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)
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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).
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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

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6.Interferon inducing activity of a 2'-modified double-stranded complex,poly(2'-azido2'-deoxyinosinic acid).poly(cytidylic acid).
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J.Pharm.Dyn.,1 62 (1978)
2'置換二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

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Nucleic Acids Res.,5 3315 (1978)
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N.Kakiuchi and M.Ikehara
Eur.J.Biochem.,88 341 (1978)
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2.Polynucleotides.XLIV.Synthesis and properties of poly(2-azaadenylic acid) and poly(2-azainosinic acid)
T.Fukui,N.Kakiuchi and M.Ikehara
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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

2′-置換ポリヌクレオチドの合成と性質に関する研究

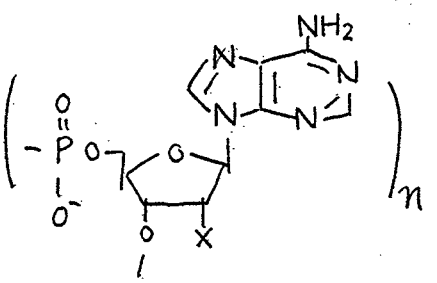
学位申請者

垣内信子

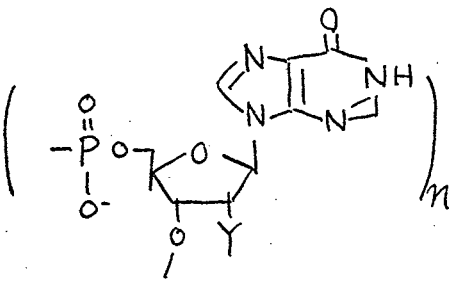
緒　論

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′位にアジド基、アミノ基、及びハロゲンを導入した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 : poly(Az)
NH_2 : poly(Aa)
F : poly(At)
Cl : poly(Acl)
Br : poly(Abr)



Y = N_3 : poly(Iz)
F : poly(It)
Cl : poly(Icl)

本　論

第 1 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

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-サイクロアデノシンを出発原料とし、2'-アジド-2'-デオキシアデノシン (Az) を合成するルートが開発された。[1] Az は Fig 1 のように ジリン酸化を行い、polynucleotide phosphorylase[4] を用いて、ポリ

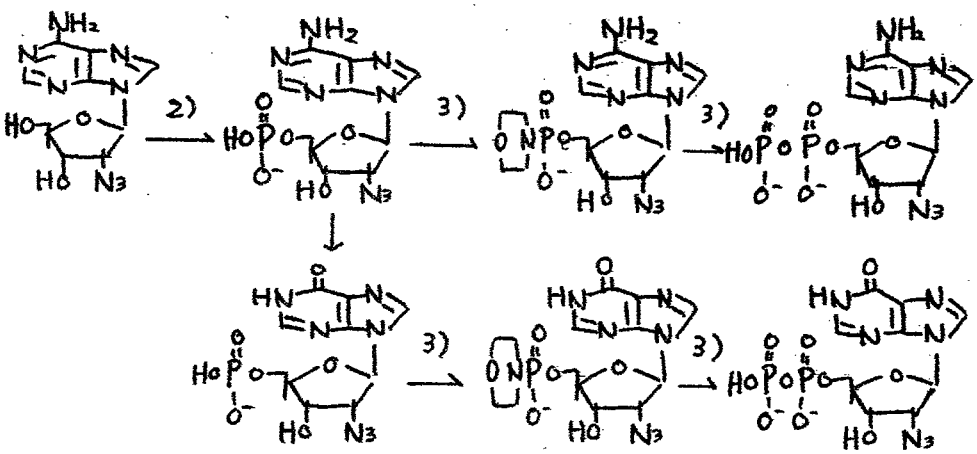
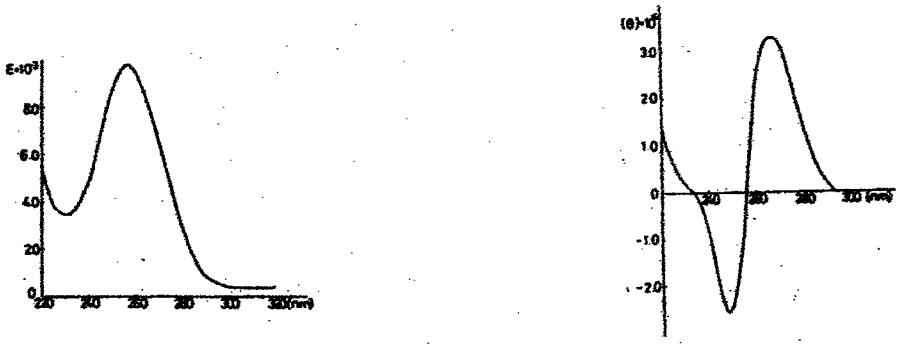


Fig 1

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

poly(Az) は、0.15M Na イオン濃度 pH 7.0 室温において 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 で、分子吸光係数 (ε) は 9780 で、モノマーよりの

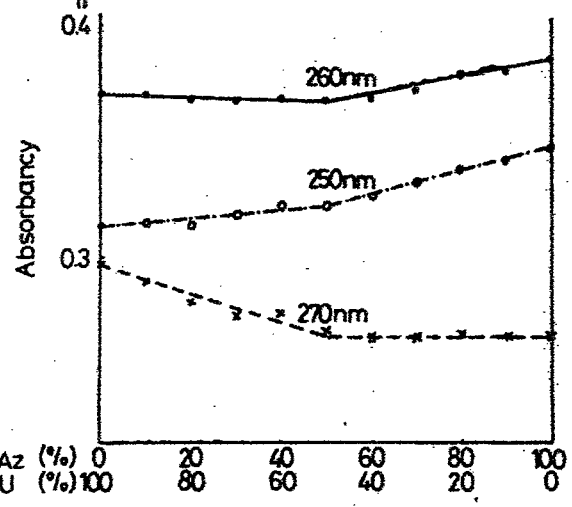


UV absorption spectrum of poly (Az) at pH 7.0. CD spectrum of poly (Az) at pH 7.0

Fig 2

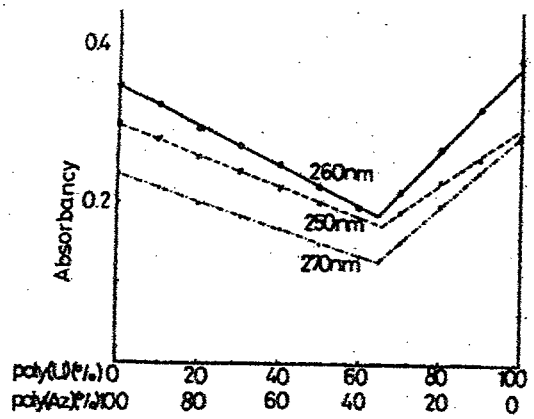
hypochromicity は 35% であった。これは poly(A) の hypochromicity と同じであり、poly(Az)は poly (A) と同程度の stacking をしていることが判る。一方 CD スペクトルの小さい円率は poly(A)の 60% 程度であった。又、poly (Az) は poly(U) と 0.04M Na イオン存在下 mixing すると 30分後の測定では 1:1, 0.15M

Fig3 a



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

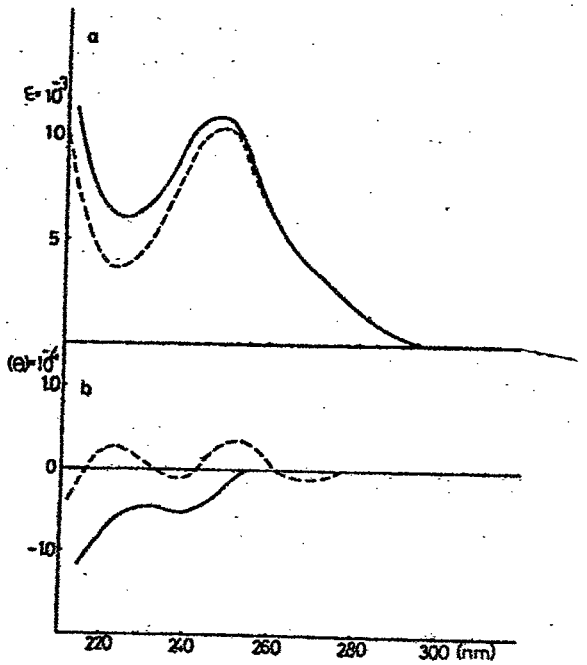
Fig 3 b



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

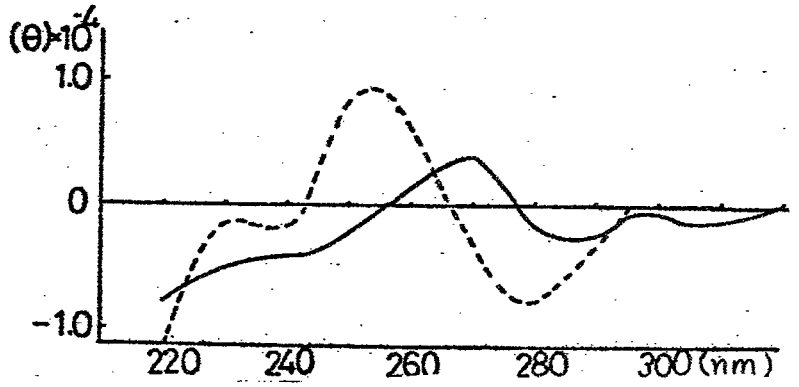
Naイオン濃度, over night の測定では 1:2 の complex の形成が観測された。これらの complex の melting temperature (Tm) は、0.04M Naイオン濃度で 46°C, 0.15M Naイオン濃度で 65°C であり、poly (A)・poly (U) complex の場合が 51°, 62° であるので、ほぼ同程度の熱的安定性を有している。

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 効果があらわれるのに対して、poly (Iz) ではほとんど正の cotton 効果はあらわれていない。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



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

Fig 5

pH 7.0 で poly (Iz) と poly (C) の mixing を行うと, 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)

complexのTm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

Fig6

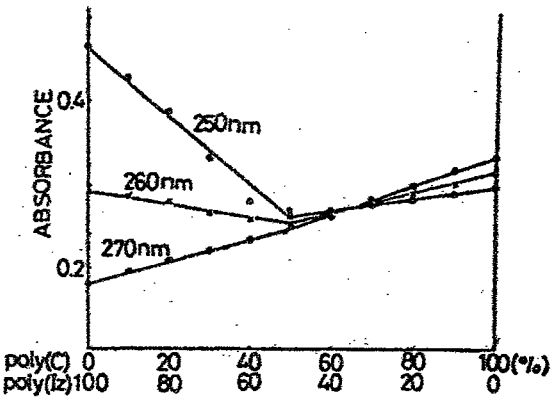


Fig7 (Na)

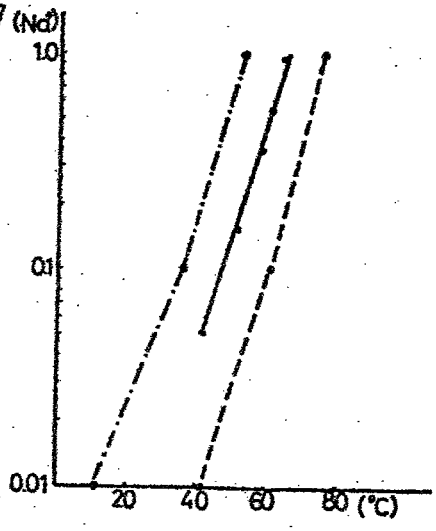


Fig 6 Mixing experiment of poly(Iz) and poly(C)

Fig 7 Relationship of Na^+ concentration and Tm 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'-アミノ-2'-デオキシアデニル酸)の合成と性質

アミノ基の電気陰性度は水酸基と大きく異っているが、分子量は

よく似ている。poly(Aa)の合成の基質となるAaDPはAzDPより接触還元によって合成し、polynucleotide phosphorylase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での UV

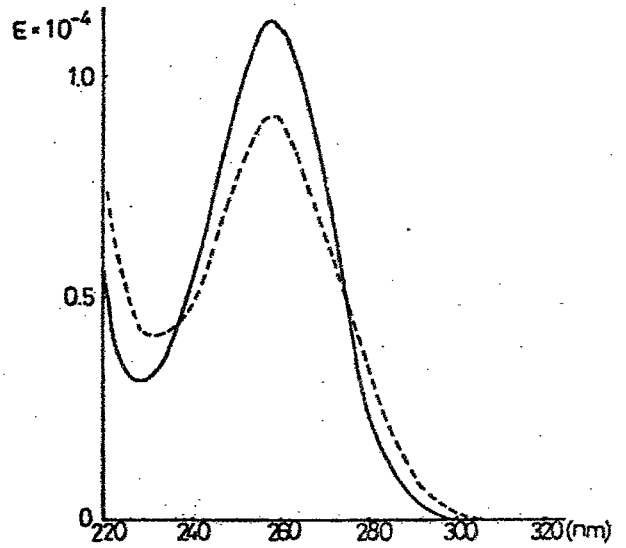


Fig8 UV absorption spectra of poly(Aa),— at pH 7.0, --- at pH 6.3.

スペクトルはFig 8のようになる。pH 7.0で λ_{max} 258 nm分子吸光係数 11200であった。ヌクレオシドよりのhypochromicityは25%で、poly(A)に比べて著しく小さい。pH7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スペクトルを示し、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、

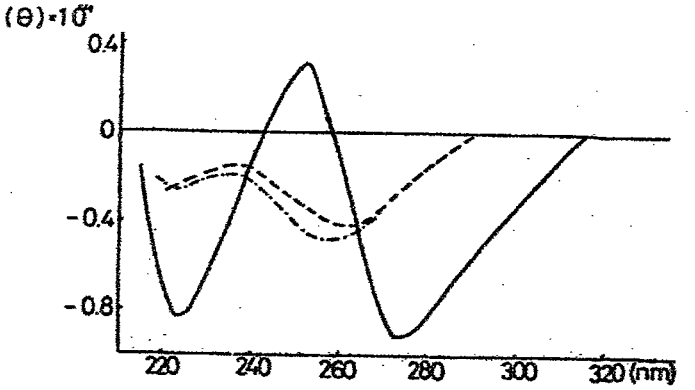


Fig9-a CD spectra of poly(Aa) taken at 20°(—), 46°(---) and 66°(-·-·-)

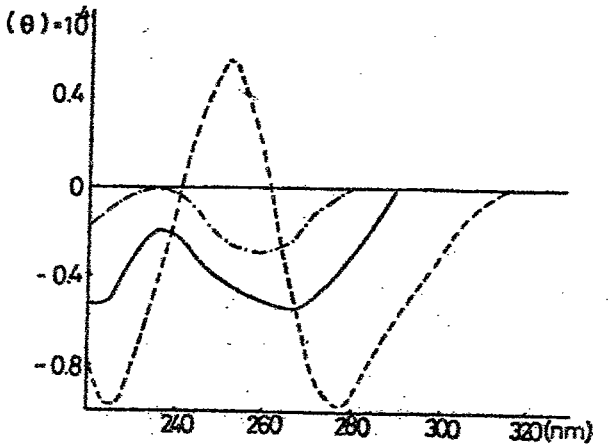


Fig9-b CD spectra of AaDP and poly(Aa), ——poly(Aa) at pH 7.0. ---poly(Aa) at pH 5.7, -·-·- AaDP at pH 7.0.

何らかのordered structureをとっているものと思われる。これは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Tm値と$\log_{10}[Na^+]$の関係はFig10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ネートした

いわゆる"acid form"ではなく、2'位アミノ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'-NH_2 は電気陰性度が水酸基と大きく異るために、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 conformation に重大な影響のあることが判った。

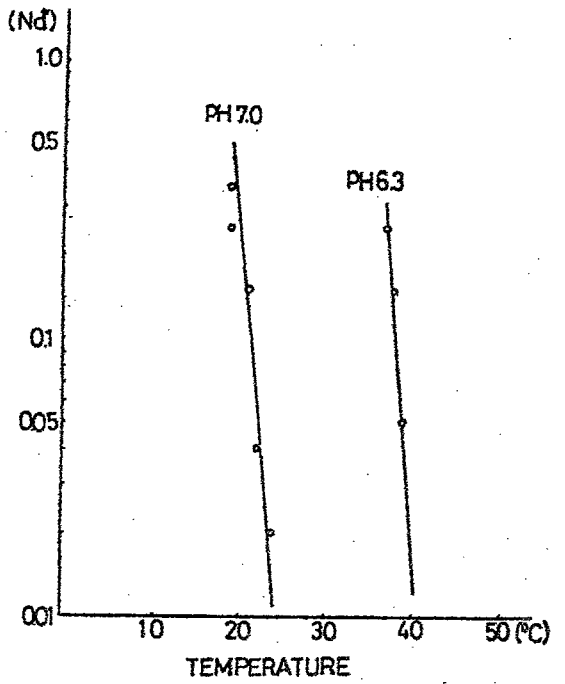
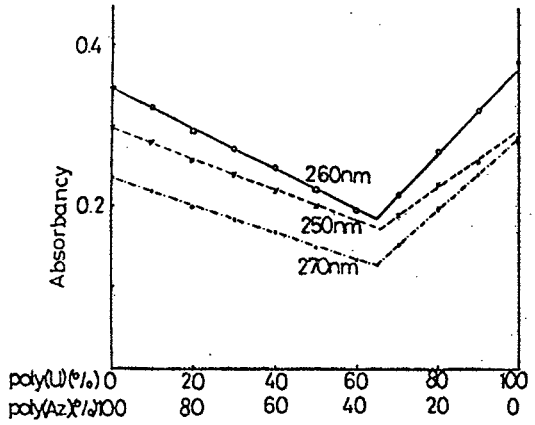


Fig.10 Dependence of Tm of Poly(Aa) on Na^+ concentration

第3章 ポリ(2'-ハロゲノ-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、大きさが水酸基と類似しているアミノ基を有する poly(Aa) は、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ジド基を有する poly(Az) が、poly(A) と似た

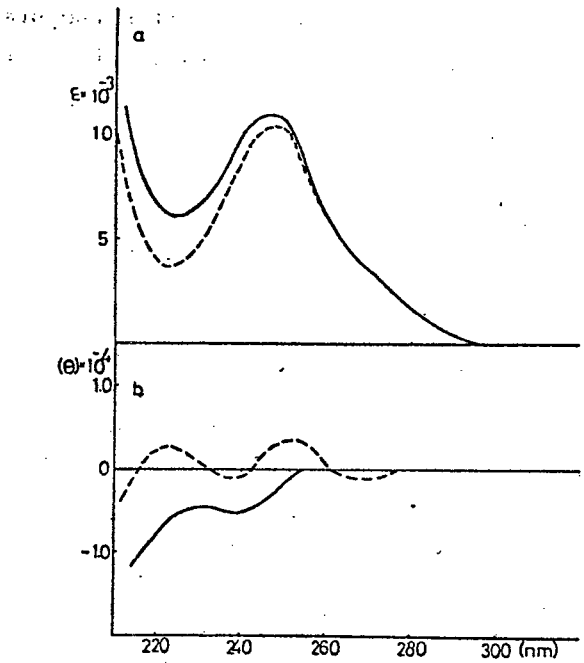
Fig 3 b



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

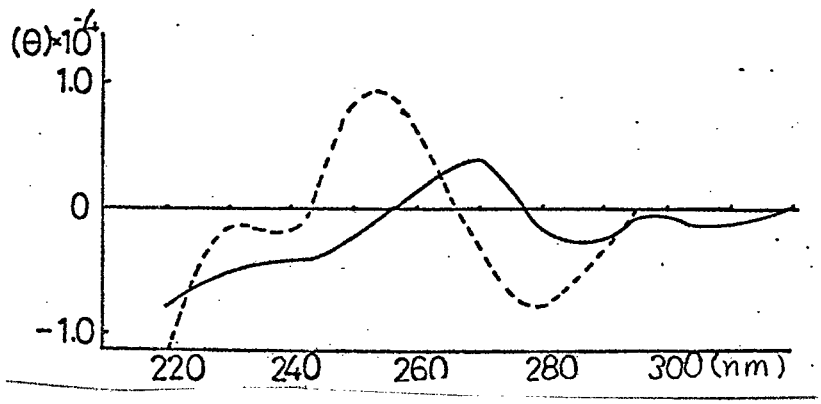
Naイオン濃度, over night の測定では 1:2 の complex の形成が観測された。これらの complex の melting temperature (Tm) は、0.04M Naイオン濃度で 46°C, 0.15M Naイオン濃度で 65°C であり、poly(A)·poly(U) complex の場合が 51°, 62° であるので、ほぼ同程度の熱的安定性を有している。

poly(Iz) の 0.15M Naイオン濃度, pH7.0, 室温での UV, CD スペクトルを Fig4 に示す。poly(I) では 225, 255 nm 付近に正の cotton 効果があらわれるのに対して、poly(Iz) ではほとんど正の Cotton 効果はあらわれていない。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



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

Fig 5

pH 7.0 で poly(Iz) と poly(C) の mixing を行うと、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。これら三者とpoly(U)とを0.04M Naイオン存在下pH7.0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、0.15M 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。これらcomplexのTm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)
Acl-U	46 (2→1)	56 (3→1)
Abr-U	45 (2→1)	53 (3→1)

熱的安定性はAf-U > Acl-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(If), poly(Icl)の0.15M Naイオン濃度, pH7.0 20°Cにおけるスペクトルは 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(If)ではpoly(I)と打全く

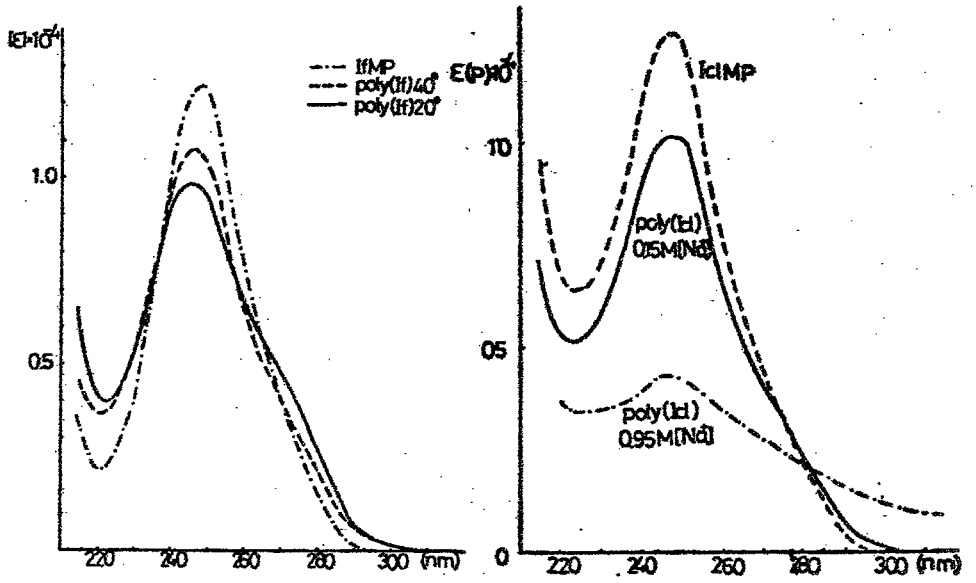
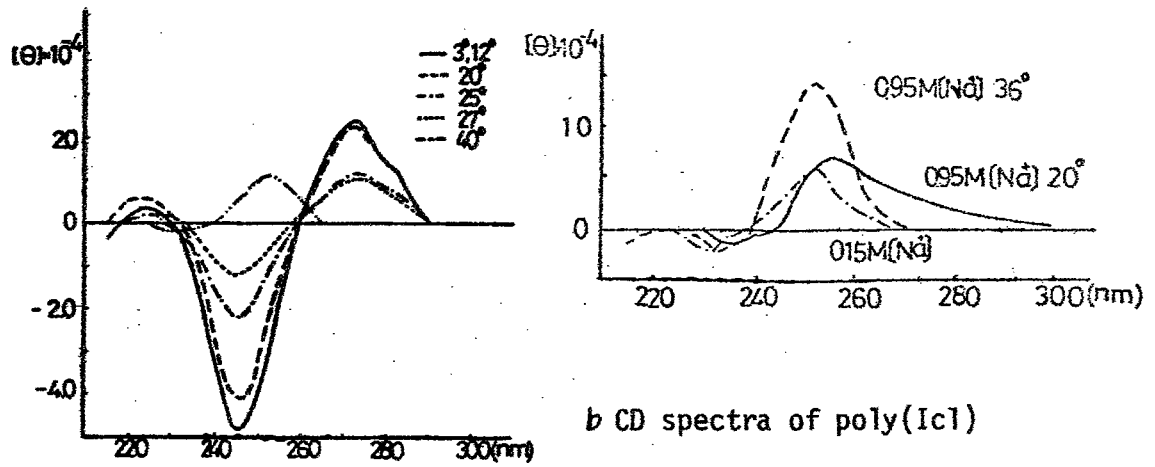


Fig13 a UV spectra of poly(If) b UV spectra of poly(Icl)



b CD spectra of poly(Icl)

Fig14 a CD spectra of poly(If)

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、Tmである27℃をこえるとCDスペクトルは逆転し、poly(I)が

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(Icα) 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(Icα) は poly(If) にみられる様な ordered structure は形成しないことがわかる。又、poly(If), poly(Icα) は poly(C) と中性条件下 1:1 の complex を形成する。これら complex

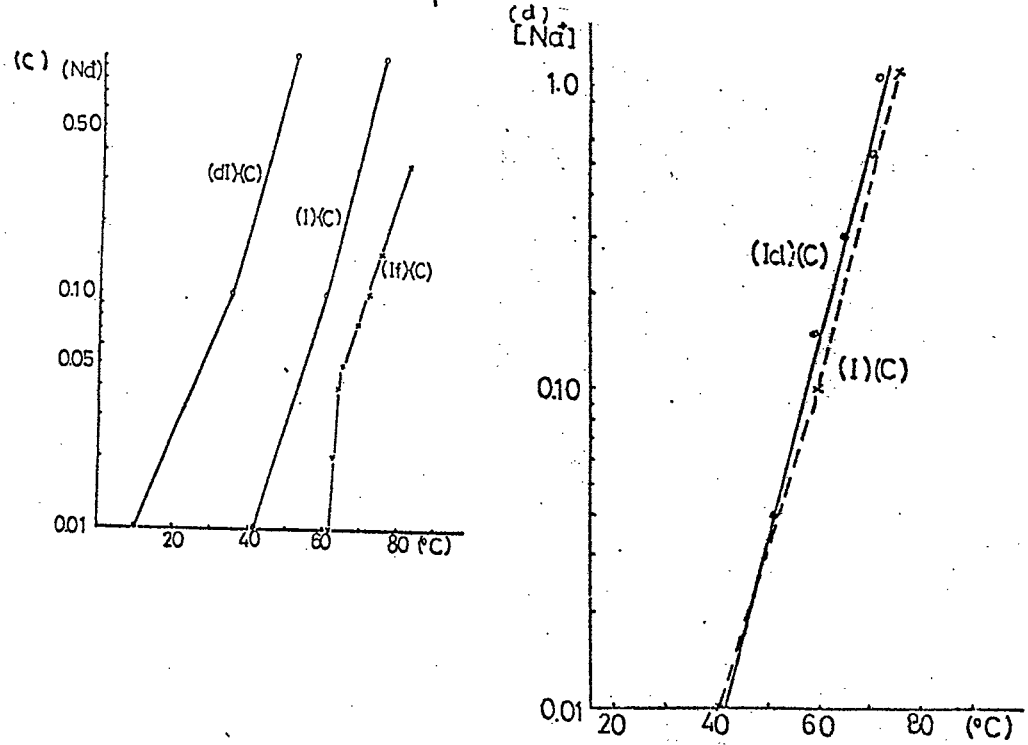


Fig 14(c)(d) Relationship between Tm of various double-stranded complexes and Na^+ concentration.

の熱的安定性を Na イオンと変化させて調べた結果が Fig 14(c)(d) である。両者ともに Tm 値と $\log_{10}[Na^+]$ の間に直線関係が成立する。poly(If)・poly(C) は poly(I)・poly(C) complex に比べ、いずれの Na イオン濃度においても、

Tm値が15～20°C高いが、poly(Ic)・poly(C)ではほとんど同じである。

前述のpoly(Ax)の場合と同様poly(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poly(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子量2～3万程度の糖タンパク質で細胞にウィ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poly(I)・poly(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poly(Az), poly(Iz), poly(It) poly(Af)についてinterferon誘発活性を調べた。Table Ⅲ, Ⅳに結果を示す。poly(Az)を含むcomplexはほとんど活性を示さない。しかし、poly(Iz)・poly(C)はhumanの系でpoly(I)・poly(C)とほとんど同じ活性を有している。poly(Af)を含むcomplexは活性は示すがpoly(A)を含むcomplexより値は小さい。一方poly(I)・poly(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[7]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** 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-(U)n*			2.9
	(A)n-(rT)n			3.4
	(A)n-(Uz)n			< 1.0
	(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)			3.9
	(Iz)n-(C)n			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

Polynucleotide	Interferon titer(log 10 units/ml) obtained at polynucleotide concentration of		
	0.1 μg/ml.	1 μg/ml.	10 μg/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)$_5$	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)$_5$	< 1.0	< 1.0	1.1
poly(Af)-poly(br^{5}U)			< 1.0
2. Human skin fibloblast 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)$_5$	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)$_5$	< 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にして、oligonucleotide primerにdNTPを付加重合する酵素である。[8]

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。Fig15がtemplate primer非存在下の結果で、

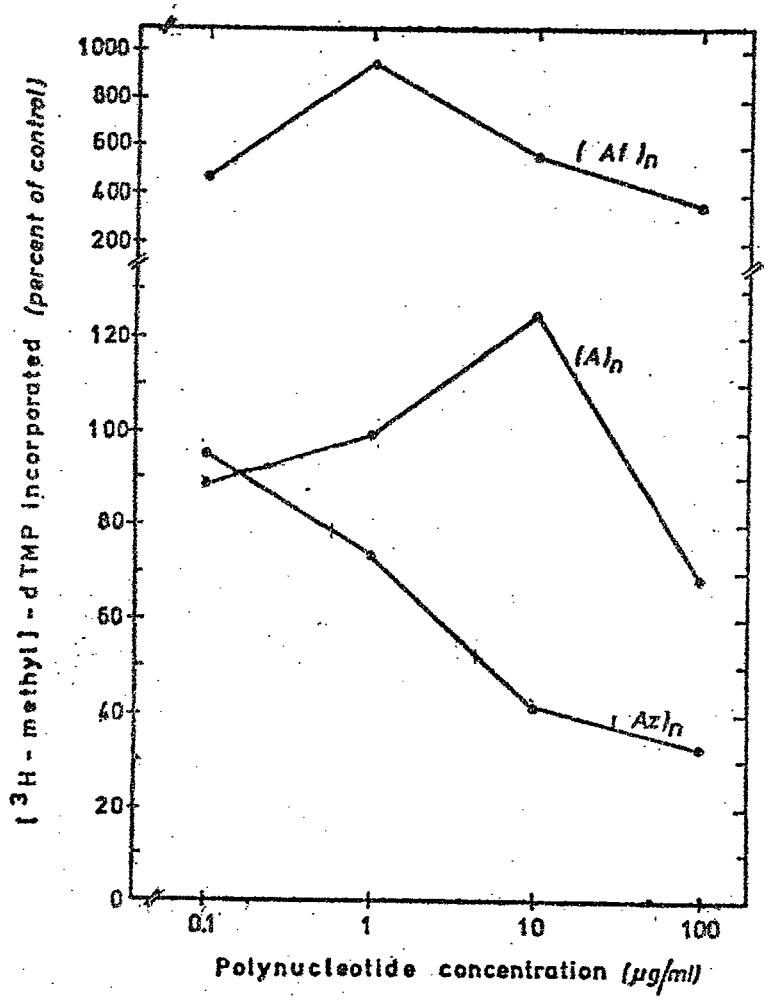


Fig 15 Effect of poly(Az),poly(Af) on MuLV reverse transcriptase in the absence of poly(A).oligo(dT) as template-primer

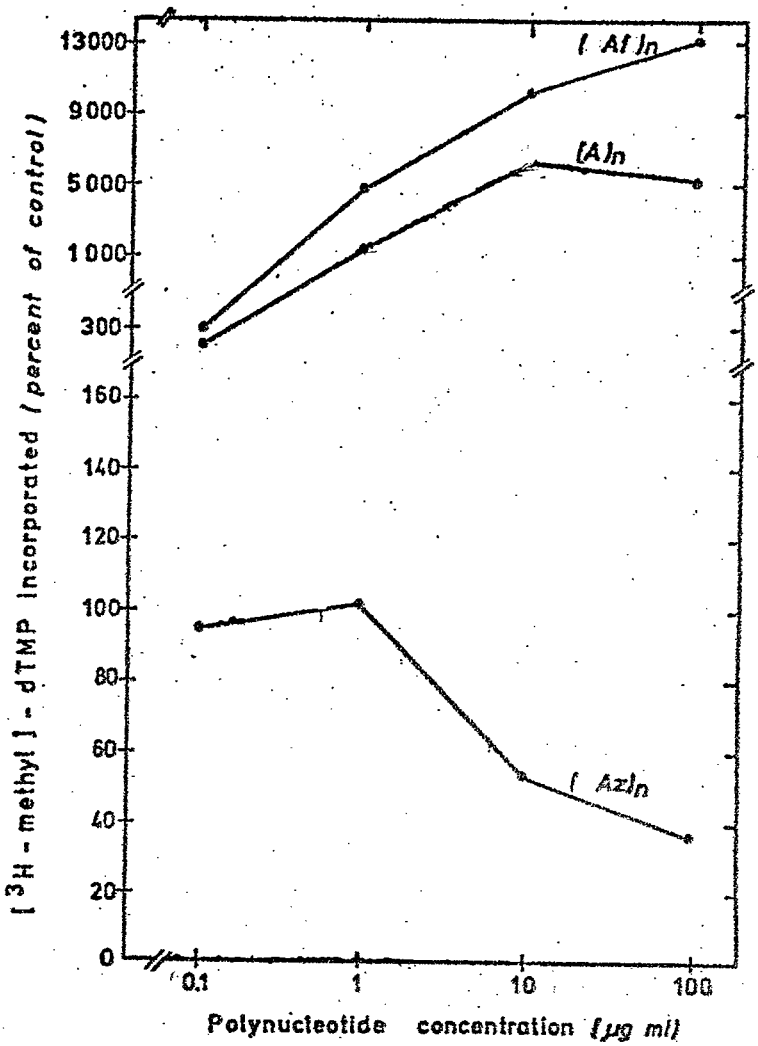


Fig 16 Effect of poly(Az) and poly(Af) on MuLV reverse transcriptase in the presence of oligo(dT) as primer

poly(Af)は, [³H]-dTMPのとりこみを促進している。

Fig16では. 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。

Fig17では. Template primer存在下でのinhibition

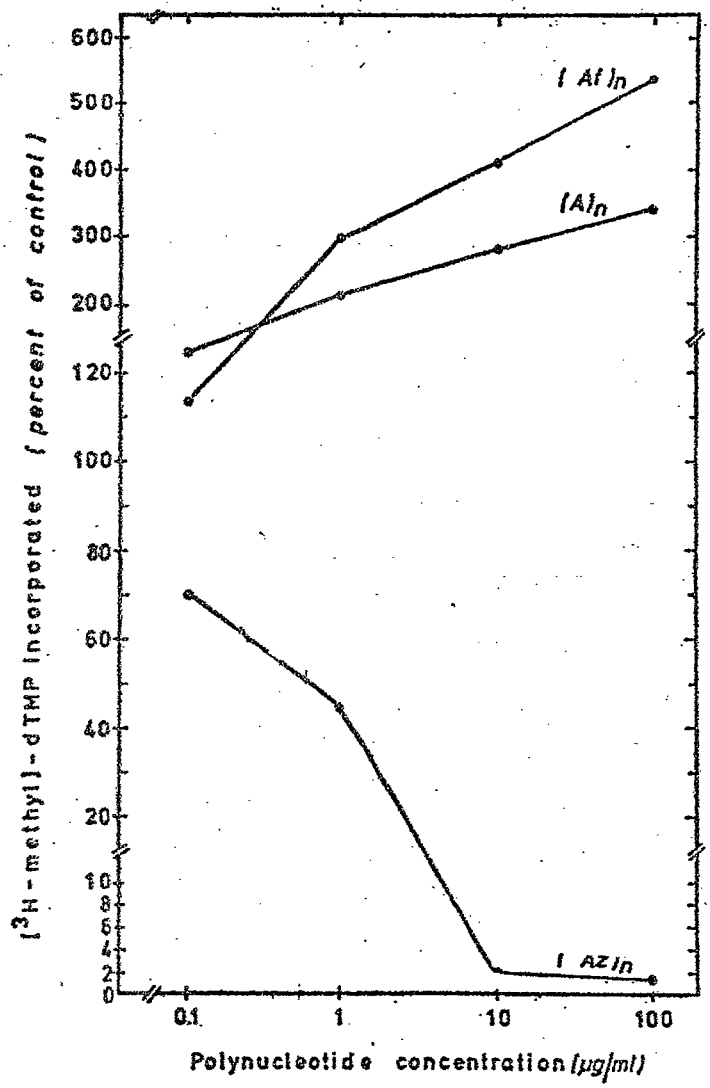


Fig 17 Effect of poly(Az) and poly(Af) on MuLV reverse transcriptase in the presence of poly(A) oligo(dT) as template-primer

効果と調べた結果である。poly(Az)が顕著な阻害作用を有しているのがわかる。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) アデノシン，イノシンの2′位をアジドで置換したpoly(Az), poly(Iz)では，poly(Az)がUVスペクトル，CDスペクトル，及びpoly(U)とのcomplexの熱的安定性がpoly(A)とよく似ているのに対して，poly(Iz)はpoly(I)におけるself-ordered structureをとり難く，poly(C)とのcomplexの熱的安定性は，poly(I)·poly(C)と，poly(dI)·poly(C)の中間に位置している．

2) 2′位にアミノ基を導入したpoly(Aa)は，弱酸性において，poly(A)では観察されなかったself-ordered structureを形成する．これは2′-アミノ基のプロトネートにより，塩基間のstackingが弱くされたものと思われる．中性条件ではstackingの程度はpoly(A)より小さく，プロトネートされない状態での2′-アミノ基はstackingを妨げる

3) アデノシンの2′位にハロゲンを導入した，poly(Af) poly(Acl), poly(Abr)では，ハロゲンの電気陰性度の増大さの減少に伴って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) イノシンの2′位にハロゲンを導入した poly (If)，poly (Ic) では、poly (If) が特異な self-ordered structure を形成し、又 poly (C) と非常に安定な complex を形成するのに対し、poly (Ic) では、そのような self-ordered structure は形成せず、poly (C) との complex の安定性は、poly (I)·poly (C) とほとんど同じである。

5) poly (Iz)·poly (C)，poly (If)·poly (C) では、poly (I)·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初めての例であり、従来の説を訂正した。

又、poly (Af) は poly (A) より高い RNA 腫瘍ウィルスの 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

垣　内　信　子

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)
Acl	2′-chloro-2′-deoxyadenosine
AclMP	2′-chloro-2′-deoxyadenosine-5′-monophosphate
AclDP	2′-chloro-2′-deoxyadenosine-5′-diphosphate
Poly(Acl)	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)
Icl	2′-chloro-2′-deoxyinosine
IclMP	2′-chloro-2′-deoxyinosine-5′-monophosphate
IclDP	2′-chloro-2′-deoxyinosine-5′-diphosphate
Poly(Icl)	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^2A)	poly(2-azaadenylic acid)
Poly(c^3A)	poly(3-deazaadenylic acid)
Poly(c^7A)	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うえで、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, アミノ基, 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。分子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って、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'-アジドウリジル酸)(poly(Uz))[1)2)]、ポリ(2'-デオキシ-2'-アジドシチジル酸)(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、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を報告している。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シ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。最近池原らによって8,2'-o-cycloadenosineを出発物質にして、arabinosyladenine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[4)27)]。

polynucleotide phosphorylase (PNPase) は Grunberg-Manago らによって発見され[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が知られている。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′-デオキシプリンヌクレオシドジホスフェートをポリヌクレオチドに導き、その物理化学的性質について研究した。

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′-デオキシアデノシン-5′-モノホスフェート (A$_2$MP) は対応するヌクレオシド[4)-7)]を小崎らの方法[5]に従って triethyl phosphate 中、$POCl_3$ を用いて 5′位をリン酸化し、活性炭

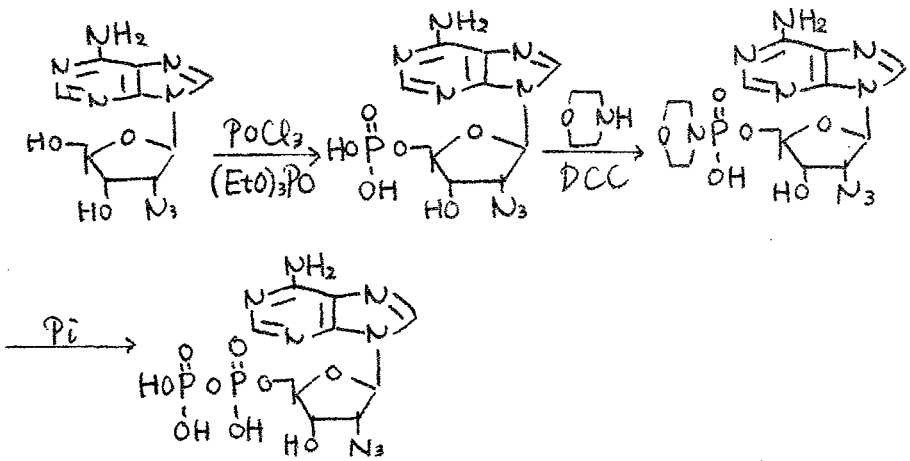


Fig 1

カラムで脱塩後、Dowex 1×2 (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の溶出パターンの1例をFig 2にあげた。

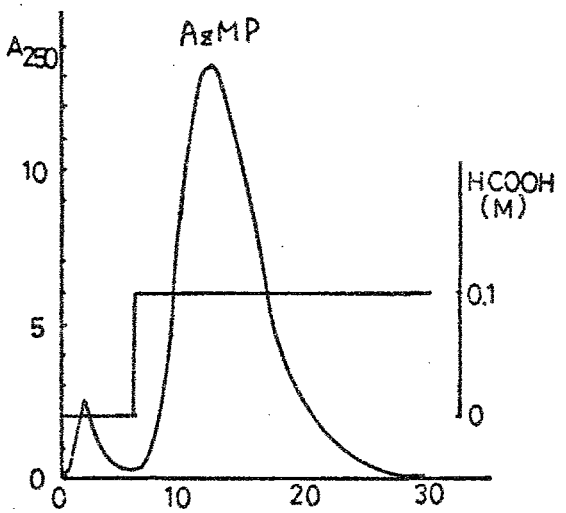


Fig 2 Dowex 1×2 (formate) カラムクロマトグラフィーによるAzMPの分離

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体をえた。そのカラムクロマトグラムの一例をFig 3にふした。AzMPおよびAzDPのクロマトグラムの性質をTable Iにふした。

Table I

	Rmp A-A		Rf		
	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

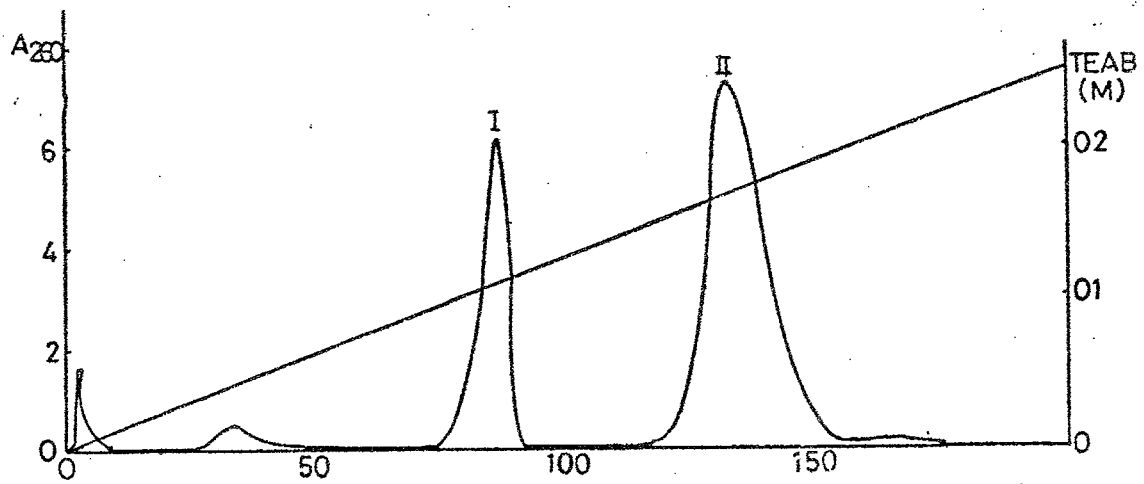


Fig 3 DEAE Sephadex A-25 (bicarbonate) による A_2DP の分離

peak I A_2MP　　peak II A_2DP

次に重合反応を行った。A_2DP 4 mM, $MgCl_2$ 2mM, Tris HCl (PH8.5) 100mM, PNPase (E. coli) 2units/ml 37°Cで反応させた。時間をおって遊離する無機リンの定量によって反応の進行をしらべるとFig4のようになる。20時間後にisoamyl alcohol - $CHCl_3$ (1:3)混液で除蛋白し反応をとめ凍結乾燥の後、Sephadex G50でゲル濾を行った。poly (A_2) は void volume 付近に

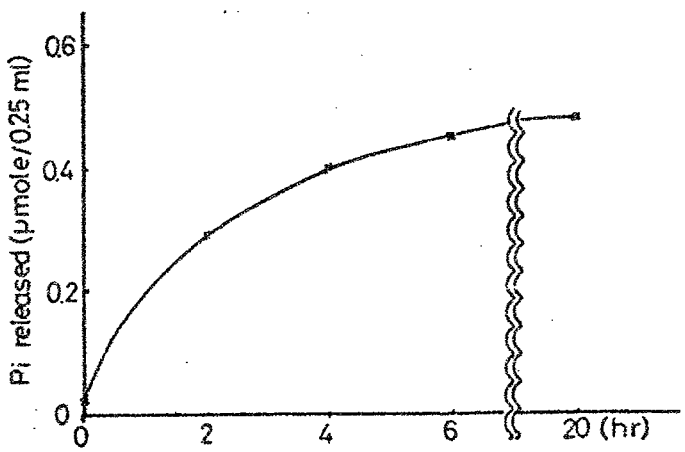


Fig4 poly (A_2) 合成の Time course

溶出される。収率は18%であった。このようにAzDPは2'位に水酸基がないにもかかわらずPNPaseの基質となることが判った。しかし、天然の基質であるADPが同じ条件で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、反応速度が遅く低収率である。

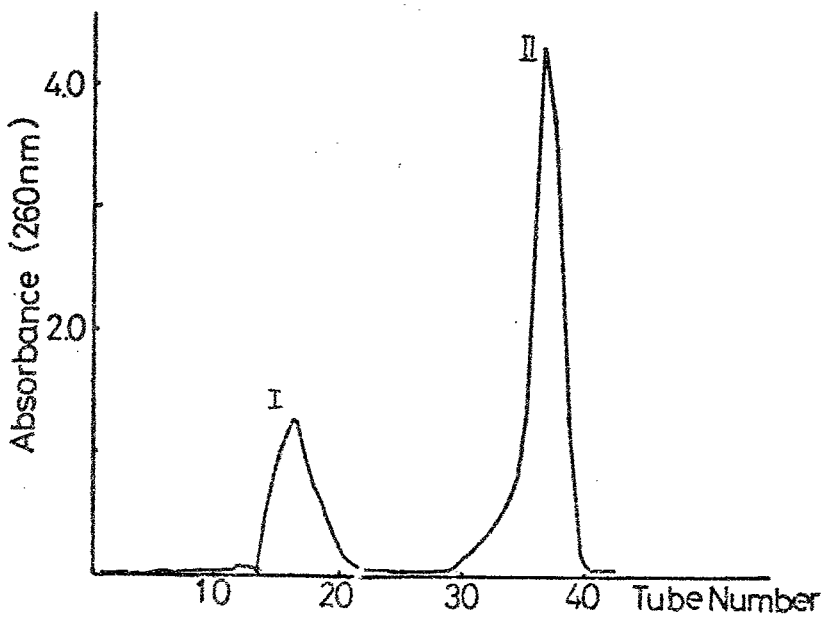


Fig 5 Sephadex G-50 gel filtration of poly(Az)
peakI;poly(Az),peakII;AzDP and AzMP

えられたpoly(Az)はsnake venom phosphodiesteraseで完全水解され、AzMPと少量のAzになった。このヌクレオシドはAzMPが酵素中に混在していると思われるが5'nucleotidaseによって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スペクトル

0.15 M Naイオン存在下 pH 7.0 でUVスペクトルを測定すると Fig 6a のようになる。 λmax は 256 nm リンの定量より

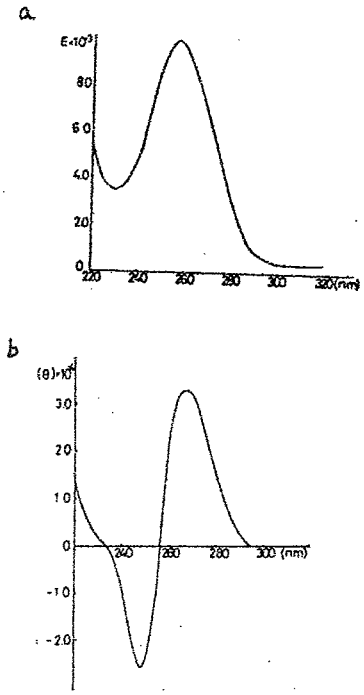


Fig.6 UV CD spectrum of poly (Az) in the presence of 0.15M Na^+ at pH 7.0 and 16°.

求めた分子吸光係数(ε(P))は9780で、hypochromicityはAzMPより35%である。このUVスペクトルは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をしているものと思われる。

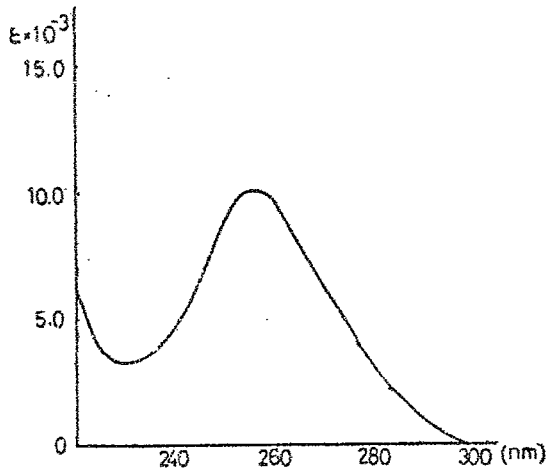


Fig. 7 a

UV absorption spectra of poly (A)

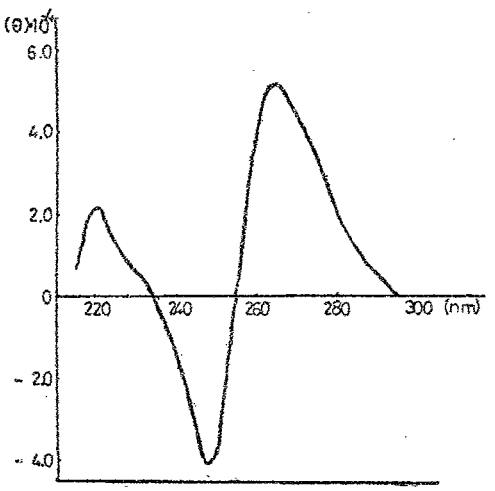


Fig. 7 b

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Fig6b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効果の分だけ円偏光度([θ])はpoly(A)の約60%程度しかない。

poly(Uε)[1)2)], poly(Cε)[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(Aε)のstackingの程度はpoly(A)とほぼ同じで

あると思われるので、この〔θ〕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思われる。poly(dA)ではhypochromicityはむしろpoly(A)より大きく[7]、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〔θ〕値は小さくなっている[8]。これはpoly(A)とpoly(dA)がconformationに違いがあるためである。poly(Az)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において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。

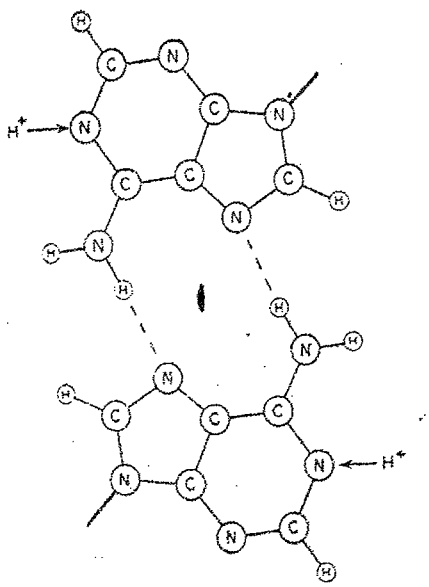


Fig 8 "acid form"の構造

そこでpoly(Az)についてもacid form形成について調べた。0.15 M Naイオン

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) に比べ 0.5 pH unit 小さくなっている。

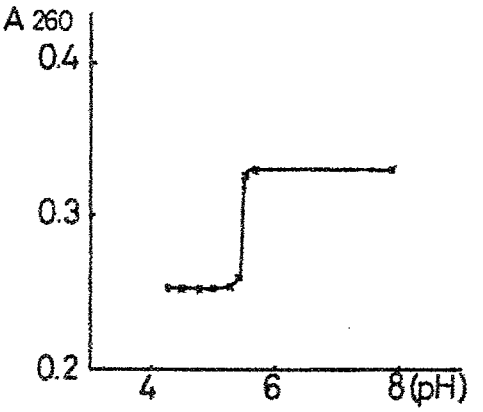


Fig 9 Acid titration curve of poly (Az).

次に 0.15 M Naイオン存在下で poly(Az)の temperature-absorbance profile を測定した。pH 7.0 では Fig 10 (a) のように co-operative な melting は観測されないが pH 5.0 では Fig 10(b) のように 33° に Tm が観察された。

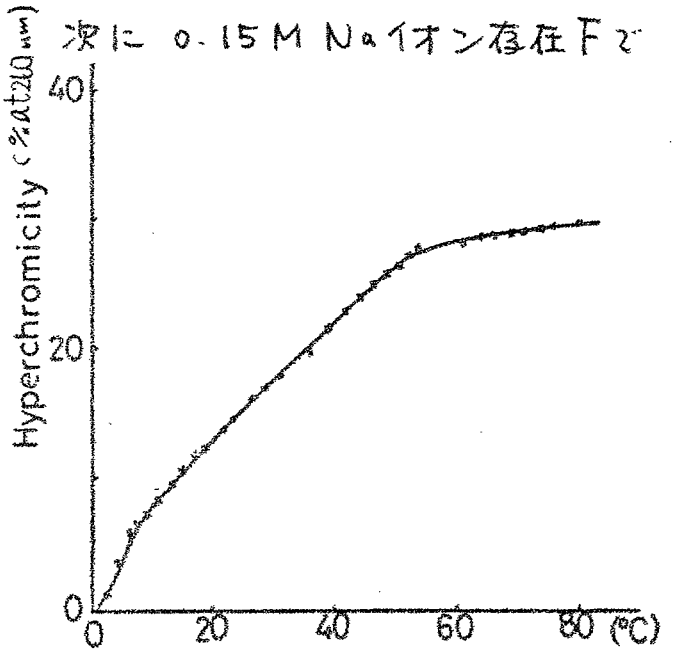


Fig 10 (a) poly(Az) の temperature-absorbance profile (pH 7.0)

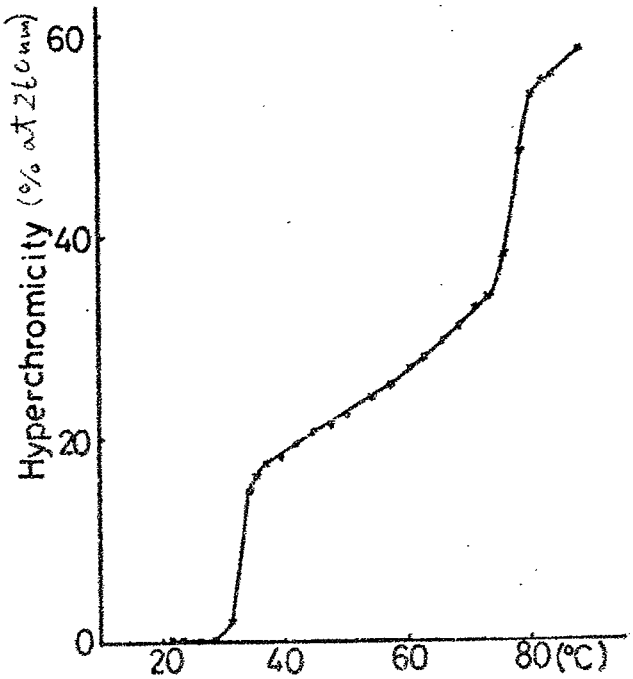


Fig 10(b) poly(A₂) の temperature - absorbance profile (pH 5.0)

80°付近の第2の transition については不明である。poly(A) は pH 5.0 で Tm が 60° であった。poly (A₂) は poly (A) に比べて、acid form が形成し難く、形成された acid form も熱的安定性が小さいことがわかる。poly(dA) でも、acid form の不安定化の傾向があるので[5]、この点においても poly (A₂) 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本鎖より[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 Naイオン濃度存在下. PH7.0で poly(Az)と poly(U)と mixingを行い, 30分後に 260nm 260nm

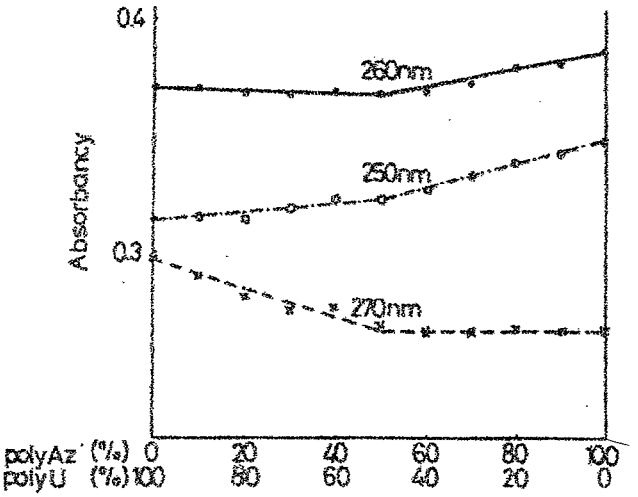


Fig 11 Mixing curves of poly (Az) and poly (U) at 0.04M Na^+.

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ので, 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 Naイオン存在下, 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

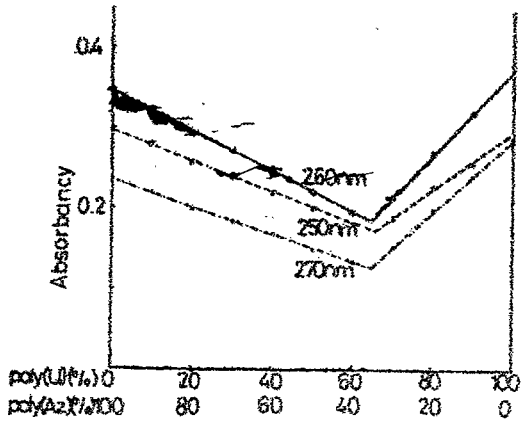


Fig 12 Mixing curves of poly (Az) and poly (U) at Na^+ concentration of 0.15M.

もっている ことが判る。又、形成した complex は Fig 14 の様な Watson-Crick[16] 型の2本鎖、及び Watson-Crick と Hoogsteen[14] 型の3本鎖を形成しているものと思われる。

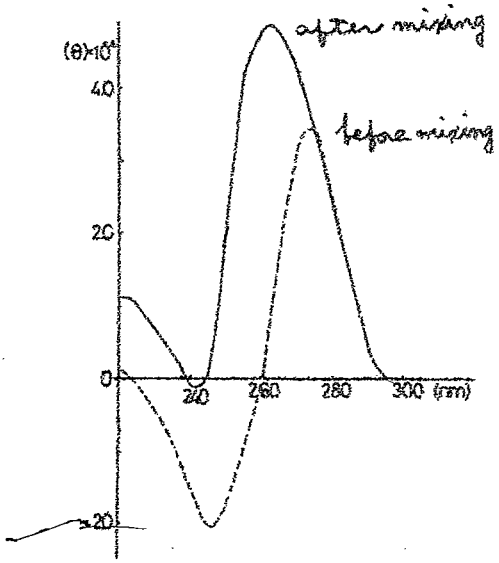


Fig 13 CD curves of poly (Az) plus poly (U) before and after mixing at 0.15 M Na^+ concentration.

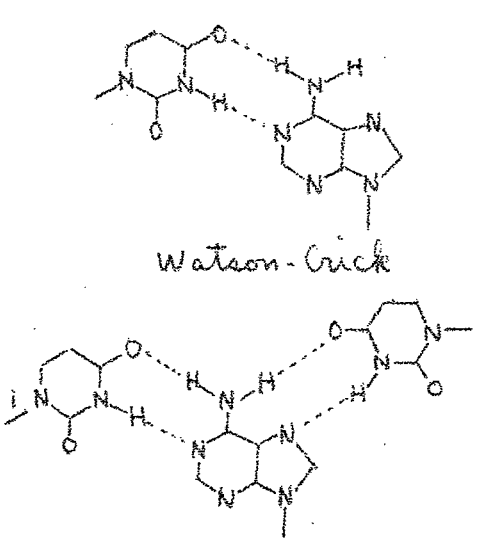
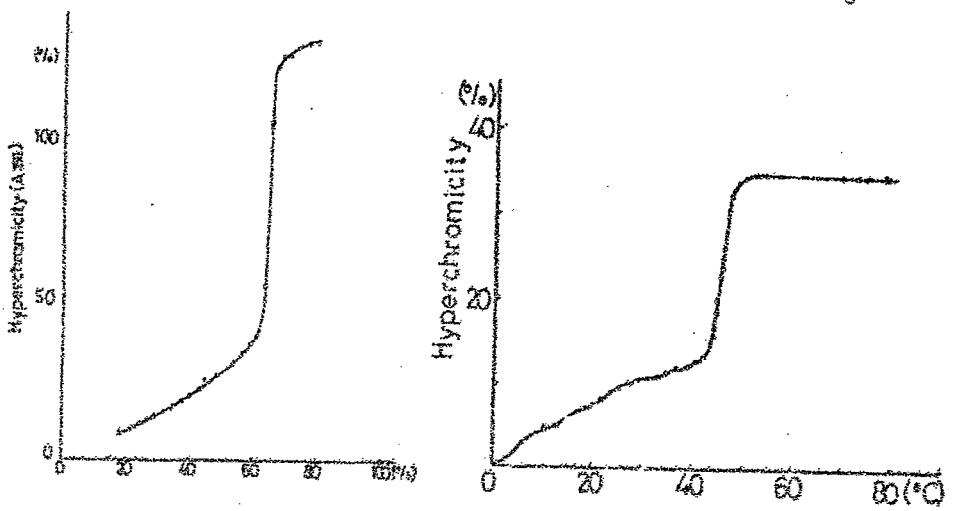


Fig 14 : (A)-(U) Complex の構造

次にそれらcomplexの熱的安定性について調べた。Fig 15が



(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 0.04 M 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Tm値である。同条件でpoly(A) poly(U) complexでは各々61℃, 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形成能を有しており、そ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形成において水酸基とほぼ同じ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思われる。

RNAのconformationの決定には、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'-デオキシイノシン酸)の合成

2'-アジド-2'-デオキシイノシン-5'-モノホスフェート(IadMP)はAadMPを酢酸々性中$NaNO_2$と反応させて合成した。反応完結は、PH3.5

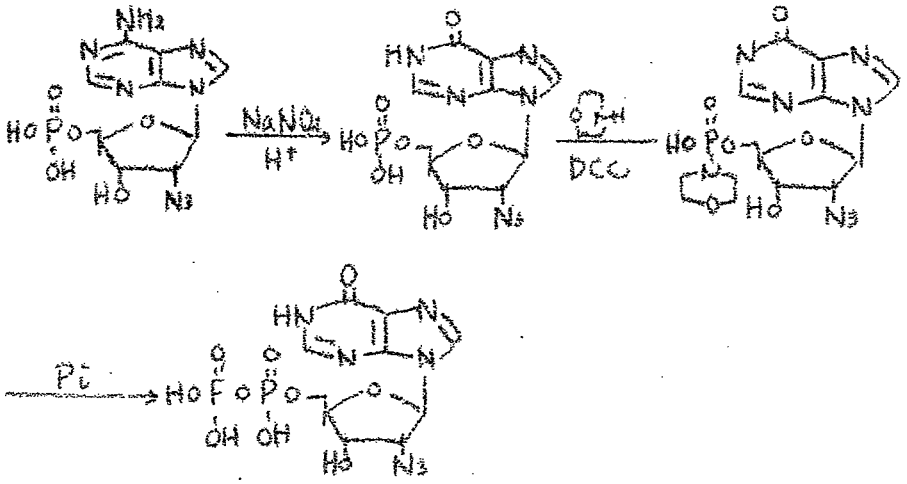


Fig 16

の電気泳動で原料のスポットが消失し、よりR$_f$値の大きな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 1×2(formate)のカラムクロマトグラムを行って80~97%の収率でIadMPを得た。次にIadMPを第1節で述べたと同様にして、5'-diphosphate体(IadDP)に導いた。精製はDEAE Sephadex A-25(Acetate)で行い、60~70%の収率でIadDPを得た。

これらのクロマトグラフ上の諸性質は Table 2 のようになる.

Table 2

	PEP (RmpA-A)		PPC (Rf)		
	PH7.5	pH 3.5	G↓	F↓	M↓
IzMP	0.98	2.00	0.13	0.67	0.93
IzDP	1.19	—	0.03	0.22	0.49

重合反応は. IzDP4 mM, MnCl₂ 2mM, TrisHCl(pH8.5) 100mM, 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って調べると

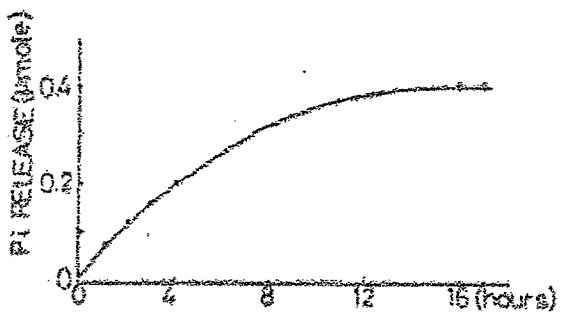


Fig 17 Time course of polymerization of 2'-azido-2'-deoxy-inosine 5'-diphosphate

Fig 17 のようになる. 16時間付近で反応は plateau に達しこれ以上は進まない. 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 μmole/0.25ml であるので, IzDP は IDP より反応の基質として劣っているといえる. 17時間後に isoamyl-alcohol : CHCl₃ = 1:3 混液で除蛋白して反応を止め, Mn イオンを除くために 0.01 M TrisHCl (pH 8.5) に対して透析し, Sephadex

G50 ゲルロ過を行った。poly(Is)は void volume 付近に溶出され、14%の収率で得ることができた。得られた poly(Is)は snake venom phosphodiesterase により完全に水解され、IsMP と trace 量の Is になった。

第4節 ポリ(2'-アミド-2'-デオシイノシン酸)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、リン酸の定量で求めた 260 nm 吸光度は 10,450 である。IsMP よりの

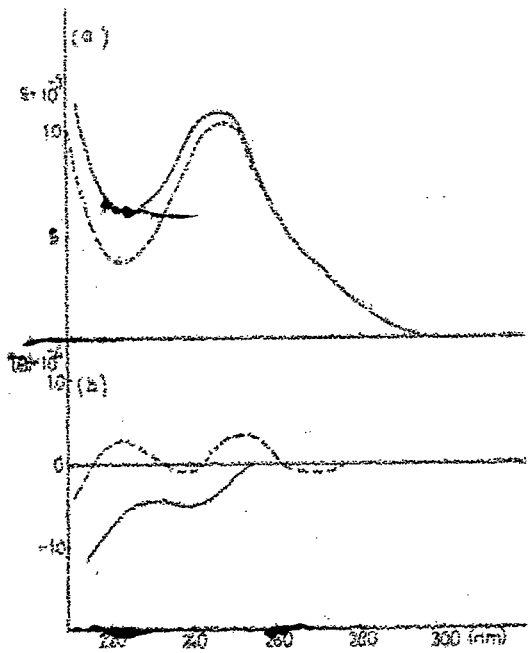


Fig 18 U.V. and C.D. spectra of poly(Is) and poly(in neutral solution containing 0.15M Na⁺.
—— poly(Is), ---- poly(I)

hypochromicity は11%であった。UVスペクトルパターンも 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(Iz) では正の Cotton 効果が観察されない。0.95M Naイオン濃度では 285nm 付近

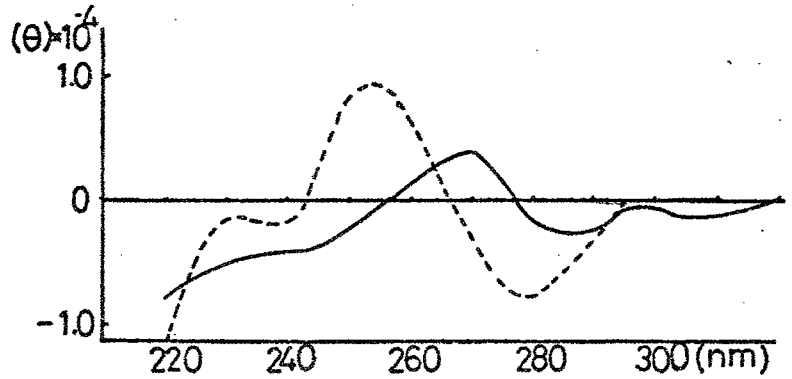


Fig 19 CD spectra of poly(Iz) and poly(I) at 0.95M Na⁺
——poly(Iz) - - - - poly(I)

に負、270nm 付近に正の cotton 効果があらわれ、poly(Iz) 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いて3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 付近に強い負の CD バンドが存在するのが特徴である。[19] 高塩濃度における poly(Iz) はこれに比べスペクトルパターンは似ているが、正負の cotton 効果が長波長側に shift して、[θ] 値もたいへんちいさい。このことから、poly(Iz) は低イオン濃度では

ordered structure をとらず、高塩濃度では類似の ordered structure をとり得るが、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 をとり難いことが判った。

・self-ordered structure の熱的安定性

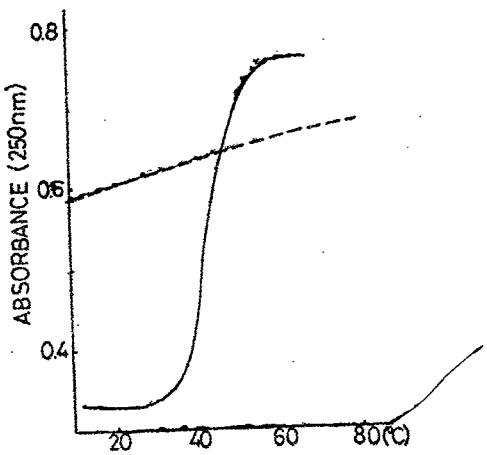


Fig 20 Tm of poly(Iz)
——0.95M Na^+
-----0.15M Na^+

Fig 20 は、Naイオン濃度が 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°~~ に Tm が観測される。同じ条件で poly(I) の Tm 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(Uz) 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 Tm 値を有していると報告されている[1]。しかし、poly(Iz) ではこのような安定化はみられなかった。

・poly(C)との complex 形成

中性条件で poly(I) は poly(C) と 1:1 complex を形成することが知られている。[20] そこで、0.15M Naイオン存在下、pH7.0 で poly(Iz) と poly(C) の mixing を行うと Fig 20 のようになる。

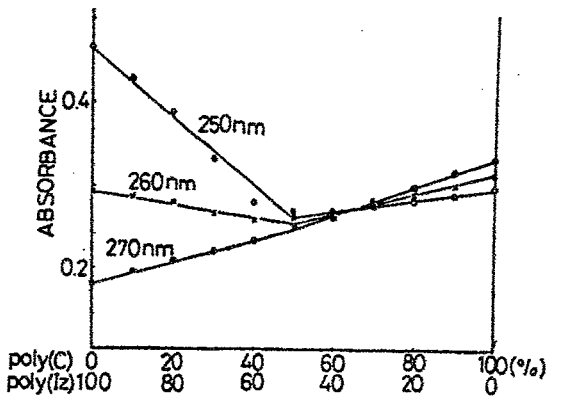


Fig 20 Mixing experiment of poly(Iz) and poly(C)

poly(Iz) 50% のところに屈曲点があらわれ poly(Iz)・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 complex 形成を示唆している。この complx

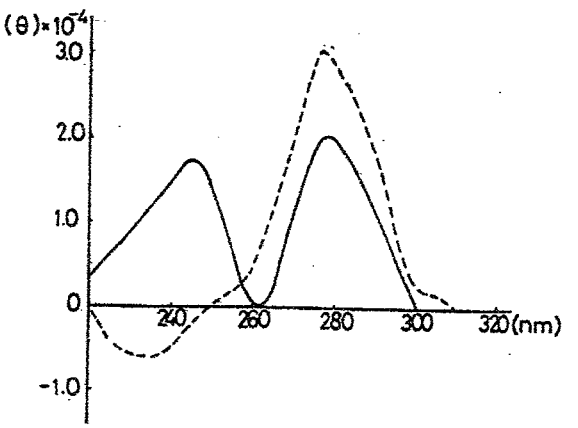


Fig 21 CD spectra of poly(Iz) and poly(C) (1:1) before and after mixing ----- before mixing, ——— after mixing

のCDスペクトルは poly(I)·poly(C) complex のそれによく似ている。

・poly (I₂)· poly(C) complexの熱的安定性

poly(I₂)·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Tm値を求めこれを $\log_{10}[Na^+]$ 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。poly(I)·poly(C) complex及びpoly(dI)·poly(C)

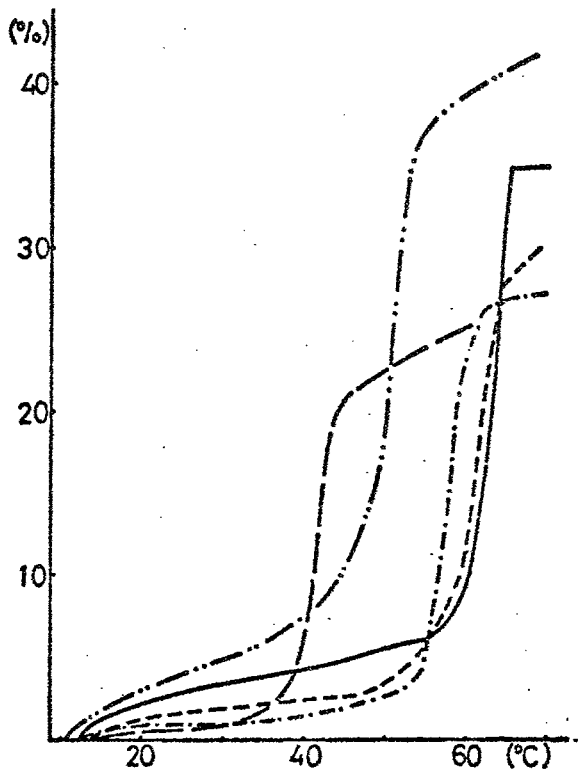


Fig 22 Temperature absorbance profile of poly(I₂)poly(C) complexes at various Na⁺ concentration
——0.04M, -·-·-0.15M, -·-·- 0.35M, - - - 0.55M, —— 0.95M,

complexにおいても同様の直線関係が成立し[21]、その傾きはほぼpoly(Iz)·poly(C) complexと同じであるので、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、poly(Iz)·poly(C) complexはpoly(I)·poly(C) complexより低いTm値を有しているが、poly(dI)·poly(C) complexよりは安定である。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、又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

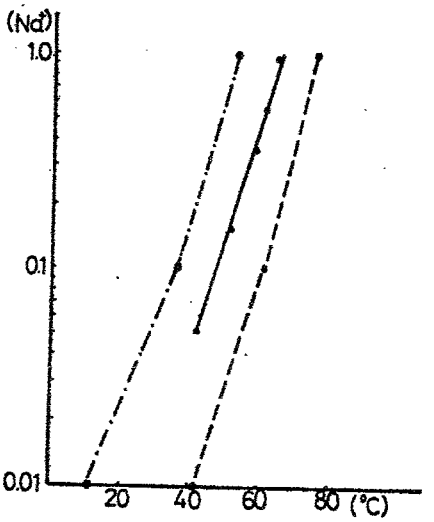


Fig23 Relationship of Na^+ concentration and Tm of poly(Iz)·poly(C) (—), Poly(I)·poly(C) (----) and poly(dI)·poly (C) (— - — - —).

2′位置換基の変化による影響をう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[58]

アミノ基は電気陰性度は2.9で水酸基と大きく異っているが分子量はよく似ている。又pKa値は水酸基より大きく、より容易にプロトネートすると考えられる。

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poly(Ua)[2], poly(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を接触還元してAaDPがえられた。これを用いてpoly(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で接触還元して合成した。反応完結はpH3.5の電気泳動でAzDPよりおくれる位置に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)で行い、80%の収率でAaDPを得た。そのクロマトグラフの性質をTable 3に示す。重合反応はAaDP 4 mM $MnCl_2$ 2 mM, Tris HCl (pH 8.5) 80 mM, PNPase (M. luteus) 5 units/mlを用いて37°Cで行った。時間をおって無機

Table 3

	PEP ($R_{m\,PA\text{-}A}$)		PPC (R_f)		
	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Fig24である。

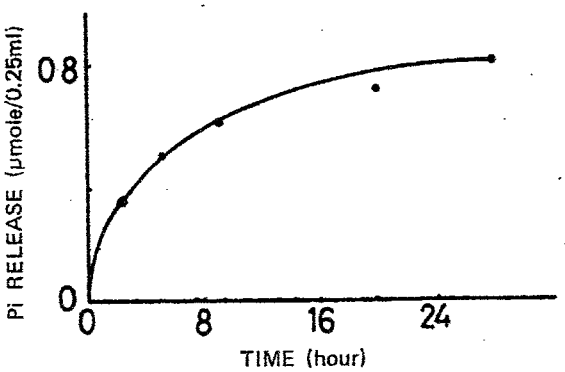


Fig 24 Time course of AaDP polymerization.

27時間後にisoamylalcohol:$CHCl_3$=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 Tris HCl (pH7.0)で透析し、更に水に対して透析した。分離精製はSephadex G-50ゲルロ過を行った。

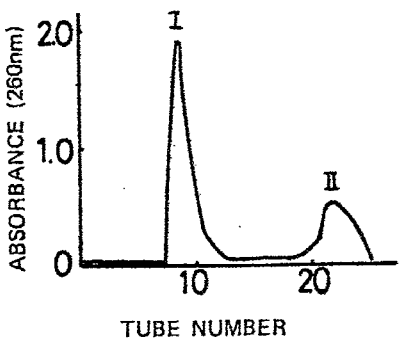


Fig 25 Sephadex G-50 column chromatography of poly (Aa).
peak I poly (Aa)
peak II AaOP

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

第2節 ポリ(2'-アミノ-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UVスペクトルはpH7.0で Fig 26のようにである. λmax 258 nm で無機リンの定量より

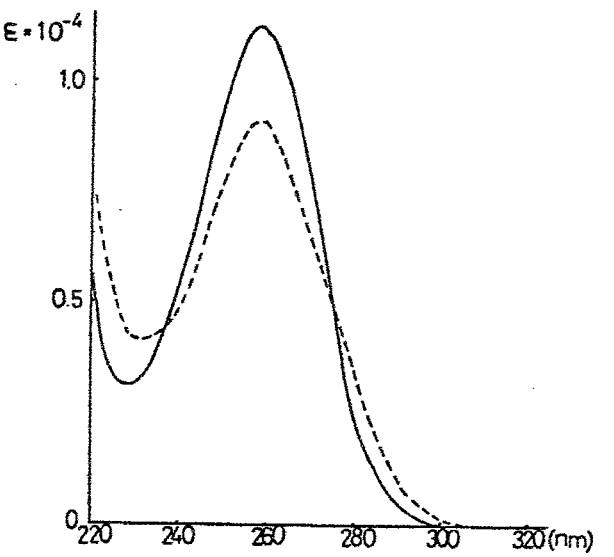


Fig 26. UV absorption spectra of poly(Aa),— at pH 7.0, --- at pH 6.3.

求めた分子吸光係数は 11200 であった. Aa が λmax 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) がこの条件では 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λmax は変化しないがε(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。λmaxの変化がないことより、N-1 位に protonate はしておらず、いわゆる"acid form"をとっていないと思われる。

・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λmax 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 が起り易い部位としては 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。

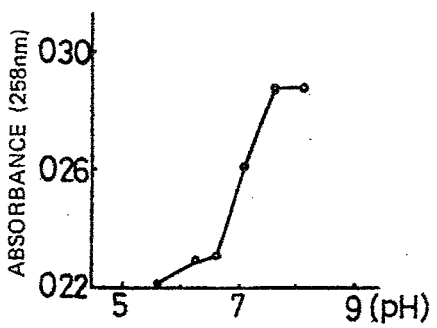


Fig 27 Acid titration of poly(Aa).

° CDスペクトル

0.15M Na1オン存在下24°Cでのpoly(Aa)のCDスペクトルをFig28に示した。pH7.0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な構造はとっていないことがわかる。これはUVスペクトルのλmax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pH5.7あるいはpH6.3ではCDスペクトルは大きく変化し、274, 225nmに大きいtrough, 252nm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でordered structureを形成しているのが判る。このCDスペクトルはpoly(A)の

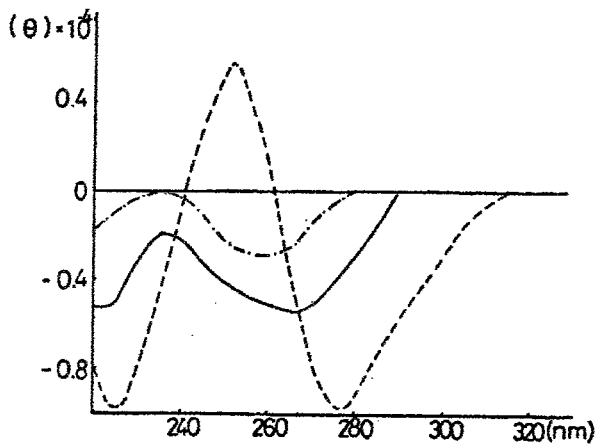


Fig28 CD spectra of AaDP and poly(Aa), ——poly(Aa) at pH 7.0. ---poly(Aa) at pH 5.7, -·-·- AaDP at pH 7.0.

acid form の CD スペクトルとは全く異っているので、この型の ordered structure でないと思われる。この ordered structure は温度を上げるとくずれて、その monomer に似た CD スペクトルに戻る。pH 6.3 の場合が Fig29 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。こういったことはピリミジンヌクレオチドでは観察されていない。

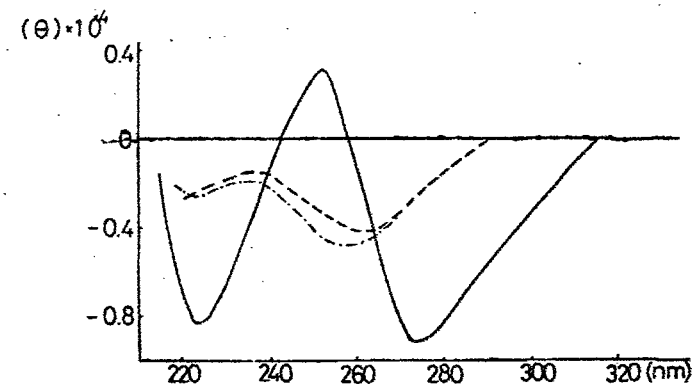


Fig29 CD spectra of poly(Aa) taken at 20°(—), 46°(---) and 66°(-·-·-).

• 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 Tm 値は

各々 22℃, 38℃, 54℃ であった. pH が下がるにつれて Tm値が上昇する傾向がある. Tm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. pH6.3, pH7.0 のいずれの場合にも, Tm値と $\log_{10}[Na^+]$ との間に直線

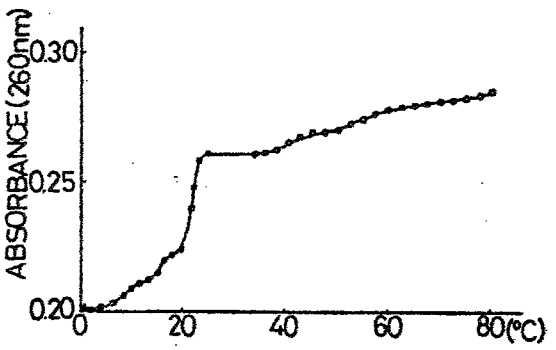


Fig 30(a) Temperature-absorption profile of poly(Aa) taken at 7.0.

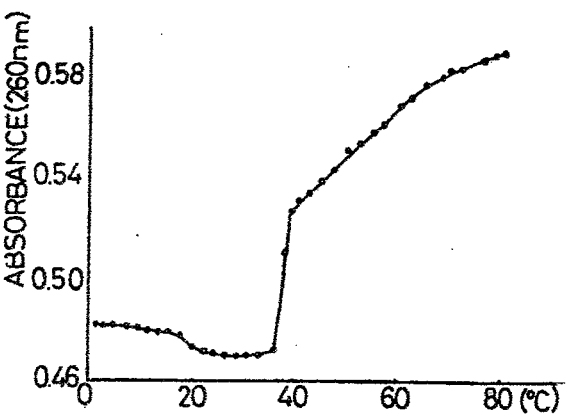


Fig 30(b) Temperature-absorption profile of poly(Aa) taken at pH 6.3.

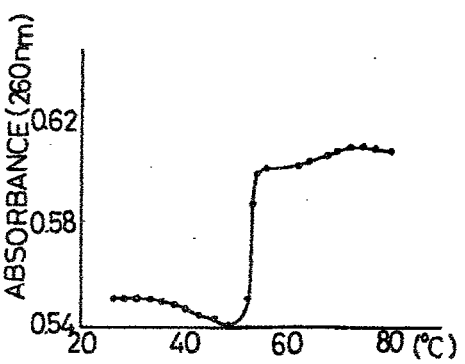


Fig 30(c) Temperature-absorption profile of of poly(Aa) taken at pH 5.7.

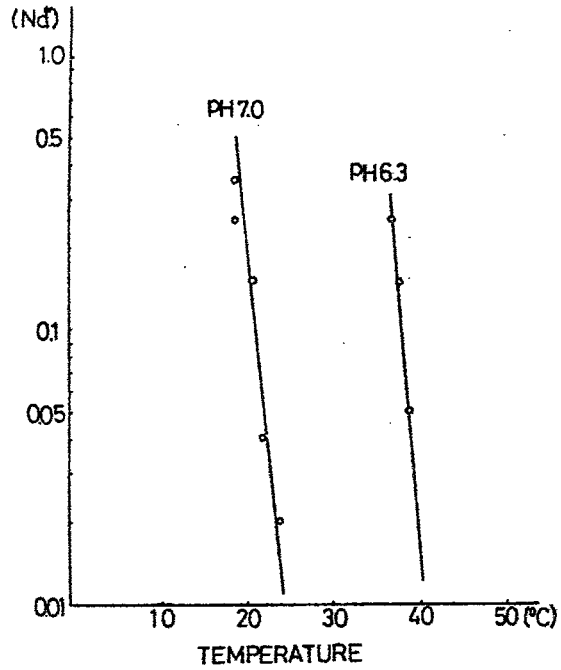


Fig 31 Depending of Tm of poly(Aa) on sodium ion concentration.

関係が成立する。そして塩濃度の上昇に伴ってTm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する。[23] 他の水素結合を含むcomplexでは塩濃度の上昇に従ってTm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Tm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位アミノ基のprotonateにより stackingが強化された

と考えられる.

・poly(U)とのcomplex形成とその動的安定性

0.15 M Na イオン存在下で, poly(Aa)とpoly(U)をmixingするとFig 32のように, poly(Aa) 33%のところに屈曲点があらわれ, poly(Aa)·poly(U)=1:2の

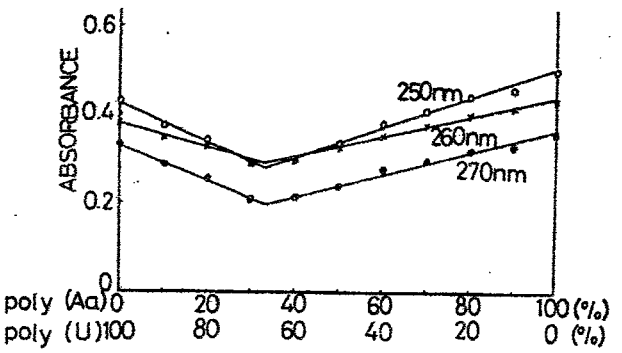


Fig 32 Mixing experiments of poly(Aa) with poly(U).

3本鎖complexの形成が観察された。これはFig 33

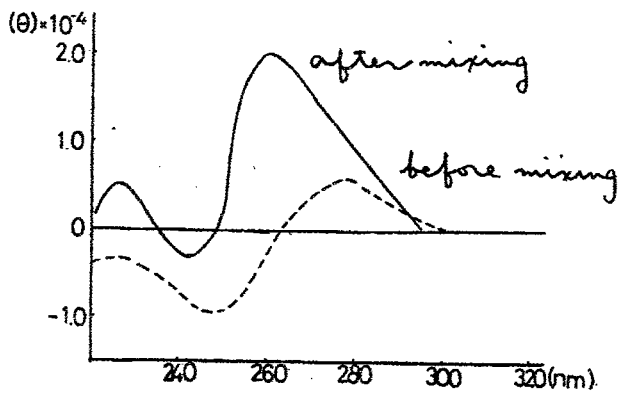


Fig 33 CD spectrum of poly(Aa)·2poly(U) complex.

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)とは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Tm値は非常に低いものである。poly(Aa)においても0.04Mイオン存在下における mixing 実験では、明らかな屈曲点があらわれず、この条件でcomplex形成が完結していないことがわかる。0.15M 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℃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Tm値より8℃低くなっている。

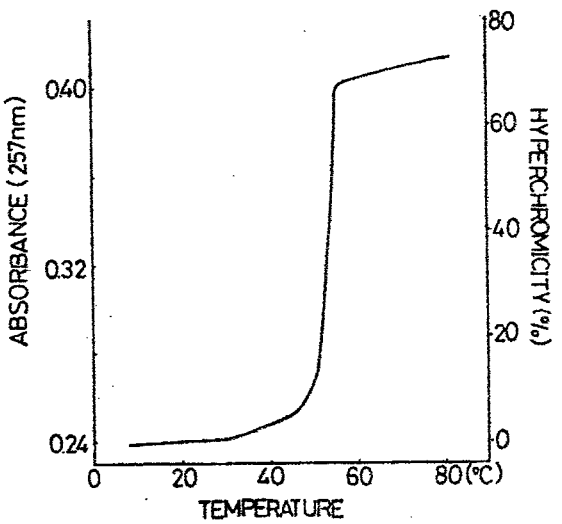


Fig 34 Temperature-absorption profile of poly(Aa)·2poly(U) complex.

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 0.10M Na イオン存在下 (pH 7.5) で 25°C と低い Tm 値を有する不安定なものである。このように 2' 位アミノ基の存在は complex 形成に不利であることが判った。

第三章 ポリ(2'-ハロゲノ-2'-デオキシプリンヌクレオチド)の合成と性質[60) 61) 63)]

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って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が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なり、電気陰性度がより水酸基に近いアジド基を有するpoly(Az)は、置換基のsizeの違いにもかかわらず、poly(A)に似ていることが判った。又、poly(I)アナログでは、その塩基のもつ特異性によってpoly(A)アナログより影響をう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(Å)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って、2'位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、沖原等により2'-ハロゲノ-2'-デオキシアデノシンの合成法が開発され[23)]、これらを5'-diphosphateに導き、PNPaseで重合することによって対応するポリヌクレオチドが得られる。

第1節 ポリ(2'-ハロゲノ-2'-デオキシアデニル酸)の合成

2'-ハロゲノ-2'-デオキシアデノシン 5'-モノホスフェート(AXMP)はすべて1章で述べたと同様に、小崎らの方法[5]に従って triethyl phosphate中 POCl3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。これらはすべて crude snake venom 5'-nucleotidase によって脱リン酸化され、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。

次にこれらを Moffat-Khorana の方法によって 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 Table4 のように示す。

次にこれらの重合反応を行った。基質 4mM, $MgCl_2$ 2mM, TrisHCl (pH8.5) 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 によるゲル濾で polynucleotide と

Table 4

	PEP(R.coli A)		PPC		
	pH7.5	pH8.5	C↓	G↓	M↓
AfMP	1.04	1.00	0.13	0.23	049
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 は poly(Abr) の gel filtration の溶出パターンを示した。えられた polynucleotide の収率は poly(Af) 55%, poly(Ace) 25%, poly(Abr) 13% であった。

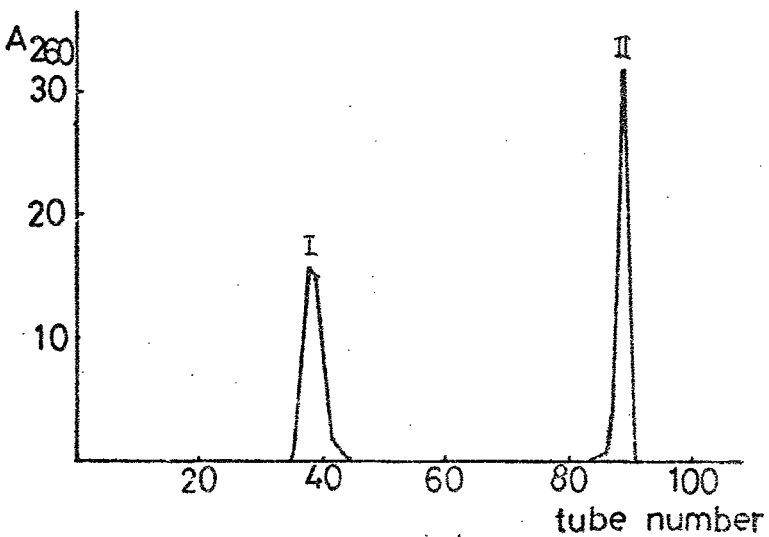


Fig.35 Sephadex G-50 gel filtration of poly(Abr).
peakI:poly(Abr), peakII:AbrDP.

前述のようにPNPaseは、2′-デオキシ体を重合反応の基質とはしない。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うと、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(Acl), 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

0.15M Naイオン存在下25°で poly(At) はFig36のようなUVスペクトルを示した。pH7.0ではλ_{max} 255nm, pH4.5で253nmであった。poly(Acl)とpoly(Abr)はたいへんよく似ており、そのうちpoly(Acl)をFig37にあした。poly(Acl), poly(Abr)ともpH7.0でλ_{max} 257nm, pH4.5でλ_{max} 255nmであった。この三者のpH7.0におけるリンの

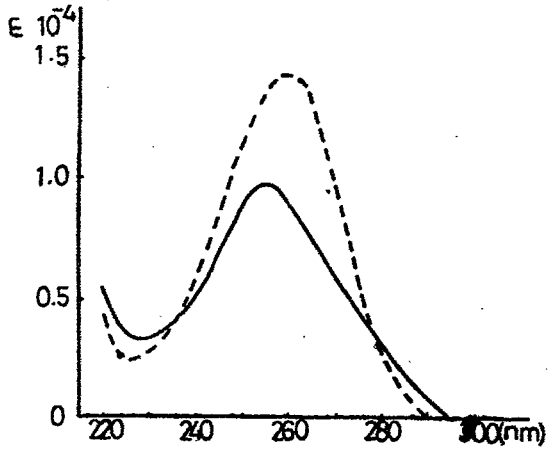


Fig 36 U. V. absorption spectra of poly(Af) and Af 5'-MP.
Poly(Af) ———, AfMP ----.

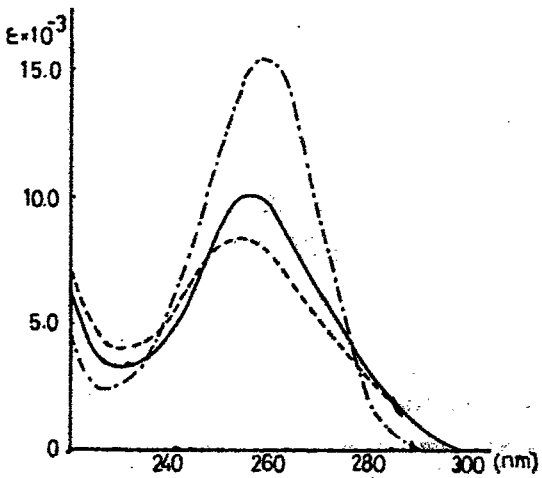


Fig. 37 UV absorption spectra of poly (AcI). ———at pH 7.0.
.....at pH 4.5, -.-.- AcIMP.

定量により求めた分子吸光係数εとhypochromicityを
Table 5 に示した。このようにhypochromicityの大きさ
は poly(I) > poly(Af) ≧ poly(Acl) > poly(Abr) となる。
hypochromicity は stacking の程度を反映しており、

Table 5

	ε(P)x10^{-4} at λmax		hypochromicity(%)
	(a)polymer	(b)monomer	[1-(a)/(b)]x100
A	1.00	1.54	35
Af	0.97	1.43	32
Acl	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, Acl 5 Hz, Abr 7.5 Hz である。[25] Altonaらは S⇌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のパーセンテージは $10 \times J_{H1'-H2'}$ で与えられると報告している。[26] これを2'-ハロゲノアデノシンの場合にあてはめれば、Af<Acl<Abrの順でS conformerの比率が大きくなる。すなわち2'-endo型をとりうる確率が大きくなる。2'-endo型であると、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。

Alderfer[27]らは、2'-O-アルキル体のシリーズで poly(dA) > poly(A) > poly(Am) > poly(Ae) と hypochromicity

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°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 poly (Af) > poly (Ace) > poly (Abr) の順で減少している。その値はTable 6に示す。hypochromicityの減少と分子楕円率 [θ] の減少がよく対応しているので、[θ] 値の減少はstackingの減少によると考えられる。

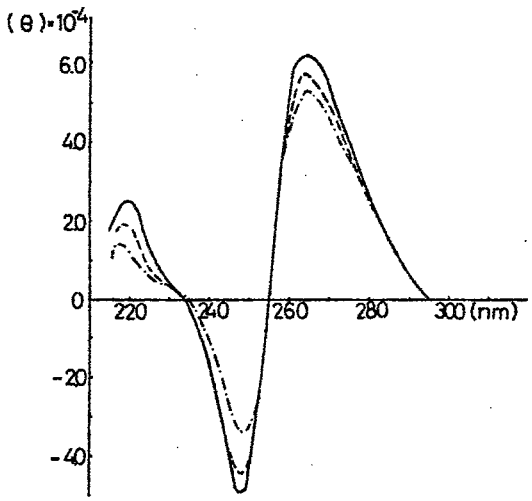
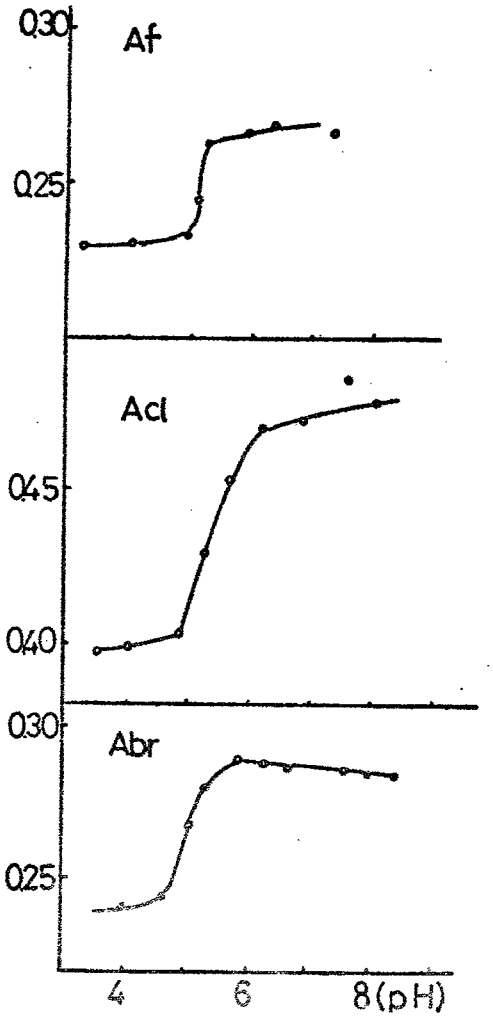


Fig 38 CD spectra of poly(Af), poly(Acl) and poly(Abr).
Poly(Af) ——, poly(Acl) ----, poly(Abr)-.-.-.

Table 6

polynucleotide	$[\theta]\times10^{-4}$		
	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 transaction 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。

0.15M NaCl 存在下で、25℃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 Tm 値を Table 7 にまとめた。

Fig 39
Acid titration curves of poly(Ax) in the presence 0.15M NaCl at 25°C

Table 7

polynucleotide	p H t1/2	Tm at pH4.5
poly(A)	5.5	60
poly(Af)	5.2	37
poly(Acl)	5.5	63
poly(Abr)	5.0	56

stackingの強いpolynucleoticleは塩基部にprotonateしにくいことが知られており、poly(Af)のpH t½、及びpH4.5のTm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、これらニつの値が小さくなっているものと考えられる。又、poly(Acl)はpoly(A)によく似ている。

- poly(U)とのcomplex形成とその安定性

0.15M Naイオン存在下、pH7.0でpoly(Af), poly(Abr) poly(Abr)とpoly(U)とmixingすると Fig40のようになる。いずれのpoly(Ax)もpoly(U)とに2の3本鎖complexを形成したことがわかる。

又、0.04M Naイオン存在下、pH7.0でmixingを行い、30分後の測定ではFig41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

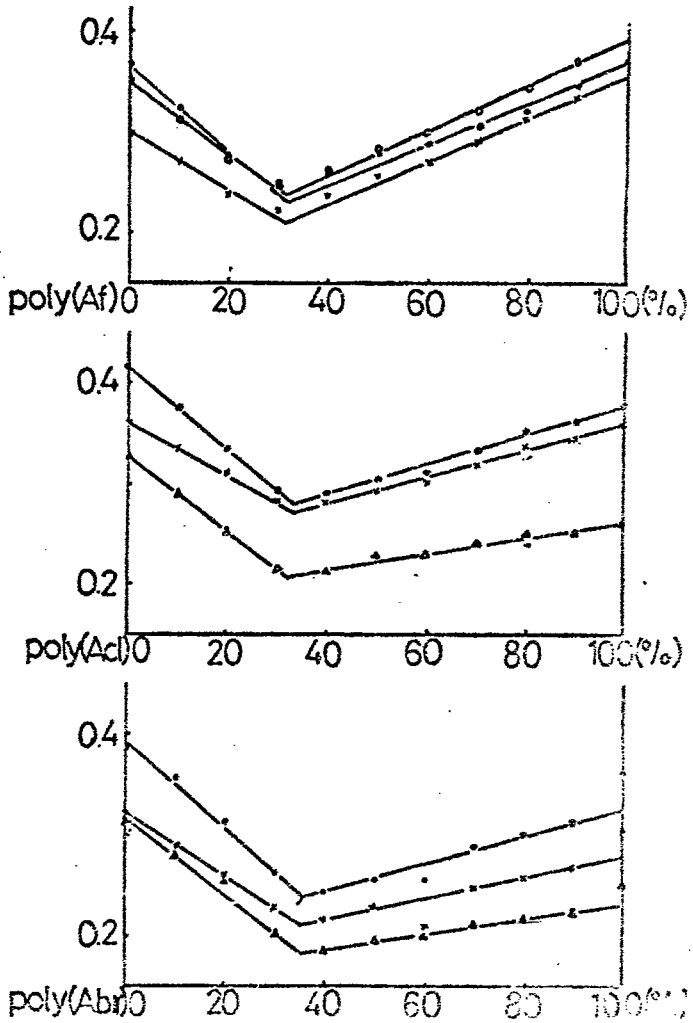


Fig 40
Mixing curves of poly(Ax) with poly(U) in the presence 0.15M Na^+

250nm
255nm
260nm
270nm

ものとなり、poly (Af)-poly (U) の系では1週間後に完全に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 (Af)-poly (U) 1:2 complex 形成における、CDスペクトルの変化である。

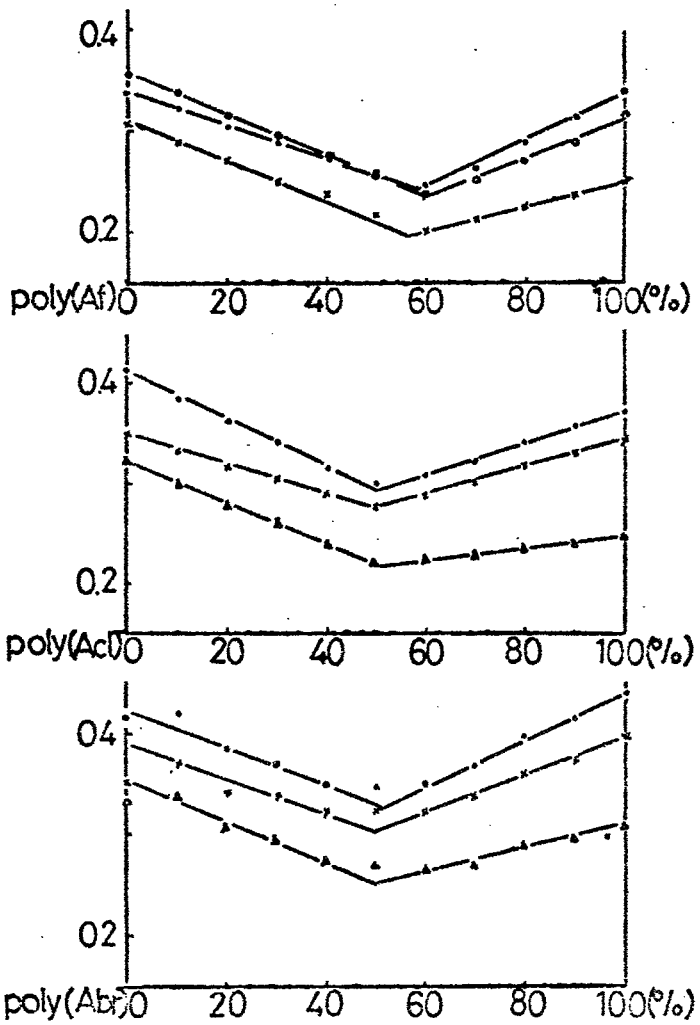


Fig41
Mixing curves of poly(Ax) with poly(U) inthe presence 0.04M Na^+

250nm
255nm
260nm
270nm

Fig 42 CD spectra of poly(Af) and poly(U) (1:2)

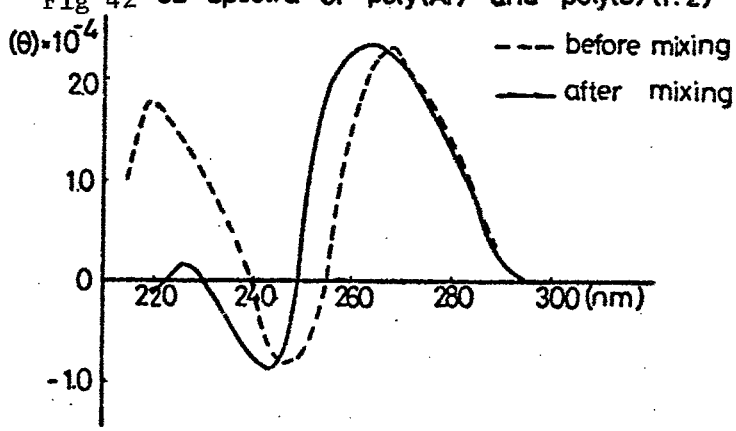


Table 8

complex	Tm 0.04M [Na^+] 30mim.	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 Tm 値は Table 8 のようになる。いずれの塩濃度においても complex の熱的安定性は poly(Af)·poly(U) > poly(Acl)·poly(U) > poly(Abr)·poly(U) であった。2'置換基の大きいほど Tm 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 で poly (Af), poly (Ac),

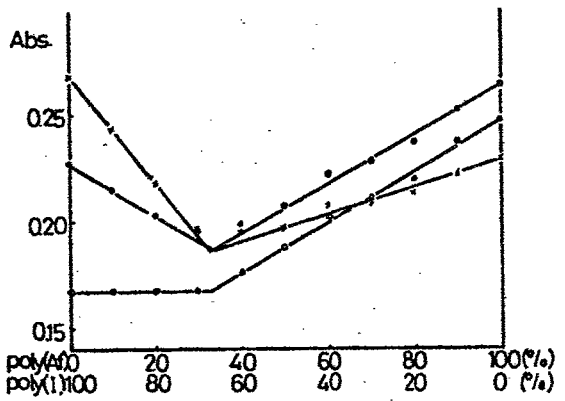


Fig43 Mixing curves of poly(Af) with poly(I) in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0) at 25°. At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

poly(Abr)とpoly(I)とmixingすると、いずれもpoly(I) 67%のところで屈曲したmixing curveがえられ、poly(Ax)·poly(I) 1:2の3本鎖complexの形成が観察された。このことは、poly(Ax)·poly(I)を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、poly(Af)の場合をあげたのがFig43, Fig44である。これらcomplexのTm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10mMのMgCl₂をかえるとわずかだが差を生じ、poly(Af)·poly(I)>poly(Acl)·poly(I)>poly(Abr)·poly(I)る。poly(Ax)·poly(U)でえられたと同様、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poly(Ax)はself-structureも、poly(U)あるいはpoly(I)とのcomplex形成もpoly(A)と似た性質であるが、F, Cl, Brの順でstackingの程度が減少し、

Fig44 CD spectra of poly(Af) and poly(I) (1:2)

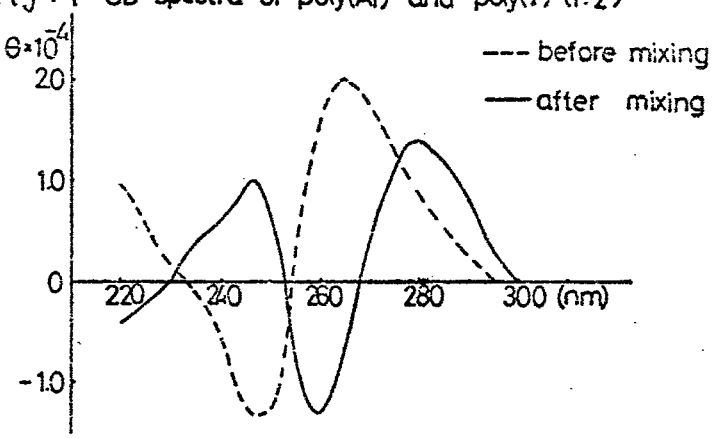


Table 9

complex	Tm(°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'-ハロゲノ-2'-デオキシイノシン酸)の合成

2'-ハロゲノ-2'-デオキシイノシン-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を酢酸々性中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の方法によって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をえた。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, $MgCl_2$ 2mM, TrisHCl (pH8.5) 100mMの条件で、poly(It)はPNPase(E. coli) 4.5 units/ml, poly(Ica)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
IclMP	1.10	2.70	0.12	0.31	0.45
IclDP	1.40		0.08	0.21	0.45

ゲルロ過で void volume 付近に溶出される。収率は poly(If) 12%, poly(Icl) 16% であった。poly(At), poly(Acl) が PNPase 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(Icl) のゲルロ過の溶出パターンを示した。

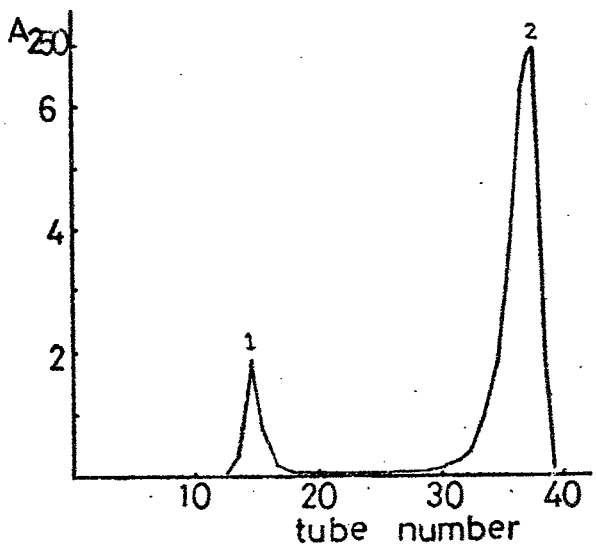


Fig.45 Sephadex G-50 gel filtration of poly(Icl)
peak1:poly(Icl),peak2:IclDP

第4節 ポリ(2'-ハロゲノ-2'-デオキシイノシン酸)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Fig46のようになる。20°Cにおいては、poly(I)のUVスペクトルに似ているが、275nm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Tm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。IfMPよりのhypochromicityは20°Cにおいて20%、40°Cで12%であった。これは、0.75M Naイオン存在下では、30°Cにおいても、又Tm値である47°C以上例えば52°Cにおいても、275nm付近にふくらみは見られない。

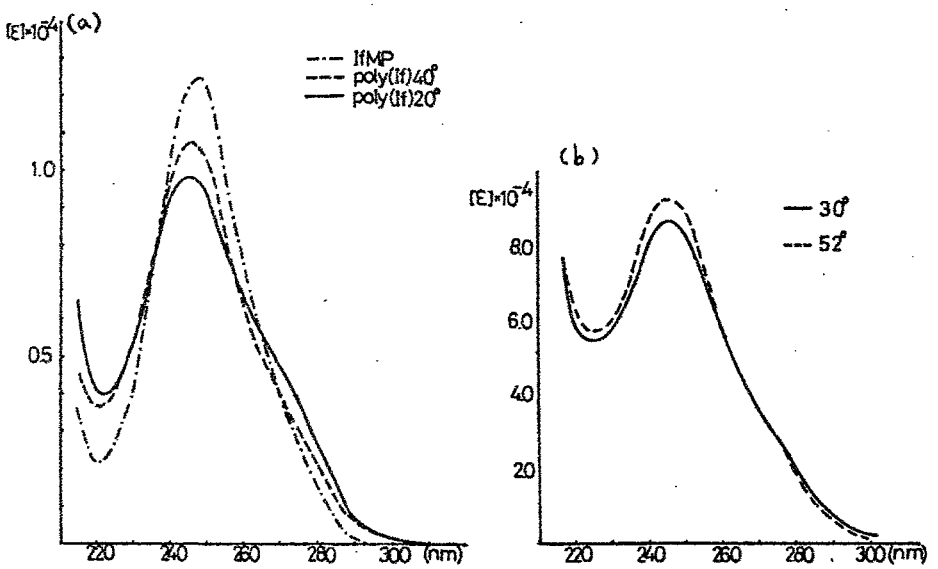


Fig.46(a) UV spectrum of poly(If) and If MP at pH 7.0 in the presence of 0.15M Na^+ion.
Fig.46(b) UV spectrum of poly(If) in the presence of 0.75M Na^+.

従って、poly(Ic)は低イオン濃度と高イオン濃度ではとりうるordered structureが違っていることが推定される。

一方poly(Icl)のUVスペクトルはFig 47のようになる。これらはたいへんpoly(I)に似ている。0.15M Naイオン濃度ではIclMPよりhypochromicityは20%である。0.95M Naイオン濃度においては、よりえきなhypochromicityが観察され、長波長側にbackgroundが高くなっている。これはaggregationによるものと思われる。

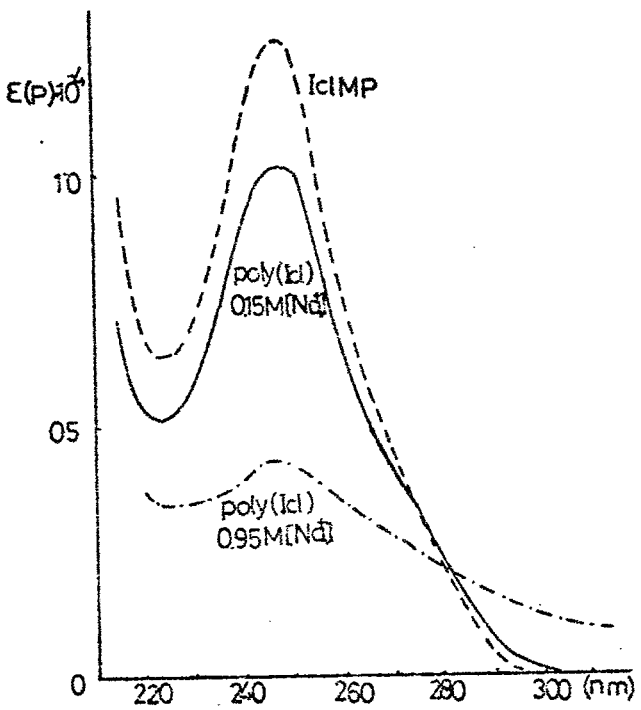


Fig 47 UV spectra of poly(Icl) and IclMP at pH7.0

・self-ordered structure の熱的安定性

poly(If) の 0.15M 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、0.15M 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ったものである。

Na イオン濃度を変化させて、Tm 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 $\log_{10}[Na^+]$ の変化に対して Tm 値の変化が大きくなっている。これより低塩濃度に

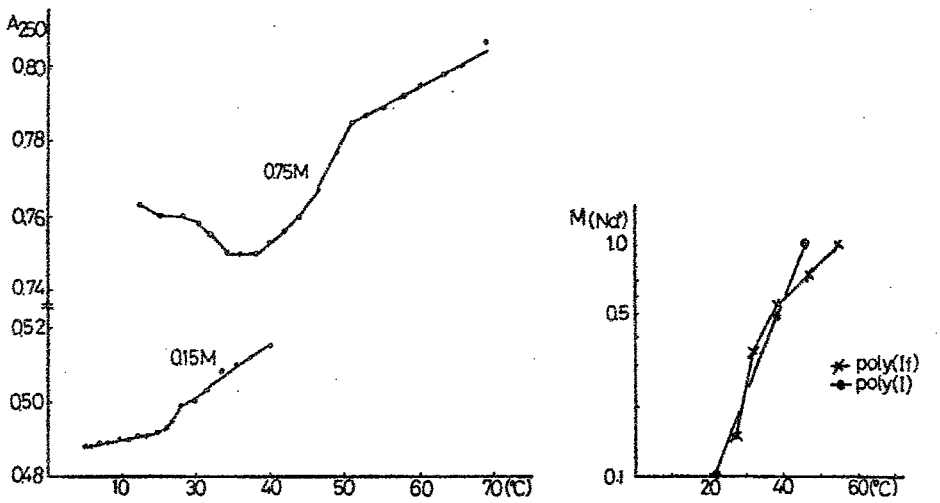
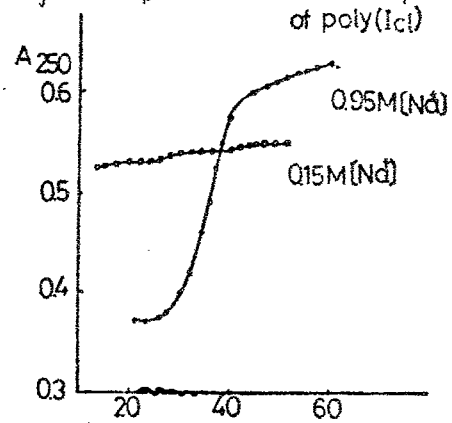


Fig 48(a) Thermal melting profiles of poly(If) at Na^+ concentration of 0.15M and 0.75M.
Fig 48(b) Relationship of Tm of poly(If) and poly(I) to Na^+ concentration.

おけるordered structureはあまり塩濃度の変化によって安定性に変化はうけないが、高塩濃度におけるordered structureでは大きく安定化をうけている。

一方、poly(Icl)の0.15M Na^+イオン濃度, 0.95M Naイオン濃度でのthermal melting curveをFig 49にしめす。0.15Mでは顕著なtransitionがみられない。0.95Mでは Tmが36°Cとなる。

Fig49 Temperature-absorbance profile of poly(Icl)



・CDスペクトル

0.15 M Naイオン濃度, pH7.0でpoly(It)のCDスペクトルを測定したものがFig 50である。Tm値より低温では長波長側に正, 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(It)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

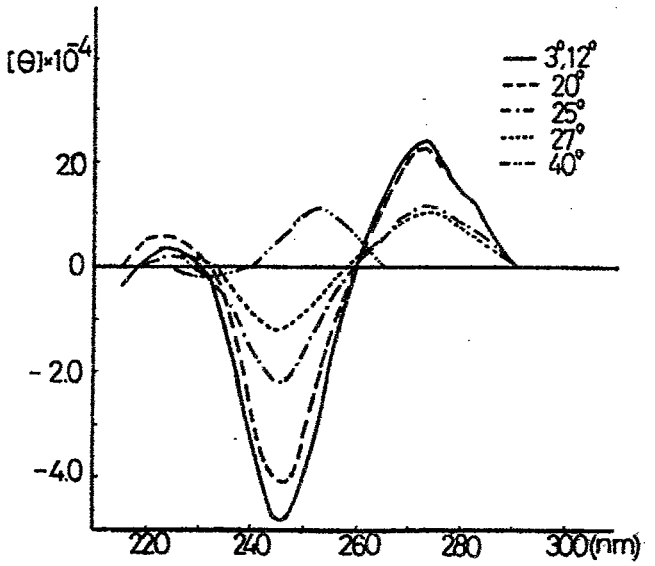


Fig50 CD spectra of poly(If) at 0.15M Na^+ concentration.

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、Tm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。

より高い塩濃度においては、275nm付近に負のcotton効果がみられる。Fig51は0.75M 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。Tm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(If) at 0.75M(Na⁺)

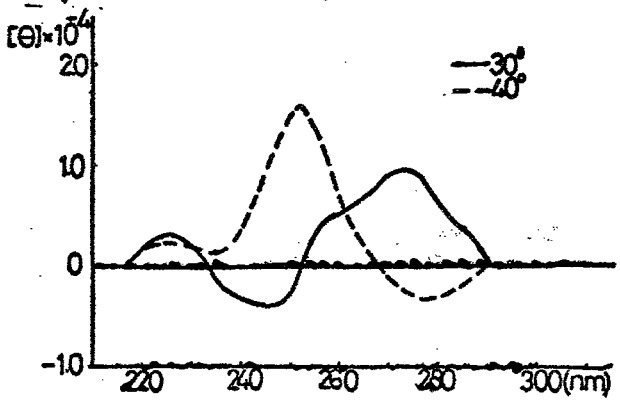
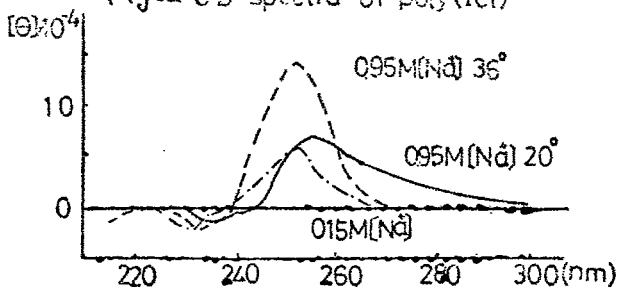


Fig 52 CD spectra of poly(Icl)



一方 poly(Icl)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(Icl)はpoly(If)にみられるようなCDスペクトルは観察されず、random coil状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(If), 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。濃度を

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(Ica) は、poly(I)、poly(It) にみられる高塩濃度における4本鎖の形成、あるいは poly(It) にみられる低塩濃度における self ordered structure の形成いずれも観測できなかった。従って、poly(Ica) は self complex を形成し難いことがわかった。これは2'位クロルの存在による self structure の不安定化によると考えられる。poly(Ax) 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

0.15 M Naイオン濃度, pH 7.0 で poly(It) と poly(C) を mixing すると Fig 53 のように poly(It) 50% のところに屈曲点が生じ、この条件下、poly(It) 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、poly(I)·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(Ica) を同様に poly(C) と mixing すると、

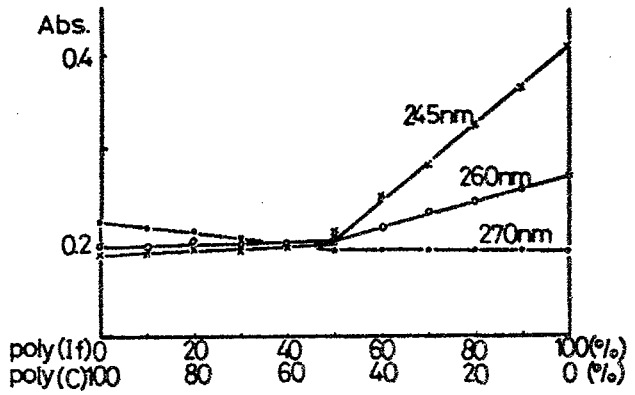


Fig 53 Mixing curves of poly(If) with poly(C)

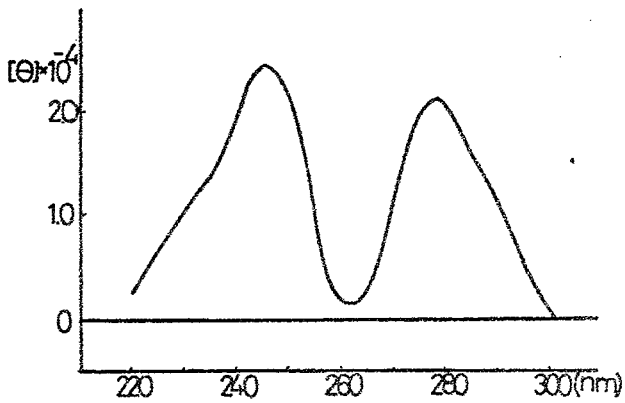


Fig. 54 CD spectrum of poly(If)·poly(C) complex.

Fig 55 のように poly (Ica)・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。

Fig 55 Mixing curves of poly(Icl) with poly(C)

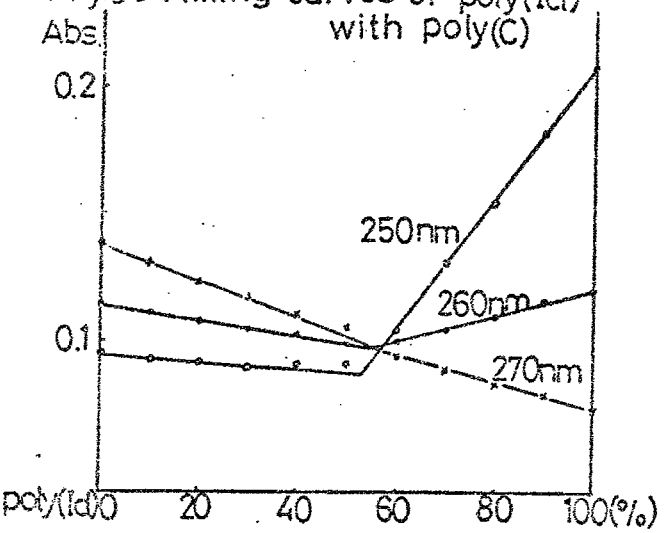
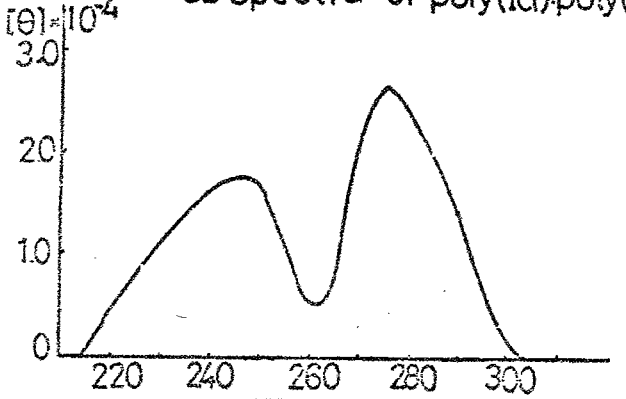
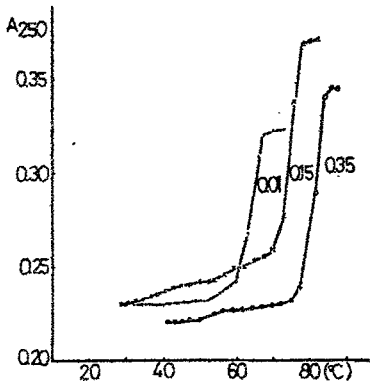


Fig 56 CD spectra of poly(Icl)·poly(C)



得られたTm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Tm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'-フロロ体では塩基により、その安定化の傾向が異っている。poly(Uf)の場合[28]、poly(Uf)·poly(A)



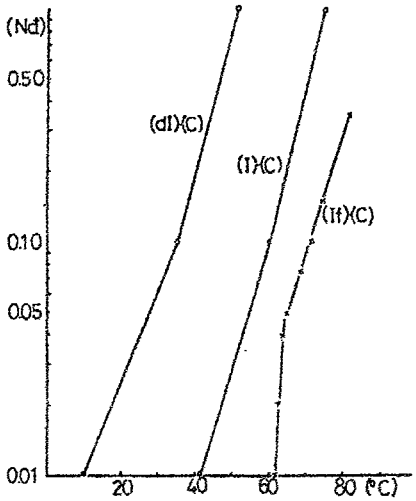


Fig. 57 Temperature absorbance profiles of poly(If)·poly(C) at various Na^+ concentrations.
Fig. 58 Relationship between Tm of various double-stranded complexes and Na^+ concentration.

complex は poly(A)·poly(U) complex より、各塩濃度で常に15～20°C高いTm値を有し、かなり安定にとうけている。しかし poly(Cf)[29] では poly(Cf)·poly(I) は poly(I)·poly(C) complex より若干低いTm値である。又、前述の poly(Af)·poly(U) complex では、poly(A)·poly(U) complex とほぼ同じTm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合わせると、2'-フロロ体が塩基部にアミノ基を有する component に含まれるとき影響はないが、塩基部に水素結合における proton acceptor の C=O を有する component に含まれるとき、complex の著しい安定化がおこる。

一方、poly(Ice)·poly(C) の complex の熱的安定性

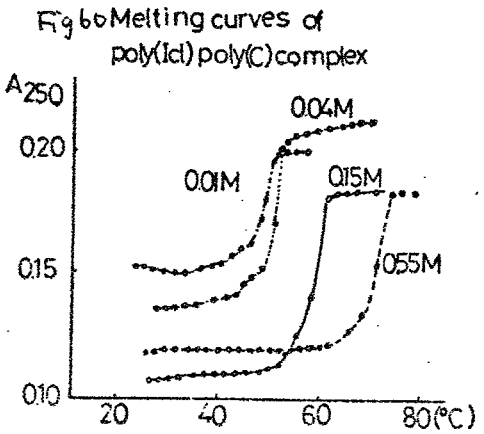
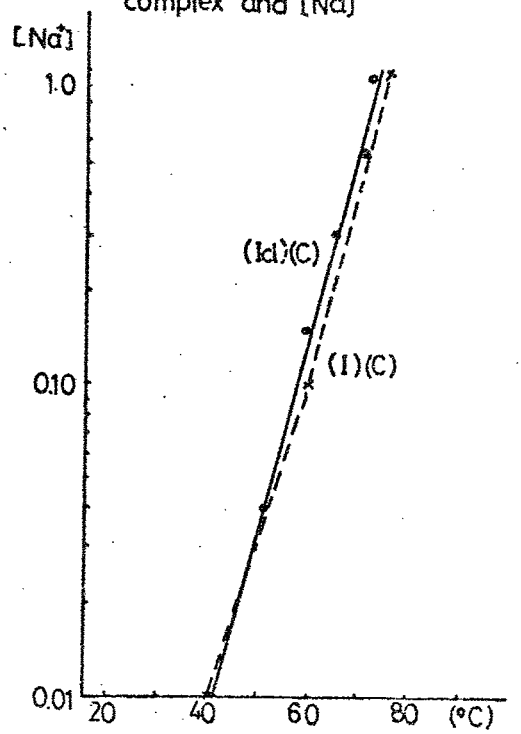


Fig 61 Relationship between Tm of (Icl)(C) complex and [Na+]



について調べた。種々のNaイオン濃度における poly(Ica)・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 Tm 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(Ica)・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。ピリミジン系においては[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 Tm 値は、対応する poly(A)・poly(U) および poly(C)・poly(I) より若干高いと報告されている。このように、2位に

Clと置換しても、complex形成能やその安定性には大きな差はみられない。又、poly(It)のそれと比較するとpoly(Icl)ゞは、complexの安定性が低下している。poly(Ax)の場合と同様に、poly(Ix)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）の刺激をうけて産生する分子量 2～3万程度の糖蛋白質[31)32)]で、細胞に作用して抗ウィルス作用を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、生体防御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ィ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ィルス核酸であろうと考えられてきた[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がインターフェロンを誘導することが判った[35)]。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)の臨床への応用にはその毒性、トレ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ナログについて、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して[36][37]

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) 10bp以上の分子量を必要とする。[39]

iii) Tm値，そしてRNaseに対する抵抗性が高い。

iv) 塩基部の窒素を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含むcomplexについての報告にもとづいている。すなわち、poly(A)·poly(U)型complexについては、A·Uf[41]，A·Uc[42]，A·Us[43]，A·Um[44]，A·Ue[45]，Ae·U[45]，Aac·U[46]である。又、poly(I)·poly(C)系では、I·dC[47][48]，I·Cc[42]，I·Cm[44]，dI·C[49]，Im·C[44]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、前章までに合成したpoly(Az)，poly(Is)，poly(At)，poly(It)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 1c

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

System	polynucleotide	interferon titer** 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-(U)n*			2.9
	(A)n-(rT)n			3.4
	(A)n-(Uz)n			≤1.0
	(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)			3.9
	(Iz)n-(C)n			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

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

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

2'-アジドアナログについて示した。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 とほとんど同じかそれ以上の活性を示している。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) では 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 μg/ml.	1 μg/ml.	10 μg/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)$_5$	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)$_5$	≤ 1.0	≤ 1.0	1.1
poly(Af)-poly(br^{5}U)			≤ 1.0
2. Human skin fibloblast 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)$_5$	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)$_5$	≤ 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 (At), poly (It) について調べた結果が Table 11 である。poly (At) を含む 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) を含む complex より低い値を示している。一方 poly (It)·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 の系で著しい。

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 (It)·poly (C) complex, poly (Ic)·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 腫瘍ウィルスの gene を合成するリバーストランスクリプターゼは Baltimore[50], Temin[51] らによって発見された。その機能は名のように RNA を template として oligonucleotide を primer に deoxyTriphosphate を付加重合することである。

これまで多くのポリヌクレオチドアナログの、この酵素に対する

影響が調べられている。そのうち poly(Am)[52], poly(Ae)[52] poly(u²A)[53], poly(C³A)[54], poly(C⁷A)[55] がこの酵素の polymerase 活性を阻害することが報告されている。

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 62 は template の poly(A), primer の oligo(dT)12-18

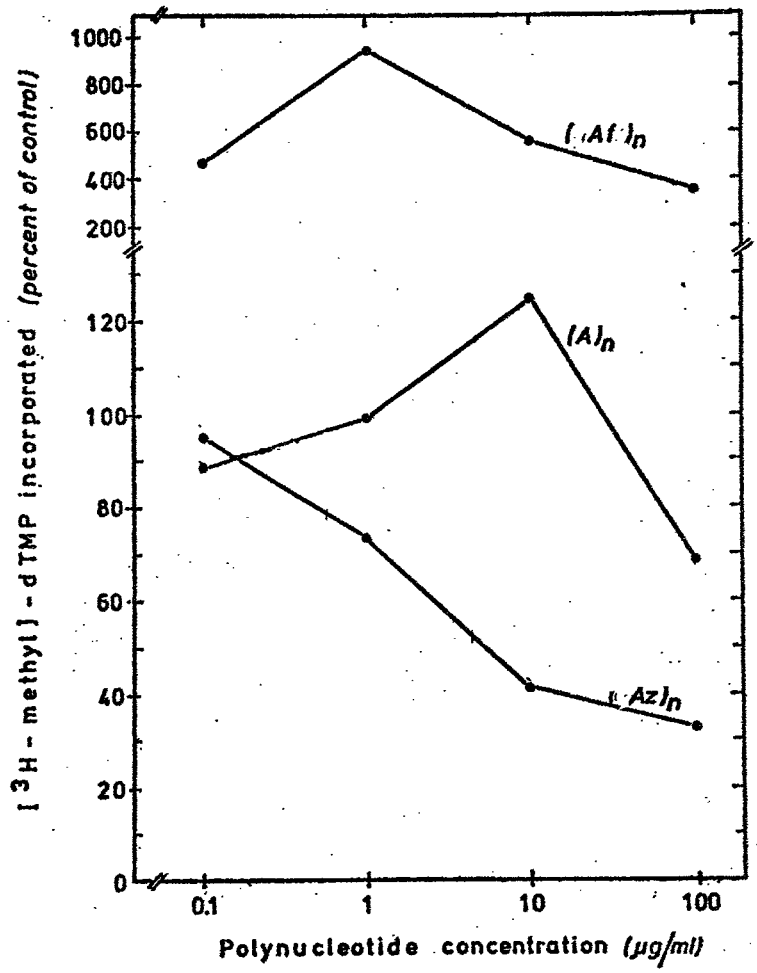


Fig 62 Effect of poly(Az) and poly(Af) on Mulv reverse-transcriptase in the absence of polu(A),oligo(dT) as template-primer

非存在下での [³H] dTMPのとりこみをみたものである. 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 活性をみたものである. poly (Af) は天然の

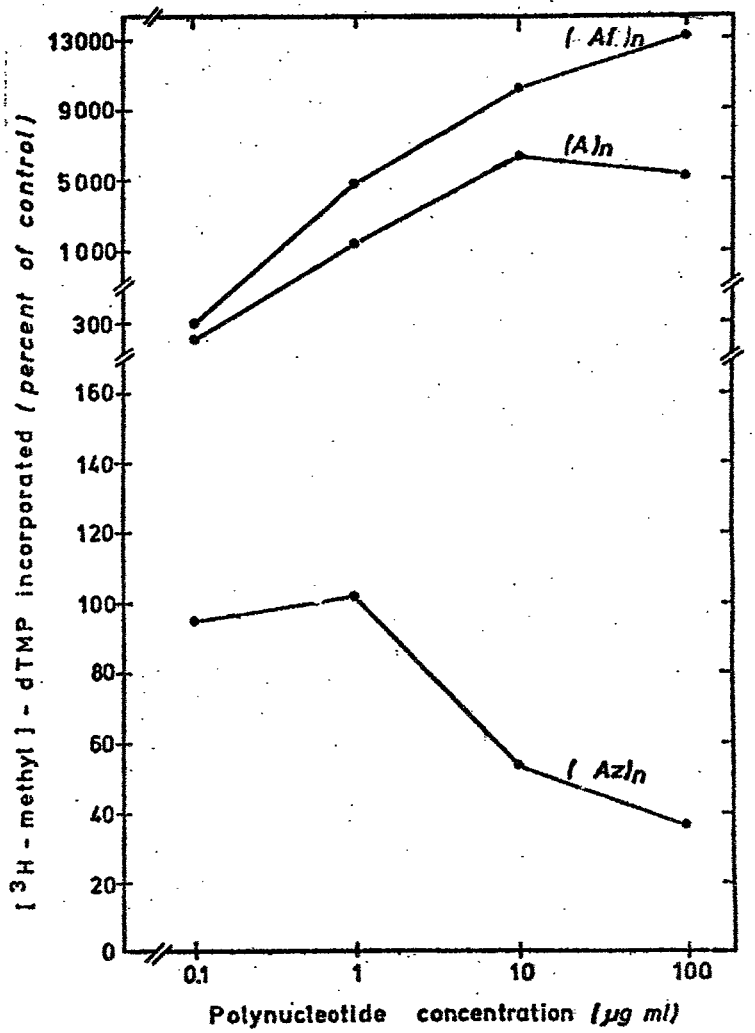


Fig 63 Effect of poly(Az) and poly(Af) on Mulv reverse transcriptase in the presence of oligo(dT) as primer

templateである poly(A) より高い [3H]dTMP のとりこみをしめしており、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 は template, primer 存在下の

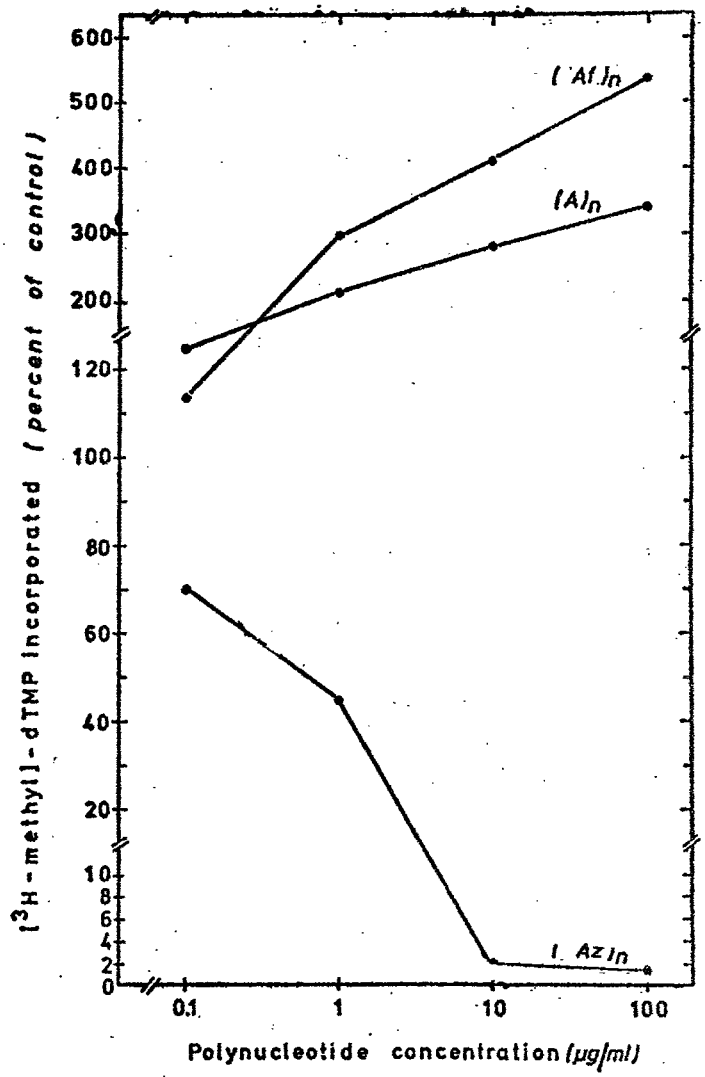


Fig 64 Effect of poly(Az) and poly(Af) on Mulv reverse transcriptase in the presence of poly(A) oligo(dT) as template-primer

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

このように、poly(At)ではreverse transcriptaseに対して、poly(A)より高いtemplate活性を有し、poly(Az)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おいて興味深い。

結　語

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

2) 2′位にアミノ基を導入したpoly(Aa)は弱酸性において，poly(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poly(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) アデノシンの2′位にハロゲンを導入した，poly(Af) poly(Acl), poly(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) イノシンの2'位にハロゲンを導入したpoly(Ift)，poly(Icl)では，poly(It)が，が特異なself-ordered structureを形成し，又poly(C)と非常に安定なcomplexを形成するのに対して，poly(Icl)では，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(Ibr)·poly(C)，poly(It)-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(At)はpoly(A)より高い．RNA腫瘍ウィルスのreverse transcriptaseのtemplate活性を有していることが判った．

謝　辞

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実験の部

・UVスペクトルの分子吸光係数〔ε(P)〕, CDスペクトルの分子だ円率([θ])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 Nacacodylate (PH7.0)-0.10 M NaCl の系を用い、buffer solution 中で測定した。又、酸性条件としては、pH 5.7 以上は Nacacodylate buffer を用い、それ以下は Naacetate 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って行った。

・UVスペクトルは日立124分光々度計、Hitachi Model 200-10 spectrophotometer, 日立323分光々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10mmのセル、又は9mm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。

・各測定用サンプルは homo complex の場合. buffer solution 中20～30分室温放置後測定を行った. hetero complex は特に断らない限り. 一夜放置後測定した.

・T_m 測定は小松電子工業の分光々度計恒温装置 Model SPD-H 124 を日立 124 形分光々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. Na cacodylate 中(pH 7.0) total 0.02～0.08 mM で各 polynucleotide をそれぞれの割合で混合し. 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 35 V/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 - 濃アンモニア水 - 水 (7 : 1 : 3)

G↓ : n-butanol - 酢酸 - 水 (5 : 2 : 3)

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

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

・PEP, PPC には東洋ろ紙 No.51-A を用いた.

・poly (A), poly (C), poly (I), poly (U) は, ADP, CDP IDP, UDP を PNPase により重合したものを用いた。

・polynucleotide phosphorylase は, M. luteus は米国シグマ社より購入したものを用いた。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 硫安分画	1910	530	3.6
$Ca_3(PO_4)_2$ 分画	1620	360	4.5
II 硫安分画 DEAE セルロース	1600	340	5.0
クロマトグラフィー	600	12	50

assay条件；incubation mixture 0.25ml

ADP 4mM, TrisHCl (pH8.5) 50mM

$MgCl_2$ 2mM, 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 1μmole の無機リンを遊離する酵素活性を1unitと定義する。

また蛋白量は Lowry法[56]により定量した。

- crude snake venom 5'-nucleotidase は鹿児島県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3OD TrisHCl (pH8.5) 100mM

$MgCl_2$ 10mM enzyme 6mg/ml

で37°C 4時間 incubate し、反応液を PPC に apply して調べた。

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

Sample 約3OD TrisHCl (pH8.5) 50~200mM

enzyme 1.5mg/ml

で、37°C 24時間反応させ、反応液を PPC に apply して調べた。

第一章の実験

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

氷冷下. triethyl phosphate 1mlに $POCl_3$ 0.067 ml (0.74 m mole) を加えて撹拌し、これに 2'-azido-2'-deoxyadenosine 107mg (0.37 m mole) を加える。1.5時間撹拌の後、氷水にあけ反応をとめ、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、残渣を水にとかし、Dowex 1×2 (formate) カラム (φ0.7×5cm) に apply する。水洗後、0.1Nのギ酸で溶出する。

収量 3885 OD260 (0.26 m mole) 70%

UV : λmax (at pH7.0) 259.5 nm

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

AzMP 3885 OD260 (0.26 m mole) (free) を t-butanol 2.5 ml, H_2O 2.5 ml, morpholine 0.1 ml (1.1 m mole) にとかし. reflux する。DCC 235mg (1.1 m mole) を t-butanol 5 ml にとかしたものと、1時間に

わたって滴下する。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DCC ureaを除き、溶媒を留去の後、水を加え、ether 3 mlで3回抽出する。水層を減圧留去し、残渣をpyridineで数回共沸した。85%リン酸 0.05 ml (0.73 mmole), tri-n-butyl amine 0.17 ml (0.73 mmole) をpyridineで数回共沸する。これをmorpholidateと合してpyridine共沸の後、pyridine 2 mlを加え室温放置する。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 : H_2O : cNH$_3$ (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 φ2.0 × 19 cm にapplyする。0 Mから0.25M triethyl ammonium bicarbonate (TEAB) buffer total 4 l の linear gradient で溶出する。main peakは2つで、目的のdiphosphateは0.20M付近で溶出される。0.14Mで溶出

される ものは monophosphate で 791 OD

AzDP の収量 2290 OD (59%)

UV: λmax (at 259.5 nm) 259.5 nm

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

AzDP 4mM, $MgCl_2$ 2mM, Tris HCl (pH 8.5) 100 mM, PNPase (E-coli) 1.9 units/ml を total volume 7.3 ml で 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 G50 カラム φ1.7 × 85 cm でゲル口過した。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 mmol) を 80% 酢酸 2ml にとかし、$NaNO_2$ 0.3g (4.3 mmole) を加えて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かめ、水でうすめて活性炭カラムに apply する。

水洗の後. ethanol : H_2O : cNH$_3$ (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. 0.3N HCOOH で溶出する。main peak は I つであった。

収量　958 OD (0.079 μmole) 97%

UV　λmax (PH 7.0) 249 nm

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

IzMP 958 OD (79 μmole) を t-butanol 2 ml H_2O 2 ml, morpholine 0.1 ml (1 mmole) にとかし reflux する。DCC 210 mg (1 mmole) を t-butanol にとかし、30 分にわたって滴下する。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、残渣を水にとかし、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% リン酸 30 μl (0.44 mmole), tri-n-butyl amine 0.1 ml (0.44 mmole) を pyridine 共沸 3 回い行い、両者を合して. pyridine 共沸、トルエン共沸の後. DMF 1 ml にとかす。室温放置 2 日後. 原料の消失を確かめ、水を加え. 反応を

とめる。活性炭カラムにapplyし水洗の後、ethanol 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 (bicarbonate) カラム φ1.3×8cmにapplyする。0から0.25M TEAB buffer total 3lのlinear gradientで溶出する。main peakは2つで目的物は0.16Mに溶出される。0.1Mに溶出されるのはmonophosphateでbOD250

収量　656 OD (53μmole) 68%

UV　λmax (pH7.0) 249nm

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

IzDP 4mM, $MnCl_2$ 2mM, TrisHCl (pH8.5) 80mM PNPase (E.coli) 2-4 unit/ml 全量12.5mlで37°C incubateする。1, 2, 3, 4, 16, 17時間後に0.25ml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 φ1.7×100cmでゲルに通す。poly(Iz)はvoid volume付近に溶出される。これを0.01M TrisHCl (pH7.0) 0.01MEDTA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 φ1.7×110 cm でゲル p 過する。void volume に溶出された poly (I8) を 75 OD260 14% で得た。

第二章の実験

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

AzDP 747 OD260 (49 μmole) を水 5 ml, 酢酸 1 ml にとかし、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 p 去し、p 液を留去し、水共沸して酢酸を除き、DEAE Sephadex A-25 (bicarbonate) φ1.7×20 cm カラムに apply する。0.15M から 0.25M TEAB buffer total 2 l の linear gradient で溶出する。main peak は 1 つで、0.20M に溶出された。

収量 60 l OD260 (39 μmole) 80.4%

UV λmax 258.5 nm

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

AaDP 4mM, $MnCl_2$ 2mM, TrisHCl(pH8.5)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 0.25 ml ずつとり、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 (pH7.0), 0.01M EDTA に透析し、蒸留水に対して透析する。凍結乾燥の後、Sephadex G50 カラム φ1.7×110 cm でゲル口過する。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

A† 40.3 mg (0.15 mmole) を $POCl_3$ 0.1 ml (1.1 mmole) triethyl phosphate 1 ml 水冷下撹拌中の混液に加え、反応させる。2時間後、電気泳動で原料の消失を確かめ3時間後に氷水約 200 ml にあけ反応をとめ、活性炭カラム

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

収量 1607 OD260 (0.113 mmole) 76%

UV λmax (pH7.0) 259.5 nm

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

AMP 1607 OD260 (0.113 mmole) (free) と t-butanol 1.5 ml, H_2O 1.5 ml, morpholine 0.1 ml (1.1 mmole) にとかし reflux 中。DCC 235 mg (1.1 mmole) を 2 ml の t-butanol にとかしたものを滴下、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 共沸3回行う。80% リン酸 40 μl (0.58 mmole) と tri-n-butyl amine 0.14 ml (0.58 mmole) を pyridine 共沸3回行い両者を合せて pyridine 共沸、トルエン共沸し、DMF 1 ml にとかし 31°C 放置する。3日後反応をとめ、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 7.7 OD (0.05 mmole) 46%

UV λmax (pH7.0) 259.5 nm

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

AfDP 4mM, $MgCl_2$ 2mM, TrisHCl(pH8.5) 100mM, E.coli PNPase 4.5 units/ml 全量5mlで37°24時間incubateした。除蛋白の後、凍結乾燥し、Sephadex G50カラムでゲル濾しpoly(Af)を得た。

収量 108 OD260 (0.011 m mole) 55%

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

Ace 32.1mg (0.11 m mole) と $POCl_3$ 50μl (0.54m mole) triethyl phosphate 2ml 氷冷下撹拌中の混液に加え反応させる。6時間後に反応をとめ、活性炭カラムで脱塩の後、Dowex 1×2 (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 1012 OD260 (61%)

UV λ_{max} (pH7.5)

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

Ace MP 915 OD (61μmole) と morpholine 30ml (0.35 m mole) H_2O 1ml t-butanol 1ml とかす。

reflux中 DCC 72mg (0.35 m mole) をt-butanol 1.5ml 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 と共沸し、これに 80% H_3PO_4 18μl (0.26 m mole) tri-n-butyl amine 62μl (0.26 m mole) をpyridine 共沸したものを合せ、さらにpyridine 共沸し、pyridine 1ml 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) のクロマトグラフィーで分離精製した。

収量 330 OD (22 μmole) 35%

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

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

収量 45 OD_{260} (4.4 μmole) 25%

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

AbrA 27.4 mg (83 μ moles) を $POCl_3$ 0.1 ml (1.1 m mole) triethyl phosphate 1 ml 氷冷下撹拌中の溶液に加え、撹拌下反応させる。4時間後、氷水にあけ活性炭カラムで脱塩する。次にDowex 1×2 (formate) カラムクロマトグラフィーで分離精製する。

収量 790 OD_{260} (53 μmole) 63%

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

AbrMP 790 OD_{260} (53 μmole) を morpholine 75 μl (0.86 m mole), t-butanol 1 ml, H_2O 1 ml にとかし加熱下、DCC 176 mg (0.86 m mole) を t-butanol 1.5 ml にとかしたものを加え、4時間還流し、室温放置した後、後処理する。pyridine 共沸し、80% リン酸 30 μl (0.4 μ mole), tri-n-butyl amine 0.1 ml (0.4 m mole) を pyridine 共沸したものを加えさらに 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 (25μmole) 48%

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

AbrDP 4mM, $MgCl_2$ 2mM, Tris HCl (pH8.5) 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 filteration した。

収量　33 OD260 (13%)

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

AfMP 2000 OD260 (0.14 mmole) と 30% 酢酸溶液 1.5 ml にとかし、$NaNO_2$ 0.3 g と 37°C 20 時間反応させる。pH3.5 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

IMP 1290 OD250 (0.11 mmole) を t-butanol 1.5ml morpholine 0.1ml (1.1 mmole) H_2O 1.5ml にとかし OD 乾下. DCC 235 mg (1.1 mmole) を t-butanol 2ml にとかしたものを OD 乙. 3-5時間還流し. 冷却し後処理する. pyridine 共沸し. 80% リン酸 40μl (0.58 mmole) と tri-n-butylamine 0.14ml (0.58 mmole) と pyridine 共沸したものを OD 乙. トルエン共沸し. DMF 1ml 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 カラムクロマトグラフィーで分離精製する.

収量 745 OD260 (65 μmole) 59%

UV λ_{max} (PH7.0) 248nm

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

ITP 4mM, $MgCl_2$ 2mM, TrisHCl (pH8.5) 100mM, PNPase (E. coli) 5.8 units/ml 全量 7.5ml 37°C 24時間反応した。除蛋白の後. Sephadex G50 gel filteration した.

収量 35 OD260 (3.6 μmole) 12%

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

AcMP 2000 OD260 (0.13 m mole) と 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 OD260 (0.10 m mole) 78%

UV　λmax (pH 7.0) 248 nm

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

IcMP 1112 OD (0.091 m mole) と morpholine 80 μl (0.9 m mole) t-butanol 1 ml, H_2O 1 ml にとかし、加熱下、DCC 186 mg (0.9 m mole) と t-butanol 1.5 ml にとかしたものを加え、4時間還流の後、冷却し後処理する。pyridine 共沸し、85% リン酸 0.09 ml (1 m mole), tri-n-butylamine 0.238 ml (1 m mole) を pyridine 共沸したものを加え、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　711 OD$_{260}$ (57 μmole) 63%

u v　(PH7.0) λmax 258 nm

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

ICDP 4mM, $MgCl_2$ 2mM, Tris HCl (pH8.5) 100 mM, PNPase (M. luteus) 40 units/ml 全量 5 ml とし 37°C 24時間反応する。除蛋白し、Sephadex G50 gel filteration する。

収量　31 OD$_{260}$ (3.1 μmole) 16%

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

ra, Toshikazu Fukui and Nobuko Kakiuchi

harmaceutical Sciences, Osaka University, Suita, Osaka, Japan 565

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ACT

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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 guanosine[7] 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., Tm 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

Morio Ikehara, Toshikazu Fukui and Nobuko Kakiuchi

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan 565

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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 Tm's somewhat higher than that of poly (A)· poly (U) complex in the same condition.

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2'-Azido-2'-deoxyadenosine (Ia) (63 mg, 0.21 mmole) was dissolved in a mixture of $POCl_3$ (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 OD_{260} units, 0.15 mmole) in a yield of 73%. UV: λ_{max}^{pH2} 257 nm, λ_{max}^{pH12} 259 nm.

2'-Azido-2'-deoxy AMP (0.15 mmole) was dissolved in a mixture of H_2O (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 H_2O-ether (1:1) mixture. Insoluble material was filtered off and the aqueous layer was separated and evaporated. The residue was azeotropically dried with pyridine several times. To the residue, inorganic phosphate (0.04 ml, of 95%), which was previously dried by azeotropical evaporation with pyridine together with tri-n-butylamine (0.14 ml) and dissolved in pyridine (1 ml), was added. The reaction mixture was kept at 30° for 5 days. The reaction mixture was evaporated in vacuo, the residue dissolved in H_2O, and applied to a column of charcoal. The column was washed with H_2O and eluted with 50% EtOH containing 5% conc. ammonia. Eluents were concentrated and applied to a column (1.0 x 17.5 cm) of DEAE-Sephadex A 25. Elution was performed with 0.1-0.3 M triethylammonium bicarbonate buffer (total 2 l) in a linear gradient and 15 ml fractions were collected. Fractions No. 65-85 were pooled and evaporated. 2'-Azido-2'-deoxy ADP (760 OD_{260} units, 35%) was obtained. UV : λ_{max}^{pH2} 257 nm, λ_{max}^{pH7} 259.5 nm, λ_{max}^{pH12} 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 $MgCl_2$ or $MnCl_2$ 0.4 mM were adjusted to 0.25 ml with H_2O and incubated at 37°

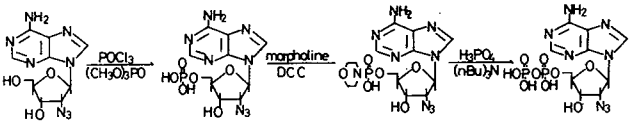
for 6 hrs. Pi release was 0.27 μmole (34%) or 0.33 μmole (41%) in the presence of Mg^{2+} or Mn^{2+} respectively.

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: $\lambda_{max}^{pH7.0}$ 256 nm (ε =8,900). This sample was completely hydrolyzed with snake venom phosphodiesterase to give only 2'-azido-2'-deoxy A5'p. UV spectra were taken with a Hitachi 124 spectrophotometer in the presence of 0.1 M NaCl and 0.05 M Na cacodylate (pH 9.0) at 12°. CD spectra were taken with a JASCO ORD/UV-5 spectrometer equipped with a CD attachment in the presence of 0.1M NaCl and 0.05M Na cacodylate (pH 7.0) at 16°. Melting temperature was measured in the presence of 0.1M NaCl and 0.05M Na cacodylate at pH 7.0 with a Hitachi spectrophotometer equipped with a thermostated cell. The temperature inside the cell was measured with a thermocouple. Mixing curves were obtained by measuring the absorbance of mixture which contained 0.04 mM total concentration of poly (Az) and poly (U) in the ratios indicated in Fig 6 . Salt concentration was 0.1 M NaCl and pH was adjusted to 7.0 with 0.05M Na cacodylate.

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

RESULTS AND DISCUSSION

As in the case with 2'-substituted pyrimidine nucleoside 5'-diphosphates[4] 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

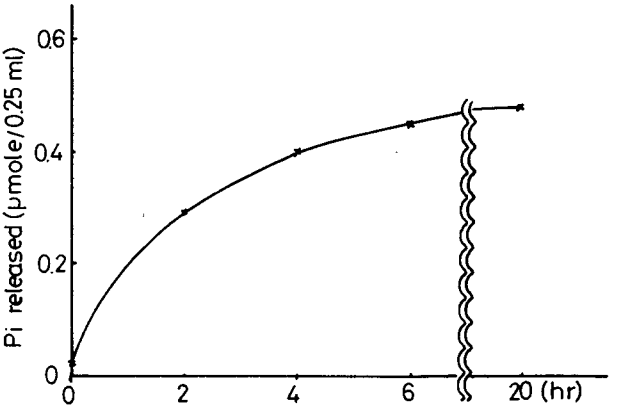


Fig. 1. Time course of polymerization of AzDP

some of the material was lost during the deproteination, 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 λmax at 256 nm. A blue shift of 4 nm of the λmax 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, $H > N_3 > OH > OMe > OEt$. This cannot be explained solely in terms of sterical distorsion by 2'-groups of the vertial stacking

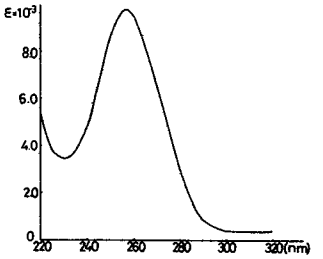


Fig. 2. UV absorption spectrum of poly (Az) at pH 7.0.

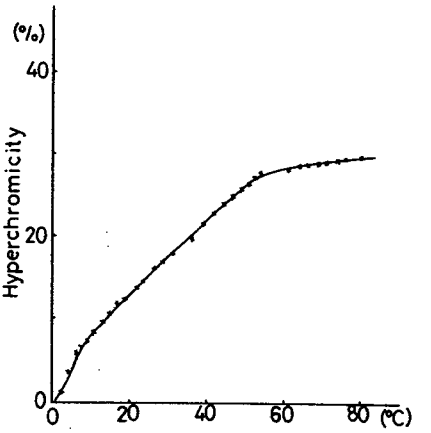


Fig. 3. Temperature-absorption profile of poly (Az) at pH 7.0.

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.

Temperance-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 (m^2A),[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 Tm was reported to be 12° in the presence of 0.01M $MgCl_2$. 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

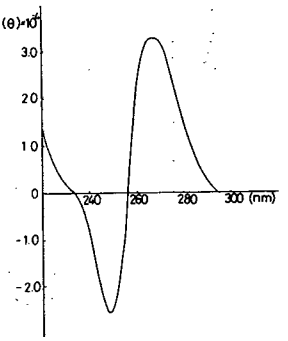


Fig. 4 . CD spectrum of poly (Az) in the presence of 0.15M Na^+ at pH 7.0 and 16°.

at 268 nm ([θ]=39,000) and a trough at 248 nm ([θ]=30,000). The cross-over point was at 256 nm, close to the absorpton 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 [θ] is significantly different. The $[\theta]_{peak}$ 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 $[\theta]_{trough}$ also shows the same tendency.

As was discussed previously[17], the magnitude of rotatory strngths 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 sterical distorsion by azido groups.

Acid titration of poly (Az)

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

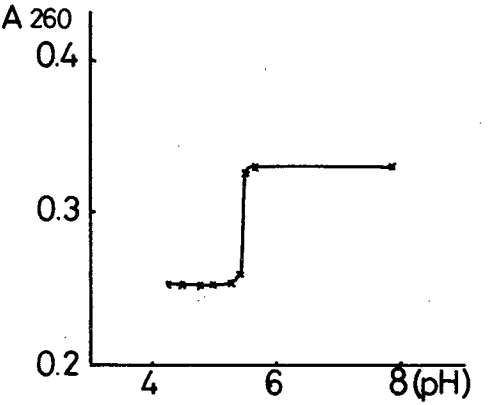


Fig 5. Acid titration curve of poly (Az).

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

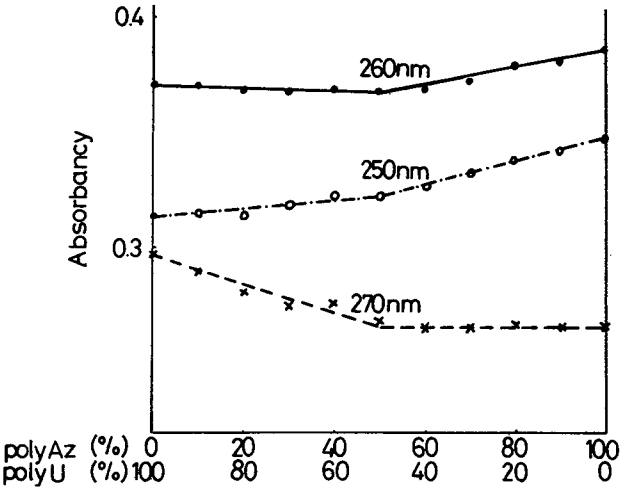


Fig. 6. Mixing curves of poly (Az) and poly (U) at 0.04M Na^+.

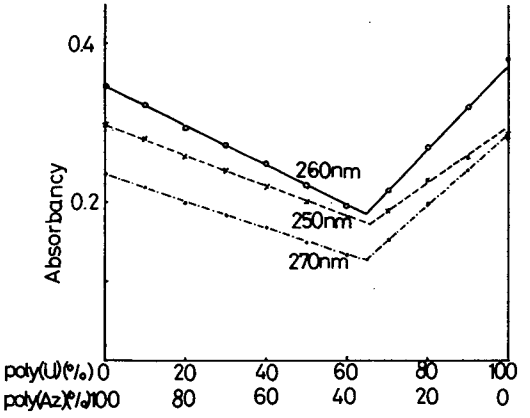


Fig. 7. Mixing curves of poly (Az) and poly (U) at Na^+ concentration of 0.15M.

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.

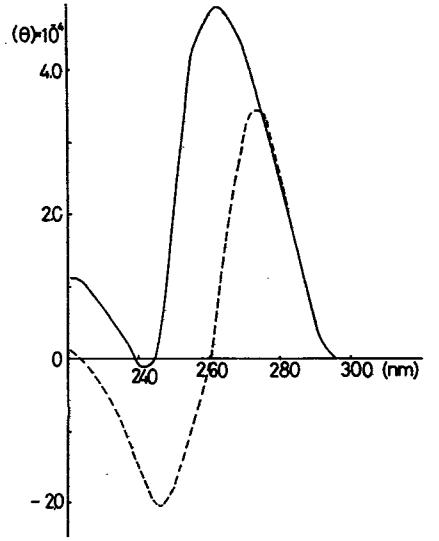


Fig. 8. CD curves of poly (Az) plus poly (U) before and after mixing at 0·15 M Na^+ concentration.

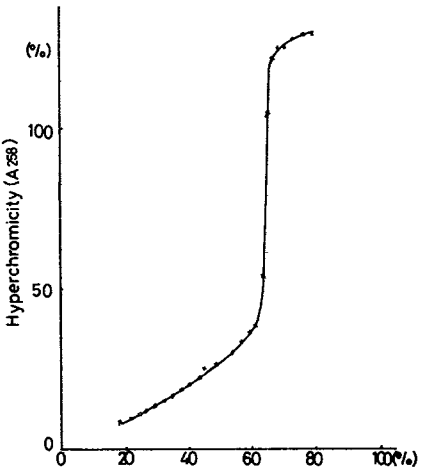


Fig. 9. Temperature-absorption profile of poly (Az). poly (U) in the presence of 0.15M Na^+.

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 Tm value 46° for the poly (Az)·poly (U) complex. As it was reported previously the poly (A)·poly (U) complex has a Tm of 47° in the same salt concentration[19], 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 palteau after 68°. This shows a Tm at 65° and a hyperchromicity as large as 46%.

This Tm value of poly (Az)· 2poly (U) is somewhat higher than that observed in poly(A)· 2poly(U) (60°).[19] Since the Tm 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 distorsion 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 Tm higher than that of poly (A)· 2poly (U). The stabilising effect of the azido group seems to be invariable by complexing. This may be bacuse 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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Volume 4 Number 4 April 1977 Nucleic Acids Research

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

Morio Ikehara, Toshikazu Fukui and Nobuko Kakiuchi

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan 565

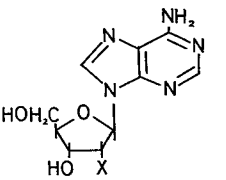
Received 22 February 1977

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 Tm 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=N_3 and NH_2) was developed.[2] Using this approach poly (2'-azido-2'-deoxyadenylic acid) [poly(Az)] has been synthesized[3] 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 (Tm) 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'-diphosphate[3] (747 OD_{260} 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 Rf 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 11 min. AaDP was eluted at the buffer concentration 0.2 M. The yield was 601 OD_{260} 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 phopshatase 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, $MnCl_2$ 2 mM, polynucleotide phosphorylase obtained from Micrococcus luteus[8] by a method described by Klee and Singer[9] 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-$CHCl_3$ (1:3, vol/vol) for

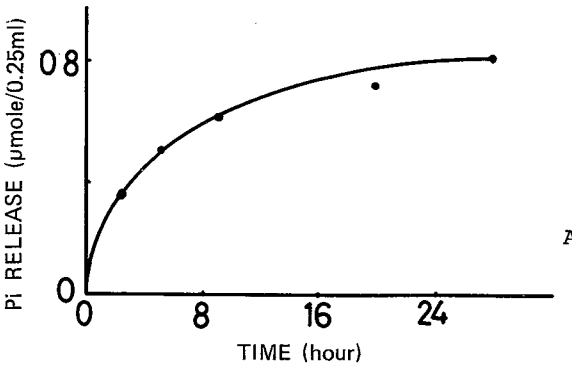


Fig. 1. Time course of AaDP polymerization.

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 OD_{260} 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 spectrometer equipped with a CD attachment. Calibration was performed with d-10-camphorsulfonic acid. Tm'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

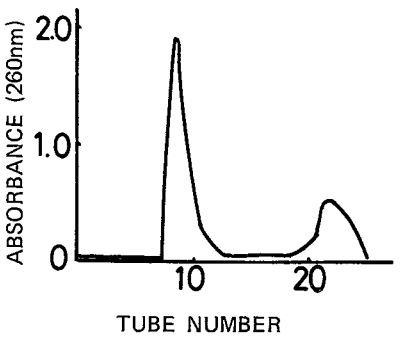


Fig. 2. Sephadex G-50 column chromatography of poly (Aa).

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 nm[2], 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

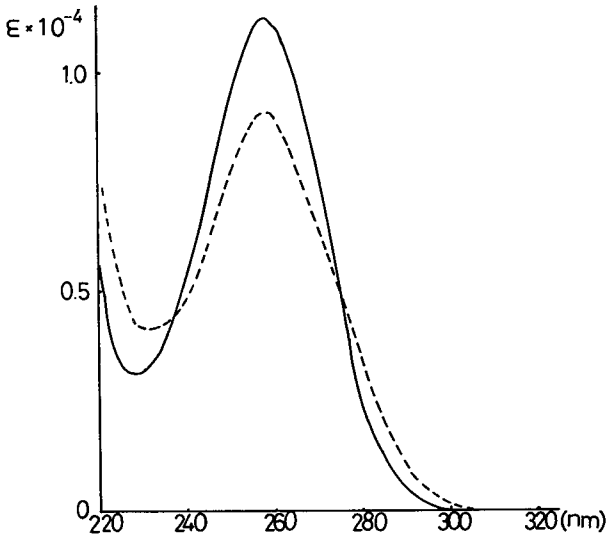


Fig. 3. UV absorption spectra of poly(Aa),— at pH 7.0, --- at pH 6.3.

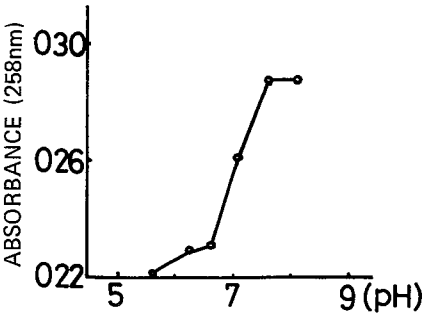


Fig. 4. Acid titration of poly(Aa).

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'-NH_2 group at around pH 6.7 and stacking of bases may be increased, despite the fact that no protonation at the N^1-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'-NH_2 group. This type

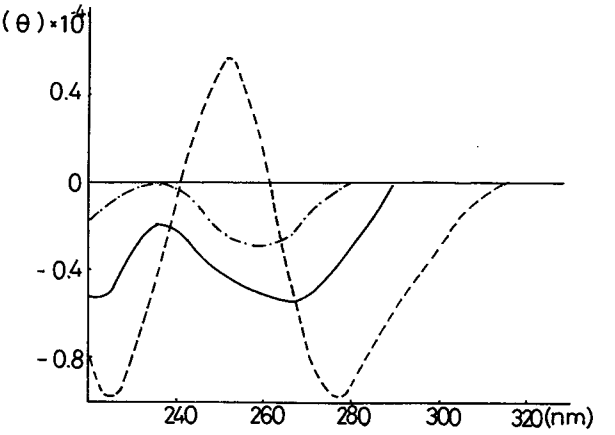


Fig. 5. CD spectra of AaDP and poly(Aa), ——poly(Aa) at pH 7.0. ---poly(Aa) at pH 5.7, -·-·- AaDP at pH 7.0.

of structure has not been reported previously with pyrimidine 2'-NH_2 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'-NH_2 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

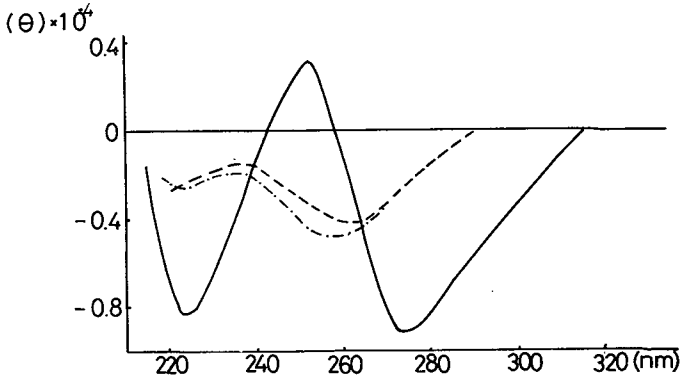


Fig. 6. CD spectra of poly(Aa) taken at 20°(—), 46°(---) and 66°(-·-·-).

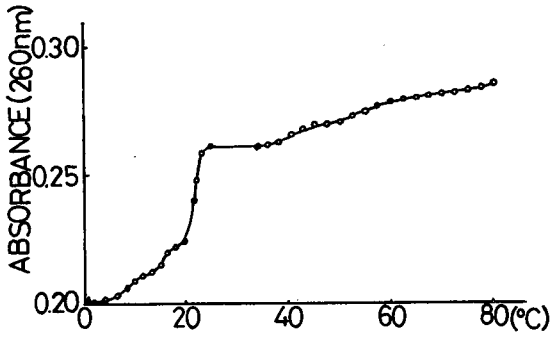


Fig. 7. Temperature-absorption profile of poly(Aa) taken at 7.0.

presence of 0.1M NaCl and 0.05M sodium cacodylate, the absorption steeply increased at 20° and reached a pleteau at 24°, then it gradually increased up to 80°. This provides a Tm 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 N^1 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

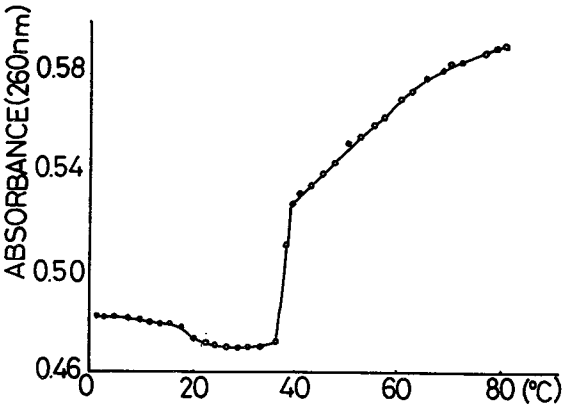


Fig.8.Temperature-absorption profile of poly(Aa) taken at pH 6.3.

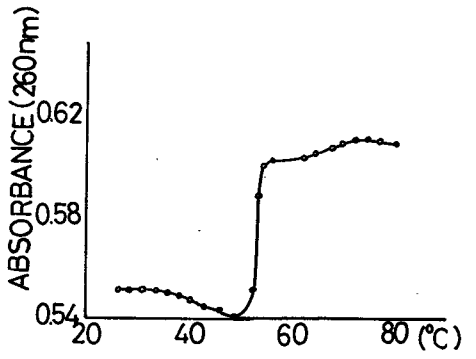


Fig. 9. Temperature-absorption profile of of poly(Aa) taken at pH 5.7.

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 Tm on cationic concentration

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

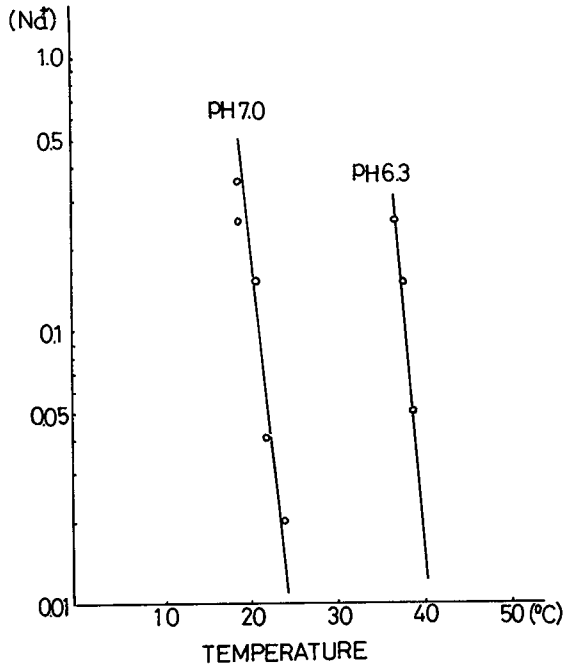


Fig. 10. Depending of Tm of poly(Aa) on sodium ion concentration.

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

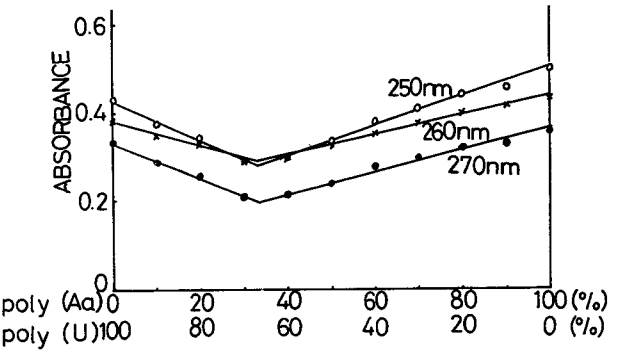


Fig. 11. Mixing experiments of poly(Aa) with poly(U).

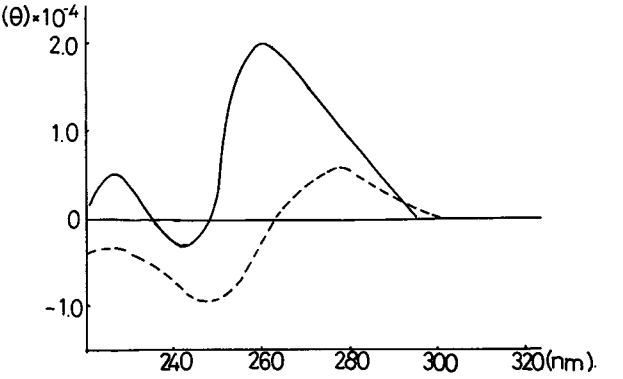


Fig. 12. CD spectrum of poly(Aa)·2poly(U) complex.

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

Tm of poly (Aa)·2 poly(U)

Tm 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 Tm 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'-NH_2 group instead of OH of poly (A) inhibits to some extent the stacking of bases.

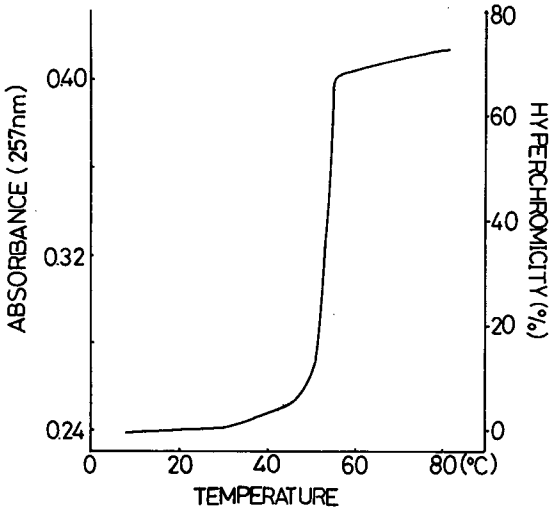


Fig. 13. Temperature-absorption profile of poly(Aa)·2poly(U) complex.

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'-NH_2 group. From the thermal stability measurement and examination of dependency of Tm on cationic strength, it may be deduced that this protonated form of poly (Aa) would be stabilized by an intrastrand interaction of 2'-NH_3^+ 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 Tm 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.

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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

Nomenclature

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Volume 4 Number 8 August 1977 Nucleic Acids Research

Polynucleotides. XLV[1] Synthesis and properties of poly(2'-azido-2'-deoxyinosinic acid)

Toshikazu Fukui, Nubuko Kakiuchi and Morio Ikehara

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan 565

Received 14 April 1977

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 Tm at 1.0M Na^+ ion concentration was 43°. Upon mixing with poly (C), poly (Iz) forms a 1:1 complex having a Tm 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'-halogeno[3], -azido[4,5], -amino[5], -methoxy[6] and -ethoxy[7] substituents have been reported[8]. We have found a new method for the synthesis of 2'-N-substituted nucleosides[9] 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]

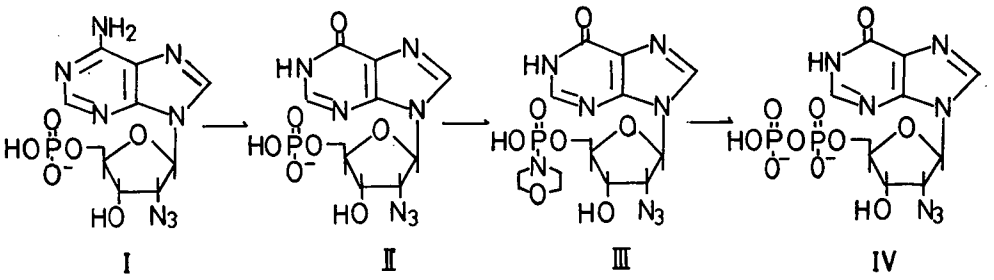
In this paper we describe the synthesis of poly(2'-azido-2'-deoxyinosinic acid)[poly(Iz)] catalyzed by E.coli polynucleotide phosphorylase together with its physical properties including UV, CD and Tm in neutral media and the results obtained on mixing with poly(C). It has been found that the introduction 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'-phosphate[10] (I, 5000 OD_{260} 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 OD_{250} units, 0.29 mmole, 87%). UV: $\lambda_{max}^{pH7.0}$ 249 nm. Paper electrophoresis: R_{AMP} 1.0.



I II III IV

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

2'-Azido-2'-deoxyinosine 5'-phosphate (3640 OD_{250} 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 H_2O-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 OD_{250} 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), $MnCl_2$ (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 analyses [15] on aliquots (0.25 ml) removed at various time intervals. (Fig 1) After 17 hrs the mixture was deproteinised with isoamylalcohol-$CHCl_3$ (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 volumn. 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 μmoles) was polymerized as described above. After purification, the yield of poly(Iz) was 75 OD_{250} units (7.04 μmoles, 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 Mn^{2+} 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)

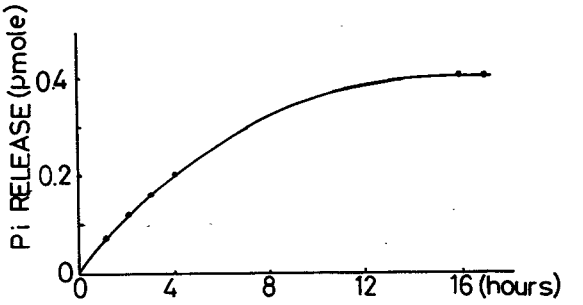


Fig. 1 Time course of polymerization of 2'-azido-2'-deoxy-inosine 5'-diphosphate

showed λ max 247 nm (ε 10,950) as compared with λ max 249 nm (ε 12,500) for IzMP. A blue shift of 2 nm of the λ 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.

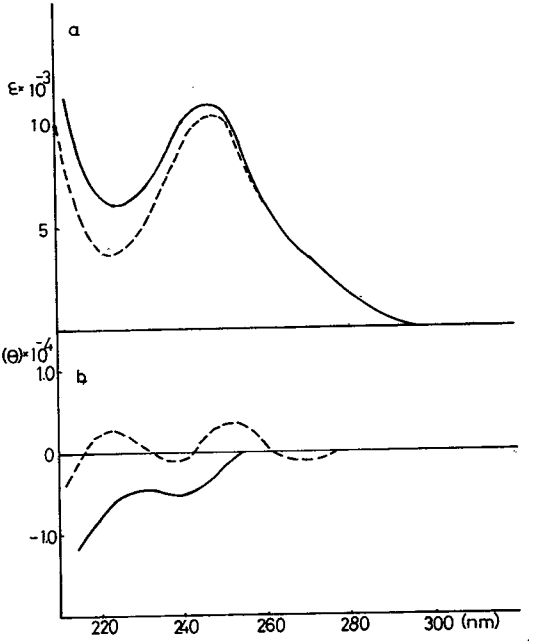


Fig 2 U.V. and C.D. spectra of poly(Iz) and poly(I) in neutral solution containing 0.15M Na^+.
—— poly(Iz), ---- poly(I)

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 ([θ]-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 Rich [17] 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 descreased 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).

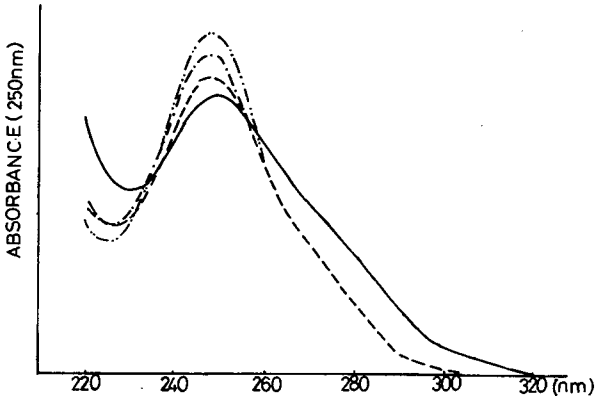


Fig.3 U.V. absorption of poly(Iz) at various Na^+ ion concentration. ——— 0.95M, --- 0.51M, —·—·—0.35M —··—··— 0.05M

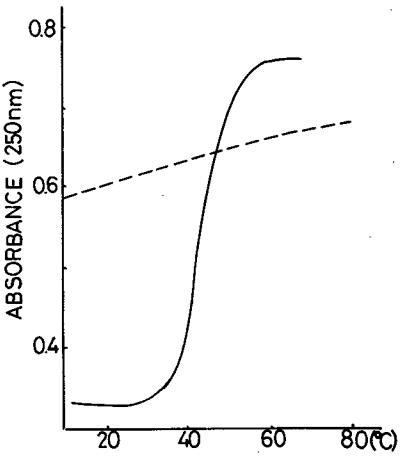


Fig.4 Tm of poly(Iz)
———0.95M Na^+,
-----0.15M Na^+

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 Tm (43°) for poly(Iz) is the same as the Tm (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, poly(Iz)·poly(C), as in the case of poly(I)·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

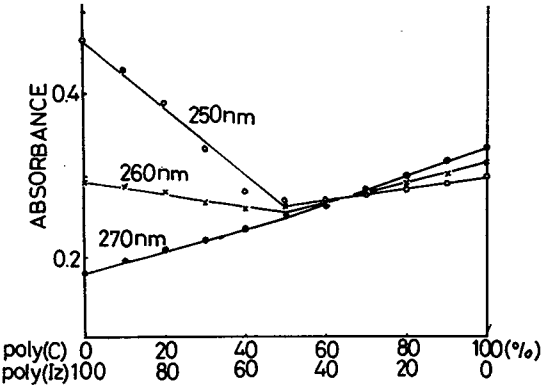


Fig.5 Mixing experiment of poly(Iz) and poly(C)

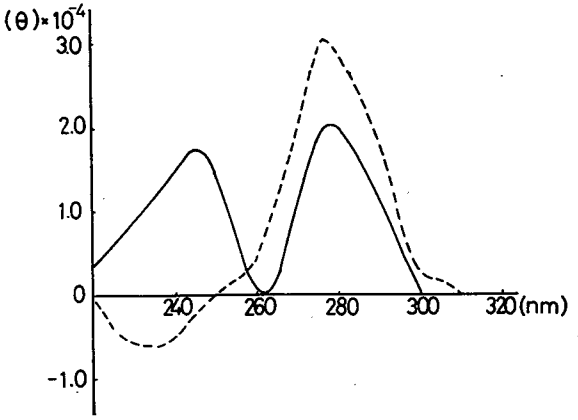


Fig. 6 CD spectra of poly(Iz) and poly(C) (1:1) before and after mixing ----- before mixing, ——— after mixing

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 descreased 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 temparature-absorption profile at various ionic strengths are recorded. The Tm 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 Tm of poly (Iz)·poly(C) complex was 11° lower than that the Tm of poly(I) ·poly(C) reported to be 62.5° at 0.15M Na^+ ion concentration. These Tm'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

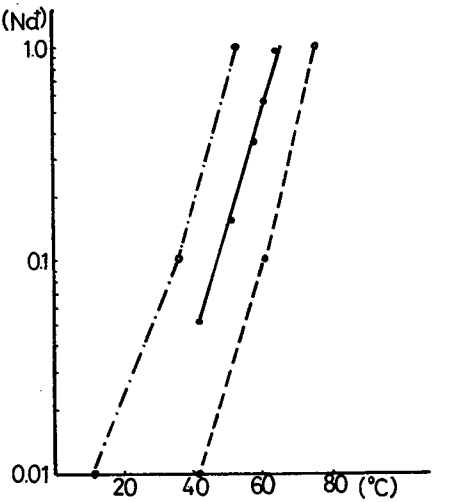


Fig.7. Relationship of Na^+ concentration and Tm of poly(Iz)·poly(C) (—), Poly(I)·poly(C) (----) and poly(dI)·poly (C) (– – – –).

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 Tm than the deoxyribose duplexes as well as the hybrid duplexes.[19] We have reported previously[10] that the poly (Az) forms a 1:2 complex with poly(U) in 0.15M Na^+ solution and this complex has a Tm 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 Tm's lower than those of the ribose duplex, poly(I)·poly(C), but has Tm'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 enchancement of interferon-inducing activity by the 2'-azido group may be ascribed to resistance of poly(Iz) to enzymatic degradation.

ACKNOWLEDGEMENT

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Volume 4 Number 12 December 1977 Nucleic Acids Research

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

Morio Ikehara, Toshikazu Fukui and Nobuko Kakiuchi

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan 565

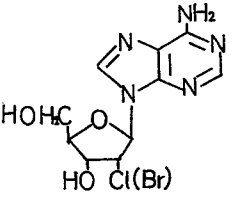
Received 9 September 1977

ABSTRACT

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

INTRODUCTION

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



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

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

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

MATERIAL AND METHODS

General Procedure

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Enzymatic digestion of polynucleotides

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

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

RESULTS AND DISCUSSION

UV absorption properties

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

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

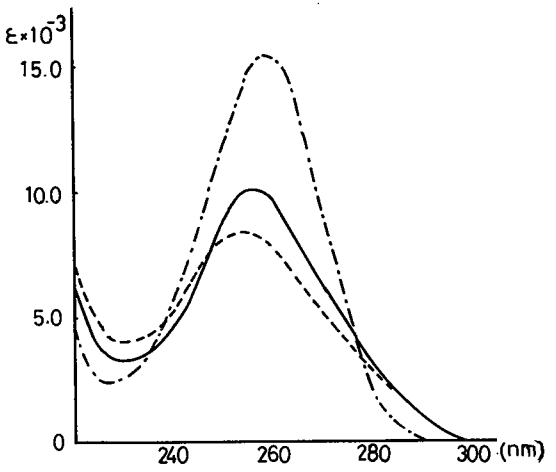


Fig. 1. UV absorption spectra of poly (Acl). ——at pH 7.0. ..o..o..o at pH 4.5, -.-.- Acl 5'-MP.

CD spectra

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

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

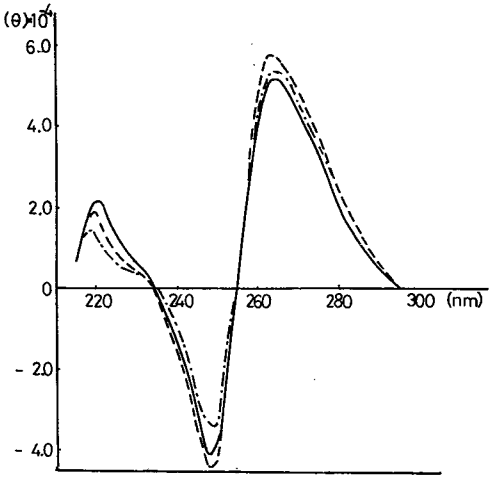


Fig. 2. CD Spectrum of Poly (Acl), Poly (Abr) and Poly(A).
——— Poly (A), ---- Poly (Acl), —.—.— Poly (Abr).

Table I Molecular Ellipticity of Polynucleotides

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

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

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

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

Table II Acid Titration of Polynucleotides

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

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

Formation of Complexes with Poly (U)

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

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

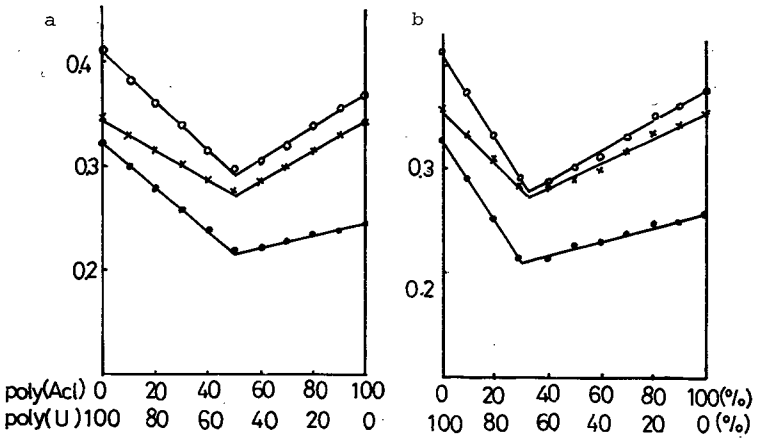


Fig. 3. Complex Formation of Poly (Acl) with Poly (U).

complex described below.

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

Thermal Transition of Complexes

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

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

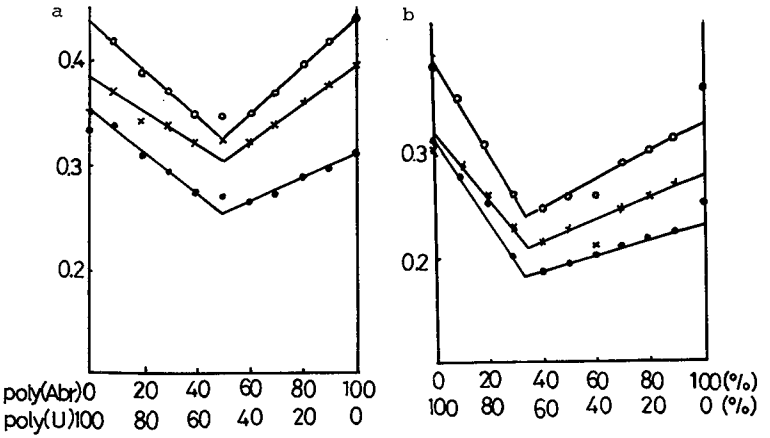


Fig. 4. Complex Formation of Poly (Abr) with Poly (U).

Table III. Thermal Transition Points of Complexes.

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

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

CONCLUSIONS

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

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

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

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

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

what different from case to case.

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

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

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Volume 5 Number 6 June 1978 Nucleic Acids Research

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

Morio Ikehara, Toshikazu Fukui and Nobuko Kakiuchi

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan

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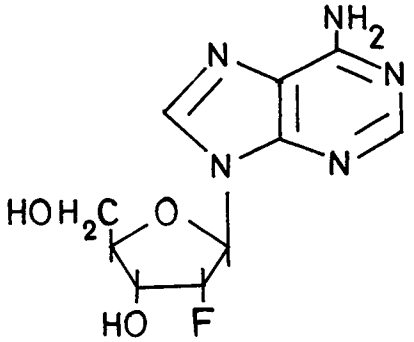
ABSTRACT

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 Tm of the acid form was 37° which was significantly lower than that of poly(A), but similar to that of poly(2'-azido-2'-deoxyadenylic aicd). Poly(Af) formed 1:1 and 1:2 complexes with poly(U) having Tm 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 Tm 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 plynucleotides 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'-fluoroadenosine[8] and data on its physical pro-



2'-Deoxy-2'-fluoro-adenosine

perties such as UV and CD spectra, Tm, 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'-fluoroadenosine[8] (40.3 mg, 0.15 mmole) was stirred with $POCl_3$ (0.1 ml, 1.1 mmole) and $(EtO)_3PO$ (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 EtOH-H_2O-c.NH_4OH (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 : Rf(A) 0.23, Rf(C) 0.13. Paper electrophoresis (at pH 7.5): $R_{A\text{-}pA}$ 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-BuOH (1.5 ml) and H_2O (1.5 ml). While this solution was refluxed, DCC (235 mg) dissolved in t-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. H_2O (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 µl, 0.58 mmole) and $(n\text{-But})_3N$ (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. H_2O (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 A_{260} (0.05 mmole, 46%). Paper chromatography : Rf(A) 0.13, Rf(B) 0.44. Paper electrophoresis (at pH 7.5) : $R_{A\text{-}pA}$ 1.35.

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

A solution (5 ml) containing 2'-deoxy-2'-fluoroadenosine 5'-DP 4 mM, $MgCl_2$ 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 µmol/0.25 ml of the incubation mixture) was liberated. The mixture was extracted with i-AmOH-$CHCl_3$ (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 wich was excluded in the void volume was collected. The yield was 108 A_{260} (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. Tm was measured with a Hitachi 124 spectrophotometer equipped with a themostated 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-H_2O (5:2:3) and B, i-PrOH-conc.NH_4OH-H_2O (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 plymerization, while AzDP[2], AclDP[3] and AbrDP[3] 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 wothwhile 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).

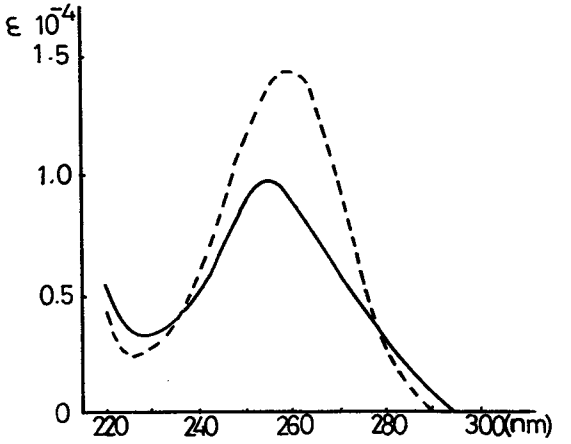


Fig. 1 U. V. absorption spectra of poly(Af) and Af 5'-MP. Poly(Af) ———, Af-5'-MP ----.

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 ε 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(Acl) and poly(Abr). The shape of the spectra closely resmble 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 $[\theta]_{peak}$ of poly(Af) at 264 nm is 6,3000 and $[\theta]_{trough}$ at 248 nm is -49,000 (see Table II).

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

Table I

Polynuclotide	ε	(λmax)	εMonomer	(λmax)	Hypochromicity (%)
Poly(A)	10,000	(257 nm)	15,400	(259 nm)	35
Poly(Af)	9,700	(255)	14,300	(259.5)	32
Poly(Acl)	10,500	(257)	15,500	(259)	32
Poly(Abr)	10,700	(257)	15,000	(259)	29

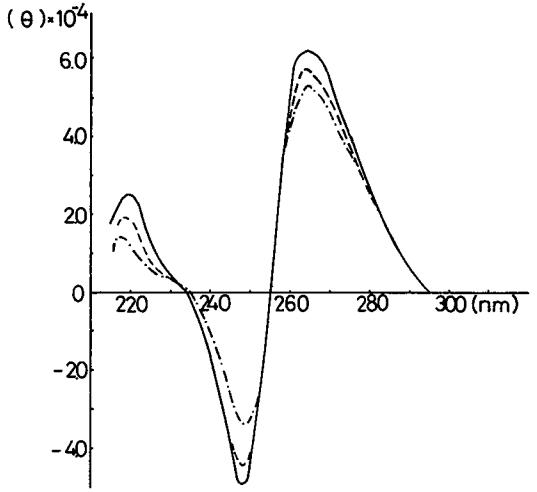


Fig. 2 CD spectra of poly(Af), poly(Acl) and poly(Abr). Poly(Af) ——, poly(Acl) ----, poly(Abr) -.-.-.

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 [θ] values in CD spectra, which are caused by coupling of transition moments[11] 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. Thia 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 [θ] 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	$[\theta]_{peakI}$ (nm)	$[\theta]_{trough}$ (nm)	$[\theta]_{peakII}$ (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)

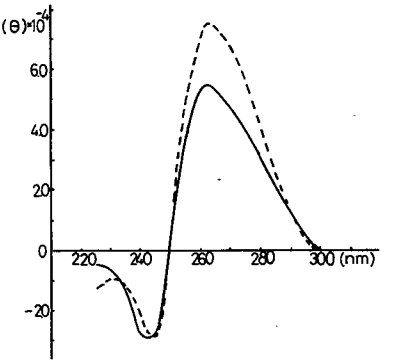


Fig. 3 CD spectra of poly(Af) and poly(A) acid form. Poly(Af) ———, poly(A) ----.

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

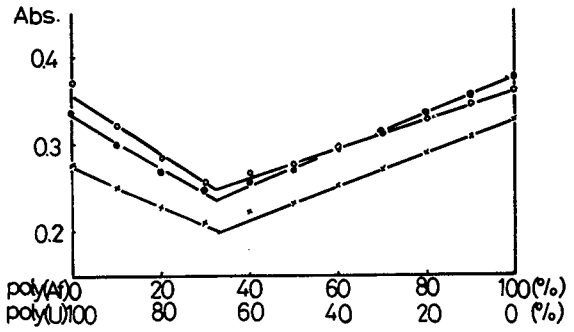


Fig. 4 Mixing curves of poly(Af) with poly(U) measured at 25° in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0).
At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

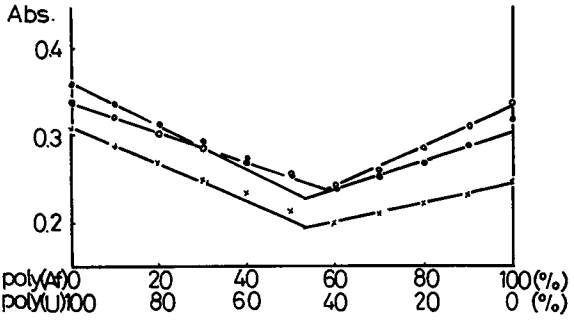


Fig. 5 Mixing curves of poly(Af) with poly(U) in the presence of 0.04M Na Cacodylate (pH 7.0) at 25°.
At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

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

Table IV

Polynucleotide	Tm at 0.04M Na^+	Tm 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 semms to influence the Tm 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

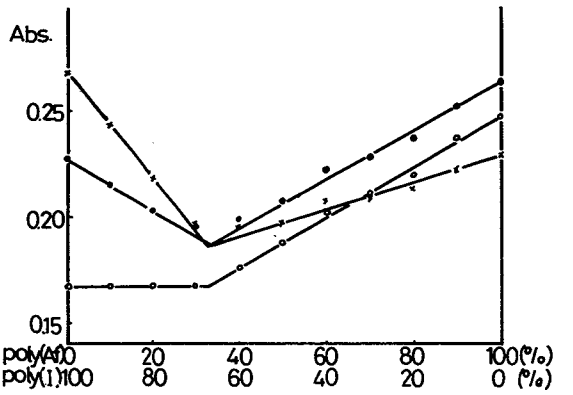


Fig. 6 Mixing curves of poly(Af) with poly(I) in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0) at 25°. At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

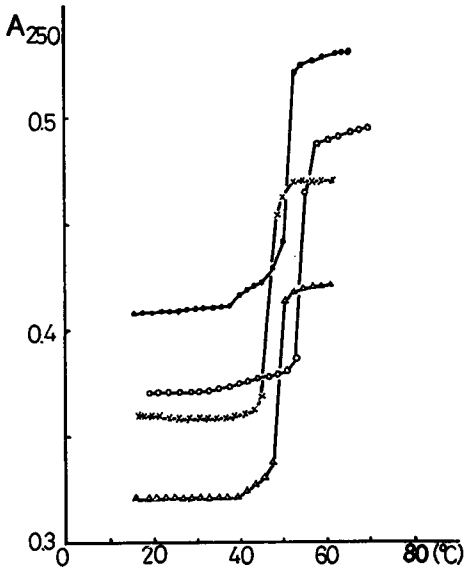


Fig. 7 Melting profiles of poly(A)-poly(I) complexes measured in the presence of 0.10M NaCl, 0.05M Na Cacodylate (pH 7.0) and 10mM $MgCl_2$.
Poly(Af)·2poly(I) ●-●-●, poly(A)·2poly(I) o-o-o, poly-(Abr)·2poly(I) x-x-x, poly(Abr)·2poly(I) △-△-△.

Table V

Complex	Tm 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°

Tm 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 Tms was Af > Acl > 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)

ERIK DE CLERCQ,* TOSHIKAZU FUKUI, NOBUKO KAKIUCHI AND MORIO IKEHARA**

*Rega Institute for Medical Research, University of Leuven, * Minderbroedersstraat 10, B-3000, Leuven, Belgium and Faculty of Pharmaceutical Sciences, Osaka University,** 133-1 Yamadakami, Suita, Osaka 565, Japan*

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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 $[(dIz)_n \cdot (C)_n]$, to be exquisitely effective in inducing interferon. Various other 2′-azido analogs, *i.e.* $(dIz) \cdot (brC)_n$, $(I)_n \cdot (dCz)_n$, $(dAz)_n \cdot (U)_n$, $(dAz)_n \cdot (rT)_n$ 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 $(dIz)_n$.[7] The latter preparation $[(dIz)_n]$ was further purified by gel filtration on a Sephadex G-100 colum (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 actinimycin 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] (units/ml) Average	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 μg/ml (-2.5×10^{-5}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 $(dIz)\cdot(C)_n$, 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 reaction[8] occurred:

$$(dIz)_n\cdot(brC)_n+(I)_n \rightarrow (I)_n\cdot(brC)_n+(Iz)_n.$$

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 μg/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 doublestranded 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 polycleotides 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 Tm of $(dIz) \cdot (brC)_n$ 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 Tm 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. LVI[1]. Synthesis and properties of poly(2'-deoxy-2'-fluoroinosinic acid)

Morio Ikehara, Nobuko Kakiuchi and Toshikazu Fukui

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan

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SUMMARY

Poly(2'-deoxy-2'-fluoroinosinic acid)[poly(If)] was synthesized by polymerization of 2'-deoxy-2'-fluoroinosine 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 psectra 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 Tm'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], chloro[5], bromo[5] and fluoro[6] 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 transcriptase[9] 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 METHODS

General procedure

The general procedures used in this study were essentially the same as reported previously[4], except that paper chromatography solvent systems were as follows : A, i-PrOH-conc. NH_4OH-H_2O (7:1:2); B, n-BuOH-AcOH-H_2O (5:2:3); C, n-PrOH-conc. NH_4OH-H_2O (55:15:35).

Synthesis of poly(If)

If 5'-MP--- 2'-Deoxy-2'-fluoroadenosine 5'-monophosphate[6] (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 : Rf(A) 0.07, Rf(B) 0.34, Rf(C) 0.32. Paper electrophoresis : $R_{A\text{-}pA}$ 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-Bu_3N (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. H_2O (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 A_{250} (59%). Paper chromatography : Rf(A) 0.04, Rf(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, $MgCl_2$ 2 mM, Tris-HCl (pH 8.5) 100 mM and *E. coli* polynucleotide phosphorylase[11] 5.8 units/ ml was incubated at 37° for 24 hrs. The mixture was extracted five times with an i-AmOH-$CHCl_3$ (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 A_{250} (3.6 µmole, 12%). Digestion of this sample (ca. 5 A_{250} 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%.

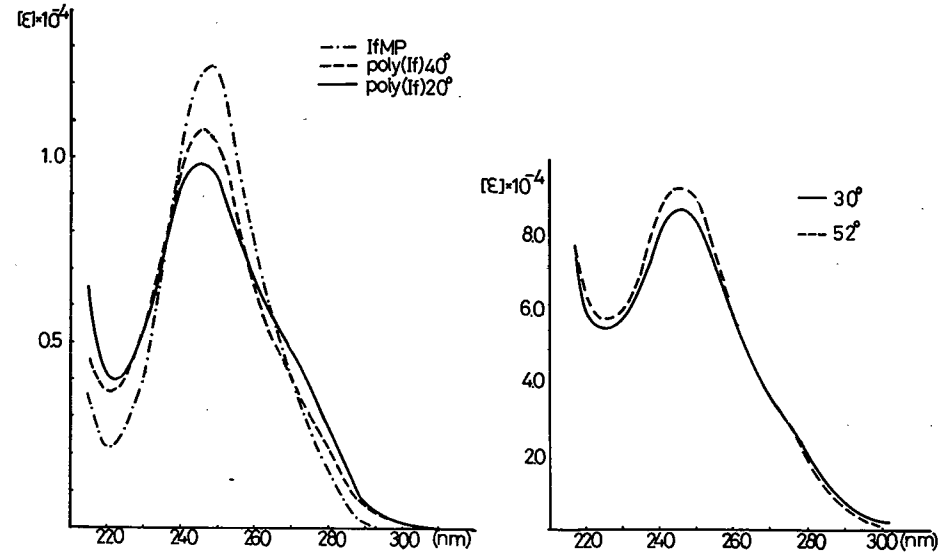
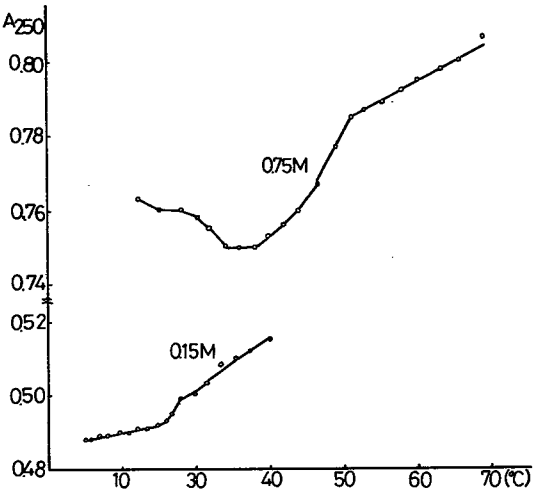


Fig. 1. UV spectrum of poly(If) and If 5'-MP at pH 7.0 in the presence of 0.15M Na^+ion.
Fig. 2. UV spectrum of poly(If) in the presence of 0.75M Na^+.

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 λ 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 Tm of 47° was obtained. However, these two states of the poly(If) ordered forms cannot be identical, because a graph of the relationship between Tm and Na^+ (Fig. 4) does not give a straight



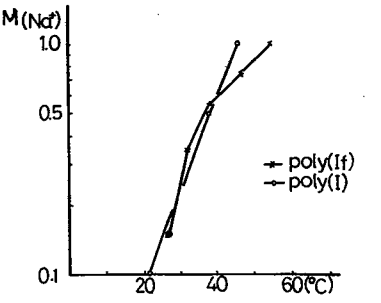


Fig. 3. Thermal melting profiles of poly(If) at Na^+ concentration of 0.15M and 0.75M.
Fig. 4. Relationship of Tm of poly(If) and poly(I) to Na^+ concentration.

line, but tends towards relatively higher Tm'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 Tm 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

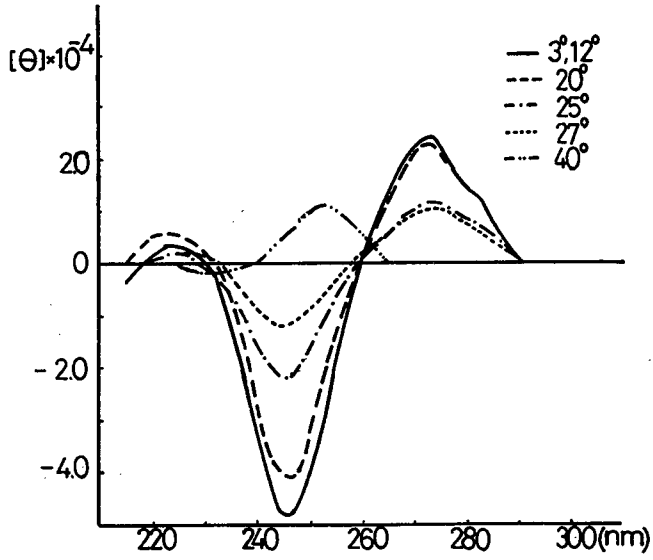


Fig. 5. CD spectra of poly(If) at 0.15M Na^+ concentration.

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

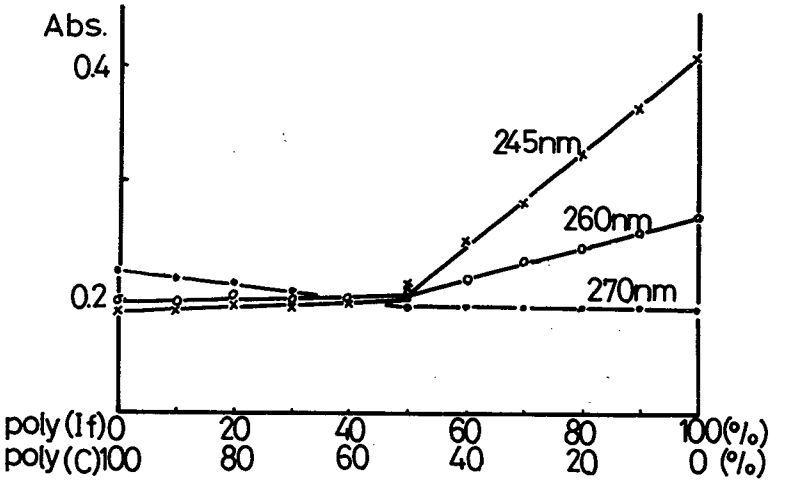


Fig. 6. Mixing curves of poly(If) and poly(C).

inducing activity.

When poly(If) 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(If) and poly(C) (Fig. 6). This indicates the formation of a 1:1 complex, poly(If)·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(If)·poly(C) has a similar conformation to that of poly(I)·poly(C) which is believed to be RNA-11 fold helix[18], but not to that of poly(dI)·poly(C).[15]

The thermal melting curves of the complex, poly(If)·poly(C) measured with various Na^+ concentrations are summarized in Fig. 8. The Tm at $[Na^+]$ = 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 Tm vs. $[Na^+]$ curves shown in Fig. 9. In the range of $[Na^+]$ of 0.01-0.35M, poly(If)·poly(C) has higher Tm values than those of poly(I)·poly(C) and poly(dI)·poly(C). Even at very low salt concentration the poly(If)·poly(C) complex has considerable thermal stability.

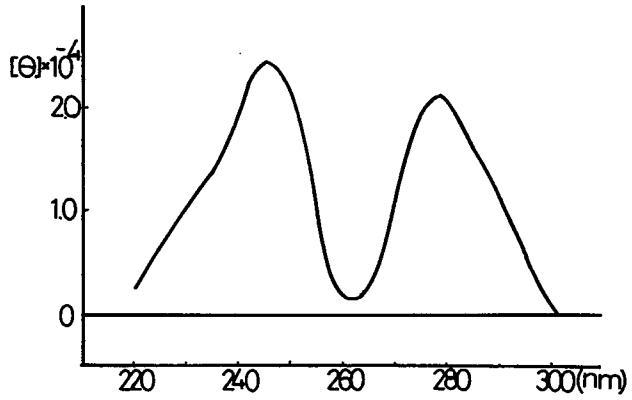
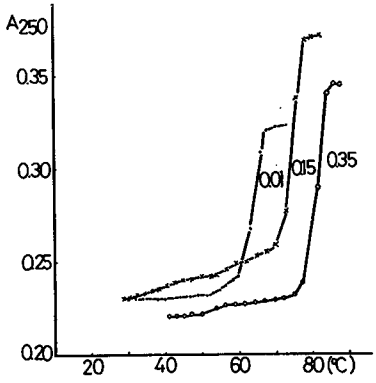


Fig. 7. CD spectrum of poly(If)·poly(C) complex.

Comparison of the Tm'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 Tm than that of poly-(I).poly(C). Furthermore, poly(Af)·poly(U)[6] has almost the same Tm 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-



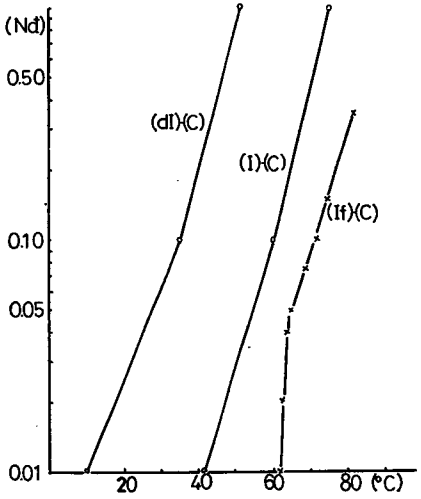


Fig. 8. Temperature absorbance profiles of poly(If).poly(C) at various Na^+ concentrations.
Fig. 9. Relationship between Tm of various double-stranded complexes and Na^+ concentration.

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 NH_2 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 NH_2 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 ^{1}H-NMR coupling of $H_{1'}$ and $H_{2'}$[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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Eur. J. Biochem. *88*, 341 – 349 (1978)

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

Erik DE CLERCQ, Paul F. TORRENCE, B. David STOLLAR, John HOBBS, Toshikazu FUKUI, Nobuko KAKIUCHI, and Morio IKEHARA

Rega Institute for Medical Research, University of Leuven; Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda; Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston; Abteilung Chemie, Max-Planck-Institut für Experimentelle Medizin, Göttingen; and Faculty of Pharmaceutical Sciences, Osaka University

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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 ^{3}H-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 ≥ 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 µl 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 µl of a solution containing various concentrations of the (unlabelled) inhibiting polynucleotide. Finally, 0.3 µg of $[^{14}C](I)_n \cdot (C)_n$ (in 50 µl) 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 µg of polynucleotide in 50 µl were placed opposite a trough containing 150 µl 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 $t_m \approx 84$ °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 $t_m \approx 55$ °C for the first transition and $t_m \approx 60$ °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 Ca^{2+} or Mg^{2+}) (Fig. 1). Under the same conditions $(I)_n \cdot (C)_n$ had $t_m = 62.5$ °C. Likewise, an equimolar mixture of $(dIn_3)_n$ and $(br^5C)_n$ showed a single transition at $t_m = 77$ °C (Fig. 1) (in $NaCl/P_i$ without Ca^{2+} or Mg^{2+}). Under the same conditions $(I)_n \cdot (br^5C)_n$ had $t_m = 87$ °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 'superinduced' with cycloheximide and actinomycin D, none

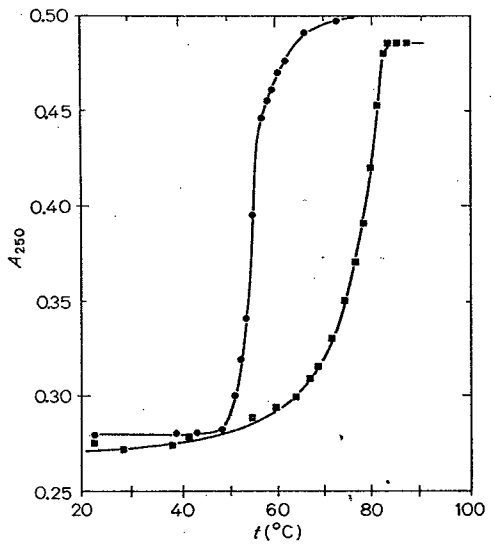

Fig. 1. *Melting profiles for an equimolar mixture of* $(dIn_3)_n + (C)_n$ (●) *and for an equimolar mixture of* $(dIn_3)_n + (br^5C)_n$ (■), *both determined in phosphate-buffered saline (without* Ca^{2+} *or* Mg^{2+}*), pH 7.2*

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 $(dIn_3)_n \cdot (C)_n$, $(dIn_3) \cdot (br^5C)_n$ 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. $(c^7I)_n \cdot (C)_n$, 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:

$$(dIn_3)_n \cdot (br^5C)_n + (I)_n \longrightarrow (I)_n \cdot (br^5C)_n + (dIn_3)_n.$$

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 cycloheximide 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. $t_m = 52$ °C (Fig. 2) as compared to 62.5 °C for $(I)_n \cdot (C)_n$ when determined under similar

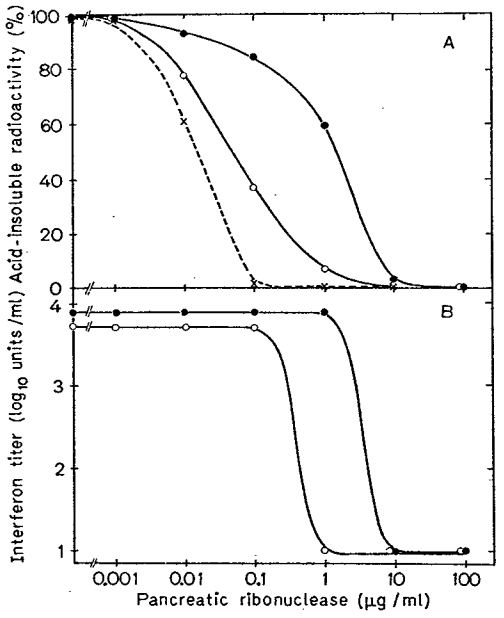

Fig. 2. *Sensitivity of $(dIn_3)_n \cdot (C)_n$ (○) and $(I)_n \cdot (C)_n$ (●) to degradation by pancreatic ribonuclease A, as monitored by (A) residual acid-insoluble radioactivity or (B) interferon-inducing activity of the polynucleotide-enzyme mixtures.* The polynucleotide-enzyme mixtures which were 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}C]-$(I)_n \cdot (C)_n$ to antibodies directed to double-stranded RNA. With antibodies induced by $(A)_n \cdot (U)_n$ and purified from a precipitate with $(I)_n \cdot (C)_n$ (Fig. 5A), or with whole antiserum to $(A)_n \cdot (U)_n$ (data not shown), $(dIn_3)_n \cdot (br^5C)_n$ competed more effectively than did $(dIn_3)_n \cdot (C)_n$. Their reactivity was similar to or greater than that of $(I)_n \cdot (br^5C)_n$, but smaller than that of $(I)_n \cdot (C)_n$ (Fig. 5A) or $(A)_n \cdot (U)_n$ (not shown).

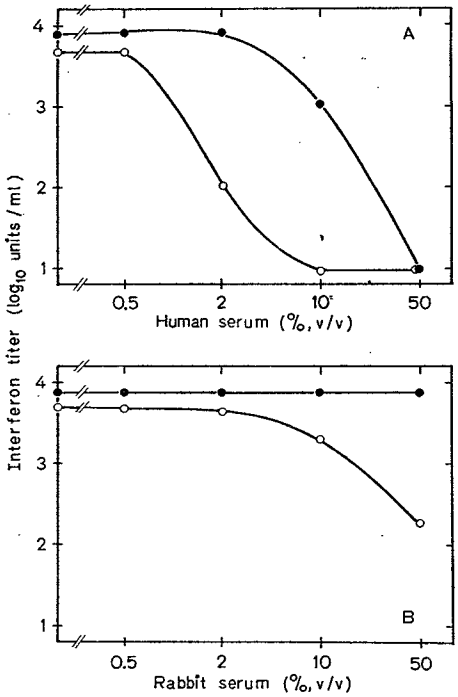

Fig. 3. *Sensitivity of $(dIn_3)_n \cdot (C)_n$ (○) and $(I)_n \cdot (C)_n$ (●) to degradation by (A) human serum and (B) rabbit serum, as monitored by residual interferon-inducing activity.* The polynucleotide-serum mixtures contained 1 µg of polynucleotide and varying concentrations of (A) human or (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

Competitive radioimmunoassays were also performed with antisera induced by $(I)_n \cdot (br^5C)_n$ (kindly provided by M. I. Johnston). Binding of [^{14}C]$(I)_n \cdot (C)_n$ to these antibodies was inhibited most effectively by $(dIn_3)_n \cdot (C)_n$ in terms of the amount of inhibitor required, but this inhibition did not exceed a maximum of about 85% (Fig. 5B). For $(I)_n \cdot (C)_n$, $(dIn_3)_n \cdot (br^5C)_n$ and $(I)_n \cdot (br^5C)_n$, 3–6-fold higher amounts of inhibitor were required, but inhibition reached 100% (Fig. 5B).

The reactivity of $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ with antibodies to double-stranded RNA was much stronger than the reactivity of other 2′-modified $(I)_n \cdot (C)_n$ or $(A)_n \cdot (U)_n$ analogues such as $(A)_n \cdot (Um)_n$, $(Um)_n$ being poly(2′-*O*-methyluridylic acid). In counterimmunoelectrophoresis, $(A)_n \cdot (Um)_n$ gave a very weak reaction with undiluted antiserum to $(A)_n \cdot (U)_n$ and no reaction at all with a 1/3 antiserum dilution. Both $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ gave a distinct precipitation at a 1/12 antiserum dilution; the $(dIn_3)_n \cdot (br^5C)_n$ precipitation line was more intense than the $(dIn_3)_n \cdot (C)_n$ line (Fig. 6, upper part). At a 1/40 serum dilution, $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$ 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 $(I)_n \cdot (br^5C)_n$. At a 1/5 serum dilution $(A)_n \cdot (Um)_n$ did not show any reaction, whereas both $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ gave distinct precipitation lines (Fig. 6, lower part).

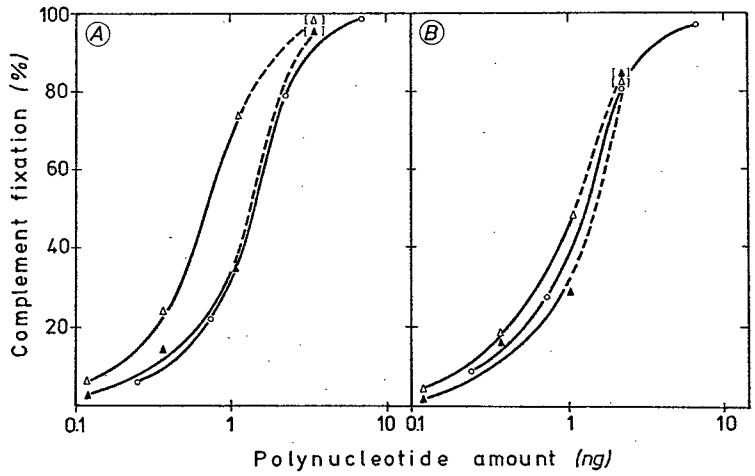

Fig. 4. *Quantitative complement fixation of $(dIn_3)_n \cdot (C)_n$ (△), $(dIn_3)_n \cdot (br^5C)_n$ (▲) and $(I)_n \cdot (C)_n$ (○) with antibody to $(A)_n \cdot (U)_n$.* The antibodies had been purified with $(I)_n \cdot (C)_n$ and were used at 1/1500 dilution (A) or 1/3000 dilution (B) from stock. Because of the anticomplementary activity of $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ at amounts higher than 1 ng, the exact amounts of $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ required for maximal complement fixation could not be determined. The complement fixation values obtained at doses at which $(dIn_3)_n \cdot (C)_n$ and $(dIn_3)_n \cdot (br^5C)_n$ showed partial anticomplementary activity are indicated in brackets

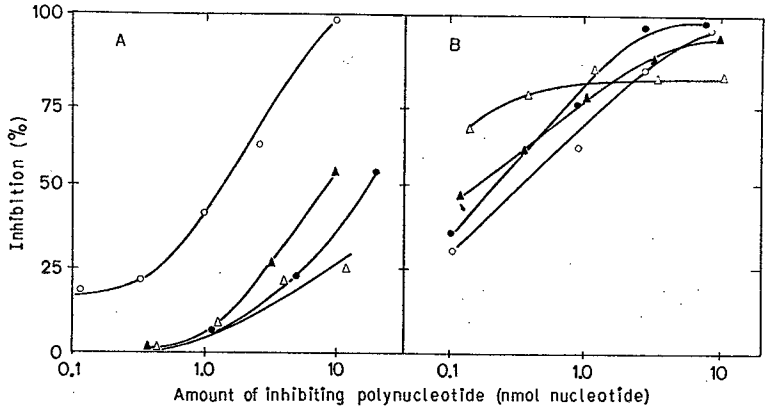

Fig. 5. *Inhibition of bindings of* $[^{14}C](I)_n \cdot (C)_n$ *to antibodies to double-stranded RNA by* $(dIn_3)_n \cdot (C)_n$ (Δ), $(dIn_3)_n \cdot (br^5C)_n$ (▲), $(I)_n \cdot (C)_n$ (○) *and* $(I)_n \cdot (br^5C)_n$ (●), *as determined with a competitive radioimmunoassay.* Amount of $[^{14}C](I)_n \cdot (C)_n$: 0.3 µg (≈ 0.9 nmol nucleotide). Amount of inhibiting polynucleotides as indicated. Source of antibodies to double-stranded RNA: (A) antibodies to $(A)_n \cdot (U)_n$, purified with $(I)_n \cdot (C)_n$; (B) antibodies to $(I)_n \cdot (br^5C)_n$. 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 µg (A) or 1/30 dilution from stock (B)

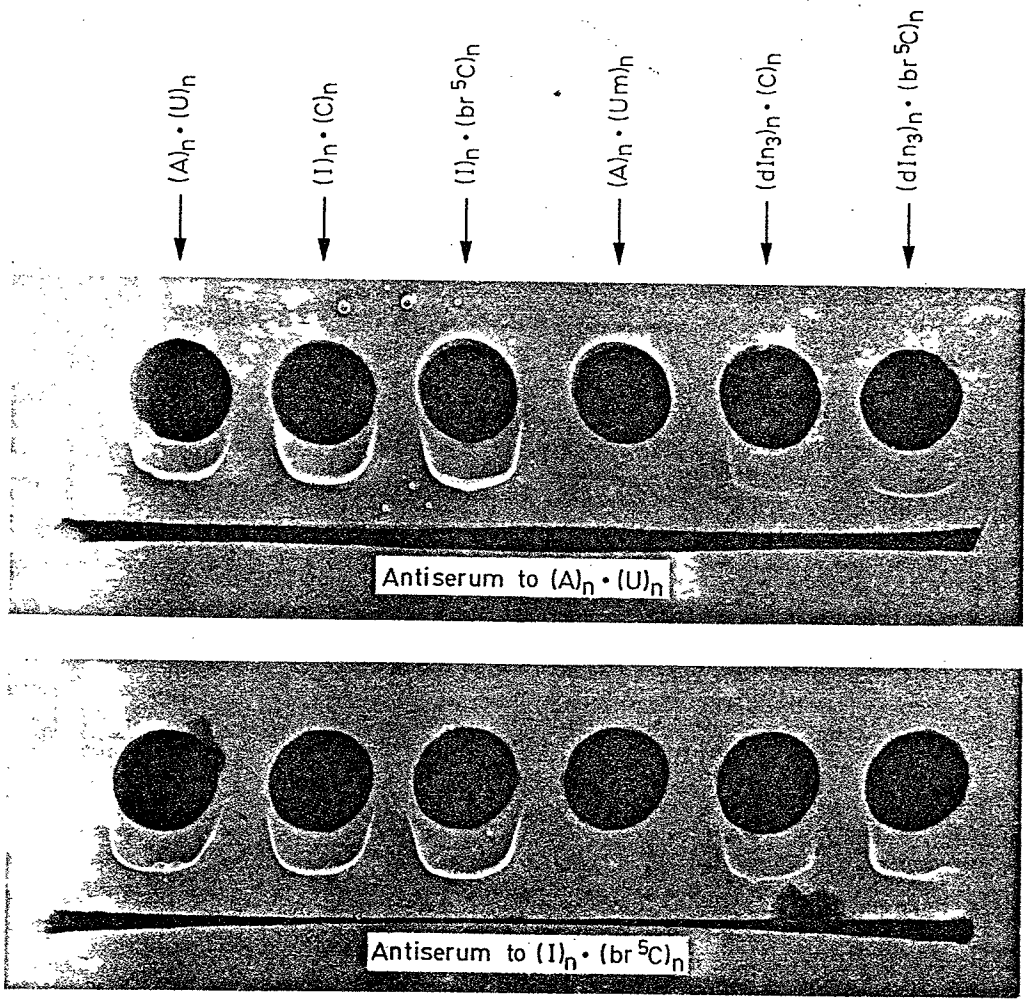

Fig. 6. *Counterimmunoelectrophoresis of* $(A)_n \cdot (U)_n$, $(I)_n \cdot (C)_n$, $(I)_n \cdot (br^5C)_n$, $(A)_n \cdot (Um)_n$, $(dIn_3)_n \cdot (C)_n$ *and* $(dIn_3)_n \cdot (br^5C)_n$ *with antibody to either* $(A)_n \cdot (U)_n$ *(upper part) or* $(I)_n \cdot (br^5C)_n$ *(lower part).* The antisera to $(A)_n \cdot (U)_n$ and $(I)_n \cdot (br^5C)_n$ were used at a dilution of 1/12 and 1/5, respectively (150 µl/trough). The antigen wells contained 0.5 µg of polynucleotide in 50 µl

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 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 [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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E. De Clercq, Rega Instituut voor Experimentele Geneeskunde, K.U.L., Minderbroedersstraat 10, B-3000 Leuven, Belgium

P. F. Torrence, Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland, U.S.A. 20014

B. D. Stollar, Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts, U.S.A. 02111

J. Hobbs, Department of Chemistry, The City University, St-John Street, London, Great Britain, EC1V 4PB

T. Fukui, N. Kakiuchi, and M. Ikehara, Faculty of Pharmaceutical Sciences, Osaka University, 133-1 Yamada-kami, Suita-shi, Osaka-fu, Japan 565

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

Fu Huang *, Paul F. Torrence *, Toshikazu Fukui+, Nobuko Kakiuchi+

lical Research, University of Leuven, Minderbroedersstraat 10, B-3000

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nylic acid) [(aza^{2}A)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. (Aza^{2}A)n formed a complex with (U)n and
(br^{5}U)n, and (aza^{2}I)n formed a complex with (C)n and (br^{5}C)n, but these complexes were markedly destabilized relative to the corresponding (A)n or (I)n complexes. The (aza^{2}A)n- and (aza^{2}I)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.(br^{5}C)n induced high amounts of interferon. Both (aza^{2}A)n and (aza^{2}I)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. (c^{7}A)n.(U)n, may seem related to the rather low Tm (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, (c^{7}A)n.(br^{5}U)n and (L)n.(br^{5}C)n fail to stimulate interferon production, although they possess a sufficiently high Tm (72°, 0.15M Na^+, pH 7) (1,3). For other (I)n.(C)n analogues, e.g. (c^{7}I)n.(br^{5}C)n, the high Tm (86°, 0.2M Na^+, pH 7) is associated with a significant interferon inducing ability.

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

Erik de Clercq, Guang-Fu Huang *, Paul F. Torrence *, Toshikazu Fukui+, Nobuko Kakiuchi+ and Morio Ikehara+

Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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ABSTRACT

Poly(2-azaadenylic acid) [(aza^{2}A)n] and poly(2-azainosinic acid [(aza^{2}I)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. (Aza^{2}A)n formed a complex with (U)n and (br^{5}U)n, and (aza^{2}I)n formed a complex with (C)n and (br^{5}C)n, but these complexes were markedly destabilized relative to the corresponding (A)n or (I)n complexes. The (aza^{2}A)n- and (aza^{2}I)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.(br^{5}C)n induced high amounts of interferon. Both (aza^{2}A)n and (aza^{2}I)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. (c^{7}A)n.(U)n, may seem related to the rather low Tm (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, (c^{7}A)n.(br^{5}U)n and (L)n.(br^{5}C)n fail to stimulate interferon production, although they possess a sufficiently high Tm (72°, 0.15M Na^+, pH 7) (1,3). For other (I)n.(C)n analogues, e.g. (c^{7}I)n.(br^{5}C)n, the high Tm (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, (c^3I)n and (c^7I)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 (aza^{2}A)n and (aza^{2}I)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 (c^7A)n with (br^{5}U)n and complexes of (I)n, (c^7I)n, (c^3I)n or (L)n with (br^{5}C)n have a markedly higher Tm than the corresponding complexes with (U)n and (C)n (1-3,5), complexes have been prepared of (aza^{2}A)n with (br^{5}U)n and of (aza^{2}I)n with (br^{5}C)n. It was reasoned that, if (aza^{2}A)n.(U)n and (aza^{2}I)n.(C)n would not show interferon inducing activity by virtue of their low Tm, (aza^{2}A)n.(br^{5}U)n and (aza^{2}I)n.(br^{5}C)n might be able to do so.

MATERIALS AND METHODS

The synthesis and physicochemical properties of (aza^{2}I)n and (aza^{2}A)n have been described recently (8); (br^{5}C)n and (br^{5}U)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); (aza^{2}A)n, poly(2-azaadenylic acid); (aza^{2}I)n, poly(2-azainosinic acid); (c^3I)n, poly(3-deazainosinic acid); (c^7A)n, poly(7-deazaadenylic acid); (c^7I)n, poly(7-deazainosinic acid); (L)n, polylaurusin or polyformycin B; (ms^{2}I)n, poly(2-methylthioinosinic acid); (dUz)n, poly-(2'-azido-2'-deoxyuridylic acid); (br^{5}C)n, poly(5-bromocytidylic acid); (br^{5}U)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); Tm, 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 (aza^{2}A)n and (aza^{2}I)n, and not to (n^{2}A)n or (n^{2}I)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^+), (aza^2I)n forms a 1:1 stoichiometric complex with (C)n (8). The Tm 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 Tm of the complexes of (C)n with (I)n, (c^7I)n, (c^3I)n or (L)n by 20-30° (1-3,5). Thus, as expected, (aza^2I)n was found to interact with (br^5C)n to give a complex that had a Tm of ∿40° in PBS (Fig. 2). (Aza^2I)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). (Aza^2A)n alone showed a rather clear transition point at ∿36° in Dulbecco's PBS (Fig. 3). A similar cooperative melting has been observed with (aza^2A)n at about 20° in 0.15M Na^+ (0.1M NaCl + 0.05M sodium cacodylate, pH 7.0) (8). In the latter solution, (aza^2A)n is assumed to form a 1:2 stoichiometric complex with (U)n, the Tm of which is ∿41° (8). For an equimolar mixture of (aza^2A)n with (br^5U)n, a biphasic melting profile was obtained (Fig. 3). The first transition corresponded to the melting of the (aza^2A)n homopolymer. The second, rather broad, transition with Tm ∿65° could be attributed

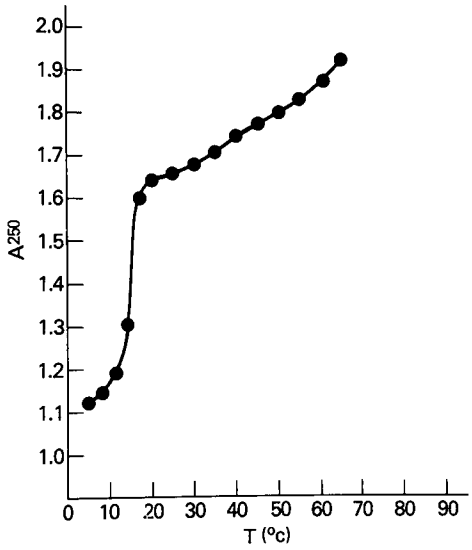
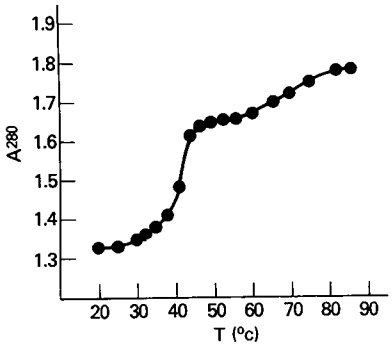


Figure 1.

Melting profile for an equimolar mixture of (aza^{2}I)n + (C)n in Dulbecco's PBS.



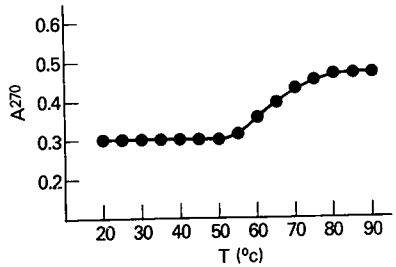


Figure 2.

Top : melting profile for an equimolar mixture of (aza^{2}I)n + (br^{5}C)n in Dulbecco's PBS.

Bottom : melting profile for the (aza^{2}I)n homopolymer in Dulbecco's PBS.

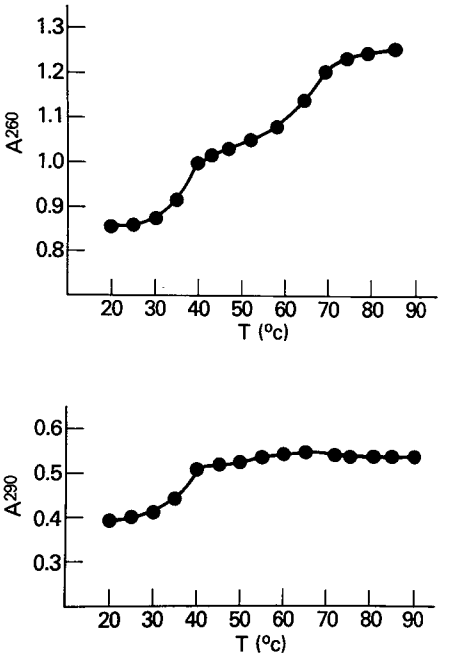


Figure 3.

Top : melting profile for an equimolar mixture of $(aza^2A)n$ + $(br^5U)n$ in Dulbecco's PBS.

Bottom : melting profile for the $(aza^2A)n$ homopolymer in Dulbecco's PBS.

to the melting of the $(aza^2A)n.(br^5U)n$ duplex (or triplex). The biphasic melting behavior of $(aza^2A)n.(br^5U)n$ may suggest that no complex formation with $(br^5U)n$ occurs until the self-structure of $(aza^2A)n$ is destroyed. This possibility may be further examined when greater amounts of $(aza^2A)n$ 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 $(aza^2A)n$- and $(aza^2I)n$-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 $\left[(aza^2A)n.(br^5U)n, (aza^2I)n.(br^5C)n\right]$ failed to trigger an interferon response. The 1:2 mixture of $(aza^2A)n$ 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 $(aza^2I)n.(C)n$ and $(aza^2I)n.(br^5C)n$ were entirely inactive in inducing interferon, they attained the level of activity characteristic for (I)n.(C)n and $(I)n.(br^5C)n$ 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

Mixture*		Interferon titer (units/ml)	
Complex+	Homopolymer	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) (aza^{2}I)n.(C)n + (I)n → (aza^{2}I)n + (I)n.(C)n

ii) (aza^{2}I)n.(br^{5}C)n + (I)n → (aza^{2}I)n + (I)n.(br^{5}C)n

(I)n.(C)n retained its full interferon inducing capacity in the presence of (aza^{2}I)n, suggesting that (aza^{2}I)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, (aza^{2}I)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 (aza^{2}I)n is mixed with (A)n.(U)n, according to the following reaction scheme :

iii) (aza^{2}I)n + (A)n.(U)n → (A)n.(U)n.(aza^{2}I)n

Upon mixing with (A)n, the interferon inducing activity of (aza^{2}A)n.(U)n increased, but not up to the level normally observed for (A)n.(U)n (Table 1). Concomitantly, (aza^{2}A)n caused a partial (∿8-fold) reduction in the interferon response to (A)n.(U)n. According to the Tm rule (13), (aza^{2}A)n should not displace (A)n from its complex with (U)n. How could (aza^{2}A)n inhibit the induction of interferon by (A)n.(U)n ? Theoretically (15), (aza^{2}A)n may inhibit interferon induction through one of the following machanisms : (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 (c^{7}A)n and (c^{7}I)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 (aza^{2}A)n.(br^{5}U)n (Table 1). Whether or not (A)n displaced (aza^{2}A)n from its complex with (br^{5}U)n could not be ascertained from our interferon induction data. Even if (A)n.(br^{5}U)n was formed in the (aza^{2}A)n.(br^{5}U)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 (aza^{2}A)n- and (aza^{2}I)n-derived complexes listed in Table 1, various mixtures of (aza^{2}A)n or (aza^{2}I)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. (Aza^{2}A)n alone and (aza^{2}I)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, (aza^{2}A)n and (aza^{2}I)n differed from (c^{7}A)n and (c^{7}I)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.(br^{5}C)n readily induce interferon titers of 10.000-20.000 units/ml. When assayed under similar conditions, (aza^{2}I)n.(C)n, (aza^{2}I)n.(br^{5}C)n and (aza^{2}A)n.(br^{5}U)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, (aza^{2}I)n also inhibited complement activity, albeit to a lesser extent than (I)n. Even (aza^{2}A)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, (c^{7}I)n, (dUz)n and (ms^{2}I)n on MuLV (Moloney) DNA polymerase activity, both (aza^{2}A)n and (aza^{2}I)n caused a distinct inhibition of DNA synthesis (Fig. 4). (Aza^{2}I)n was more inhibitory than (aza^{2}A)n (Fig. 4A and B). The inhibitory activity of (aza^{2}I)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 ... µg/ml of the polynucleotide)				
	0	10	20	40	100
(A)n	160	160	160	160	160
(aza^2A)n	160	160	160	160	80
(I)n	160	160	80	20-40	10-20
(aza^2I)n	160	160	160	80-160	40-80

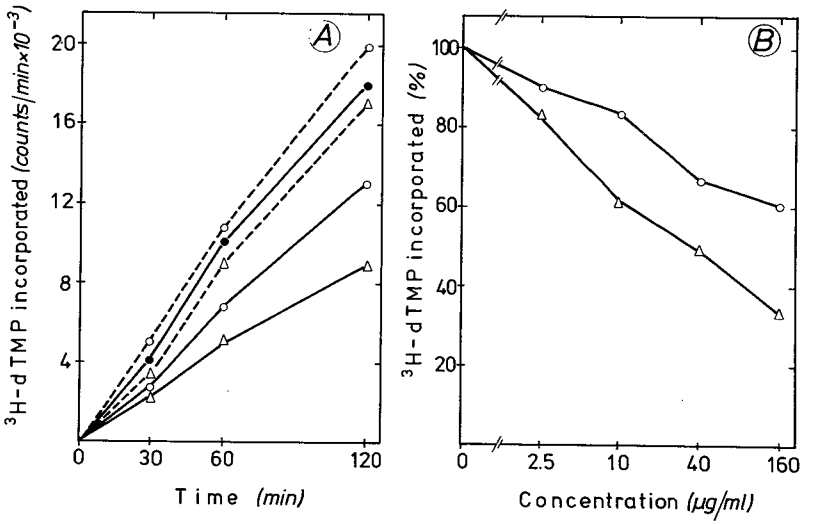


Figure 4. Effect of (aza^2A)n and (aza^2I)n on DNA polymerase activity of MuLV (Moloney). A : DNA synthesis measured at different times of incubation of the assay mixture. Final concentration of the polymers in the assay mixture : 70 µg/ml. B : DNA synthesis measured at different concentrations of the polymers in the assay mixture. Incubation time of the assay mixture : 60 min. ●——● : control; o---o : (A)n; Δ---Δ : (I)n; o——o : (aza^2A)n; Δ——Δ : (aza^2I)n.

template:primer such as poly(A).oligo(dT) (18,19).

The inhibition of MuLV (Moloney) reverse transcriptase activity by (aza^2I)n showed a dose-response relationship (Fig. 4B) which was almost identical to that obtained previously for (ms^2I)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

(Aza^{2}A)n formed a complex with (U)n and (br^{5}U)n, and (aza^{2}I)n formed a complex with (C)n and (br^{5}C)n. Neither of these complexes proved capable of inducing interferon in PRK or HSF cell cultures. The inactivity of the (aza^{2}I)n-derived complexes may be ascribed to the low Tm of these complexes, insuring that they would not remain intact under physiological conditions. The inactivity of the (aza^{2}A)n.(U)n complex (presumably triplex) may be attributed to the low Tm and/or triple-helical structure of the complex. The lack of ability of the (aza^{2}A)n.(br^{5}U)n complex to induce interferon cannot be ascribed to a low Tm. Whether the inactivity of (aza^{2}A)n.(br^{5}U)n may be rationalized by assuming a conformational shift [as postulated for (A)n.(br^{5}U)n (1)] is not clear yet.

The inhibitory effects of (aza^{2}I)n and (aza^{2}A)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 (aza^{2}A)n or (aza^{2}I)n and their complementary polynucleotides are markedly destabilized relative to the parent (A)n or (I)n complexes [by up to 50° for the (aza^{2}I)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 pKa 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.

* Laboratory of Chemistry, National Institute of Arthritis and Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014, USA.

+ Faculty of Pharmaceutical Sciences, Osaka University, 133-1 Yamada-Kami, Suita, Osaka 565, Japan.

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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

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BBA 99249

POLYNUCLEOTIDES

XLIV. SYNTHESIS AND PROPERTIES OF POLY(2-AZAADENYLIC ACID) AND POLY(2-AZAINOSINIC ACID) *

TOSHIKAZU FUKUI, NOBUKO KAKIUCHI and MORIO IKEHARA

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka (Japan)

(Received April 22nd, 1977)
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Summary

Chemically synthesized 2-azaadenosine 5′-diphosphate (n^2ADP) and 2-azainosine 5′-diphosphate (n^2IDP) were polymerized to yield poly(2-azaadenylic acid), poly(n^2A), and poly(2-azainosinic acid), poly(n^2I), using *Escherichia coli* polynucleotide phosphorylase. In neutral solution, poly(n^2A) and poly(n^2I) had hypochromicities of 32 and 5.5%, respectively. Poly(n^2A) 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^2A) formed a 1 : 2 complex with T_m of 41°C at 0.15 M salt concentration. Poly(n^2A) and poly(n^2I) formed three-stranded complexes with poly(I) and poly(A), respectively. Poly(n^2A) · 2poly(I), poly(A) · 2poly(n^2I), and poly(n^2A) · 2poly(n^2I) complexes had T_m values of 23, 48, and 31°C at 0.15 M salt concentration, respectively.

Poly(n^2I) 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^1A [6], c^3A [6], c^7A [7] and c^7I [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 atomes, 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

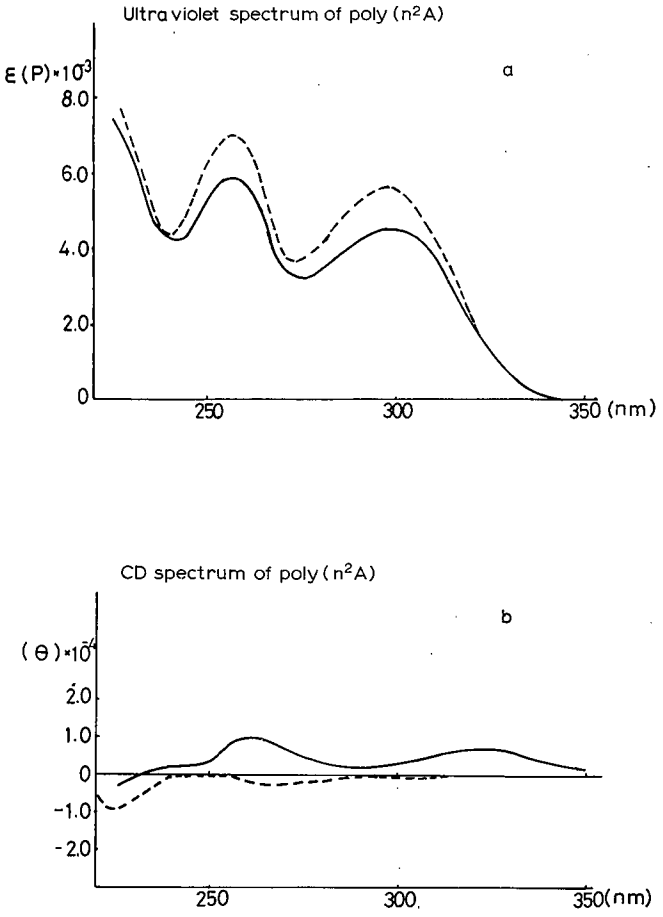

Fig. 1. Ultraviolet absorption and CD spectra of poly(n^2A). ———, poly(n^2A) at 0.1 M Na^+ and 0.05 M sodium cacodylate, pH 7.0; - - - - - -, n^2AMP under the same conditions.

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,

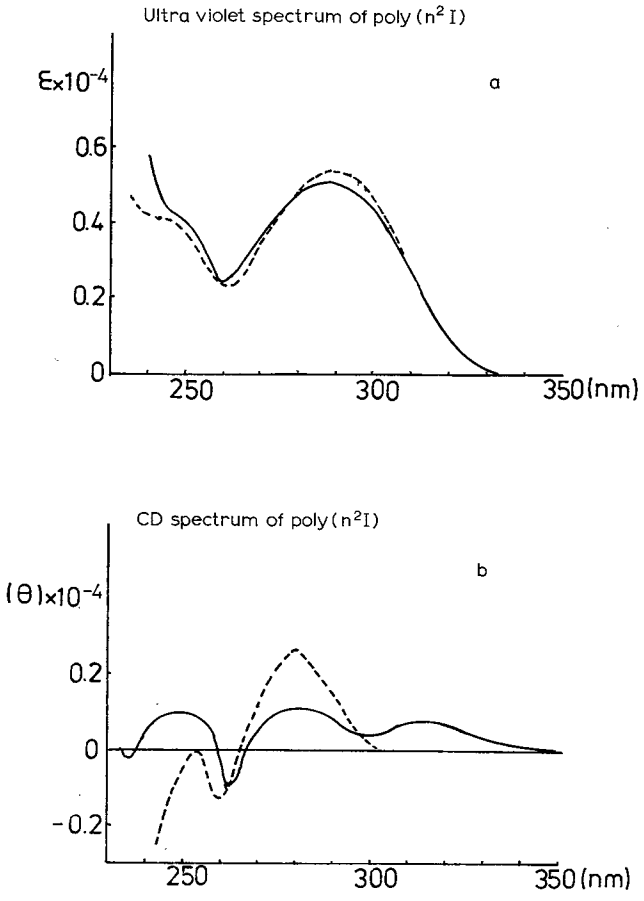

Fig. 2. Ultraviolet absorption and CD spectra of poly(n^2I). ———, poly(n^2I) at 0.1 M Na^+ and 0.05 M sodium cacodylate, pH 7.0; - - - - - -, n^2IMP under the same conditions.

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(n^2A) 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(n^2I) is shown in Fig. 2b. Peaks appeared at 320, 282, and 249 nm, and throughs 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(n^2A) and poly(n^2I) in neutral solution containing 0.15 M Na^+ are shown in Fig. 3. In the case of poly(n^2A), a cooperative melting was observed at about

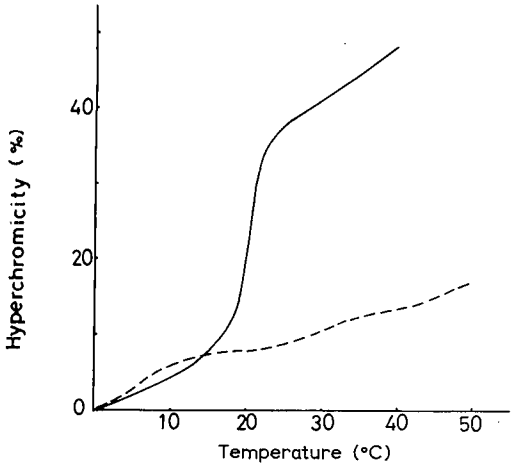

Fig. 3. Thermal melting of poly(n^2A) and poly(n^2I). ———, poly(n^2A) at 0.15 M Na^+ and pH 7.0; - - - - - -, poly(n^2I) under the same conditions.

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 bout 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

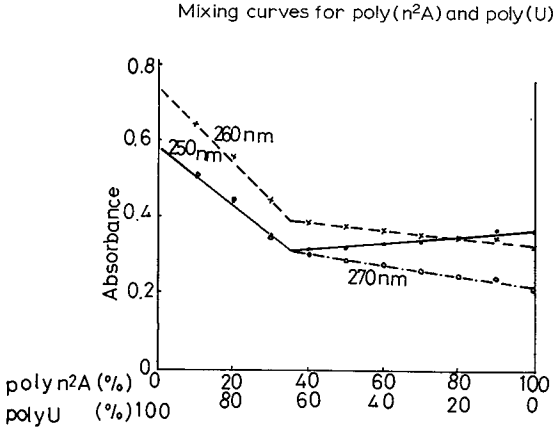

Fig. 4. Mixing curve of poly(n^2A) and poly(U). X- - - - -X, absorbance at 260 nm; ●- - - - -●, 250 nm; o- - - - -o, 270 nm.

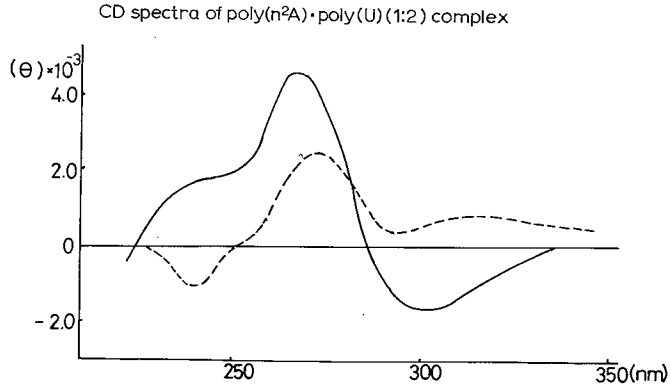

Fig. 5. CD spectra of poly(n^2A)-poly(U) (1 : 2), measured in the presence of 0.15 M Na^+ (pH 7.0). ———, observed curve; - - - - -, calculated curve from CD curves of components.

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.

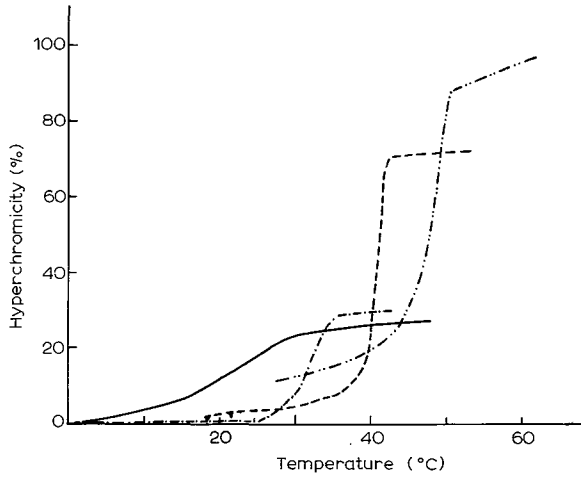

Fig. 6. Thermal melting of poly(n^2A) · poly(U) (1 : 2) complex. Poly(n^2A) · poly(I) (1 : 2) complex, poly(n^2I) · poly(A) (1 : 2) complex and poly(n^2A) · poly(n^2I) (1 : 2) complex. - - - - - -, poly(n^2A)-poly(U), measured at 260 nm; ———, poly(n^2A)-poly(I), measured at 249 nm; — ·· — ·· —, poly(n^2I)-poly(A), 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^2I) 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^2I) and poly(C) could not form the double-stranded complex in the present condition.

Complex formation of poly(n^2A) or poly(A) with poly(I) or poly(n^2I)

It was reported that poly(I) forms a triple-stranded complex with poly(A) [23]. We tested the complex formation of poly(n^2A) with poly(I). Results in the mixing experiment are shown in Fig. 7. This curve clearly showed that poly(n^2A) 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^2A) · 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^2I) to form a complex with poly(A), poly(n^2I) was mixed in various ratios with poly(A) in the presence of 0.15 M

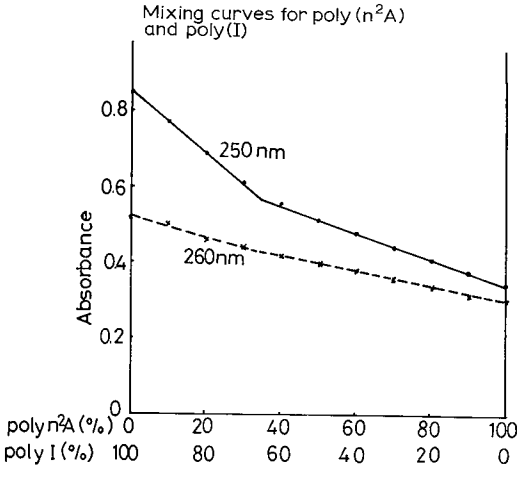

Fig. 7. Mixing curve of poly(n^2A) and poly(I). X- - - - -X, absorbance at 260 nm; ●———●, at 250 nm.

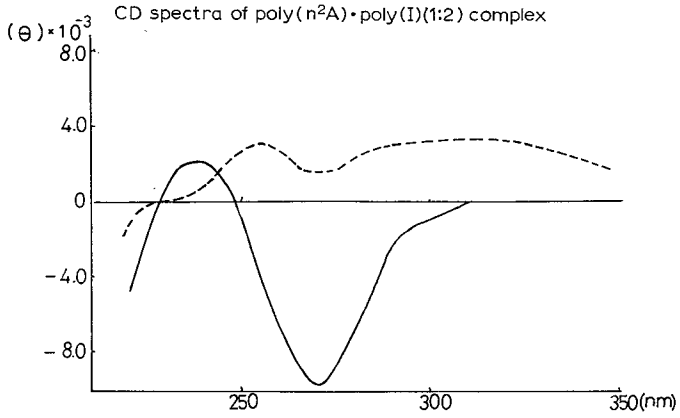

Fig. 8. CD spectra of poly(n^2A) · poly(I) (1 : 2) complex. Measured in the presence of 0.15 M Na^+ (pH 7.0). ———, observed curve; - - - - - -, calculated curve from CD curves of components.

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 poly (n^2I) and poly(A) formed a triple-stranded complex as observed in the case of poly(n^2A) · poly(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 poly(n^2I) and poly(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 poly(A) and poly(I). The temperature-absorption profile of this

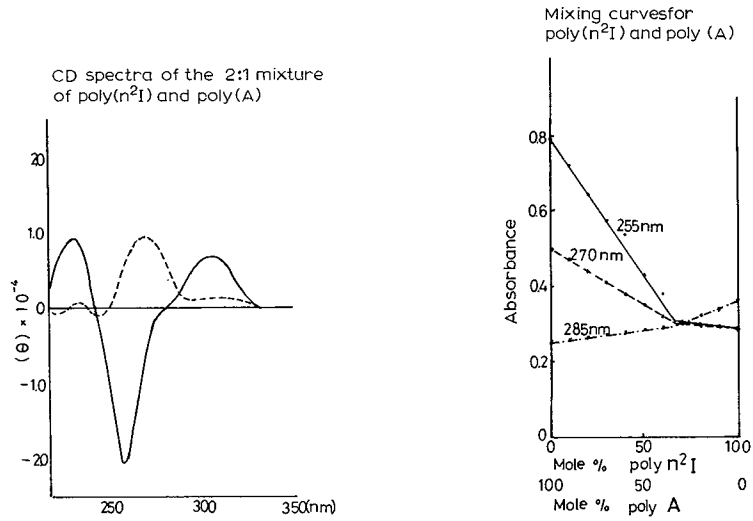

Fig. 9. Mixing curve of poly(n^2I) and poly(A). ●———●, absorbance at 255 nm; ●———●, at 270 nm, X - - - - - - X at 285 nm.

Fig. 10. CD spectra of poly(n^2I) · poly(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.

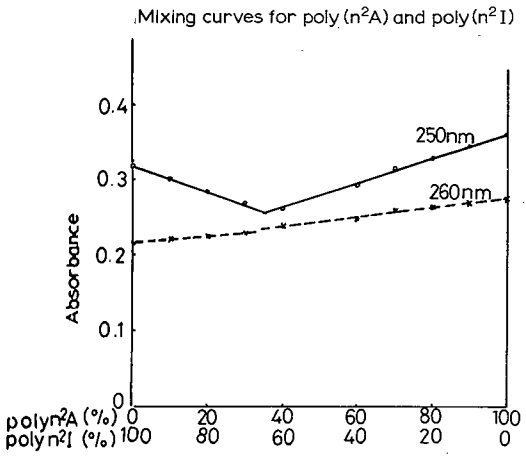

Fig. 11. Mixing curve of poly(n^2A) and poly(n^2I). o———o, absorbance at 250 nm; X- - - - -X, at 260 nm.

complex is shown in Fig. 6, from which the T_m at 0.15 M Na^+ concentration was shown to be 48°C. The T_m of the 2poly(n^2I) · poly(A) complex was 8°C higher than the T_m of 2 poly(I) · 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°C, which is 9°C lower than the T_m of poly(A) · 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) · 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-

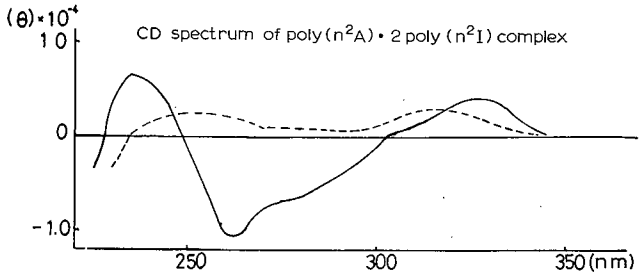

Fig. 12. CD spectra of poly(n^2I) · poly(n^2I) (1 : 2) complex. Measured in the presence of 0.15 M Na^+ (pH 7.0). ———, observed curve; - - - - -, calculated curve from CD curves of components.

zation whereas in the case of the hypoxanthine ring, a stabilizing effect upon replacement of C_2 atom by N_2 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(n^2A) 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 N_2-atom. In the case of poly(n^2I) 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(n^2A) can form triple-stranded complexes with poly(U) or poly(I), and poly(n^2I) can form triple-stranded complexes with poly(A) or poly(n^2A). 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(n^2A) with poly(U) or poly(I) are lower by 17—19°C compared to the corresponding poly(A) · 2poly(U) complex (T_m = 60°C) and poly(A) · 2poly(I) complex (T_m = 40°C).

In contrast to the case of poly(n^2A), poly(n^2I) 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(n^2A) with poly(n^2I), 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 C_2 atom of the adenine ring by an N_2 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, NMe_2, 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(n^2I) can not form a double-stranded complex with poly(C), because of repulsion between the lone pair electrons of the 2-N= of poly(n^2I) and 2-C=O group of poly(C). In addition, the same steric distortion could be involved in the case of the poly(n^2A)-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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