermal stability of polynucleotides not only for steric reasons, but also by their polarizability causing association of solvent molecules. These effects in the anti-parallel double stranded poly (A)·poly (U) analogs and Watson-Crick-Hoogsteen (antiparallel) arrangements in poly (A)·2 poly (U) type complexes may be some-

what different from case to case.

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

ACKNOWLEDGEMENTS

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- 1 Razzell, W.E. and Khorana, H.G. (1958) *Biochim. Biophys. Acta* 28, 562-566
- 2 Davidson, J.N. (1969) *The Biochemistry of the Nucleic Acids*, 6th edn. pp. 177-178. Methuen, London
- 3 Burdon, R.H. (1971) in *Progress in Nucleic Acid Research and Molecular Biology*, Davidson, J.N. and Cohn, W.E., Eds., Vol. II, pp. 33-79. Academic Press, New York

Nomenclature

As far as possible, authors should follow the Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, and particularly the abbreviations for nucleic acids, polynucleotides and their constituents (1971) *J. Mol. Biol.* 55, 299-305.

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Polynucleotides. LII.¹ Synthesis and properties of poly(2'-deoxy-2'-fluoroadenylic acid)

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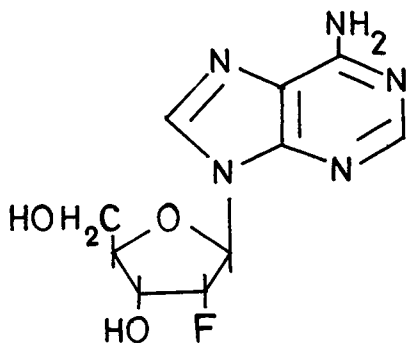
ABSTRACT

2'-Deoxy-2'-fluoroadenosine was chemically transformed to its 5'-diphosphate and polymerized with polynucleotide phosphorylase to give poly(2'-deoxy-2'-fluoroadenylic acid) [poly(Af)]. Polymerization proceeded smoothly as in the case of poly(A) and the yield of the polymerization was 55%. The UV absorption spectra of poly(Af) closely resembled those of poly(A) and the hypochromicity was 32% at pH 7.0. The CD profile at 25° and neutrality showed similar pattern to that of other poly(2'-deoxy-2'-halogenoadenylic acids) with somewhat larger [θ] values both in the positive and negative maxima. Acid titration of poly(Af) showed a transition point at pH 5.2 and the T_m of the acid form was 37° which was significantly lower than that of poly(A), but similar to that of poly(2'-azido-2'-deoxyadenylyc acid). Poly(Af) formed 1:1 and 1:2 complexes with poly-(U) having T_m of 49° and 62° at 0.04M and 0.15M Na⁺ concentration, respectively. Poly(Af) also formed a 1:2 complex with poly(I) and its T_m was 36° at 0.05M Na⁺ concentration. These data showed that poly(Af) has rather similar properties to those of poly(A), but not to poly(dA).

INTRODUCTION

Recently we have reported the synthesis and properties of poly(2'-deoxy-2'-azido-², chloro-³ and bromoadenylic acid).³ The general feature on introducing aprotic and polarizable group such as azido, chloro or bromo at the 2'-position instead of the OH of poly(A) is that the physical properties of these polynucleotides are rather similar in spite of their lacking proton donors, which are thought to stabilize ribopolynucleotide conformations.^{4,5} Moreover, it was found that poly(2'-azido-2'-deoxyinosinic acid)⁶ was active as an interferon inducer when complexed with poly(C).⁷

In this paper we report a method for synthesis of poly(Af) from 2'-deoxy-2'-fluoroadenosine⁸ and data on its physical pro-



2'-Deoxy-2'-fluoro-
adenosine

perties such as UV and CD spectra, T_m , acid titration of single stranded form and formation of complexes with poly(U) and poly(I).

MATERIALS AND METHODS

2'-Deoxy-2'-fluoroadenosine 5'-monophosphate

2'-Deoxy-2'-fluoroadenosine⁸ (40.3 mg, 0.15 mmole) was stirred with POCl_3 (0.1 ml, 1.1 mmole) and $(\text{EtO})_3\text{PO}$ (1 ml) at 0° for 3 hrs. The mixture was poured in ice-water (ca. 200 ml) and the solution was applied to a column of charcoal (ca. 2 ml). After the water-wash the nucleotide was eluted with a mixture of $\text{EtOH-H}_2\text{O-c.NH}_4\text{OH}$ (50:50:1, vol/vol, 50 ml) and eluents were evaporated in vacuo. The residue was dissolved in H_2O and applied to a column of Dowex 1x2 (formate form, 2 ml). After the water-wash, elution with 0.1N formic acid gave a peak of Af 5'-MP. The yield was 1607 A_{260} (0.11 mmole, 76%). Paper chromatography : $R_f(\text{A})$ 0.23, $R_f(\text{C})$ 0.13. Paper electrophoresis (at pH 7.5): $R_{\text{A-pA}}$ 1.04. When Af5'-MP (3 A_{260} units) was incubated with 0.1M MgCl_2 5 μl , 1M Tris.HCl (pH 8.5) 4 μl , crude snake venom (10 mg/ml) 30 μl and H_2O 10 μl at 37° for 4 hrs, it was completely dephosphorylated. Thus the position of phosphorylation was confirmed as 5'.

2'-Deoxy-2'-fluoroadenosine 5'-diphosphate

Af 5'-MP (1600 A_{260} , 0.11 mmole) and morpholine (0.1 ml, 1.1 mmole) were dissolved in $t\text{-BuOH}$ (1.5 ml) and H_2O (1.5 ml). While this solution was refluxed, DCC (235 mg) dissolved in $t\text{-BuOH}$ (2 ml) was added dropwise in 40 min. After 1.5 hrs refluxing, morpholine (0.1 ml, 1.1 mmol) and DCC (235 mg) were

added and the refluxing was continued for a further 2 hrs. H₂O (ca. 20 ml) was added, dicyclohexylurea was removed by filtration, and the solution was extracted with ether (10 ml x 3). The aqueous solution was evaporated in vacuo and evaporated three times with added pyridine. Inorganic phosphoric acid (80%, 40 μ l, 0.58 mmole) and (n-But)₃N (0.14 ml, 0.58 mmole) were rendered anhydrous by evaporation three times with pyridine. Both residues were dissolved in DMF (1 ml) and kept at 31° for 3 days. H₂O (ca. 60 ml) was added and the acidic solution was applied to a column of charcoal (20 ml). After a water-wash, the nucleotide was eluted with methanolic ammonia (50 ml) as was described before. Eluents were evaporated in vacuo and the residue applied to a column (1.5 x 38 cm) of DEAE-Sephadex A-25 (bicarbonate form). Elution with triethylammonium bicarbonate buffer of 0M to 0.3M in a linear gradient gave three peaks. The last peak which eluted at 0.15M buffer concentration was pooled and evaporated in vacuo. The yield was 705 A₂₆₀ (0.05 mmole, 46%). Paper chromatography : R_f(A) 0.13, R_f(B) 0.44. Paper electrophoresis (at pH 7.5) : R_{A-pA} 1.35.

Poly(2'-deoxy-2'-fluoroadenylic acid) [poly(Af)]

A solution (5 ml) containing 2'-deoxy-2'-fluoroadenosine 5'-DP 4 mM, MgCl₂ 2 mM, Tris.HCl (pH 8.5) 100 mM and E. coli polynucleotide phosphorylase 4.5 units/ml was incubated at 37° for 24 hrs. Inorganic phosphate (0.68 μ mol/0.25 ml of the incubation mixture) was liberated. The mixture was extracted with i-AmOH-CHCl₃ (1:3, vol/vol) mixture and the water-layer was lyophilized. The powder thus obtained was filtered through a column (2.7 x 95 cm) of Sephadex G-50 (540 ml). The material which was excluded in the void volume was collected. The yield was 108 A₂₆₀ (0.011 mmole, 55% regardless of hypochromicity). Digestion of this polynucleotide with snake venom phosphodiesterase showed only Af 5'-P and 2'-deoxy-2'-fluoroadenosine was not detected on a paper chromatogram of the digest. This means that the chain length of the polynucleotide is greater than 100 nucleotide units.

Physical measurements

U.V. absorption spectra were taken with a Hitachi 124

spectrophotometer equipped with a thermostated cell. CD spectra were measured with a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment. T_m was measured with a Hitachi 124 spectrophotometer equipped with a thermostated cell. The temperature inside the cell was measured with a thermocouple.

Paper chromatography and electrophoresis

Paper chromatography was performed in solvent systems: A, n-BuOH-AcOH-H₂O (5:2:3) and B, i-PrOH-conc.NH₄OH-H₂O (7:1:2) by the descending technique. Paper electrophoresis was performed in 0.05M triethylammonium bicarbonate buffer (pH 8.5) at 900 V/20 cm for 1 hr.

Enzymes

Polynucleotide phosphorylase was prepared by the method described by Williams and Grunberg-Manago.⁹ Crude snake venom was a gift from Kagoshima Prefecture Hygienic Institute to which our thanks are due. Purified snake venom phosphodiesterase was purchased from Worthington Biochem. Co.

RESULTS AND DISCUSSION

Polymerization of AfDP catalyzed by polynucleotide phosphorylase

The polymerization of AfDP using *E. coli* polynucleotide phosphorylase proceeded smoothly to the extent of 65% in 24 hrs. This rate is comparable to that of ADP polymerization, while AzDP², AcIDP³ and AbrDP³ polymerized in the same conditions to extents of only 13-25%. This may mean that the conformation of AfDP in the incubation mixture is very similar to that of ADP. It is worthwhile mentioning that in spite of the small size of the 2'-fluoro atom, AfDP completely mimics ADP rather than dADP, which is known to be an inhibitor of the polymerization.¹⁰

U.V. absorption properties of poly(Af)

The U.V. absorption spectrum of poly(Af) at pH 7.0 and 25° is shown in Fig. 1 together with that of Af 5'-MP. The spectrum of poly(Af) has λ_{max} at 255 nm and the ϵ_{max} was 9,700. The hypsochromic shift (4 nm) is significantly larger than that of poly(A) and ϵ value is smaller than that of poly(A) measured in the same conditions (Table I).

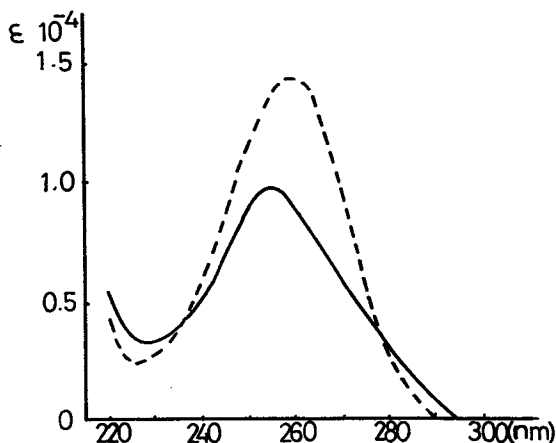


Fig. 1 U. V. absorption spectra of poly(Af) and Af 5'-MP.
Poly(Af) —, Af-5'-MP ----.

These facts may indicate that in poly(Af) the adenine residues stacked more strongly than in poly(A), presumably because of the large polarity and small size of fluorine atom at the 2'-position. The hypochromicity calculated from the ζ value of the monomer is 32%, which is also somewhat smaller than that of poly(A). This is in accordance with our previous findings^{2,3} that the size and polarity of 2'-substituents influence the UV spectral properties of polynucleotides.

CD spectrum of poly(Af)

The CD spectrum of poly(Af) is shown in Fig. 2 together with that of poly(Ac1) and poly(Abr). The shape of the spectra closely resemble each other and that of poly(A), suggesting that these 2'-hogenated polynucleotides are in very similar conformations in solution at pH 7.0, which is analogous to that of poly(A). The $[\theta]_{\text{peak}}$ of poly(Af) at 264 nm is 6,3000 and $[\theta]_{\text{trough}}$ at 248 nm is -49,000 (see Table II).

Comparing these values with those of poly(Ac1) and poly-(Abr), it was shown that the total $[\theta]$ values are in the order

Polynucleotide	ζ	(λ_{max})	ζ_{Monomer}	(λ_{max})	Hypochromicity (%)
Poly(A)	10,000	(257 nm)	15,400	(259 nm)	35
Poly(Af)	9,700	(255)	14,300	(259.5)	32
Poly(Ac1)	10,500	(257)	15,500	(259)	32
Poly(Abr)	10,700	(257)	15,000	(259)	29

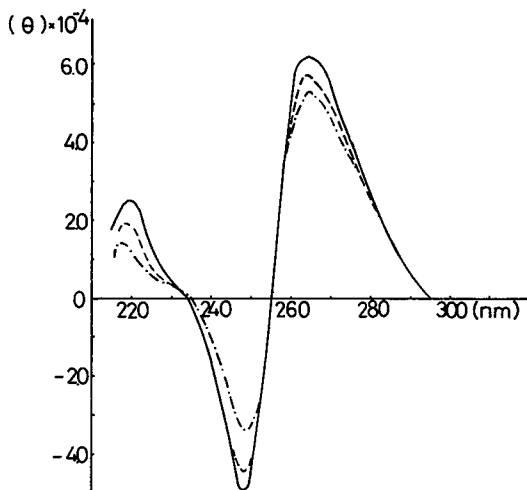


Fig. 2 CD spectra of poly(Af), poly(Acl) and poly(Abr).
Poly(Af) —, poly(Acl) ----, poly(Abr) -.-.-.

Af > Acl > Abr, which is inversely parallel to the size of the 2'-substituents. This fact may indicate that, if the nature of the substituents is similar, the size of the 2'-atom is an important factor for determining $[\theta]$ values in CD spectra, which are caused by coupling of transition moments¹¹ of adenine bases stacked one on another in the polynucleotide array.

Acid titration of poly(Af)

When poly(Af) was titrated with 0.1N HCl at 25° in the presence of 0.15M Na⁺, a titration curve having a transition point at pH 5.2 was obtained. This fact indicated that poly(Af) changed from its neutral form to an acid form as has been found in the case of poly(A).¹² As shown in Fig. 3, the CD curve of poly(Af) acid form showed a curve of similar shape, but different $[\theta]$ values to that of poly(A) acid form.

This may indicate that the introduction of the strongly electron-withdrawing fluoro atom at the 2'-position instead of OH, changed the stacking conformation of the acid form significantly. This may be seen in Table III by comparing the Tm

Table II

Polynucleotide	$[\theta]_{\text{peak I}} (\text{nm})$	$[\theta]_{\text{trough}} (\text{nm})$	$[\theta]_{\text{peak II}} (\text{nm})$
Poly(Af)	63,000 (264)	-49,000 (248)	25,000 (220)
Poly(Acl)	57,000 (264)	-44,000 (248)	19,000 (218)
Poly(Abr)	53,000 (264)	-34,000 (249)	14,000 (217)

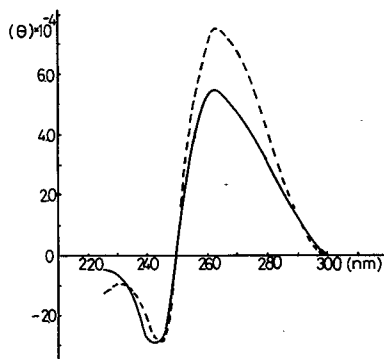


Fig. 3 CD spectra of poly(Af) and poly(A) acid form.
Poly(Af) —, poly(A) ----.

values of the acid form of poly(Af) with that of other polynucleotides. Introduction of aprotic substituents such as halogen or azido groups markedly decreases the T_m of the acid forms by as much as 20–40°. This may suggest that the proton at 2'-OH plays an important role in stabilizing the conformation of the poly(A) acid form.

Complex formation of poly(Af) with poly(U)

As all 2'-substituted polynucleotides so far examined formed two- and three-stranded complexes^{2,3} with poly(U) as in the case of poly(A),¹³ the complex formation of poly(Af) was studied.

In conditions of 0.15M Na^+ concentration, pH 7.0 and 25° poly(Af) formed a three-stranded complex with poly(U) as indicated by the continuous variation curves shown in Fig. 4. Measurement of U.V. spectra at percentages from 0–30% poly(Af) showed isosbestic points at 232, 283 and 300 nm, while at percentages between 40–100% isosbestic points were observed at 222, 281 and 300 nm. This suggests that only one three-stranded complex is present in this solution.

Table III

Polynucleotide	T_m at pH 4.5 in the presence of 0.10M NaCl (at 24–26°), over 80°
Poly(A)	
Poly(Af)	37°
Poly(Acl)	63°
Poly(Abr)	56°
Poly(Az)	38°

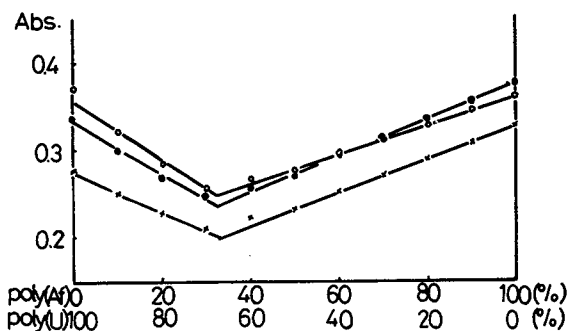


Fig. 4 Mixing curves of poly(Af) with poly(U) measured at 25° in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0).
At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

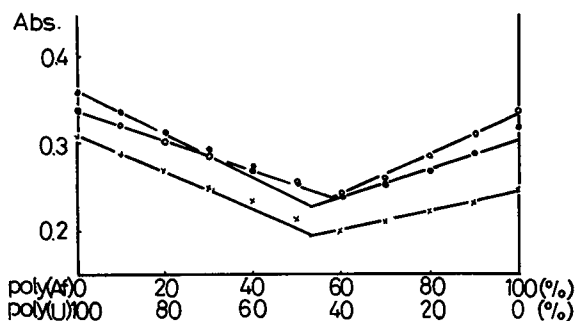


Fig. 5 Mixing curves of poly(Af) with poly(U) in the presence of 0.04M Na Cacodylate (pH 7.0) at 25°.
At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

At Na^+ concentration of 0.05M mixing curves (Fig. 5) showed 1:1 complexing of poly(Af) and poly(U) only after 30 min annealing. These curves gradually changed to those of a 1:2 complex after 7 days at 25°. Therefore, the 1:1 complex is relatively unstable in this condition and gradually rearranges to the 1:2 complex even in these low salt conditions.

CD curves before and after the mixing of poly(Af) and poly-(U) showed significant changes (data not shown) and the complex formation has been substantially confirmed.

The T_m 's of A-U complexes of various 2'-halogeno polynucleotides are summarized in Table IV. It is shown that the sizes of T_m values are inversely parallel to those of the halogen atoms in the order of $\text{F} > \text{Cl} > \text{Br}$ for both 1:1 and 1:2

Table IV

Polynucleotide	T _m at 0.04M Na ⁺	T _m at 0.15M Na ⁺
Poly(Af)+poly(U)	49°	64°
Poly(Acl)+poly(U)	46°	56°
Poly(Abr)+poly(U)	45°	53°

complexes. Again the size of 2'-substituents seems to influence the T_m of the hetero complexes. Electronegativity of the halogen atoms might also be a consideration of the stacking conformations.

Complex formation of poly(Af) with poly(I)

Poly(A) was reported to form a triple stranded complex with poly(I).¹⁴ When poly(Af)-poly(I) complexing was tested by the continuous variation method, curves as shown in Fig. 6 were obtained. These curves clearly showed inflection points at poly(Af):poly(I) ratios equal to 33:67 indicating that a 1:2 complex was formed. U. V. absorption curves measured in poly(Af) concentration ranges 0-30% showed isosbestic points at 227, 260 and 300 nm and those between 40-100% showed isosbestic points at 246, 283 and 300 nm. These facts indicated that only a 2:1 complex was present in the mixture.

Fig. 7 shows the melting curves of various complexes of poly(A) analogs with poly(I). All these complexes had sharp transition points and hypochromicities before and after the melting are of the same order. As summarized in Table V, the

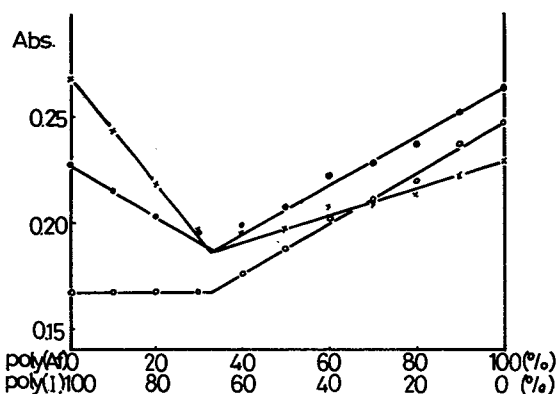


Fig. 6 Mixing curves of poly(Af) with poly(I) in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0) at 25°. At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

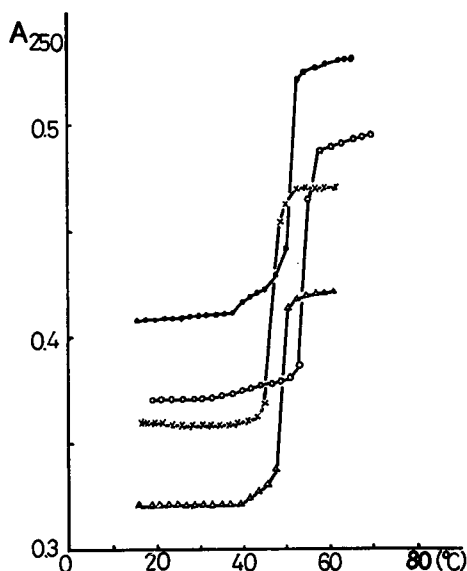


Fig. 7 Melting profiles of poly(A)-poly(I) complexes measured in the presence of 0.10M NaCl, 0.05M Na Cacodylate (pH 7.0) and 10mM MgCl_2 . Poly(Af)·2poly(I) ●-●-●, poly(A)·2poly(I) ○-○-○, poly-(Abr)·2poly(I) x-x-x, poly(Abr)·2poly(I) △-△-△.

Table V		
Complex	T_m at 0.15M Na^+	+10mM Mg^{2+}
Poly(A)·2poly(I)	42°	54°
Poly(Af)·2poly(I)	36°	51°
Poly(Acl)·2poly(I)	36°	49°
Poly(Abr)·2poly(I)	35°	47°

T_m differences among the 2'-halogeno polynucleotide complexes are rather small and somewhat lower than that of poly(A)·2poly(I). However, in the presence of 10mM Mg^{2+} in addition to 0.15M Na^+ , the differences increased and the order of T_m s was $\text{Af} > \text{Acl} > \text{Abr}$. The tendency for a decrease in thermal stability with size of the substituents was again observed in these three-stranded, all purine polynucleotide complexes.

CONCLUDING REMARKS

We have presented evidence that poly(A) analogs having 2'-halogen atoms instead of OH show quite similar physical properties to those of poly(A). Even when the size of the

halogen is very small (as in the case of F), the polynucleotide poly(Af) showed similar properties to poly(rA) rather than poly(dA). These facts clearly demonstrate that 2'-substituents of nucleosides in polynucleotides must be involved as governing factor(s) of polynucleotide conformation and the size as well as polarity of these substituents have influence on their stability. If the nature of the 2'-substituents is similar, for instance halogen atoms, the conformational stability decreases as the size of the substituent increases.

Although the true mechanism by which 2'-substituents exert their stabilization effects must await further investigations, some involvement of solvent molecules cannot be excluded.

ACKNOWLEDGEMENTS

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INTERFERON INDUCING ACTIVITY OF A 2'-MODIFIED DOUBLE-STRANDED COMPLEX, POLY (2'-AZIDO-2'-DEOXYINOSINIC ACID)·POLY (CYTIDYLIC ACID)

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Although the presence of free 2'-hydroxyl groups in both strands of a double-stranded RNA complex has been recognized as one of the major requisites for the interferon inducing activity of double-stranded RNAs, we have found a particular analog of $(I)_n \cdot (C)_n$, in which the 2'-hydroxyls of the purine strand were replaced by azido groups $[(dlz)_n \cdot (C)_n]$, to be exquisitely effective in inducing interferon. Various other 2'-azido analogs, *i.e.* $(dlz) \cdot (brC)_n$, $(I)_n \cdot (dCz)_n$, $(dAz)_n \cdot (U)_n$, $(dAz)_n \cdot (rT)_n$ and $(dAz)_n \cdot (dUz)_n$ were inactive as inducers of interferon. Thus we come to the conclusion that the interferon inducing system recognizes total three-dimensional conformation of the double-stranded RNA.

Keywords—interferon induction; poly (2'-azido-2'-deoxyinosinic acid); double-stranded complex; primary rabbit kidney cells; human skin fibroblast cells

INTRODUCTION

The presence of intact 2'-hydroxyl groups in both strands of the dsRNA (double-stranded RNA) complex has been considered as an absolute requirement for the interferon inducing ability of synthetic polynucleotides.¹⁾ Indeed, various attempts to replace the 2'-hydroxyl group in either strand of $(I)_n \cdot (C)_n$ or $(A)_n \cdot (U)_n$ by one or another substituent have invariably produced duplexes with little, if any, interferon inducing activity. The lack of interferon inducing activity of the 2'-modified RNA complexes could be interpreted to mean that the cellular receptor site for interferon induction specifically recognizes the 2'-hydroxyl groups of the dsRNA molecules. Alternatively, the presence of the 2'-OH groups may generate a particular steric configuration which allows the dsRNA to interact with the interferon-inducer receptor system.

MATERIALS AND METHODS

Polynucleotides — $(I)_n \cdot (C)_n$, $(A)_n \cdot (U)_n$ and

other complexes employed in the interferon induction assays were constituted with homopolymers obtained from P-L Biochemicals (Milwaukee, Wisconsin). The sedimentation values (s_{20} , w) of $(I)_n$, $(C)_n$, $(A)_n$, and $(U)_n$ were 9.4, 10.0, 9.8 and 7.0 S, respectively. The complex $(I)_n \cdot (brC)_n$ and $(A)_n \cdot (rT)_n$ have been described previously.²⁾ The origin of the 2'-azido polynucleotides was as follows: $(dUz)_n$,⁴⁾ $(dCz)_n$,⁵⁾ $(dAz)_n$,⁶⁾ and $(dlz)_n$.⁷⁾ The latter preparation $[(dlz)_n]$ was further purified by gel filtration on a Sephadex G-100 column (2×25 cm, 0.04M NH_4HCO_3 , pH 7.5). Only the polymer that eluted in the void volume was pooled and lyophilized to dryness.

Interferon Induction — The production of interferon was measured in four systems: (i) primary rabbit kidney (PRK) cells "superinduced" with cycloheximide and actinomycin D; (ii) human skin fibroblast (HSF) cells (VGS strain) "primed" with human fibroblast interferon and "superinduced" with cycloheximide and actinomycin D;

TABLE I. Interferon Induction by Various 2'-Azido Analogs of $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$ in Primary Rabbit Kidney Cells "Superinduced" with Cycloheximide and Actinomycin D

Polynucleotide ^{a)}	Interferon titer ^{b)}	
	Average	(units/ml) Range
$(A)_n \cdot (U)_n$	750	600—1000
$(A)_n \cdot (rT)_n$	2400	2000—3000
$(A)_n \cdot (dUz)_n$	10	<10—20
$(dAz)_n \cdot (U)_n$	<10	
$(dAz)_n \cdot (rT)_n$	<10	
$(dAz)_n \cdot (dUz)_n$	<10	
$(I)_n \cdot (C)_n$	6000	3000—10000
$(I)_n \cdot (brC)_n$	8000	6000—10000
$(I)_n \cdot (dCz)_n$	10	
$(dIz)_n \cdot (C)_n$	1900	1000—3000
$(dIz)_n \cdot (brC)_n$	<10	

a) Applied to the cells at 10 $\mu\text{g/ml}$ ($-2.5 \times 10^{-5}\text{M}$ in duplex).

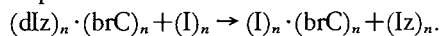
b) For at least 3 separate determinations (up to 10 separate determinations for these polynucleotides for which the range of activity is indicated).

(iii) mouse L-929 cells "primed" with mouse interferon; and (iv) intact rabbits weighing approximately 1 kg. The exact methodology for monitoring interferon production in these systems has been described.³⁾

RESULTS

When assayed in primary rabbit kidney cells "superinduced" with cycloheximide and actinomycin D, none of the 2'-azido polynucleotides [$(dAz)_n$, $(dCz)_n$, $(dIz)_n$, $(dUz)_n$] or their complexes except $(dIz)_n \cdot (C)_n$, proved active as an interferon inducer (Table I). The interferon inducing ability of $(dIz)_n \cdot (C)_n$ was confirmed at different occasions with different preparations of polynucleotide: $(dIz)_n \cdot (C)_n$ was almost active as $(A)_n \cdot (rT)_n$ and only 3 times less active than $(I)_n \cdot (C)_n$. Unlike $(dIz)_n \cdot (C)_n$, $(dIz)_n \cdot (brC)_n$ failed to stimulate the production of interferon in rabbit kidney cells. The inactivity of $(dIz)_n \cdot (brC)_n$ is quite unexpected, as other $(I)_n \cdot (C)_n$ analogs, *viz.* $(c^7I)_n \cdot (C)_n$ and $(I)_n \cdot (C)_n$ itself become more potent interferon inducers upon substitution of a bromine at C-5 of the pyrimidine ring.²⁾ When mixed with $(I)_n$, $(dIz)_n \cdot (brC)_n$ attained the level of interferon inducing activity normally observed for $(I)_n \cdot (brC)_n$

(8000 units/ml, Table I), suggesting that under mixing conditions employed, the following displacement reaction⁸⁾ occurred:



With $(dIz)_n \cdot (C)_n$ and $(dIz)_n \cdot (brC)_n$, additional interferon induction tests were run in human diploid cells "primed" with interferon and "superinduced" with cycloheximide and actinomycin D. In this highly sensitive induction system which is generally applied for the large-scale production of human fibroblast interferon, $(dIz)_n \cdot (C)_n$ and $(I)_n \cdot (C)_n$ proved equally effective in inducing interferon, irrespective of the doses at which they were tested (Table II).

The efficacy of $(dIz)_n \cdot (C)_n$ as an interferon inducer in rabbit and human cell cultures prompted the extension of our interferon induction studies with $(dIz)_n \cdot (C)_n$ to other assay systems: mouse L-929 cells ("primed" with interferon) and intact rabbits. In the first system a close-response relationship was established for the amounts of interferon produced, whereas in the second system interferon production was followed as a function of time. In both test systems, $(dIz)_n \cdot (C)_n$ was less active than $(I)_n \cdot (C)_n$.

TABLE II. Interferon Induction by $(dIz)_n \cdot (C)_n$ and $(dIz)_n \cdot (brC)_n$ in Human Skin Fibroblasts "Primed" with Interferon and "Superinduced" with Cycloheximide and Actinomycin D

Polynucleotide	Interferon titer ($\log_{10}$ units*/ml) obtained with $\mu\text{g/ml}$ of polynucleotide ^{a)}		
	0.1	1	10
$(I)_n \cdot (C)_n$	3.0 (2.8—3.5)	3.7 (3.6—3.8)	3.9 (3.7—4.0)
$(I)_n \cdot (brC)_n$	3.5 (3.4—3.7)	3.9 (3.9—4.0)	4.2 (4.1—4.4)
$(dIz)_n \cdot (C)_n$	3.0 (2.8—3.2)	3.7 (3.5—3.8)	4.1 (4.0—4.4)
$(dIz)_n \cdot (brC)_n$	<1.3	<1.3	<1.3

a) Mean values for 3 or 4 determinations. Range of individual values is indicated in parentheses.

DISCUSSION

The most remarkable feature of the results reported herein is the interferon inducing activity of $(dIz)_n \cdot (C)_n$ which equalled that of $(I)_n \cdot (C)_n$, at least in human diploid cell cultures (Table II). $(dIz)_n \cdot (C)_n$ represents the first 2'-modified double-stranded RNA (with all 2'-hydroxyl group substituted in one strand of the duplex) which has been found to be an effective interferon inducer. The interferon stimulating ability of $(dIz)_n \cdot (C)_n$ indicates that (i) the presence of free 2'-OH groups in both strands is not an absolute requirement for the interferon inducing capacity of doublestranded RNA complexes and (ii) the receptor site for interferon induction does not specifically recognize the 2'-OHs *per se*, but rather the steric configuration conferred by the presence of these hydroxyls. Apparently, the steric configuration resulting from substitution at 2'-azido for 2'-OH in the $(I)_n$ strand is also recognized by the interferon induction receptor. Our results reinforce the concept that interferon induction by double-stranded polynucleotides is dependent on the recognition of a particular conformation rather than the binding of specific functional groups such as 2'-OH or purine N-7.⁹⁾ The double-strand character of $(dIz)_n \cdot (C)_n$ was suggested by UV absorption-mixing curves and monophasic melting curves.⁷⁾ In view of the interferon inducing potency of $(dIz)_n \cdot (C)_n$ the inactivity of $(dIz)_n \cdot (brC)_n$ is quite unexpected, the more so as the T_m of $(dIz)_n \cdot (brC)_n$ is about 25° higher (results not shown) than that of $(dIz)_n \cdot (C)_n$.

While $(dIz)_n \cdot (C)_n$ was effective as an interferon inducer, $(I)_n \cdot (dCz)_n$ was not (Table I). A low thermal stability could not be held responsible for the lack of the activity, since its T_m was almost identical to that of $(I)_n \cdot (C)_n$ and considerably higher than that of $(dIz)_n \cdot (C)_n$. The difference in interferon inducing activity between these complexes suggests that the interferon stimulating potency is more tolerant to substitutions at C-2' of the $(I)_n$ strand than it is to similar modifications of the $(C)_n$ strand. However, other modifications such as strand interruption by unpaired bases or bond breakage, are better tolerated by the $(C)_n$ strand than by the $(I)_n$ strand.¹¹⁾

Considering these results it may be deduced that the interferon inducing system recognizes the total three-dimensional conformation of the double-stranded polynucleotides. This point may be clarified by X-ray fiber diffraction studies of the appropriate polynucleotide complexes.

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Polynucleotides. LVI¹. Synthesis and properties of poly(2'-deoxy-2'-fluorinosinic acid)

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SUMMARY

Poly(2'-deoxy-2'-fluorinosinic acid) [poly(If)] was synthesized by polymerization of 2'-deoxy-2'-fluorinosine 5'-diphosphate catalyzed by *Escherichia coli* polynucleotide phosphorylase. Although the UV absorption properties of poly(If) closely resembled those of poly(I), thermal melting curves at Na⁺ concentrations of 0.15M and 0.75M suggested two ordered structures for poly(If) neutral form. CD spectra taken at 0.15M Na⁺ concentration showed rather large amplitudes in both a peak at 273 nm and a trough at 246 nm, suggesting rather strong vertical stacking of bases. When complexed with poly-(C), poly(If) forms a double-stranded complex, poly(If).poly-(C) which has T_m's higher by 10-20° than those of poly(If).poly(C) measured under the same conditions. The CD spectrum of this complex resembled that of poly(I).poly(C). The effect of the fluorine atom at the 2'-position on thermal stability of polynucleotides is discussed.

INTRODUCTION

We have reported the synthesis and properties of various 2'-substituted 2'-deoxypolynucleotides.²⁻⁶ A general feature of these polynucleotides is that though they lack 2'-OH groups and have substituents such as azido^{2,4}, chloro⁵, bromo⁵ and fluoro⁶ atoms at the 2'-position, these polynucleotides revealed rather similar physical and biological properties to those of polyribonucleotides. Poly(2'-azido-2'-deoxyinosinic acid), for example, had a strong interferon inducing ability when complexed with poly(C).^{7,8} Moreover, poly(2'-fluoro-2'-deoxyadenylic acid) serves as a good template for viral reverse transcriptase⁹ and as a messenger for protein synthesis.¹⁰

In this paper we report the synthesis of poly(2'-fluoro-2'-deoxyinosinic acid) [poly(If)] and some of its physical properties.

MATERIALS AND METHODSGeneral procedure

The general procedures used in this study were essentially the same as reported previously⁴, except that paper chromatography solvent systems were as follows : A, i-PrOH-conc. $\text{NH}_4\text{OH}-\text{H}_2\text{O}$ (7:1:2); B, n-BuOH-AcOH- H_2O (5:2:3); C, n-PrOH-conc. $\text{NH}_4\text{OH}-\text{H}_2\text{O}$ (55:15:35).

Synthesis of poly(If)

If 5'-MP--- 2'-Deoxy-2'-fluoroadenosine 5'-monophosphate⁶ (2,000 A_{260} , 0.14 mmole) was dissolved in 30% AcOH (1.5 ml) and NaNO_2 (0.3g) was added. The solution was kept at 37° for 20 hrs. H_2O (ca. 100 ml) was added and the mixture was applied to a column of active charcoal. The column was thoroughly washed with H_2O and eluted with EtOH- H_2O -conc. NH_4OH (50:50:1) mixture (ca. 50 ml). Eluents were evaporated in vacuo, the residue dissolved in H_2O , and applied to a column of Dowex 1x2 (formate form, 20 ml). After the water-wash, elution with 0.3N formic acid gave two peaks. Fractions in the second peak were pooled and evaporated in vacuo. Traces of formic acid were removed by evaporation several times with added water. The yield of 2'-deoxy-2'-fluoroinosine 5'-monophosphate was 1,290 A_{250} (0.11 mmole, 76%). Paper chromatography : $R_f(\text{A})$ 0.07, $R_f(\text{B})$ 0.34, $R_f(\text{C})$ 0.32. Paper electrophoresis : R_{A-pA} 2.5 (at pH 3.5) and 1.04 (at pH 7.5).

If 5'-DP --- If 5'-MP (1,290 A_{250} , 0.11 mmole) was refluxed in a mixture of H_2O (1.5 ml) and t-BuOH (1.5 ml) with morpholine (1.1 mmole). Into the solution DCC (235 mg, 1.1 mmole) dissolved in t-BuOH (2 ml) was added dropwise during 1 hr. The refluxing was maintained for 3.5 hrs and the cooled solution was extracted with ether (3 ml x 3). The water-layer was evaporated in vacuo and the residue was rendered anhydrous by evaporation three times with added pyridine. 80% phosphoric acid (40 μl , 0.58 mmole) and n-Bu₃N (0.14 ml, 0.58 mmole) were rendered anhydrous by evaporation three times with added pyridine, the residue taken up in pyridine, and added to the anhydrous morpholidate. The mixture was further evaporated three times each with added pyridine and toluene. The

residue was dissolved in DMF (1 ml) and kept at 30° for 2 days. H₂O (100 ml) was added and the solution was made slightly acidic with 1N HCl. The solution was applied to a column (1.4 x 8 cm) of DEAE-Sephadex A-25 (12 ml, bicarbonate form). The column was eluted with a linear gradient of 0 to 0.3M triethylammonium bicarbonate. Three peaks were eluted at buffer concentrations of 0.03M, 0.13M and 0.19 M, respectively. Peak I contained IfDP in a yield of 745 A₂₅₀ (59%). Paper chromatography : R_f(A) 0.04, R_f(B) 0.10. Paper electrophoresis : R_{A-pA} 1.41 (at pH 7.5). Peak II and III were confirmed to be 5'-5' pyrophosphate and the unreacted morpholidate.

Poly(If) ----- An incubation mixture (7.5 ml) containing IfDP 4 mM, MgCl₂ 2 mM, Tris-HCl (pH 8.5) 100 mM and E. coli polynucleotide phosphorylase¹¹ 5.8 units/ml was incubated at 37° for 24 hrs. The mixture was extracted five times with an i-AmOH-CHCl₃ (1 : 3) mixture and lyophilized. Filtration through a column (2.7 x 95 = 540 ml) of Sephadex G-50 gave poly(If) as a single peak, which was eluted at the void volume, indicating that this poly(If) had a chain length of at least 50 nucleotide units. The yield was 35 A₂₅₀ (3.6 μ mole, 12%). Digestion of this sample (ca. 5 A₂₅₀ units) with snake venom phosphodiesterase gave only IfMP as examined by paper chromatography.

RESULTS AND DISCUSSION

UV spectra of poly(If)

The UV absorption spectra of poly(If) taken at pH 7.0 in the presence of 0.15M Na⁺ are shown in Fig. 1 together with that of If 5'-MP. At 20° the curve has a maximum at 245 nm having ϵ of 9,800 and a shoulder at around 273 nm. The hypochromicity calculated from ϵ (12,500) of the monomer, If 5'-MP was 20%. These values were comparable to those of poly(I), except that the shoulder at 273 nm is smaller in poly(I). At 40° the spectrum of poly(If) changed slightly to that shown in Fig. 1. This curve had a maximum at 246 nm and the shoulder at 274 nm disappeared. The hypochromicity calculated from ϵ value of the monomer was 12%.

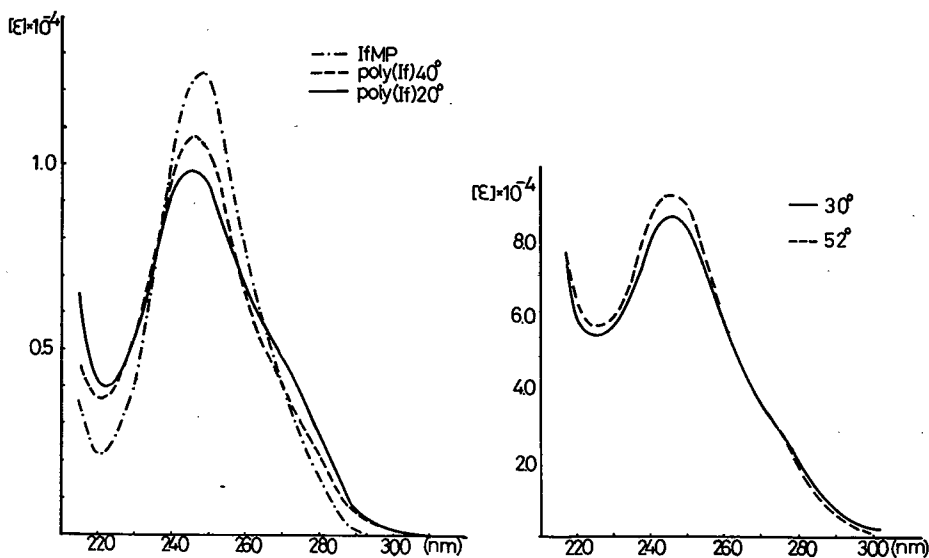


Fig. 1. UV spectrum of poly(If) and If 5'-MP at pH 7.0 in the presence of 0.15M Na⁺ ion.

Fig. 2. UV spectrum of poly(If) in the presence of 0.75M Na⁺.

When the UV spectra of poly(If) were taken at neutrality in the presence of 0.75M Na⁺, the curves shown in Fig. 2 were observed. At 30° the curve had $\lambda_{\max}$ at 247 nm as in the case of 0.15M Na⁺, but the ϵ value decreased to 8,650. On raising the temperature to 52° some hyperchromic change was observed as indicated in Fig. 2 (dotted line). As discussed later, these two types of poly(If) neutral form may be the result of differing salt concentrations of 0.15M and 0.75M and are corresponding to those of poly(I) neutral forms.^{12,13}

Thermal melting of poly(If)

In the presence of 0.15M Na⁺, the melting curve of poly-(If) shows an abrupt increase at 27° (see Fig. 3, lower line). This may indicate that poly(If) has a semistable ordered form at this Na⁺ concentration. The thermal melting curve taken in the presence of 0.75M Na⁺ (Fig. 3, upper curve) shows a steep increase between 40° and 52° and a T_m of 47° was obtained. However, these two states of the poly(If) ordered forms cannot be identical, because a graph of the relationship between T_m and Na⁺ (Fig. 4) does not give a straight

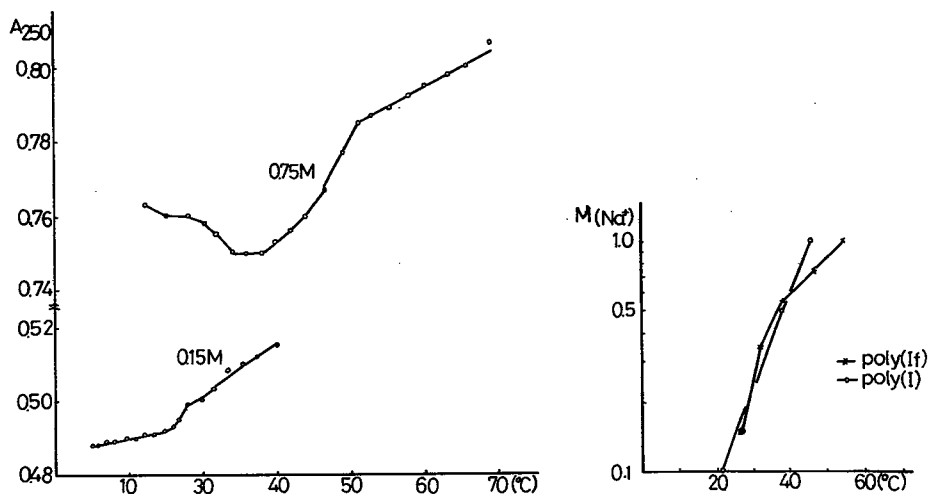


Fig. 3. Thermal melting profiles of poly(If) at Na⁺ concentration of 0.15M and 0.75M.

Fig. 4. Relationship of T_m of poly(If) and poly(I) to Na⁺ concentration.

line, but tends towards relatively higher T_m's as Na⁺ concentration is increased. It may be noted that in the case of poly(I) this curve is almost straight (see Fig., o-o-o) and the effect of salt concentration on thermal stability is much larger in poly(If) than in the case of poly(I). Furthermore, the T_m of the high salt form of poly(If) is higher by 4° than that of the poly(I) four-stranded form.¹²

CD spectra of poly(If)

CD spectra of poly(If) taken at neutrality with 0.15M Na⁺ are shown in Fig. 5. Curves taken below 20° have two maxima at 274 nm and 223-224 nm and a large trough at 246 nm. This spectrum is significantly different from that of poly(I) neutral form which has three peaks at 282, 254 and 223 nm and two troughs at 267 and 238 nm in the same conditions.¹⁴ Moreover, the [θ] values are much smaller in poly(I) than those of poly(If). The spectra taken at 25° and 27° showed half melting of the ordered form and finally the spectrum taken at 40° showed complete breakdown of this form. When the CD of poly(If) was measured in the presence of 0.75M Na⁺ at pH 7.0, the curve at 30° showed a similar features to those

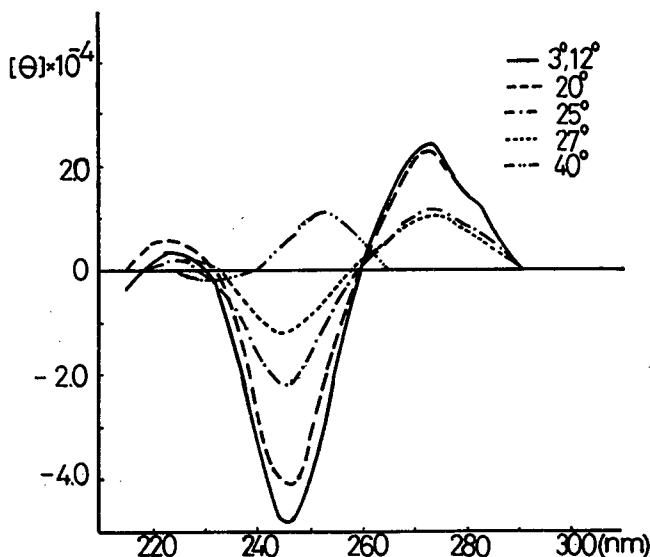


Fig. 5. CD spectra of poly(If) at 0.15M Na⁺ concentration.

with 0.15M Na⁺, but its amplitudes were somewhat smaller. On heating this solution to 40°, the curve changed to one which resembled that observed in Fig. 5 at high temperature.

We may conclude, therefore, that poly(If) has two types of ordered structure according to the Na⁺ concentration, one observed at the low Na⁺ concentration (0.15M) and one observed at high (0.75M) Na⁺ concentration. Although the latter may have the same quadruple-stranded form as that observed in the case of poly(I) at high salt concentration, the structure which is present at 0.15M Na⁺ concentration may be a semi-stable structure stabilized by vertical stacking of neighboring bases. Considering that the same base moiety, hypoxanthine is present in both poly(I) and poly(If), this effect of enhanced stability must be due to the 2'-fluoro atom.

Complex formation of poly(If) with poly(C)

Poly(I) is known to form a 1:1 complex with poly(C)¹⁵ and the poly(I)·poly(C) complex is widely used as an inducer of interferon in mammalian cells.¹⁶ Recently we found that poly-(2'-azido-2'-deoxyinosinic acid) formed a complex with poly(C) and exerted the first non-ribo type polynucleotide interferon

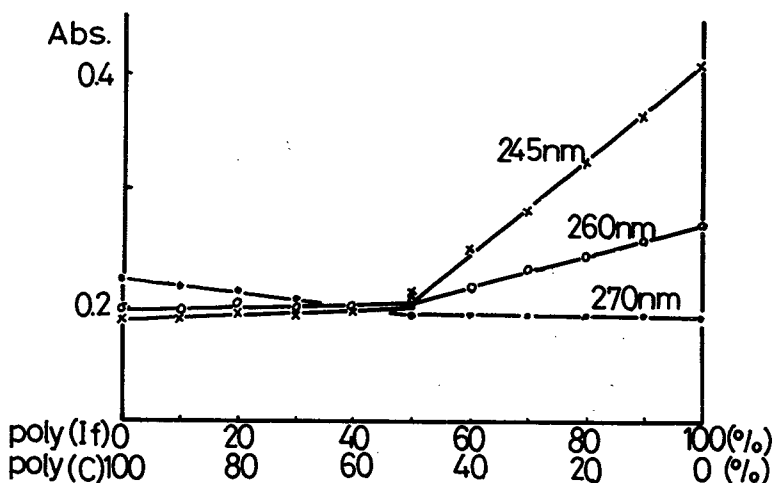


Fig. 6. Mixing curves of poly(I) and poly(C).

inducing activity.

When poly(I) was mixed with poly(C) in various ratios and the UV absorbance of these mixtures measured at 245, 260 and 270 nm were plotted, we observed inflection points at ratios of 50:50 for poly(I) and poly(C) (Fig. 6). This indicates the formation of a 1:1 complex, poly(I)·poly(C). The CD spectrum of this complex was as shown in Fig. 7. and the overall profile of this curve closely resembles that of poly(I)·poly(C) complex.¹⁷ These facts suggest that poly(I)·poly(C) has a similar conformation to that of poly(I)·poly(C) which is believed to be RNA-11 fold helix¹⁸, but not to that of poly(dI)·poly(C).¹⁵

The thermal melting curves of the complex, poly(I)·poly(C) measured with various Na^+ concentrations are summarized in Fig. 8. The T_m at $[\text{Na}^+] = 0.15$ was 75° which is higher by 12° than that of poly(I)·poly(C) in the same conditions. This tendency may be seen in the T_m vs. $[\text{Na}^+]$ curves shown in Fig. 9. In the range of $[\text{Na}^+]$ of 0.01-0.35M, poly(I)·poly(C) has higher T_m values than those of poly(I)·poly(C) and poly(dI)·poly(C). Even at very low salt concentration the poly(I)·poly(C) complex has considerable thermal stability.

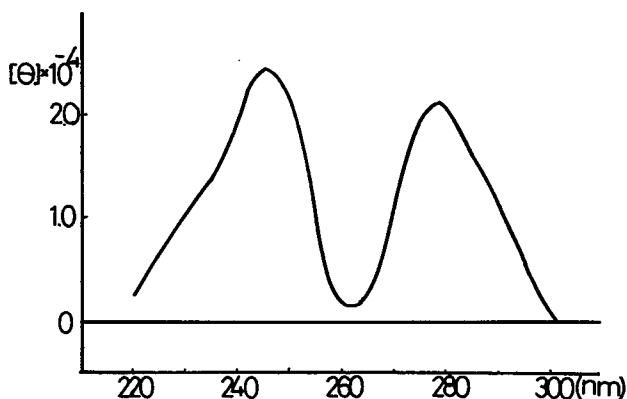


Fig. 7. CD spectrum of poly(If)·poly(C) complex.

Comparison of the T_m 's of double-stranded complexes of other 2'-fluoro polynucleotides show that of poly(A)·poly(Uf) is higher by 15–20° than that of poly(A)·poly(U)¹⁹, whereas poly(I)·poly(Cf)²⁰ has a somewhat lower T_m than that of poly(I)·poly(C). Furthermore, poly(Af)·poly(U)⁶ has almost the same T_m as that of poly(A)·poly(U). (Table I) From this comparison it emerges that the stabilization of ordered structure by the introduction of F atom instead of OH in ribopolynucleo-

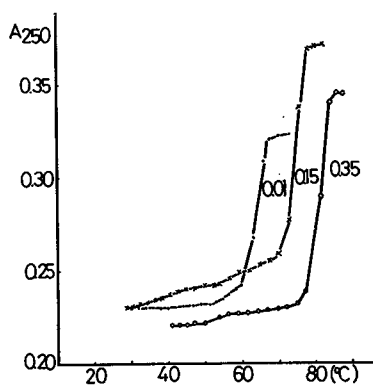


Fig. 8. Temperature absorbance profiles of poly(If)·poly(C) at various Na^+ concentrations.

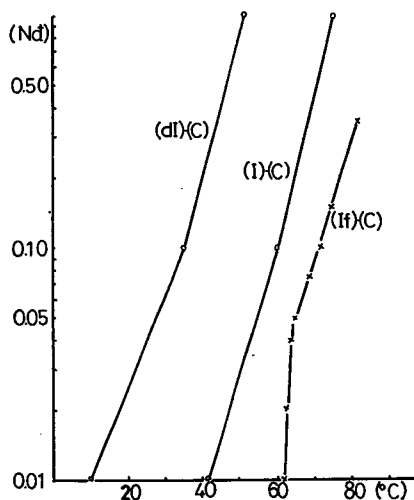


Fig. 9. Relationship between T_m of various double-stranded complexes and Na^+ concentration.

Table I. Melting temperature of various double-stranded complexes (° C).

	0.10M Na ⁺	0.15M Na ⁺
Poly(A)·poly(U)	57	62
Poly(Af)·poly(U)		64
Poly(A)·poly(Uf)	75.2	
Poly(I)·poly(C)	60.2	63
Poly(If)·poly(C)	72	75
Poly(I)·poly(Cf)	57-58	

tides may be different in strands having C=O groups at the hydrogen-bonding site (6-position of purines or 4-positions of pyrimidines) to those bearing NH₂ groups in the same position. If the F atom is introduced to a C=O containing strand, as in the case of poly(If) or poly(Uf), the stability of the complex is increased. In contrast, if the F atom is introduced to an NH₂ containing strand, as in poly(Af) and poly(Cf), the stability of complexes decreased relative to their ribo counterparts. Thus the F atom when introduced to the 2'-position, exerts its effects by strong electron-withdrawal which favors solvation as hydrogen-bonding acceptor and decreases base pKa through bonds or solvent molecules. Recent finding that in 2'-fluoro-2'-deoxyadenosine the 2'-carbon is more axial than in the case of ribo-adenosine as studied by ¹H-NMR coupling of H₁, and H₂,²¹ may be relevant to these facts.

Thus far it was found that the introduction of fluorine atoms to the 2'-position of polynucleotides altered their properties and may be interesting in biological studies.

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As far as possible, authors should follow the Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, and particularly the abbreviations for nucleic acids, polynucleotides and their constituents (1971) *J.Mol.Biol.* 55, 299-305.

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Interferon Induction by a 2'-Modified Double-Helical RNA, Poly(2'-azido-2'-deoxyinosinic acid) · polycytidylic acid

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Although the presence of free 2'-hydroxyl groups in both strands of a double-stranded RNA complex has been recognized as one of the major requisites for the interferon-inducing activity of double-stranded RNAs, we have found a particular analogue of $(I)_n \cdot (C)_n$ in which the 2'-hydroxyls of the purine nucleotide strand were replaced by azido groups, $(dIn_3)_n \cdot (C)_n$, to be highly effective in inducing interferon. Various other 2'-azido analogues of $(I)_n \cdot (C)_n$ and $(A)_n \cdot (U)_n$, i.e. $(dIn_3)_n \cdot (br^5C)_n$, $(I)_n \cdot (dCn_3)_n$, $(dAn_3)_n \cdot (U)_n$, $(A)_n \cdot (dUn_3)_n$, $(dAn_3)_n \cdot (rT)_n$ and $(dAn_3)_n \cdot (dUn_3)_n$, were inactive as inducers of interferon. In human fibroblast cultures, the interferon-inducing activity of $(dIn_3)_n \cdot (C)_n$ equalled that of $(I)_n \cdot (C)_n$. In other interferon-induction systems (primary rabbit kidney cells, mouse L-929 cells, intact rabbits), $(dIn_3)_n \cdot (C)_n$ was less active than $(I)_n \cdot (C)_n$. As assessed by both radiochemical and biological means, $(dIn_3)_n \cdot (C)_n$ was more susceptible to degradation by pancreatic ribonuclease and human serum nucleases than was $(I)_n \cdot (C)_n$. The t_m of $(dIn_3)_n \cdot (C)_n$ was 52.5 °C, as compared to 62.5 °C for $(I)_n \cdot (C)_n$, both determined in 0.15 M Na⁺, pH 7.0. Under the same conditions, $(dIn_3)_n \cdot (br^5C)_n$ had a t_m of 77 °C and $(I)_n \cdot (br^5C)_n$ had a t_m of 87 °C. The reactivity of $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ towards antibodies to double-stranded RNA was evaluated by quantitative complement fixation, counterimmunoelectrophoresis and competitive radioimmunoassay. In these tests, $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ showed an immunoreactivity pattern comparable to that of $(I)_n \cdot (C)_n$ and $(I)_n \cdot (br^5C)_n$.

The presence of intact 2'-hydroxyl groups in both strands of the dsRNA (double-stranded RNA) complex has been considered as an absolute requirement for the interferon-inducing ability of synthetic polynucleotides. Indeed, various attempts to replace the 2'-hydroxyl group in either strand of $(I)_n \cdot (C)_n$ or $(A)_n \cdot (U)_n$ by one or another substituent have invariably produced duplexes with little, if any, interferon-inducing activity. Modifications thus far executed include: 2'-hydrogen in the $(I)_n$ strand of $(I)_n \cdot (C)_n$ [1–3], 2'-hydrogen in the $(C)_n$ strand of $(I)_n \cdot (C)_n$ [1, 2, 4, 5], 2'-fluoro in the $(U)_n$ strand of $(A)_n \cdot (U)_n$ [6],

2'-chloro in the $(U)_n$ strand of $(A)_n \cdot (U)_n$ [5], 2'-chloro in the $(C)_n$ strand of $(I)_n \cdot (C)_n$ [5], 2'-azido in the $(U)_n$ strand of $(A)_n \cdot (U)_n$ [7], 2'-O-methyl in the $(U)_n$ strand of $(A)_n \cdot (U)_n$ [4], 2'-O-methyl in the $(I)_n$ strand of $(I)_n \cdot (C)_n$ [8], 2'-O-methyl in the $(C)_n$ strand of $(I)_n \cdot (C)_n$ [4, 8], 2'-O-ethyl in the $(A)_n$ and $(U)_n$ strands of $(A)_n \cdot (U)_n$ [9], and 2'-O-acetyl in the $(A)_n$ strand of $(A)_n \cdot (U)_n$ and in the $(I)_n$ and $(C)_n$ strands of $(I)_n \cdot (C)_n$ [10]. The lack of interferon-inducing activity of the 2'-modified RNA complexes could be interpreted to mean that the cellular receptor site for interferon induction specifically recognizes the 2'-hydroxyl groups of the dsRNA molecules. Alternatively, the presence of the 2'-OH groups may generate a particular steric configuration which allows the dsRNA to interact with the interferon-inducer receptor system. If this is the case, there may exist 2' substituents which imitate the effect of 2'-OH on the overall conformation of the double helix, and, accordingly, do not annihilate the interferon-inducing potency of the complex. In this report we describe such a complex, $(dIn_3)_n \cdot (C)_n$, an analog of $(I)_n \cdot (C)_n$ in which 2'-OH of the $(I)_n$

Abbreviations. Abbreviations for synthetic polynucleotides conform to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [Eur. J. Biochem. 15, 203–208 (1970)]. Less commonly used abbreviations are: $(dIn_3)_n$, poly(2'-azido-2'-deoxyinosinic acid); $(dCn_3)_n$, poly(2'-azido-2'-deoxycytidylic acid); $(dAn_3)_n$, poly(2'-azido-2'-deoxyadenylic acid); $(dUn_3)_n$, poly(2'-azido-2'-deoxyuridylic acid); $(rT)_n$, poly(ribothymidylic acid); $(br^5C)_n$, poly(5-bromocytidylic acid); $(c^7I)_n$, poly(7-deazainosinic acid); $(s^2C)_n$, poly(2-thiocytidylic acid); t_m , temperature at the midpoint of the absorbance change obtained when heating the polynucleotide.

strand is replaced by a 2'-azido group. $(dIn_3)_n \cdot (C)_n$ was found to induce significant amounts of interferon in both primary rabbit kidney and human skin fibroblast cultures. Under the same conditions, other 2'-azido substituted polynucleotide complexes, viz. $(dIn_3)_n \cdot (br^5C)_n$, $(I)_n \cdot (dCn_3)_n$, $(dAn_3)_n \cdot (U)_n$, $(A)_n \cdot (dUn_3)_n$, $(dAn_3)_n \cdot (dUn_3)_n$ and $(dAn_3)_n \cdot (rT)_n$ were ineffective as inducers of interferon.

MATERIALS AND METHODS

Polynucleotides

$(I)_n \cdot (C)_n$, $(A)_n \cdot (U)_n$ and other complexes employed in the interferon-induction assays were constituted with homopolymers obtained from P-L Biochemicals (Milwaukee, Wisconsin). The sedimentation values ($s_{20,w}$) of $(I)_n$, $(C)_n$, $(A)_n$ and $(U)_n$ were 9.4, 10.0, 9.8 and 7.0 S, respectively. The 3H -labelled $(C)_n$ ($s_{20,w} = 6.5$ S) was obtained from Miles Laboratories (Elkhart, Indiana). Its specific activity was 65.6 Ci/mol P or 1 Ci/6.1 g polymer. The complexes $(I)_n \cdot (br^5C)_n$ and $(A)_n \cdot (rT)_n$ have been described previously [11,12]. The origin of the 2'-azido polynucleotides was as follows: $(dUn_3)_n$ [13], $(dCn_3)_n$ [14], $(dAn_3)_n$ [15] and $(dIn_3)_n$ [16]. The latter preparation, $(dIn_3)_n$, was further purified by gel filtration on a Sephadex G-100 column (2×25 cm, 0.04 M NH_4HCO_3 , pH 7.5). Only the polymer that eluted in the void volume was pooled and lyophilized to dryness. A portion of this material was degraded overnight at 37 °C with a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase. The product was applied onto a silica gel GF thin-layer plate and developed with isopropyl alcohol/0.1 M boric acid/water (80/10/10, v/v/v). Under these conditions, the R_F of 2'-azido-2'-deoxyinosine (dIn_3) was 0.47 whereas the R_F of inosine was 0.37. The enzymatic hydrolysate contained dIn_3 as the only detectable nucleoside (under ultraviolet light). On this basis, it was estimated to contain $\geq 95\%$ dIn_3 .

Antisera to Double-Stranded RNA

These were obtained from New Zealand white rabbits immunized with complexes of $(A)_n \cdot (U)_n$ or $(I)_n \cdot (br^5C)_n$ with methylated bovine serum albumin [17]. Antiserum to $(A)_n \cdot (U)_n$ was absorbed with $(A)_n$ and antiserum to $(I)_n \cdot (br^5C)_n$ was absorbed with $(I)_n$ to remove small amounts of antibodies that reacted with the single homopolymers. The antibodies were immunospecifically purified by dissociation of precipitates formed with $(I)_n \cdot (C)_n$ [18].

Ultraviolet Spectroscopic Measurements

Temperature absorbance (melting) profiles were determined as described previously [13].

Interferon Induction

The production of interferon was measured in four systems: (a) primary rabbit kidney cells 'superinduced' with cycloheximide and actinomycin D; (b) human skin fibroblast cells (VGS strain) 'primed' with human fibroblast interferon and 'superinduced' with cycloheximide and actinomycin D; (c) mouse L-929 cells 'primed' with mouse interferon; (d) intact rabbits weighing approximately 1 kg. The exact technique for monitoring interferon production in these systems has been described [12,19,20].

Ribonuclease Susceptibility

The sensitivity to degradation by pancreatic ribonuclease (bovine pancreatic ribonuclease A, crystallized five times, 80 Kunitz U/mg, Sigma Chemical Co., St Louis, Missouri) and human serum (nucleases) was determined by measuring either residual interferon-inducing activity (in human skin fibroblasts, as indicated above) or acid-insoluble radioactivity [21].

Quantitative Complement Fixation

This was performed according to the method of Wasserman and Levine [22], in a buffer of 0.14 M NaCl, 0.01 M Tris · HCl, pH 7.4, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 0.1% gelatin (total volume of reaction mixture 1.4 ml).

Competitive Radioimmunoassay

Binding of the labelled polynucleotide [^{14}C]- $(I)_n \cdot (C)_n$ was performed in a buffer containing 0.14 M NaCl, 0.01 M Tris · HCl, pH 8.0 and 0.2% gelatin [23]. To determine the competitive effect of other double-stranded RNAs, 100 μ l of the antiserum dilution was heated for 15 min at 56 °C to inactivate non-specific binding proteins, then incubated for 30 min at 37 °C with 100 μ l of a solution containing various concentrations of the (unlabelled) inhibiting polynucleotide. Finally, 0.3 μ g of [^{14}C] $(I)_n \cdot (C)_n$ (in 50 μ l) was added, and the mixtures were incubated for 45 min at 37 °C. Upon addition of 0.5 ml buffer, the reaction mixtures were filtered through Whatman GF/C glass fiber filters. The filters were washed three times with 1 ml buffer (at room temperature), dried and counted in a toluene-based scintillant.

Counterimmunoelectrophoresis

This was performed with 0.8% agar in 0.05 M Tris · HCl, pH 8.0 on 5×7.5 -cm glass slides. Antigen wells containing 0.5 μ g of polynucleotide in 50 μ l were placed opposite a trough containing 150 μ l of the antiserum dilution. Electrophoresis was carried out at 250 V (14 mA per two slides) for 1 h.

Table 1. Thermal transitions of various 2'-azido analogues of $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$

Polynucleotide	t_m	Nature of transition	Medium	References
	°C			
$(A)_n \cdot (U)_n$	48.5	2→1	0.04 M Na ⁺ , pH 7.0	[24]
	49	2→3	0.15 M Na ⁺ , 1 mM Mg ²⁺ , pH 6.95	[12]
	62	3→1	0.15 M Na ⁺ , 1 mM Mg ²⁺ , pH 6.95	[12]
$(A)_n \cdot (rT)_n$	62	2→3	0.05 M Na ⁺ , pH 7.0	[25]
	53	2→3	0.15 M Na ⁺ , 1 mM Mg ²⁺ , pH 6.95	[12]
	76	3→1	0.15 M Na ⁺ , 1 mM Mg ²⁺ , pH 6.95	[12]
$(A)_n \cdot (dUn_3)_n$	59	2→1	0.21 M Na ⁺ , pH 7.0	[13]
	59	3→1		
$(dAn_3)_n \cdot (U)_n$	46	2→1 ^a	0.04 M Na ⁺ , pH 7.0	[15]
	65	3→1 ^b	0.15 M Na ⁺ , pH 7.0	[15]
$(dAn_3)_n \cdot (rT)_n$	84	— ^c	0.15 M Na ⁺ , 1 mM Mg ²⁺ , 1 mM Ca ²⁺ , pH 7.0	this report
$(dAn_3)_n \cdot (dUn_3)_n$	55	— ^c	0.15 M Na ⁺ , 1 mM Mg ²⁺ , 1 mM Ca ²⁺ , pH 7.0	this report
$(I)_n \cdot (C)_n$	57	2→1	0.1 M Na ⁺ , pH 7.5	[26]
	62.5	2→1	0.15 M Na ⁺ , pH 7.0	[27,16]
$(I)_n \cdot (br^5C)_n$	87	2→1	0.15 M Na ⁺ , pH 7.0	[27]
$(I)_n \cdot (dCn_3)_n$	56	2→1	0.1 M Na ⁺ , pH 7.5	[14]
$(dIn_3)_n \cdot (C)_n$	51	2→1	0.15 M Na ⁺ , pH 7.0	[16]
	52.5	2→1	0.15 M Na ⁺ , pH 7.2	this report
$(dIn_3)_n \cdot (br^5C)_n$	77	2→1	0.15 M Na ⁺ , pH 7.2	this report

^a Possibility of triple-stranded intermediate has not been eliminated.^b Possibility of double-stranded intermediate has not been eliminated.^c Exact nature of transition remains to be established.

RESULTS

Thermal Stability of 2'-Azido Analogues of $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$

The t_m values of the various 2'-azido analogues of $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$ are reviewed in Table 1.

As assessed previously [13], $(A)_n$ can form 1:1 and 1:2 complexes with $(dUn_3)_n$, i.e. $(A)_n \cdot (dUn_3)_n$ and $(A)_n \cdot 2(dUn_3)_n$. Both complexes undergo monophasic transitions directly to the constituent homopolymers (2→1 and 3→1, respectively) [13]. Likewise, $(dAn_3)_n$ forms 1:1 and 1:2 complexes with $(U)_n$ [15]. Both complexes may melt out directly to the constituent homopolymers, although prior rearrangements (2→3 or 3→2) have not been ruled out. The t_m values of $(A)_n \cdot (dUn_3)_n$ and $(dAn_3)_n \cdot (U)_n$ are remarkably similar to that of $(A)_n \cdot (U)_n$ (Table 1), suggesting that introduction of an azido group into the C-2' position of either $(U)_n$ or $(A)_n$ does not significantly affect the thermal stability of the $(A)_n \cdot (U)_n$ duplex.

An equimolar mixture of $(dAn_3)_n$ and $(rT)_n$ gave rise to a complex which melted over a wide range with $t_m \approx 84^\circ\text{C}$ in Dulbecco's phosphate-buffered saline (data not shown). Complex formation was also observed for an equimolar mixture of $(dAn_3)_n$ and $(dUn_3)_n$. This complex exhibited a biphasic transition

in Dulbecco's phosphate-buffered saline (NaCl/P_i) with $t_m \approx 55^\circ\text{C}$ for the first transition and $t_m \approx 60^\circ\text{C}$ for the second transition (data not shown). The exact nature of the complexes formed between $(dAn_3)_n$ and $(rT)_n$ or $(dUn_3)_n$ remains to be determined.

The thermal stability of $(I)_n \cdot (C)_n$ was not markedly altered upon substitution of 2'-azido for 2'-OH in the $(C)_n$ strand of $(I)_n \cdot (C)_n$ [14,26]. However, introduction of an azido group into C-2' of the $(I)_n$ strand destabilized the $(I)_n \cdot (C)_n$ structure by approximately 10°C [16]. The melting profile obtained for an equimolar mixture of $(dIn_3)_n$ and $(C)_n$ showed but one transition with midpoint (t_m) = 52.5°C in NaCl/P_i (without Ca²⁺ or Mg²⁺) (Fig. 1). Under the same conditions $(I)_n \cdot (C)_n$ had $t_m = 62.5^\circ\text{C}$. Likewise, an equimolar mixture of $(dIn_3)_n$ and $(br^5C)_n$ showed a single transition at $t_m = 77^\circ\text{C}$ (Fig. 1) (in NaCl/P_i without Ca²⁺ or Mg²⁺). Under the same conditions $(I)_n \cdot (br^5C)_n$ had $t_m = 87^\circ\text{C}$. Thus, $(dIn_3)_n \cdot (br^5C)_n$ also was destabilized by 10°C compared to its parent $(I)_n \cdot (br^5C)_n$.

Interferon Induction by 2'-Azido Analogues of $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$

When assayed in primary rabbit kidney cells 'super-induced' with cycloheximide and actinomycin D, none

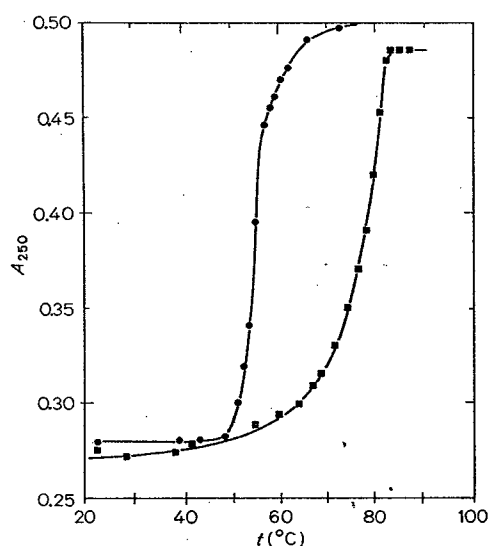


Fig. 1. Melting profiles for an equimolar mixture of $(dIn_3)_n + (C)_n$ (●) and for an equimolar mixture of $(dIn_3)_n + (br^5C)_n$ (■), both determined in phosphate-buffered saline (without Ca^{2+} or Mg^{2+}), pH 7.2

of the 2'-azido polynucleotides, $(dAn_3)_n$, $(dCn_3)_n$, $(dIn_3)_n$ and $(dUn_3)_n$, or their complexes except $(dIn_3)_n \cdot (C)_n$, proved active as an interferon inducer (Table 2). The interferon-inducing ability of $(dIn_3)_n \cdot (C)_n$ was confirmed at different occasions with different preparations of polynucleotide; $(dIn_3)_n \cdot (C)_n$ was almost as active as $(A)_n \cdot (rT)_n$ and only three times less active than $(I)_n \cdot (C)_n$ (Table 2). Unlike $(dIn_3)_n \cdot (C)_n$, $(dIn_3)_n \cdot (br^5C)_n$ failed to stimulate the production of interferon in rabbit kidney cells. The inactivity of $(dIn_3)_n \cdot (br^5C)_n$ was quite unexpected, as other $(I)_n \cdot (C)_n$ analogues, viz. $(c'I)_n \cdot (C)_n$, become more potent interferon inducers upon substitution of a bromine at C-5 of the pyrimidine ring [11]. When mixed with $(I)_n$, $(dIn_3)_n \cdot (br^5C)_n$ attained the level of interferon-inducing activity normally observed for $(I)_n \cdot (br^5C)_n$ (8000 units/ml, Table 2), suggesting that, under the mixing conditions employed, the following displacement reaction occurred:

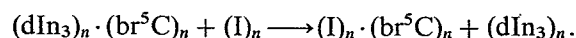


Table 2. Interferon-inducing activity of various 2'-azido analogues of $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$

All data represent average values for at least three separate determinations, except for the L-929 cells and rabbits (only two determinations). The range of individual values is indicated in parentheses. For the intact rabbit experiments the polynucleotide concentrations indicated correspond to the doses injected per rabbit. Serum interferon titers were measured at the peak of the interferon response [19], that is 2 h after intravenous injection of the polynucleotide

System	Polynucleotide	Interferon titer at polynucleotide concn of:		
		0.1 µg/ml	1 µg/ml	10 µg/ml
		$\log_{10}$ (units/ml)		
Primary rabbit kidney cells 'superinduced' with cycloheximide and actinomycin D	$(A)_n \cdot (U)_n$	—	—	2.9 (2.8–3.0)
	$(A)_n \cdot (rT)_n$	—	—	3.4 (3.3–3.5)
	$(A)_n \cdot (dUn_3)_n$	—	—	≤ 1.0
	$(dAn_3)_n \cdot (U)_n$	—	—	< 1.0
	$(dAn_3)_n \cdot (rT)_n$	—	—	< 1.0
	$(dAn_3)_n \cdot (dUn_3)_n$	—	—	< 1.0
	$(I)_n \cdot (C)_n$	—	—	3.8 (3.5–4.0)
	$(I)_n \cdot (br^5C)_n$	—	—	3.9 (3.8–4.0)
	$(dIn_3)_n \cdot (C)_n$	—	—	3.3 (3.0–3.5)
	$(dIn_3)_n \cdot (br^5C)_n$	—	—	< 1.0
	$(I)_n \cdot (dCn_3)_n$	—	—	≤ 1.0
Human skin fibroblast cells 'primed' with interferon and 'superinduced' with cyclo- heximide and actinomycin D	$(I)_n \cdot (C)_n$	3.0 (2.8–3.5)	3.7 (3.6–3.8)	3.9 (3.7–4.0)
	$(I)_n \cdot (br^5C)_n$	3.5 (3.4–3.7)	3.9 (3.9–4.0)	4.2 (4.1–4.4)
	$(dIn_3)_n \cdot (C)_n$	3.0 (2.8–3.2)	3.7 (3.5–3.8)	4.1 (4.0–4.4)
	$(dIn_3)_n \cdot (br^5C)_n$	< 1.3	< 1.3	< 1.3
L-929 cells 'primed' with interferon	$(I)_n \cdot (C)_n$	1.5	2.3	2.3
	$(I)_n \cdot (br^5C)_n$	1.5	1.8	2.0
	$(dIn_3)_n \cdot (C)_n$	< 0.5	0.8	1.0
	$(dIn_3)_n \cdot (br^5C)_n$	< 0.5	< 0.5	< 0.5
Intact rabbits	$(I)_n \cdot (C)_n$	—	3.8	4.7
	$(I)_n \cdot (br^5C)_n$	—	3.5	4.7
	$(dIn_3)_n \cdot (C)_n$	—	< 1.0	1.7

This displacement reaction, as many other displacement reactions established previously [27], is directed towards the formation of the double helix with the higher t_m .

With $(I)_n \cdot (C)_n$, $(I)_n \cdot (br^5C)_n$, $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ additional interferon-induction tests were run in human diploid cells 'primed' with interferon and 'superinduced' with cycloheximide and actinomycin D. In this sensitive interferon-induction system [20], which is generally applied for the large-scale production of human fibroblast interferon, $(dIn_3)_n \cdot (C)_n$ and $(I)_n \cdot (C)_n$ proved equally effective in inducing interferon, irrespective of the doses at which they were tested (Table 2); $(dIn_3)_n \cdot (br^5C)_n$, however, was entirely ineffective in inducing interferon in human cells.

The efficacy of $(dIn_3)_n \cdot (C)_n$ as an interferon inducer in rabbit and human cell cultures prompted the extension of our interferon-induction studies with $(dIn_3)_n \cdot (C)_n$ to two other assay systems: mouse L-929 cells ('primed' with interferon) and intact rabbits (Table 2). In both test systems, $(dIn_3)_n \cdot (C)_n$ was considerably less effective than $(I)_n \cdot (C)_n$ (Table 2).

Sensitivity of $(dIn_3)_n \cdot (C)_n$ to Degradation by Nucleases

The relatively poor interferon-inducing behavior of $(dIn_3)_n \cdot (C)_n$ in intact rabbits (Table 2) could obviously be attributed to premature degradation of the polynucleotide by nucleases present in the plasma (serum). Sera indeed contain a ribonuclease activity that specifically degrades double-stranded RNAs [28, 29]. On the other hand, substitution of 2'-azido for 2'-OH markedly decreases the susceptibility of $(C)_n$ and $(U)_n$ to various nucleases (e.g. snake venom phosphodiesterase) [14], and both $(U)_n$ and $(A)_n \cdot (U)_n$ become totally resistant to pancreatic ribonuclease A upon introduction of 2'-azido in the $(U)_n$ strand [7, 14].

However, $(dIn_3)_n \cdot (C)_n$ proved considerably more susceptible to degradation by pancreatic ribonuclease A than did its parent compound $(I)_n \cdot (C)_n$, whether the integrity of the polymers was monitored by acid-insoluble radioactivity or interferon-inducing activity (Fig. 2). $(dIn_3)_n \cdot (C)_n$ was also more susceptible to degradation by nucleases present in human serum than was $(I)_n \cdot (C)_n$ (Fig. 3). Whereas $(dIn_3)_n \cdot (C)_n$ showed a partial loss in interferon-inducing activity after incubation with rabbit serum, $(I)_n \cdot (C)_n$ proved completely resistant to the inactivating effect of rabbit serum (Fig. 3). The latter observations may, at least partially, explain the differences in serum interferon titers obtained when $(I)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (C)_n$ are administered intravenously to rabbits (Table 2).

In view of the relatively low thermal stability of $(dIn_3)_n \cdot (C)_n$, i.e. $t_m = 52^\circ\text{C}$ (Fig. 2) as compared to 62.5°C for $(I)_n \cdot (C)_n$ when determined under similar

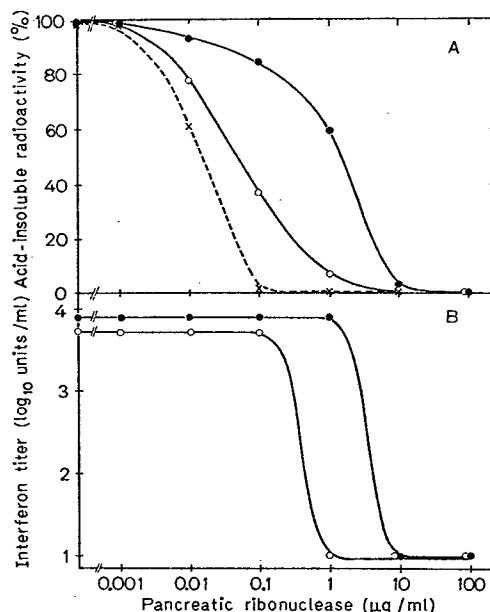


Fig. 2. Sensitivity of $(dIn_3)_n \cdot (C)_n$ (○) and $(I)_n \cdot (C)_n$ (●) to degradation by pancreatic ribonuclease A, as monitored by (A) residual acid-insoluble radioactivity or (B) interferon-inducing activity of the polynucleotide-enzyme mixtures. The polynucleotide-enzyme mixtures which were assayed for acid-insoluble radioactivity (A) contained, per 1 ml of Eagle's minimum essential medium, 0.1 µg of either $(dIn_3)_n \cdot [^3H](C)_n$ or $(I)_n \cdot [^3H](C)_n$ (approximately 6500 counts/min) and varying concentrations of pancreatic ribonuclease A (as indicated). The sensitivity of $[^3H](C)_n$ (0.05 µg/ml) to degradation by pancreatic ribonuclease was also determined (×). The polynucleotide-enzyme mixtures which were assayed for interferon-inducing activity (B) contained, per 1 ml of Eagle's minimum essential medium, 1 µg of either $(dIn_3)_n \cdot (C)_n$ or $(I)_n \cdot (C)_n$ and varying concentrations of pancreatic ribonuclease A (as indicated). All mixtures were first incubated for 1 h at 37°C and then assayed for acid-insoluble radioactivity or interferon-inducing activity. The interferon-inducing activity was assessed in human skin fibroblasts 'primed' with interferon and 'superinduced' with cycloheximide and actinomycin D.

conditions (Table 1), the differences in the susceptibility of $(dIn_3)_n \cdot (C)_n$ and $(I)_n \cdot (C)_n$ to degradation by nucleases (such as pancreatic ribonuclease A) are not unexpected. One may assume that pancreatic ribonuclease first denatures the double helix before it digests the single strand, $(C)_n$.

Immunoreactivity of $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$

As determined with a quantitative complement fixation assay, both $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ were readily recognized by specific antibody to double-stranded RNA (Fig. 4). However, full dose response curves could not be obtained and indexes of dissimilarity [30] could not be calculated, since $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ were anticomplementary (in the absence of antibody) at low concentrations.

Next, the immunoreactivity of $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ was measured in a radioimmunoassay

in which they competed with the binding of $[^{14}\text{C}]$ -($\text{I}_n \cdot (\text{C})_n$) to antibodies directed to double-stranded RNA. With antibodies induced by ($\text{A}_n \cdot (\text{U})_n$) and purified from a precipitate with ($\text{I}_n \cdot (\text{C})_n$) (Fig. 5A),

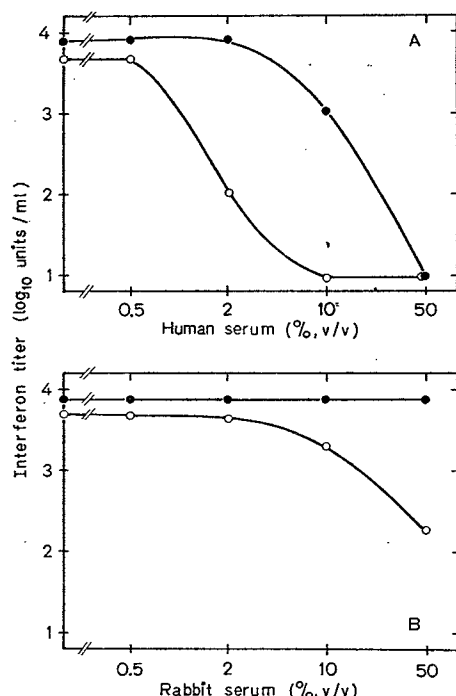


Fig. 3. Sensitivity of $(\text{dIn}_3)_n \cdot (\text{C})_n$ (○) and $(\text{I})_n \cdot (\text{C})_n$ (●) to degradation by: (A) human serum and (B) rabbit serum, as monitored by residual interferon-inducing activity. The polynucleotide-serum mixtures contained 1 μg of polynucleotide and varying concentrations of (A) human or (B) rabbit serum (as indicated) per ml Eagle's minimum essential medium. The mixtures were incubated for 1 h at 37 °C and then assayed for interferon induction in human skin fibroblasts 'primed' with interferon and 'superinduced' with cycloheximide and actinomycin D.

or with whole antiserum to ($\text{A}_n \cdot (\text{U})_n$) (data not shown), $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ competed more effectively than did $(\text{dIn}_3)_n \cdot (\text{C})_n$. Their reactivity was similar to or greater than that of $(\text{I})_n \cdot (\text{br}^5\text{C})_n$, but smaller than that of $(\text{I})_n \cdot (\text{C})_n$ (Fig. 5A) or ($\text{A}_n \cdot (\text{U})_n$) (not shown).

Competitive radioimmunoassays were also performed with antisera induced by $(\text{I})_n \cdot (\text{br}^5\text{C})_n$ (kindly provided by M. I. Johnston). Binding of $[^{14}\text{C}](\text{I})_n \cdot (\text{C})_n$ to these antibodies was inhibited most effectively by $(\text{dIn}_3)_n \cdot (\text{C})_n$ in terms of the amount of inhibitor required, but this inhibition did not exceed a maximum of about 85% (Fig. 5B). For $(\text{I})_n \cdot (\text{C})_n$, $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ and $(\text{I})_n \cdot (\text{br}^5\text{C})_n$, 3–6-fold higher amounts of inhibitor were required, but inhibition reached 100% (Fig. 5B).

The reactivity of $(\text{dIn}_3)_n \cdot (\text{C})_n$ and $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ with antibodies to double-stranded RNA was much stronger than the reactivity of other 2'-modified $(\text{I})_n \cdot (\text{C})_n$ or ($\text{A}_n \cdot (\text{U})_n$) analogues such as $(\text{A}_n \cdot (\text{Um})_n$, ($\text{Um})_n$ being poly(2'-O-methyluridylic acid). In counterimmunoelectrophoresis, $(\text{A}_n \cdot (\text{Um})_n$ gave a very weak reaction with undiluted antiserum to $(\text{A}_n \cdot (\text{U})_n$ and no reaction at all with a 1/3 antiserum dilution. Both $(\text{dIn}_3)_n \cdot (\text{C})_n$ and $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ gave a distinct precipitation at a 1/12 antiserum dilution; the $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ precipitation line was more intense than the $(\text{dIn}_3)_n \cdot (\text{C})_n$ line (Fig. 6, upper part). At a 1/40 serum dilution, $(\text{A}_n \cdot (\text{U})_n$ and $(\text{I})_n \cdot (\text{C})_n$ were still reacting, while the two 2'-azido complexes were not (data not shown).

Similar results were obtained if the counterimmunoelectrophoresis assays were performed with antiserum to $(\text{I})_n \cdot (\text{br}^5\text{C})_n$. At a 1/5 serum dilution $(\text{A}_n \cdot (\text{Um})_n$ did not show any reaction, whereas both $(\text{dIn}_3)_n \cdot (\text{C})_n$ and $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ gave distinct precipitation lines (Fig. 6, lower part).

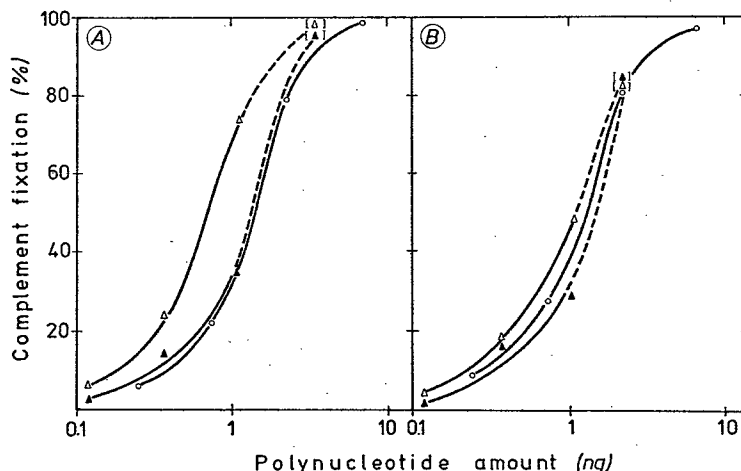


Fig. 4. Quantitative complement fixation of $(\text{dIn}_3)_n \cdot (\text{C})_n$ (Δ), $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ (▲) and $(\text{I})_n \cdot (\text{C})_n$ (○) with antibody to $(\text{A})_n \cdot (\text{U})_n$. The antibodies had been purified with $(\text{I})_n \cdot (\text{C})_n$ and were used at 1/1500 dilution (A) or 1/3000 dilution (B) from stock. Because of the anti-complementary activity of $(\text{dIn}_3)_n \cdot (\text{C})_n$ and $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ at amounts higher than 1 ng, the exact amounts of $(\text{dIn}_3)_n \cdot (\text{C})_n$ and $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ required for maximal complement fixation could not be determined. The complement fixation values obtained at doses at which $(\text{dIn}_3)_n \cdot (\text{C})_n$ and $(\text{dIn}_3)_n \cdot (\text{br}^5\text{C})_n$ showed partial anticomplementary activity are indicated in brackets.

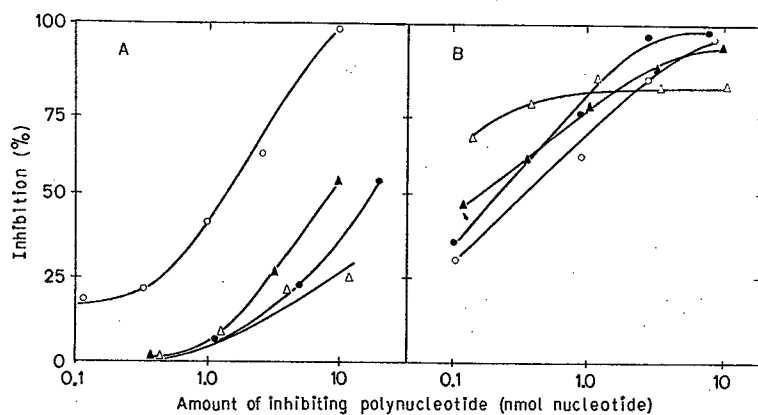


Fig. 5. Inhibition of bindings of $[^{14}\text{C}](\text{I})_n \cdot (\text{C})_n$ to antibodies to double-stranded RNA by $(d\text{In}_3)_n \cdot (\text{C})_n$ (Δ), $(d\text{In}_3)_n \cdot (\text{br}^5\text{C})_n$ ($\blacktriangle$), $(\text{I})_n \cdot (\text{C})_n$ ($\circ$) and $(\text{I})_n \cdot (\text{br}^5\text{C})_n$ ($\bullet$), as determined with a competitive radioimmunoassay. Amount of $[^{14}\text{C}](\text{I})_n \cdot (\text{C})_n$: $0.3 \mu\text{g}$ ($\approx 0.9 \text{ nmol}$ nucleotide). Amount of inhibiting polynucleotides as indicated. Source of antibodies to double-stranded RNA: (A) antibodies to $(\text{A})_n \cdot (\text{U})_n$, purified with $(\text{I})_n \cdot (\text{C})_n$; (B) antibodies to $(\text{I})_n \cdot (\text{br}^5\text{C})_n$. Control binding in the absence of inhibiting polynucleotide: 41% and 53% for A and B, respectively. Control negative serum binding: 0.1% and 2% for A and B, respectively. Amount of antibody used: $3 \mu\text{g}$ (A) or 1/30 dilution from stock (B)

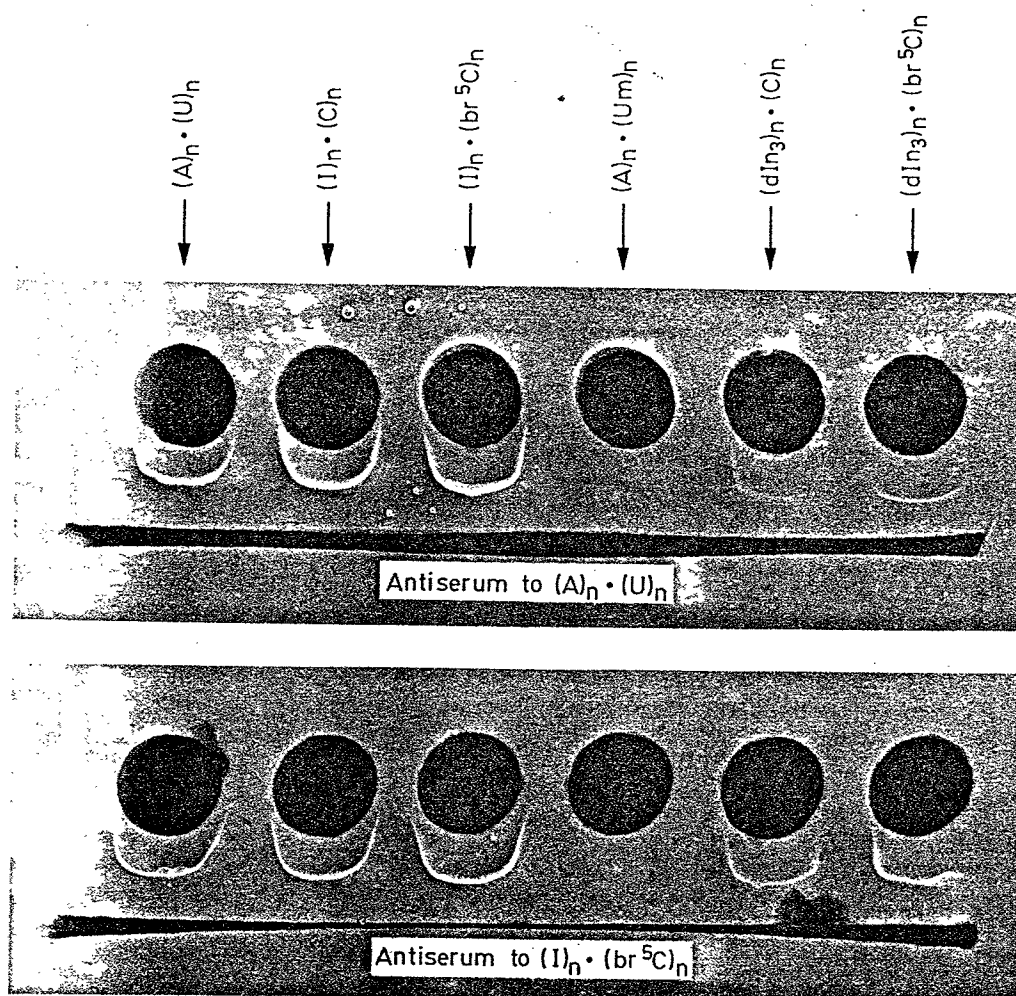


Fig. 6. Counterimmunoelectrophoresis of $(\text{A})_n \cdot (\text{U})_n$, $(\text{I})_n \cdot (\text{C})_n$, $(\text{I})_n \cdot (\text{br}^5\text{C})_n$, $(\text{A})_n \cdot (\text{Um})_n$, $(d\text{In}_3)_n \cdot (\text{C})_n$ and $(d\text{In}_3)_n \cdot (\text{br}^5\text{C})_n$ with antibody to either $(\text{A})_n \cdot (\text{U})_n$ (upper part) or $(\text{I})_n \cdot (\text{br}^5\text{C})_n$ (lower part). The antisera to $(\text{A})_n \cdot (\text{U})_n$ and $(\text{I})_n \cdot (\text{br}^5\text{C})_n$ were used at a dilution of 1/12 and 1/5, respectively ($150 \mu\text{l}/\text{trough}$). The antigen wells contained $0.5 \mu\text{g}$ of polynucleotide in $50 \mu\text{l}$

DISCUSSION

The most remarkable feature of the results reported here is the interferon-inducing ability of $(dIn_3)_n \cdot (C)_n$ which equalled that of $(I)_n \cdot (C)_n$, at least in human diploid cell cultures (Table 2). $(dIn_3)_n \cdot (C)_n$ represents the first 2'-modified double-stranded RNA (with all 2'-hydroxyl group substituted in one strand of the duplex) which has been found to be an effective interferon inducer. The interferon-stimulating ability of $(dIn_3)_n \cdot (C)_n$ indicates that (a) the presence of free 2'-OH groups in both strands is not an absolute requirement for the interferon-inducing capacity of double-stranded RNA complexes and (b) the receptor site for interferon induction does not specifically recognize the 2'-hydroxyls *per se*, but rather the steric configuration conferred by the presence of these 2'-hydroxyls. Apparently, the steric configuration resulting from substitution of 2'-azido for 2'-OH in the $(I)_n$ strand of $(I)_n \cdot (C)_n$ is also recognized by the interferon-induction receptor. Our results reinforce the concept that interferon induction by double-stranded polynucleotides is dependent on the recognition of the overall conformation of the polynucleotide rather than on the binding of specific functional groups such as 2'-OH or purine N-7 [31]. Studies of Fukui et al. [16] have indicated that there is a close resemblance in the circular dichroism spectra of $(dIn_3)_n \cdot (C)_n$ and $(I)_n \cdot (C)_n$.

The double-stranded character of $(dIn_3)_n \cdot (C)_n$ was suggested by ultraviolet absorbance mixing curves [16] and the monophasic melting profile (Fig. 1). It was further ascertained by the reactivity of $(dIn_3)_n \cdot (C)_n$ towards antibodies to double-stranded RNA (Fig. 4–6). In three different immunological assays (complement fixation, counterimmunoelectrophoresis and competitive radioimmunoassay) $(dIn_3)_n \cdot (C)_n$ reacted as a true double-stranded RNA. Similar immunoreactivity was noted for $(dIn_3)_n \cdot (br^5C)_n$ but this complex was inactive as an interferon inducer. It is at present unclear why $(dIn_3)_n \cdot (br^5C)_n$, unlike $(dIn_3)_n \cdot (C)_n$, failed to stimulate the production of interferon. There may be some differences in the conformation of $(dIn_3)_n \cdot (br^5C)_n$ and $(dIn_3)_n \cdot (C)_n$, as revealed by competitive radioimmunoassays (Fig. 5). X-ray diffraction and circular dichroism studies need to be undertaken to further explore conformational differences between $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$.

While $(dIn_3)_n \cdot (C)_n$ was effective as an interferon inducer, $(I)_n \cdot (dCn_3)_n$ was not (Table 2). The differences in interferon-inducing activity between these two complexes, as well as those noted previously for a series of partially 2'-O-methylated derivatives of $(I)_n \cdot (C)_n$ [8], suggest that the interferon-stimulating potency of $(I)_n \cdot (C)_n$ is more tolerant to substitutions at C-2' of the $(I)_n$ strand than it is to similar modifications of the $(C)_n$ strand. However other modifica-

tions, such as strand interruption by unpaired bases or bond breakage, are better tolerated by the $(C)_n$ strand than by the $(I)_n$ strand [32].

The interferon-inducing activity of $(dIn_3)_n \cdot (C)_n$ varied considerably from one assay system to another. In human fibroblast cultures which had been 'primed' with interferon and were 'superinduced' with metabolic inhibitors, $(dIn_3)_n \cdot (C)_n$ proved as active as $(I)_n \cdot (C)_n$ (Table 2); in mouse L-929 cells which had also been 'primed' with interferon, $(dIn_3)_n \cdot (C)_n$ was definitely less active than $(I)_n \cdot (C)_n$ (Table 2). The latter is not surprising, as various other double-stranded RNAs which are nearly as effective as $(I)_n \cdot (C)_n$ in inducing interferon in the human fibroblast assay, i.e. $(A)_n \cdot (U)_n$, $(A)_n \cdot (rT)_n$, $(I)_n \cdot (s^2C)_n$ [33], $(I)_{2.5} \cdot (C)_{13.2}$ and $(I)_{2.5} \cdot (C)_{3.1}$ (where the subscripts refer to the $s_{20,w}$ values of the homopolymers), show little, if any, interferon-inducing activity in interferon-primed L-929 cells [20, 34]. Hence, one should be cautious in defining the structural requirements of a polynucleotide inducer of interferon, if these requirements are deduced from one particular assay system. These requirements may not necessarily apply to other interferon-induction systems.

Does $(dIn_3)_n \cdot (C)_n$ conform to the premises of a potentially useful interferon inducer? Provided that the time required for triggering the interferon response is sufficiently different from the time required to induce the other, often noxious, physiological responses, as suggested by Ts'o et al. [35] and Carter et al. [36], one should be able to develop an interferon inducer more efficient than $(I)_n \cdot (C)_n$. This $(I)_n \cdot (C)_n$ analogue should persist in biological fluids, so as to trigger the interferon response, but it should not persist too long, so as not to induce the undesirable physiological responses. $(dIn_3)_n \cdot (C)_n$ may fulfil the second condition, in as far as it is degraded more readily by human serum than $(I)_n \cdot (C)_n$ (Fig. 3). Whether it is also less toxic than $(I)_n \cdot (C)_n$, remains to be established.

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oly (2-azaadenylic acid) and poly (2-azainosinic acid)

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参 考 論 文

nylic acid) ((aza²A)n) and poly(2-azainosinic acid) wly synthesized analogues of (A)n and (I)n, in which CH-2 is replaced by a nitrogen atom, have been evaluated in assay systems. (Aza²A)n formed a complex with (U)n and (A)n, and (aza-I)n formed a complex with (C)n and (br⁵C)n, but these complexes were markedly destabilized relative to the corresponding (A)n or (I)n complexes. The (aza²A)n- and (aza²I)n-derived complexes failed to stimulate the production of interferon in primary rabbit kidney cells and human diploid fibroblasts, under conditions (A)n.(U)n, (I)n.(C)n and (I)n.(br⁵C)n induced high amounts of interferon. Both (aza²A)n and (aza²I)n exerted a marked inhibitory effect on the endogenous RNA directed DNA polymerase (reverse transcriptase) activity associated with murine leukemia virus. They caused a relatively mild inhibition of complement activity in an hemolytic assay system.

INTRODUCTION

The interferon inducing capacity of double-stranded polyribonucleotides ((I)n.(C)n, (A)n.(U)n, ...) depends on a number of structural determinants one of which is the presence of intact purine-pyrimidine base pairs in the interior of the double helix. Substitution of CH for either N-1, N-3, N-7 or N-9 of the purine moiety of (A)n or (I)n leads to a dramatic, if not complete, reduction of the interferon inducing ability of (A)n.(U)n and (I)n.(C)n (1-5). The lack of interferon inducing activity of some of these analogues, e.g. (c⁷A)n.(U)n, may seem related to the rather low T_m (thermal stability) of the complex (1), but this contention does not hold for most other analogues of (A)n.(U)n and (I)n.(C)n. For example, (c⁷A)n.(br⁵U)n and (L)n.(br⁵C)n fail to stimulate interferon production, although they possess a sufficiently high T_m (72°, 0.15M Na⁺, pH 7) (1,3). For other (I)n.(C)n analogues, e.g. (c⁷I)n.(br⁵C)n, the high T_m (86°, 0.2M Na⁺, pH 7) is associated with a significant interferon inducing ability.

Biologic activities of poly (2-azaadenylic acid) and poly (2-azainosinic acid)

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ABSTRACT

Poly(2-azaadenylic acid) ((aza²A)n) and poly(2-azainosinic acid) ((aza²I)n), two newly synthesized analogues of (A)n and (I)n, in which CH-2 of the purine ring is replaced by a nitrogen atom, have been evaluated in various biological assay systems. (Aza²A)n formed a complex with (U)n and (br⁵U)n, and (aza²I)n formed a complex with (C)n and (br⁵C)n, but these complexes were markedly destabilized relative to the corresponding (A)n or (I)n complexes. The (aza²A)n- and (aza²I)n-derived complexes failed to stimulate the production of interferon in primary rabbit kidney cells and human diploid fibroblasts, under conditions (A)n.(U)n, (I)n.(C)n and (I)n.(br⁵C)n induced high amounts of interferon. Both (aza²A)n and (aza²I)n exerted a marked inhibitory effect on the endogenous RNA directed DNA polymerase (reverse transcriptase) activity associated with murine leukemia virus. They caused a relatively mild inhibition of complement activity in an hemolytic assay system.

INTRODUCTION

The interferon inducing capacity of double-stranded polyribonucleotides ((I)n.(C)n, (A)n.(U)n, ...) depends on a number of structural determinants one of which is the presence of intact purine-pyrimidine base pairs in the interior of the double helix. Substitution of CH for either N-1, N-3, N-7 or N-9 of the purine moiety of (A)n or (I)n leads to a dramatic, if not complete, reduction of the interferon inducing ability of (A)n.(U)n and (I)n.(C)n (1-5). The lack of interferon inducing activity of some of these analogues, e.g. (c⁷A)n.(U)n, may seem related to the rather low T_m (thermal stability) of the complex (1), but this contention does not hold for most other analogues of (A)n.(U)n and (I)n.(C)n. For example, (c⁷A)n.(br⁵U)n and (L)n.(br⁵C)n fail to stimulate interferon production, although they possess a sufficiently high T_m (72°, 0.15M Na⁺, pH 7) (1,3). For other (I)n.(C)n analogues, e.g. (c⁷I)n.(br⁵C)n, the high T_m (86°, 0.2M Na⁺, pH 7) is associated with a significant interferon inducing ability.

While ineffective as interferon inducers (upon annealing to (C)n), N-substituted analogues of (I)n may exhibit other biological activities, including anti-complement and anti-reverse transcriptase activity. Two such analogues, (c³I)n and (c⁷I)n have indeed been shown to inhibit complement activity in an hemolytic assay system and to reduce the *in vitro* reverse transcriptase (RNA directed DNA polymerase) activity of oncornaviruses (5-7).

Herein we describe the biological implications of another nuclear modification, substitution of nitrogen for CH-2 in the purine ring of (A)n and (I)n. The resulting (aza²A)n and (aza²I)n were examined for both anti-complement and anti-reverse transcriptase activity. Their complexes with (U)n and (C)n were assayed for interferon inducing capacity. As complexes of (A)n or (c⁷A)n with (br⁵U)n and complexes of (I)n, (c⁷I)n, (c³I)n or (L)n with (br⁵C)n have a markedly higher T_m than the corresponding complexes with (U)n and (C)n (1-3,5), complexes have been prepared of (aza²A)n with (br⁵U)n and of (aza²I)n with (br⁵C)n. It was reasoned that, if (aza²A)n.(U)n and (aza²I)n.(C)n would not show interferon inducing activity by virtue of their low T_m, (aza²A)n.(br⁵U)n and (aza²I)n.(br⁵C)n might be able to do so.

MATERIALS AND METHODS

The synthesis and physicochemical properties of (aza²I)n and (aza²A)n have been described recently (8); (br⁵C)n and (br⁵U)n were prepared as reported before (2,9). (I)n.(C)n, (A)n.(U)n and other complexes employed in our interferon induction assays were constituted with homopolymers obtain-

Abbreviations

(A)n, poly(adenylic acid); (C)n, poly(cytidylic acid); (I)n, poly(inosinic acid); (U)n, poly(uridylic acid); (G)n, poly(guanylic acid); (X)n, poly(xanthylic acid); (aza²A)n, poly(2-azaadenylic acid); (aza²I)n, poly(2-azainosinic acid); (c³I)n, poly(3-deazainosinic acid); (c⁷A)n, poly(7-deazaadenylic acid); (c⁷I)n, poly(7-deazainosinic acid); (L)n, polylaurusin or polyformycin B; (ms²I)n, poly(2-methylthioinosinic acid); (dUz)n, poly(2'-azido-2'-deoxyuridylic acid); (br⁵C)n, poly(5-bromocytidylic acid); (br⁵U)n, poly(5-bromouridylic acid); MuLV (Monoley), Moloney strain of murine leukemia virus; PRK, primary rabbit kidney; HSF, human skin fibroblast; MEM, minimal essential medium (Eagle's); PBS, phosphate buffered saline (Dulbecco's); T_m, temperature of thermal transition.

Note : since the IUPAC-IUB Commission on Biochemical Nomenclature has already designated the "n" notation for amino, poly(2-azaadenylic acid) and poly(2-azainosinic acid) are abbreviated to (aza²A)n and (aza²I)n, and not to (n²A)n or (n²I)n. The latter abbreviations might be reserved for poly(2-aminoadenylic acid) and poly(2-aminoinosinic acid).

ed from P-L Biochemicals (Milwaukee, Wisconsin). The sedimentation values ($s_{20,w}$) of these homopolymers were as follows : 9.4 S for (I)n, 10.0 S for (C)n, 9.8 S for (A)n and 7.0 S for (U)n. The homopolyribonucleotides employed in the reverse transcriptase and complement assays were purchased from Miles Laboratories (Elkhart, Indiana). The sedimentation values ($s_{20,w}$) of these homopolymers were as follows : 4.8 S for (I)n and 5.5 or 8.9 S for (A)n. Ultraviolet spectra and melting profiles were determined as described previously (10). Interferon production was measured in (i) PRK (primary rabbit kidney) cells "superinduced" with cycloheximide and actinomycin D, and (ii) HSF (human skin fibroblast) cells (VGS strain) "primed" with human fibroblast interferon and "superinduced" with cycloheximide and actinomycin D. The exact methodology for monitoring interferon induction in PRK and HSF cultures has been described (4,5,11). The techniques for evaluating the inhibitory effects of the compounds on complement activity and reverse transcriptase activity have also been described (6,12). The Moloney strain of murine leukemia virus (MuLV (Moloney)) (Electro-Nucleonics Laboratories, Bethesda, Maryland) served as source of both the reverse transcriptase and its template.

RESULTS AND DISCUSSION

Poly(2-azainosinic acid).poly(5-bromocytidylic acid). As revealed by mixing curves constructed at 10° (in 0.15M Na⁺), (aza²I)n forms a 1:1 stoichiometric complex with (C)n (8). The T_m of this complex is ~15° in Dulbecco's PBS (Fig. 1). Introduction of bromine at C-5 of (C)n has been shown to increase the T_m of the complexes of (C)n with (I)n, (c⁷I)n, (c³I)n or (L)n by 20-30° (1-3,5). Thus, as expected, (aza²I)n was found to interact with (br⁵C)n to give a complex that had a T_m of ~40° in PBS (Fig. 2). (Aza²I)n itself underwent a broad hypochromic change with a mid-point of ~70° in PBS (Fig. 2).

Poly(2-azaadenylic acid).poly(5-bromouridylic acid). (Aza²A)n alone showed a rather clear transition point at ~36° in Dulbecco's PBS (Fig. 3). A similar cooperative melting has been observed with (aza²A)n at about 20° in 0.15M Na⁺ (0.1M NaCl + 0.05M sodium cacodylate, pH 7.0) (8). In the latter solution, (aza²A)n is assumed to form a 1:2 stoichiometric complex with (U)n, the T_m of which is ~41° (8). For an equimolar mixture of (aza²A)n with (br⁵U)n, a biphasic melting profile was obtained (Fig. 3). The first transition corresponded to the melting of the (aza²A)n homopolymer. The second, rather broad, transition with T_m ~65° could be attributed

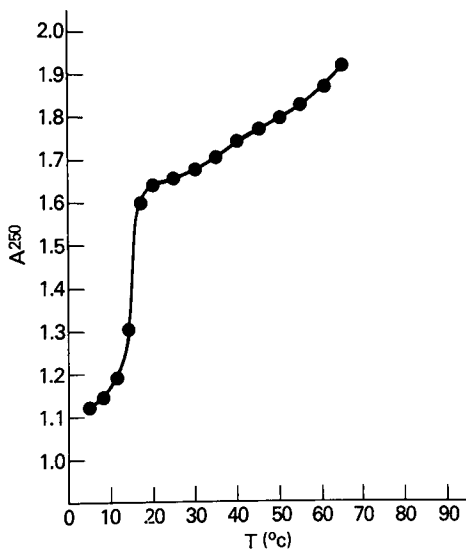


Figure 1.

Melting profile for an equimolar mixture of (aza²I)n + (C)n in Dulbecco's PBS.

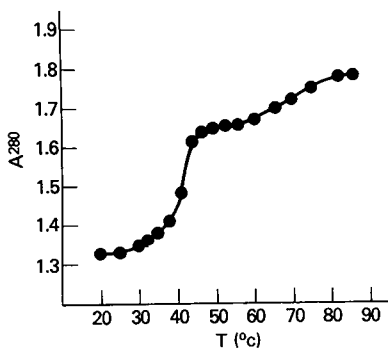
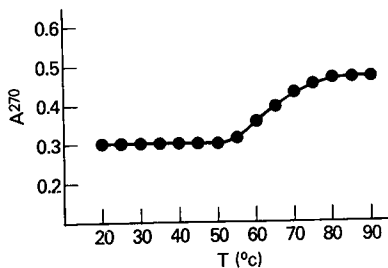


Figure 2.

Top : melting profile for an equimolar mixture of (aza²I)n + (br⁵C)n in Dulbecco's PBS.



Bottom : melting profile for the (aza²I)n homopolymer in Dulbecco's PBS.

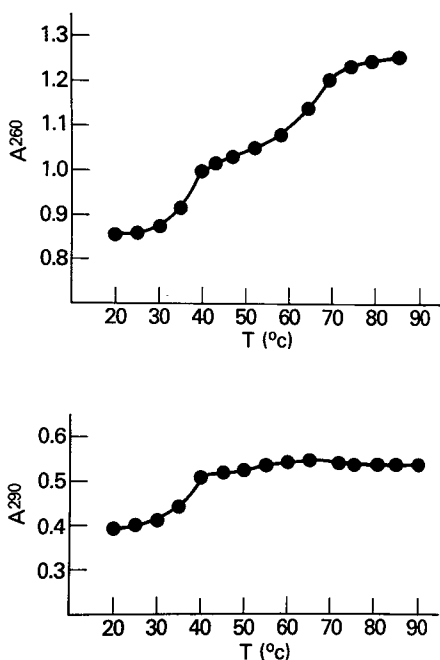


Figure 3.

Top : melting profile for an equimolar mixture of (aza²A)n + (br⁵U)n in Dulbecco's PBS.

Bottom : melting profile for the (aza²A)n homopolymer in Dulbecco's PBS.

to the melting of the (aza²A)n.(br⁵U)n duplex (or triplex). The biphasic melting behavior of (aza²A)n.(br⁵U)n may suggest that no complex formation with (br⁵U)n occurs until the self-structure of (aza²A)n is destroyed. This possibility may be further examined when greater amounts of (aza²A)n will become available.

Interferon Induction. In contrast with (A)n.(U)n and (I)n.(C)n which induced up to 10,000 units/ml of interferon when exposed to PRK cells at a concentration of 10 µg duplex per ml, the (aza²A)n- and (aza²I)n-derived complexes were devoid of any interferon inducing ability (Table 1). Even complexes which were relatively stabilized by the introduction of a bromine at C-5 of the pyrimidine strand [(aza²A)n.(br⁵U)n, (aza²I)n.(br⁵C)n] failed to trigger an interferon response. The 1:2 mixture of (aza²A)n with (U)n was also ineffective, which is not unexpected in view of the well-established inadequacy of triple-stranded complexes to induce interferon (1,9).

Although (aza²I)n.(C)n and (aza²I)n.(br⁵C)n were entirely inactive in inducing interferon, they attained the level of activity characteristic for (I)n.(C)n and (I)n.(br⁵C)n after they had been mixed with (I)n (Table 1). These data suggest that, under the mixing conditions employed, the initial

TABLE 1. INDUCTION OF INTERFERON IN PRK CELLS SUPERINDUCED
WITH CYCLOHEXIMIDE AND ACTINOMYCIN D

Complex ⁺	Mixture [*] Homopolymer	Interferon titer (units/ml)	
		Average [§]	Range [§]
(aza ² A)n.(U)n	MEM	<10	
(aza ² A)n.(U)n	(A)n	60	30-100
(aza ² A)n.2(U)n	MEM	<10	
(aza ² A)n.(2(U)n	(A)n	<10	
(A)n.(U)n	MEM	750	600-1000
(A)n.(U)n	(aza ² A)n	95	80-100
(A)n.(U)n	(aza ² I)n	15	10-30
(A)n.(U)n	(A)n	350	200-600
(A)n.(U)n	(I)n	20	10-30
(A)n.2(U)n	MEM	<10	
(A)n.2(U)n	(aza ² A)n	<10	
(aza ² A)n.(br ⁵ U)n	MEM	<10	
(aza ² A)n.(br ⁵ U)n	(A)n	<10	
(A)n.(br ⁵ U)n	MEM	<10	
(aza ² I)n.(C)n	MEM	<10	
(aza ² I)n.(C)n	(I)n	7000	6000-10000
(I)n.(C)n	MEM	6500	6000-10000
(I)n.(C)n	(aza ² I)n	6500	6000-10000
(aza ² I)n.(br ⁵ C)n	MEM	<10	
(aza ² I)n.(br ⁵ C)n	(I)n	8000	6000-10000
(I)n.(br ⁵ C)n	MEM	8000	6000-10000

* Final concentration of each homopolynucleotide in the assay mixture was 5 µg/ml. All mixtures were prepared in (Eagle's) MEM, incubated for 1 hr at 37°C and applied onto the cells immediately thereafter or after an additional incubation period of 1 week at 4°C. Quite similar results were obtained with mixtures which were tested immediately and mixtures which were incubated at 4°C for an additional week.

+ Complexes refer to 1:1 or 1:2 stoichiometric mixtures of the homopolymer components. The exact nature of the complex (whether duplex or triplex) formed under our experimental conditions was not verified.

§ For 3 to 6 separate determinations.

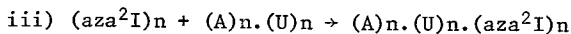
complexes dismutated according to the following reaction schemes :

- i) (aza²I)n.(C)n + (I)n → (aza²I)n + (I)n.(C)n
- ii) (aza²I)n.(br⁵C)n + (I)n → (aza²I)n + (I)n.(br⁵C)n

(I)n.(C)n retained its full interferon inducing capacity in the presence of (aza²I)n, suggesting that (aza²I)n did not displace (I)n from its complex with (C)n. The reactions i and ii obey the general rule established before (13) that polynucleotide displacement reactions are invariably directed towards the formation of the helix with the higher thermal stability.

In analogy to (I)n, (aza²I)n brought about a significant (50-fold)

decrease in the interferon inducing capacity of (A)n.(U)n (Table 1). For (I)n the reason of the decreased interferon response was determined to be the formation of the triple-helical complex (A)n.(U)n.(I)n (14). A similar triplex might be formed if (aza²I)n is mixed with (A)n.(U)n, according to the following reaction scheme :



Upon mixing with (A)n, the interferon inducing activity of (aza²A)n.(U)n increased, but not up to the level normally observed for (A)n.(U)n (Table 1). Concomitantly, (aza²A)n caused a partial (~8-fold) reduction in the interferon response to (A)n.(U)n. According to the T_m rule (13), (aza²A)n should not displace (A)n from its complex with (U)n. How could (aza²A)n inhibit the induction of interferon by (A)n.(U)n ? Theoretically (15), (aza²A)n may inhibit interferon induction through one of the following mechanisms : (a) formation of a triple-stranded complex with the inducing molecule [(A)n.(U)n], (b) inhibition of cellular RNA and protein synthesis (as has been noted for (c⁷A)n and (c⁷I)n (7)), or (c) an hitherto undefined mechanism (similar to the mechanism involved in the antagonizing effects of high salt concentrations and single-stranded polynucleotides at 4° (16)). Further studies are needed to distinguish between these possibilities.

No increase in interferon production was observed if (A)n was mixed with (aza²A)n.(br⁵U)n (Table 1). Whether or not (A)n displaced (aza²A)n from its complex with (br⁵U)n could not be ascertained from our interferon induction data. Even if (A)n.(br⁵U)n was formed in the (aza²A)n.(br⁵U)n + (A)n mixture, it could not be detected due to its lack of interferon inducing activity (Table 1) (see also ref. 1).

In addition to the (aza²A)n- and (aza²I)n-derived complexes listed in Table 1, various mixtures of (aza²A)n or (aza²I)n with either (I)n, (A)n, (U)n, (C)n, (X)n or (G)n were evaluated for interferon induction in PRK cells superinduced with cycloheximide and actinomycin D. None of these mixtures exhibited any interferon inducing activity when assayed at 10 µg/ml. (Aza²A)n alone and (aza²I)n alone were also ineffective as inducers of interferon. They failed to induce direct resistance to (vesicular stomatitis) virus infection in PRK cell cultures when applied to the cells at 10 µg/ml 24 hr before virus challenge. In this aspect, (aza²A)n and (aza²I)n differed from (c⁷A)n and (c⁷I)n which were found to inhibit viral cytopathogenicity at a concentration of 0.3 and 10 µg/ml, respectively (7).

Interferon induction tests have also been performed with human diploid cells "primed" with human fibroblast interferon and "superinduced" with cycloheximide and actinomycin D (Table 2). In this highly sensitive induction system (11), synthetic homopolyribonucleotide duplexes such as (I)n. (C)n, (A)n. (U)n and (I)n. (br⁵C)n readily induce interferon titers of 10,000-20,000 units/ml. When assayed under similar conditions, (aza²I)n. (C)n, (aza²I)n. (br⁵C)n and (aza²A)n. (br⁵U)n proved unable to induce any interferon at all (Table 2).

Anti-complement Activity. (I)n is a potent inhibitor of complement (6). This anti-complement activity is fully retained upon substitution of CH for N-7 and only slightly reduced upon substitution of CH for N-3 (5). As shown in Table 3, (aza²I)n also inhibited complement activity, albeit to a lesser extent than (I)n. Even (aza²A)n displayed a slight anti-complement activity (Table 3). This contrasts with (A)n which is known not to affect complement activity at concentrations up to 400 µg/ml (6).

Inhibition of Reverse Transcriptase Activity. In a standard reverse transcriptase assay, which has been employed before (7,12,17) to demonstrate the inhibitory effects of (I)n, (c⁷I)n, (dUz)n and (ms²I)n on MuLV (Moloney) DNA polymerase activity, both (aza²A)n and (aza²I)n caused a distinct inhibition of DNA synthesis (Fig. 4). (Aza²I)n was more inhibitory than (aza²A)n (Fig. 4A and B). The inhibitory activity of (aza²I)n was dose-dependent, at least in the range of 2.5 to 160 µg/ml (Fig. 4B) and compared favorably to the inhibitory activity of (I)n (Fig. 4A). Yet, (I)n is considered to be a relatively strong inhibitor of reverse transcriptase activity in assays in which the DNA polymerase is directed by an exogenous

TABLE 2. INDUCTION OF INTERFERON IN HSF CELLS PRIMED WITH INTERFERON AND SUPERINDUCED WITH CYCLOHEXIMIDE AND ACTINOMYCIN D

Complex	Interferon titer* (units/ml)				
	Complex added to the cells at ... µg/ml				
	0.01	0.1	1	10	100
(aza ² A)n. (br ⁵ U)n	...	<10	<10	<10	...
(aza ² I)n. (C)n	...	<10	<10	<10	...
(aza ² I)n. (br ⁵ C)n	...	<10	<10	<10	...
(A)n. (U)n	...	300	2500	10000	15000
(I)n. (C)n	30	600	5000	10000	20000
(I)n. (br ⁵ C)n	1000	2500	8000	15000	...

* Average values for 3 separate determinations.

TABLE 3. ANTI-COMPLEMENT ACTIVITY

Polynucleotide	Hemolytic complement titer				
	(complement diluted 1/10 in PBS, incubated for 1 hr at 37°C in the presence of ... $\mu\text{g/ml}$ of the polynucleotide)				
	0	10	20	40	100
(A)n	160	160	160	160	160
(aza ² A)n	160	160	160	160	80
(I)n	160	160	80	20-40	10-20
(aza ² I)n	160	160	160	80-160	40-80

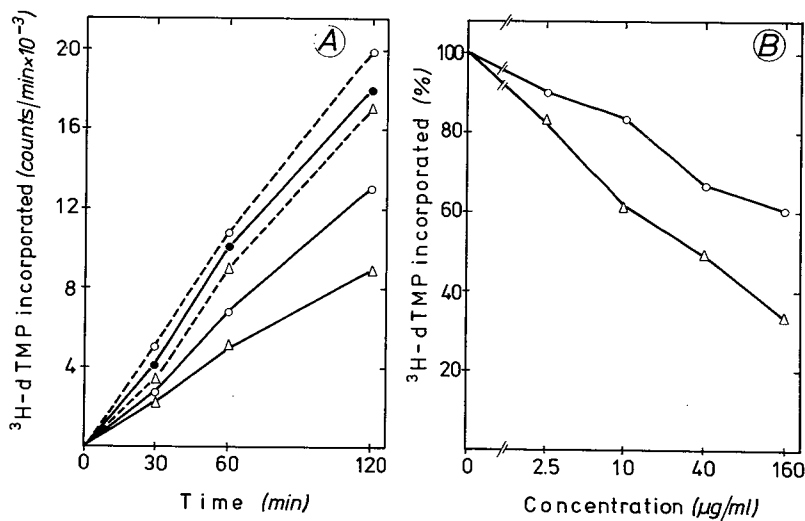


Figure 4. Effect of (aza²A)n and (aza²I)n on DNA polymerase activity of MuLV (Moloney). A : DNA synthesis measured at different times of incubation of the assay mixture. Final concentration of the polymers in the assay mixture : 70 $\mu\text{g/ml}$. B : DNA synthesis measured at different concentrations of the polymers in the assay mixture. Incubation time of the assay mixture : 60 min. ●—● : control; ○---○ : (A)n; △---△ : (I)n; ○—○ : (aza²A)n; △—△ : (aza²I)n.

template:primer such as poly(A).oligo(dT) (18,19).

The inhibition of MuLV (Moloney) reverse transcriptase activity by (aza²I)n showed a dose-response relationship (Fig. 4B) which was almost identical to that obtained previously for (ms²I)n (17). The latter (I)n analogue not only inhibited the in vitro reverse transcriptase activity of murine leukemia and sarcoma viruses but also their replication in vivo, in cultured cells (17).

CONCLUSIONS

(Aza²A)n formed a complex with (U)n and (br⁵U)n, and (aza²I)n formed a complex with (C)n and (br⁵C)n. Neither of these complexes proved capable of inducing interferon in PRK or HSF cell cultures. The inactivity of the (aza²I)n-derived complexes may be ascribed to the low T_m of these complexes, insuring that they would not remain intact under physiological conditions. The inactivity of the (aza²A)n.(U)n complex (presumably triplex) may be attributed to the low T_m and/or triple-helical structure of the complex. The lack of ability of the (aza²A)n.(br⁵U)n complex to induce interferon cannot be ascribed to a low T_m. Whether the inactivity of (aza²A)n.(br⁵U)n may be rationalized by assuming a conformational shift [as postulated for (A)n.(br⁵U)n (1)] is not clear yet.

The inhibitory effects of (aza²I)n and (aza²A)n on complement and reverse transcriptase activity may be related to the ordered structures formed by these homopolymers in solution. It should be pointed out that several polyribonucleotides (e.g. (I)n, (G)n and (X)n) which have been reported to inhibit complement (6) and reverse transcriptase activity (18-20), all exhibit a high tendency toward self-aggregation.

The complexes formed between (aza²A)n or (aza²I)n and their complementary polynucleotides are markedly destabilized relative to the parent (A)n or (I)n complexes (by up to 50° for the (aza²I)n series). The origin of this destabilization is unclear but may be related to one or more of the following considerations :

- (a) the decreased basicity of 2-azaadenosine and 2-azainosine relative to adenosine and inosine (as reflected by the precipitous drop in pK_a of N-3 : 6.8 for 2-azainosine compared to 8.9 for inosine (21));
- (b) repulsive forces involving the lone-pair electrons of N-2 of the purine ring;
- (c) changes in base-stacking interactions, as a result of electronic alterations (e.g., in dipole moment) caused by introduction of N at C-2 of the purine ring.

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- 3 Burdon, R.H. (1971) in Progress in Nucleic Acid Research and Molecular Biology, Davidson, J.N. and Cohn, W.E., Eds., Vol. II, pp. 33-79. Academic Press, New York

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POLYNUCLEOTIDES

XLIV. SYNTHESIS AND PROPERTIES OF POLY(2-AZAADENYLIC ACID) AND POLY(2-AZAINOSINIC ACID) *

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Summary

Chemically synthesized 2-azaadenosine 5'-diphosphate (n^2 ADP) and 2-azainosine 5'-diphosphate (n^2 IDP) were polymerized to yield poly(2-azaadenylic acid), poly(n^2 A), and poly(2-azainosinic acid), poly(n^2 I), using *Escherichia coli* polynucleotide phosphorylase. In neutral solution, poly(n^2 A) and poly(n^2 I) had hypochromicities of 32 and 5.5%, respectively. Poly(n^2 A) formed an ordered structure, which had a melting temperature (T_m) of 20°C at 0.15 M salt concentration. Upon mixing with poly(U), poly(n^2 A) formed a 1 : 2 complex with T_m of 41°C at 0.15 M salt concentration. Poly(n^2 A) and poly(n^2 I) formed three-stranded complexes with poly(I) and poly(A), respectively. Poly(n^2 A) : 2poly(I), poly(A) · 2poly(n^2 I), and poly(n^2 A) · 2poly(n^2 I) complexes had T_m values of 23, 48, and 31°C at 0.15 M salt concentration, respectively.

Poly(n^2 I) formed a double-stranded complex with poly(C), but its T_m was very low.

Introduction

Synthesis of ribopolynucleotides from naturally occurring ribonucleotide diphosphates as well as from a variety of analog diphosphates catalyzed by polynucleotide phosphorylase [2] has been extensively investigated [3–5].

We have reported the synthesis and properties of polynucleotides containing c^1 A [6], c^3 A [6], c^7 A [7] and c^7 I [8,9]. In these polynucleotides, lacking the

* A previous paper in this series (Ikehara, M., Limn, W. and Fukui, T. (1977) Chem. Pharm. Bull. 25, 1702–2707) was recently published.

1-, 3-, or 7-N atoms, we found unique features in their complex formation with poly(U) and poly(I).

In this paper we describe the synthesis of poly(2-azaadenylic acid) poly(n^2A), and poly(2-azainosinic acid), poly(n^2I), catalyzed by polynucleotide phosphorylase and their properties as studied by ultraviolet absorption, circular dichroism, thermal melting and mixing experiments with poly(U), poly(I), and poly(C). Because of the anti-leukemic activity of 2-azaadenosine [10], and the anticancer activity of 2-azainosine [11], the properties of these polynucleotides may be interesting with regard to nucleic acids containing 2-azanucleotide. Furthermore, the fact that poly(n^2A) and poly(n^2I) act as inhibitors of tumor virus reverse transcriptase [12] is extremely interesting.

Materials and Methods

Preparation of substrates. n^2ADP and n^2IDP were prepared in 60–65% yield from n^2AMP [13] and n^2IMP , respectively by the phosphoromorpholidate method described by Moffatt and Khorana [14].

Enzymatic polymerization of diphosphates. The polymerization mixture contained Tris · HCl (pH 8.5, 0.1 M), $MgCl_2$ (2 mM), nucleoside diphosphate (4 mM) and 2 units of *E. coli* polynucleotide phosphorylase [15] per milliliter of solution. Polymerization of n^2ADP was performed at 37°C for 7.5 h and that of n^2IDP for 21 h. The mixture was deproteinized by extraction with $CHCl_3$ /isoamyl alcohol (3 : 1, v/v) and the water layer was lyophilized. The residue was dissolved in 2 ml of water and applied to a column of Sephadex G-50 (1.7×110 cm). Elution with water gave poly(n^2A) (yield was $110A^{252}$ units, 30%), and poly(n^2I) (yield $70 A^{280}$ units, 31%), which were eluted in the void volume. These facts proved that chain length of these polynucleotides must be over 50 nucleotide units.

Physical methods. Ultraviolet melting curves were measured with a Hitachi 124 spectrophotometer, as described in a previous report [6].

CD spectra were taken with a JASCO ORD/UV5 spectrophotometer equipped with a CD attachment. The temperature of the measurements was 20–25°C and calibration was performed with d-10-camphorsulfonic acid.

Results

Polymerization of n^2ADP and n^2IDP

n^2ADP and n^2IDP were substrates for polynucleotide phosphorylase from *E. coli* on incubation at pH 8.5 in the presence of Mg^{2+} . The rates of reaction were retarded and the yields of polymers decreased in comparison with the cases of ADP and IDP, respectively. After 7.5 h incubation at 37°C the amount of inorganic phosphate liberated was 55% of the initial n^2ADP . The yield of poly(n^2A) was 30%. In the case of n^2IDP , the amount of inorganic phosphate liberated after 21 h incubation at 37°C was 54% of the initial amount of diphosphate. The yield of poly(n^2I) was 31%.

Ultraviolet absorption spectra of poly(n^2A) and poly(n^2I)

Ultraviolet-absorption spectra taken at pH 7.0 in the presence of 0.1 M NaCl

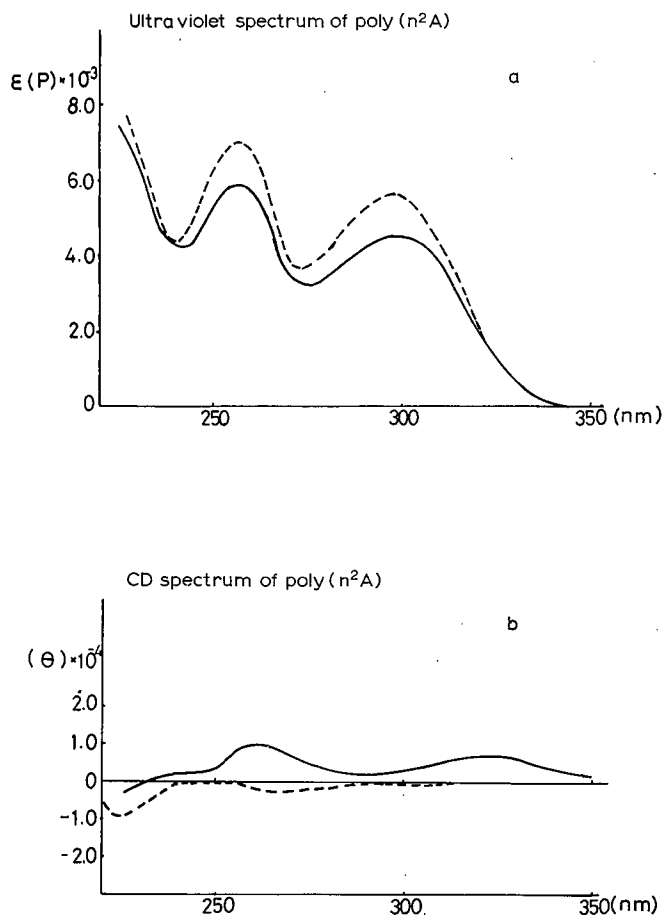


Fig. 1. Ultraviolet absorption and CD spectra of poly(n^2A). —, poly(n^2A) at 0.1 M Na^+ and 0.05 M sodium cacodylate, pH 7.0; ----, n^2AMP under the same conditions.

and 0.05 M sodium cacodylate are shown in Figs. 1a and 2a together with those of the monomers. Comparing λ_{max} of monomer and polymer, a significant red shift of λ_{max} at around 300 nm was observed in the case of 2-azaadenosine. Hypochromism observed at 254 nm was 32% and at 302 nm was 35%. These high hypochromicities suggest that poly(n^2A) exists in a highly stacked conformation in the present conditions. In the case of poly(n^2I), a significant shift was not observed and hypochromism observed at 288 nm was only 5.5%.

Circular dichroism of poly(n^2A) and poly(n^2I)

CD spectra of polynucleotides in 0.1 M NaCl and 0.05 M sodium cacodylate (pH 7.0) solution are shown in Figs. 1b and 2b. It is known that a helical polynucleotide can give two Cotton effects of equal magnitude and opposite sign centered at the λ_{max} value corresponding to an absorption band[16].

This splitting of the Cotton effects is typically observed in oligo(A) and poly(A)[17]. In this case of poly(n^2A), two well resolved absorption bands,

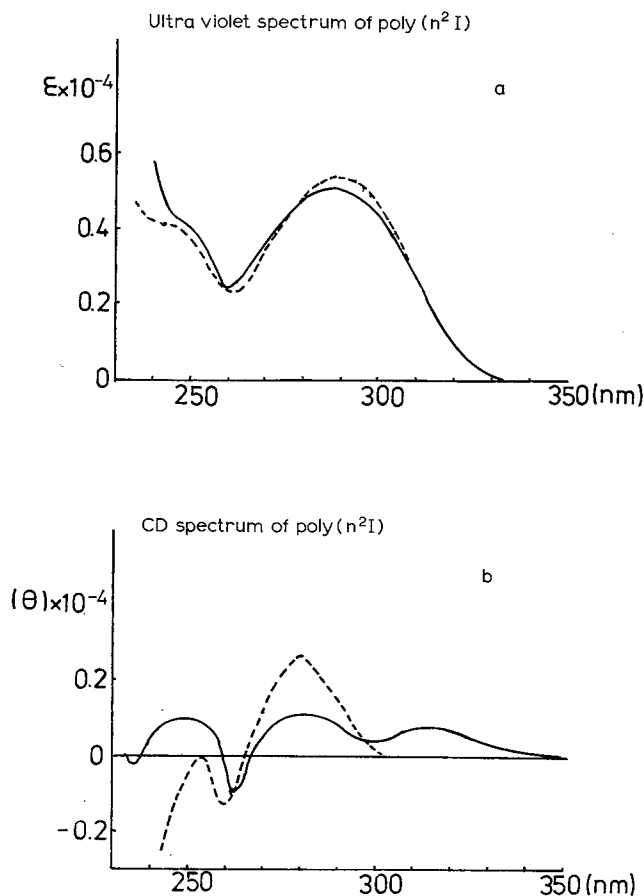


Fig. 2. Ultraviolet absorption and CD spectra of poly(n²I). —, poly(n²I) at 0.1 M Na⁺ and 0.05 M sodium cacodylate, pH 7.0; ----, n²IMP under the same conditions.

around 255 and 300 nm, were observed in the longer length region, as shown in Fig. 1b.

The CD spectrum again suggests the existence of base stacking of poly(n²A) in neutral solution. From the splitting pattern of the Cotton effects, a helical structure similar to that of poly(A) seems suggested.

The CD spectra of poly(n²I) is shown in Fig. 2b. Peaks appeared at 320, 282, and 249 nm, and troughs at 263 and 302 nm. These bands suggest the occurrence of stacking of adjacent bases, though this may not be strong.

Thermal denaturation in neutral solution

Poly(A) is known to show a non-cooperative melting curve in neutral solution. The absorption of poly(A) increased gradually from 10 to 90°C and showed no clear transition point [18]. The temperature-absorption profiles of poly(n²A) and poly(n²I) in neutral solution containing 0.15 M Na⁺ are shown in Fig. 3. In the case of poly(n²A), a cooperative melting was observed at about

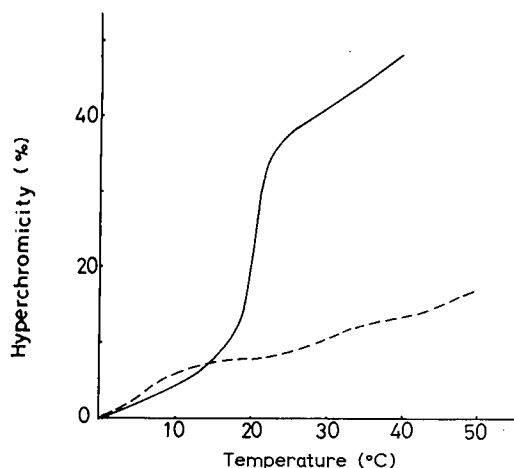


Fig. 3. Thermal melting of poly(n^2A) and poly(n^2I). —, poly(n^2A) at 0.15 M Na^+ and pH 7.0; - - - -, poly(n^2I) under the same conditions.

20°C. This result suggested that an ordered structure different from that of poly(A) may exist in neutral solution. In the case of poly(n^2I), the absorbance at 287 nm increased gradually with increasing temperature and hyperchromicity at 287 nm was about 7% on heating from 24 to 65°C. This fact suggests that poly(n^2I) exists in a random coil structure in the 0.15 M neutral salt solution as found in the case of poly(I) [19].

Complex formation of poly(n^2A) with poly(U)

Poly(A) forms complexes with poly(U) in ratios of 1 : 1 or 1 : 2 according to the ionic strength or presence of divalent cations [20,21]. When poly(n^2A) was mixed in various ratios with poly(U) at the same salt concentration, we obtained a mixing curve as shown in Fig. 4. This curve clearly showed that a 1 : 2

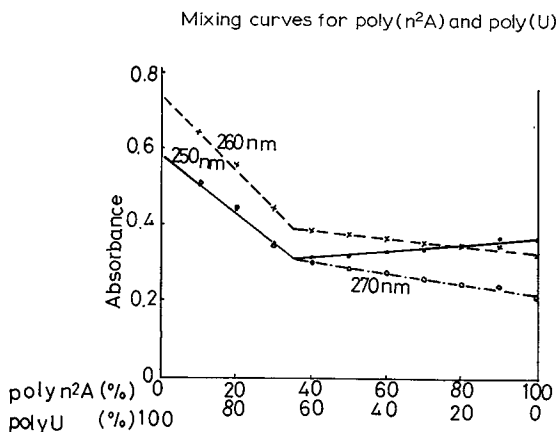


Fig. 4. Mixing curve of poly(n^2A) and poly(U). X-----X, absorbance at 260 nm; ●-----●, 250 nm; ○-----○, 270 nm.

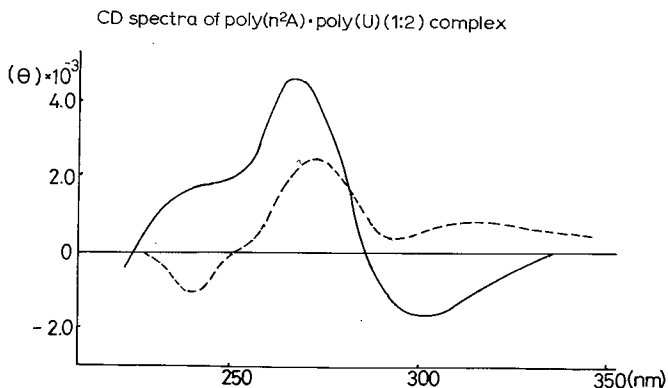


Fig. 5. CD spectra of poly(n^2A)-poly(U) (1 : 2), measured in the presence of 0.15 M Na^+ (pH 7.0). —, observed curve; ----, calculated curve from CD curves of components.

complex was formed in these conditions. As shown in Fig 5, this complex formation was also supported by measurements of CD before and after mixing of the two components. The CD curve before mixing showed two peaks at 273 and 315 nm, and a trough at 295 nm. After mixing the curve changed to a completely different one, which had a peak at 267 nm and a trough at 300 nm. The thermal transition curves of the poly(n^2A) · 2poly(U) complex (Fig. 6) in the presence of 0.15 M Na^+ at pH 7.0 showed a T_m of 41°C. The T_m for the poly(A) · 2poly(U) complex in these conditions was reported to be 60°C [22]. The T_m of the poly(n^2A) · poly(U) (1 : 2) complex was thus 9°C lower than the T_m of the poly(A) · poly(U) (1 : 2) complex. These results suggest that a three-stranded complex of poly(n^2A) and poly(U) has low thermal stability.

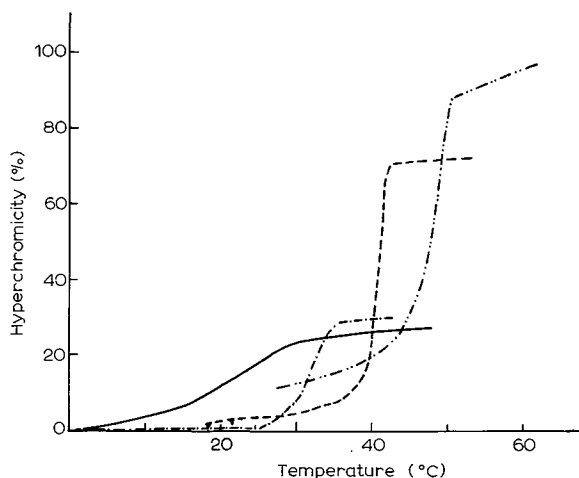


Fig. 6. Thermal melting of poly(n^2A) · poly(U) (1 : 2) complex, Poly(n^2A) · poly(I) (1 : 2) complex, poly(n^2I) · poly(A) (1 : 2) complex and poly(n^2A) · poly(n^2I) (1 : 2) complex. ----, poly(n^2A)-poly(U), measured at 260 nm; —, poly(n^2A)-poly(I), measured at 249 nm; - · - · -, poly(n^2I)-poly(A), measured at 260 nm; - · - · -, poly(n^2A)-poly(n^2I), measured at 250 nm.

Complex formation of poly(n^2I) with poly(C)

Poly(I) is known to form a double-stranded complex with poly(C) at 0.15 M Na^+ concentration and pH 7.0 [23,24]. On the other hand, poly(ms^2I), poly(m^2G), and poly(m_2^2G) could not form a double-stranded complex with poly(C) [27,29]. When poly($n^2\text{I}$) was mixed at 10°C in various ratios with poly(C) at the same concentration, ultraviolet absorption linearly changed with variation of the ratio at various wave lengths, and the CD spectrum of this 1 : 1 mixture showed little difference when the calculated sum of each component and the observed curves were compared (data were not shown). Therefore, it would be concluded that poly($n^2\text{I}$) and poly(C) could not form the double-stranded complex in the present condition.

Complex formation of poly($n^2\text{A}$) or poly(A) with poly(I) or poly($n^2\text{I}$)

It was reported that poly(I) forms a triple-stranded complex with poly(A) [23]. We tested the complex formation of poly($n^2\text{A}$) with poly(I). Results in the mixing experiment are shown in Fig. 7. This curve clearly showed that poly($n^2\text{A}$) complexed with poly(I) in a 1 : 2 ratio in the presence of 0.15 M Na^+ at pH 7.0. In Fig. 8 is shown the observed CD spectrum of the 1 : 2 complex and the calculated sum of the spectra of its component polynucleotides. The thermal stability of the complex was studied and the temperature-hypochromicity profile is recorded in Fig. 6. The T_m was 23°C at 0.15 M Na^+ concentration. The T_m of the poly($n^2\text{A}$) · 2poly(I) complex was 17°C lower than the T_m of poly(A) · 2poly(I) which was reported to be 40°C at 0.15 M Na^+ concentration.

In order to test the ability of poly($n^2\text{I}$) to form a complex with poly(A), poly($n^2\text{I}$) was mixed in various ratios with poly(A) in the presence of 0.15 M

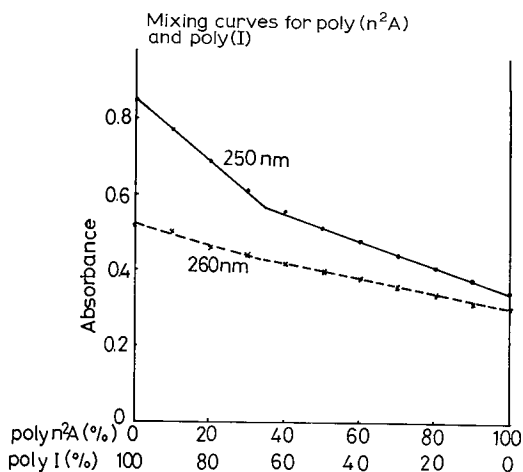


Fig. 7. Mixing curve of poly($n^2\text{A}$) and poly(I). X- - - -X, absorbance at 260 nm; ●—●, at 250 nm.

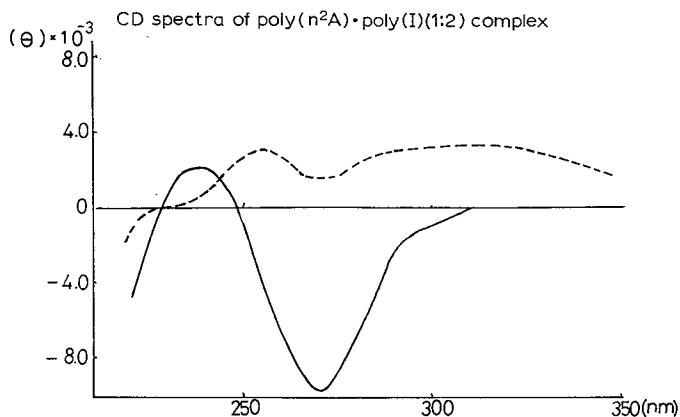


Fig. 8. CD spectra of $\text{poly}(\text{n}^2\text{A}) \cdot \text{poly}(\text{I})$ (1 : 2) complex. Measured in the presence of 0.15 M Na^+ (pH 7.0). —, observed curve; ----, calculated curve from CD curves of components.

Na^+ at pH 7.0. As shown in Fig. 9, inflections occurred at the points of 2 : 1 ratio in the mixing curve at 255, 270, and 285 nm. This suggested that $\text{poly}(\text{n}^2\text{I})$ and $\text{poly}(\text{A})$ formed a triple-stranded complex as observed in the case of $\text{poly}(\text{n}^2\text{A}) \cdot \text{poly}(\text{I})$. The CD spectrum of this 2 : 1 complex is shown in Fig. 10. The observed curve was clearly different from the curve obtained from the calculated sum of $\text{poly}(\text{n}^2\text{I})$ and $\text{poly}(\text{A})$ in a 2 : 1 ratio. Therefore, it seems that these two polynucleotides actually formed a 2 : 1 complex as was observed in the case of $\text{poly}(\text{A})$ and $\text{poly}(\text{I})$. The temperature-absorption profile of this

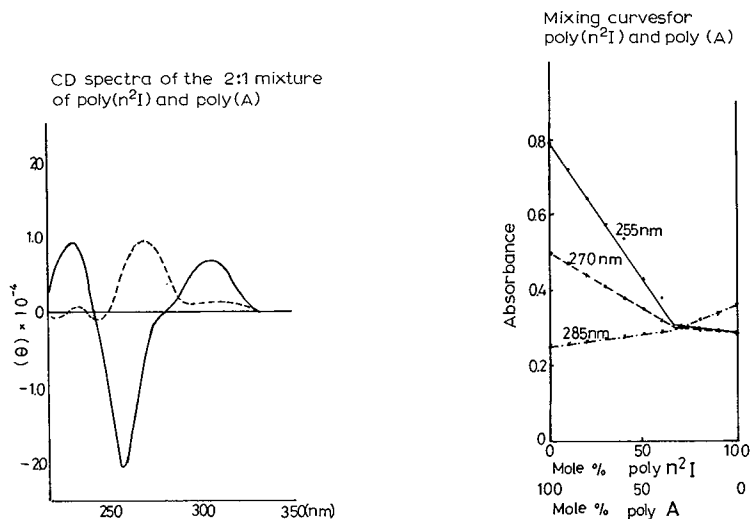


Fig. 9. Mixing curve of $\text{poly}(\text{n}^2\text{I})$ and $\text{poly}(\text{A})$. ●—●, absorbance at 255 nm; ●—●, at 270 nm, X—X at 285 nm.

Fig. 10. CD spectra of $\text{poly}(\text{n}^2\text{I}) \cdot \text{poly}(\text{A})$ (2 : 1) complex. Measured in the presence of 0.15 M Na^+ (pH 7.0). —, observed curve; ----, calculated curve from CD curves of components.

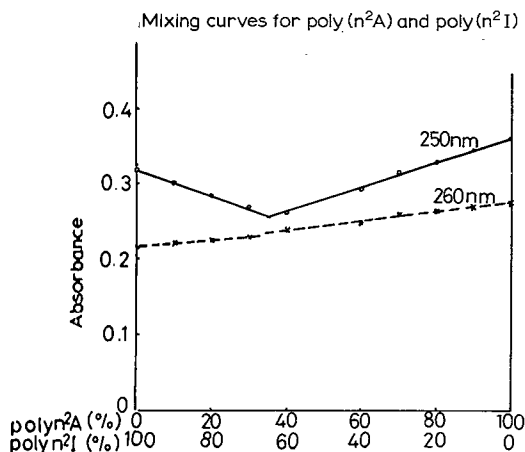


Fig. 11. Mixing curve of poly(n^2A) and poly(n^2I). $\circ$ — $\circ$, absorbance at 250 nm; X—X, at 260 nm.

complex is shown in Fig. 6, from which the T_m at 0.15 M Na^+ concentration was shown to be $48^\circ C$. The T_m of the $2poly(n^2I) \cdot poly(A)$ complex was $8^\circ C$ higher than the T_m of $2poly(I) \cdot poly(A)$.

We tested the complex formation of poly(n^2A) with poly(n^2I). Results of the mixing experiment are shown in Fig. 11. This curve also showed that poly(n^2A) complexed with poly(n^2I) in a 1:2 ratio in the presence of 0.15 M Na^+ at pH 7.0. In Fig. 12, the CD spectrum of this 1:2 complex is shown. The observed curve was clearly different from the curve obtained from the calculated sum of poly(n^2I) and poly(n^2A) in a 2:1 ratio. The temperature-absorption profile of this complex is shown in Fig. 6. The T_m at 0.15 M Na^+ concentration was $31^\circ C$, which is $9^\circ C$ lower than the T_m of $poly(A) \cdot 2poly(I)$. These results showed that poly(n^2A) formed a 1:2 complex with poly(I) or poly(n^2I) and poly(n^2I) formed a 2:1 complex with poly(A) or poly(n^2A). Poly(n^2A) had a low thermal stability in the triple-stranded complex with poly(I). Poly(n^2I), however, had a higher thermal stability in the triple-stranded complex with poly(A) than the $poly(A) \cdot poly(I)$ (1:2) complex. Therefore, the replacement of the C_2 atom of the adenine ring by the N atom had a tendency for destabili-

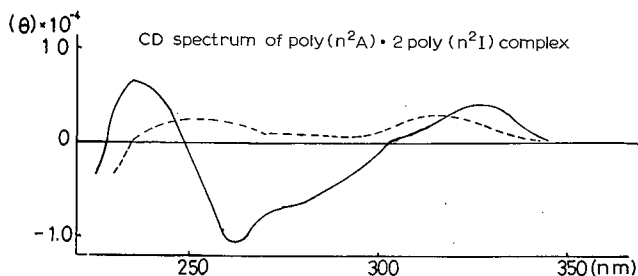


Fig. 12. CD spectra of $poly(n^2I) \cdot poly(n^2I)$ (1:2) complex. Measured in the presence of 0.15 M Na^+ (pH 7.0). —, observed curve; ----, calculated curve from CD curves of components.

zation whereas in the case of the hypoxanthine ring, a stabilizing effect upon replacement of C₂ atom by N₂ could be observed.

Discussion

The use of any modified polynucleotides in determining the effect of the specific structural feature in physicochemical or biological systems is worthy of investigation. 2-Azapurine nucleosides are known to be active against cancer cells [11,22] and studies of ribopolynucleotides containing these nucleoside phosphates are interesting to denote their roles in biological activities. As the neutral molecule poly(n²A) revealed a different feature for that of poly(A) showing a rather clear melting curve. This may be due to stabilization of an ordered structure by water molecules associated to N₂-atom. In the case of poly(n²I) this stabilization effect might be cancelled by a lower pK value of the 2'-azahypoxanthine base.

The continuous variation method in construction of mixing curves leads to the conclusion that poly(n²A) can form triple-stranded complexes with poly(U) or poly(I), and poly(n²I) can form triple-stranded complexes with poly(A) or poly(n²A). It appears that in these triple-stranded complexes the melting proceeds directly to the constituent homopolymers. The T_m values of the triple-stranded complexes of poly(n²A) with poly(U) or poly(I) are lower by 17–19°C compared to the corresponding poly(A) · 2poly(U) complex ($T_m = 60^\circ\text{C}$) and poly(A) · 2poly(I) complex ($T_m = 40^\circ\text{C}$).

In contrast to the case of poly(n²A), poly(n²I) can form a triple-stranded complex with poly(A) which shows a significantly elevated T_m value (48°C) when compared to the poly(A) · 2poly(I) triplex (40°C). In the case of the triple-stranded complex of poly(n²A) with poly(n²I), its T_m 31°C is lower by 9°C as compared to that of poly(A) · 2poly(I). These results suggested that the replacement of the C₂ atom of the adenine ring by an N₂ atom had a tendency to destabilize the complex but in contrast, in the case of the hypoxanthine ring a tendency to stabilize the complex appeared. These phenomena may be interpreted in terms of a lower basicity of 2-azaadenosine relative to adenosine and a higher basicity of 2-azainosine relative to inosine [11].

It has been shown previously [25,28], that bulky groups such as NHMe, NMe₂, SMe, or Me at position 2 of purine polynucleotides were sterically unfavorable for the formation of a Watson-Crick type double-helical complex with pyrimidine polynucleotides having 2-keto groups, presumably because of steric distortion. We conclude that poly(n²I) can not form a double-stranded complex with poly(C), because of repulsion between the lone pair electrons of the 2-N= of poly(n²I) and 2-C=O group of poly(C). In addition, the same steric distortion could be involved in the case of the poly(n²A)-poly(U) interaction. In the former case the distortion caused by interacting 2-C=C and 2-N atoms may overcome the stabilizing effect of the basicity change. These facts may imply that the altered properties of RNA containing 2-azapurines from that of natural RNA may be relevant to the cause of anticancer activities of 2-azapurine nucleosides.

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