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A Potent Interferon Inducer Derived from Poly(7-deazainosinic acid)†

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ABSTRACT: To determine whether or not purine N-7 of poly(I) plays a significant role in the induction of interferon by poly(I) · poly(C), poly(7-deazainosinic acid)[poly(c^7I)] was prepared by the *Micrococcus luteus* polynucleotide phosphorylase catalyzed polymerization of 7-deazainosine 5′-diphosphate, synthesized from 7-deazainosine (7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ol). Poly(c^7I) was, like poly(I), degraded to the nucleoside or nucleotide level by T_1 ribonuclease, bovine spleen phosphodiesterase, snake venom phosphodiesterase, micrococcal nuclease, and 0.3 N KOH but was totally resistant to degradation by pancreatic ribonuclease A. Unlike poly(I), poly(c^7I) showed little temperature-dependent hyperchromicity in 1.0 M NaCl with an indication of structure only below room temperature. Mixing curves as a function of wavelength, isosbestic points, and sedimentation velocity studies demonstrated that poly(c^7I) forms only 1:1 stoichiometric complexes with both poly(C) and poly(br^5C). Poly(C) · poly(c^7I) had a T_m of 49° (0.2 M NaCl, pH 7) and poly(br^5C) · poly(c^7I) had a T_m of 86° (0.2 M NaCl, pH 7). For comparison purposes, the previously reported poly(br^5C) · poly(I) complex was also prepared. These complexes were evaluated for antiviral activity and interferon inducing ability. With primary rabbit kidney cells, the following sequence (in order of decreasing activity) was established when direct inhibition of vesicular stomatitis virus cytopathogenic effect and interferon production in normal, interferon primed and superinduced (cycloheximide and actinomycin D) rabbit kidney cells were measured: poly(c^7I) · poly(br^5C) > poly(I) · poly(br^5C) > poly(I) · poly(C) > poly(c^7I) · poly(C). On the other hand, if the components of the complexes were administered sequentially followed by measurement of inhibition of virus cytopathogenic effect, the sequence (in order of decreasing activity) changed to: poly(I) · poly(C) > poly(c^7I) · poly(br^5C) > poly(I) · poly(br^5C) > poly(c^7I) · poly(C), if either poly(I) or poly(c^7I) were added to the cells first. If either poly(br^5C) or poly(C) were administered first, the order of decreasing activity was: poly(I) · poly(C) > poly(I) · poly(br^5C) > poly(c^7I) · poly(br^5C) > poly(c^7I) · poly(C).

The synthesis and biological evaluation of a number of modified polynucleotides have rendered possible the definition of several structural features required for an effective interferon inducer (Vilcek *et al.,* 1968; Colby and Chamberlin, 1969; De Clercq *et al.,* 1969, 1970, 1972a, 1974b; Steward *et al.,* 1972; Black *et al.,* 1972; Torrence *et al.,* 1973a,b; De Clercq and Janik, 1973). While a number of modifications have involved the pyrimidine base of poly(I) · poly(C) (Colby and Chamberlin, 1969; De Clercq *et al.,* 1972a; Reuss, K. and Scheit, K. H., personal communication, 1973; Folayan and Hutchinson, 1974; Johnston *et al.,* 1974) or poly(A) · poly(U) (Torrence *et al.,* 1973a; De Clercq *et al.,* 1974b), with one exception (De Clercq *et al.,* 1974), no nuclear modification involving the purine base of either complex has been reported. Since there is evidence to indicate that the purine member of poly(I) · poly(C) may be of greater importance in the induction process (De Clercq and De Somer, 1972; Carter *et al.,* 1972; Mohr *et al.,* 1972; De Clercq *et al.,* 1973), we have initiated an investigation into the effects of such nuclear modifications on the ability of polynucleotides to function as interferon inducers. In this paper, we report the synthesis, physical properties, and biological activity of one such modified polynucleotide in which N-7 of the hypoxanthine base of poly(I)[1]

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[1] Abbreviations for synthetic polynucleotides conform to the recommendations of the IUPAC-IUB Commission ((1970), *Biochemistry 9,* 4025). Thus poly(I) represents poly(inosinic acid), poly(I) · poly(C) represents the two-stranded complex with poly(C), poly(c^7I) is poly(7-deazainosinic acid), poly(c^7A) is poly(7-deazaadenylic acid), etc. Other abbreviations are as follows: T_m, temperature at the midpoint of the absorbancy change; CPE, cytopathogenic effect; PRK cells, primary rabbit kidney cells; VSV, vesicular stomatitis virus; MEM, minimal Eagle's medium; MIC, minimum inhibitory concentration; PFU, plaque forming units.

is replaced by CH; *viz.*, poly(7-deazainosinic acid)[poly-(c^7I)].

After our preliminary report on this system appeared (Waters *et al.*, 1973), another report on the synthesis and properties of poly(c^7I) was published (Ikehara *et al.*, 1974). Where relevant, the results embodied in this latter paper will be related to the present experimental findings.

Materials and Methods

Preparation of 7-Deazainosine 5′-Diphosphate. 7-Deazainosine (I) was prepared from tubercidin by deamination with nitrous acid (Suzuki and Marumo, 1961). After recrystallization from water, I was obtained as pale-yellow needles, mp 245–247°; lit mp 242–243° (Suzuki and Marumo, 1961); $\lambda_{max}(H_2O)$ 259 nm (ϵ 9000); lit (Mizuno *et al.*, 1963) 259 nm (8500).

2′,3′-*O*-ISOPROPYLIDENE-7-DEAZAINOSINE (II). To a suspension of I (440 mg, 1.6 mmol) in acetone (16 ml), 2,2-dimethoxypropane (1.6 ml) and then *p*-toluenesulfonic acid (2.75 g, 16 mmol) were added with constant stirring. The dark brown solution was allowed to react for 17 hr at room temperature. The solution was then cooled to 0° and an ice-cold solution of sodium bicarbonate (2 g) in water (50 ml) was added. The resulting solution was maintained at room temperature for 30 min and then concentrated *in vacuo*. After addition and evaporation of two portions of benzene, the residue was extracted three times with chloroform (50 ml each). Evaporation of the chloroform extracts gave a yellow solid (546 mg) which was crystallized from methanol–ether (5:3). The isopropylidene derivative (II) (287 mg, 58%) was obtained as colorless flakes: mp 195–196°; λ_{max}(MeOH) 258 nm (ϵ 9450). *Anal.* Calcd for $C_{14}H_{17}N_3O_5$: C, 54.72; H, 5.58; N, 13.68. Found: C, 54.83; H, 5.74; N, 13.96.

7-DEAZAINOSINE 5′-MONOPHOSPHATE (III). The isopropylidene derivative, II (307 mg, 1 mmol), was converted to the 5′-monophosphate (III) by a modification of the method of Kochetkov *et al.* (1968). The yield (as barium salt) was 517 mg (82%). It was, except for a trace impurity at the origin, homogeneous by tlc (silica gel GF; 1-butanol–methanol–water–ammonia, 6:2:2:0.1). III had $\lambda_{max}(H_2O)$ 258 nm (ϵ 9500). *Anal.* Calcd for $C_{11}H_{12}N_3O_8PBa \cdot 2H_2O$: P, 5.97. Found: P, 6.24.

7-DEAZAINOSINE 5′-DIPHOSPHATE (IV). The morpholidate method (Moffatt and Khorana, 1961) was employed to convert the monophosphate (III) to the diphosphate (IV). Thus III (134 mg, 0.26 mmol), after conversion to the free acid, was reacted with morpholine and DCC to give the morpholidate which was converted to the diphosphate by mono(tri-*n*-butyl)amine orthophosphate, and the lithium salt of the 5′-diphosphate was isolated after Dowex-1(Cl^-) chromatography (Torrence *et al.*, 1973a). The yield of diphosphate was 33 mg (25%). The product was homogeneous by paper chromatography in two different solvent systems (Whatman No. 3MM) [ethanol–ammonium acetate (pH 7.5) (5:2), $R_{F(IDP)}$ = 1.15; isobutyric acid–1 M ammonium hydroxide–0.2 M EDTA (100:60:0.9), $R_{F(IDP)}$ = 1.10]; $\lambda_{max}(H_2O)$ 258 nm (ϵ 9700). The diphosphate was degraded by bacterial alkaline phosphatase to give 7-deazainosine (I) as the only uv absorbing spot on silica gel tlc (methanol–chloroform). *Anal.* Calcd for $C_{11}H_{12}N_3O_{11}P_2Li_3 \cdot 3H_2O$: N, 8.42; P, 12.40. Found: N, 8.12; P, 12.33.

Preparation of Poly(c^7I). The incubation mixture contained (per ml): 0.1 mmol of Tris (pH 8.8), 15 mol of c^7IDP, 15 μmol of $MgCl_2$, and 10 phosphorolysis units of *M. luteus* polynucleotide phosphorylase. When after about 3 hr of incubation at 37°, phosphate release had reached a maximum (40–45%), the reaction was terminated by cooling to 0°. After dilution with one volume of 0.04 M NH_4HCO_3, the mixture was extracted (six to seven times) with $CHCl_3$–isoamyl alcohol (5:3, v/v) and then with ether (two times). After residual ether was removed by a stream of nitrogen, the deproteinized aqueous layer was applied to a Sephadex G-100 or G-200 column which was eluted with 0.04 M NH_4HCO_3. Poly(c^7I) appeared in the void volume which was lyophilized to dryness. The polymer was taken up in water and dialyzed successively against 2-l. changes of 0.001 M EDTA–0.1 M NaCl (pH 7.0), 1 M NaCl, and exhaustively against distilled water. The aqueous solution was then filtered through a Millipore filter and the filtrate lyophilized. Yields based on starting diphosphate ranged from 30 to 35%.

The extinction coefficient of poly(c^7I) was determined by ashing aliquots from a stock solution followed by determination of inorganic phosphate (Howard *et al.*, 1971). Triplicate analyses gave ϵ_{max} 9430 ± 150 (1 σ) in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0). A value for ϵ_{max} of 5560 (0.15 M Na^+) was obtained by Ikehara *et al.* (1974), but this leads to a hypochromicity which does not melt out even in a buffer of twice the above ionic strength (*cf.* Ikehara *et al.* (1974), Figures 1A and 2).

Other Methodology. Methods employed in the construction of mixing curves, the determination of T_m's, and enzymic degradation have been previously described (Torrence *et al.*, 1973a). Sedimentation constants were determined with a Beckman Model E analytical ultracentrifuge equipped with an AN-G titanium six-hole rotor at 40,000 rpm in 0.15 M NaCl–0.02 M Tris–0.001 M EDTA (pH 7.5) as buffer (except where otherwise indicated). The poly(c^7I) used in the antiviral assays was from two different preparations which had s_{20}'s of 3.7 S and 4.3 S. Poly(br^5C) used in the antiviral assays had s_{20} = 11.5 S.

Evaluation of the Antiviral Activity and Interferon Inducing Activity of Polynucleotides. Complexes were prepared by mixing appropriate stoichiometric quantities of the homopolymers in distilled water. After lyophilization, the resulting solid was dissolved in 0.1 M Tris–0.2 M NaCl (pH 7.0) at a concentration of 1 mg of polymer/ml. The complexes dissolved immediately and were incubated at 25° for 1 hr and then stored at 4° for at least 48 hr. Before antiviral evaluation, they were diluted in MEM (minimal Eagle's essential medium) to 10 μg/ml.

The assay systems and methodology used to evaluate the antiviral and interferon-inducing activities of the polynucleotides were the same as those described previously (De Clercq *et al.*, 1974b).

Poly(5-bromocytidylic acid). 5-Bromocytidine 5′-diphosphate was prepared according to the method of Howard *et al.* (1969) and polymerized by polynucleotide phosphorylase under conditions similar to those used by these authors. The material was purified by the same techniques of deproteinization and gel filtration as described above for poly(c^7I). Poly(br^5C) thus obtained possessed ultraviolet spectral properties which were in excellent agreement with the literature (Howard *et al.*, 1969) and, in addition, could be degraded by venom phosphodiesterase and alkaline phosphatase to give but one uv absorbing product which was indistinguishable from authentic 5-bromocytidine by paper chromatography (descending) in 1-propanol–concentrated

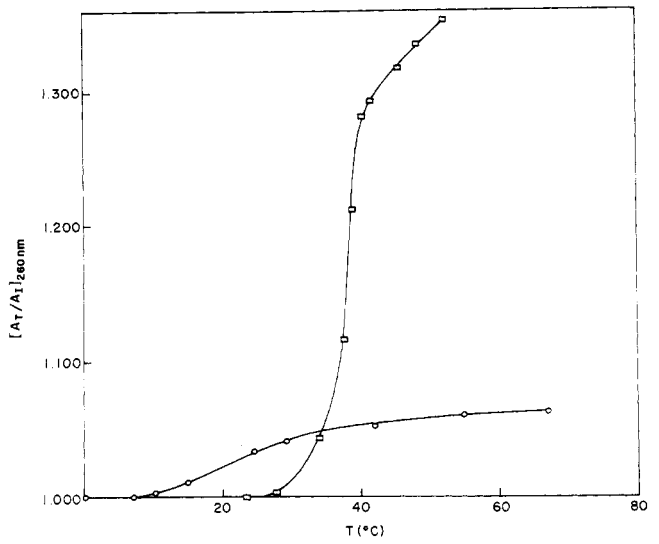

FIGURE 1: Ultraviolet absorbance-temperature profile of poly(c^7I) (○) and poly(I) (□) both in 1.0 M NaCl-0.01 M sodium cacodylate (pH 7.0). A_T/A_1 is the ratio of absorbance at some given temperature, T, to that at the initial temperature.

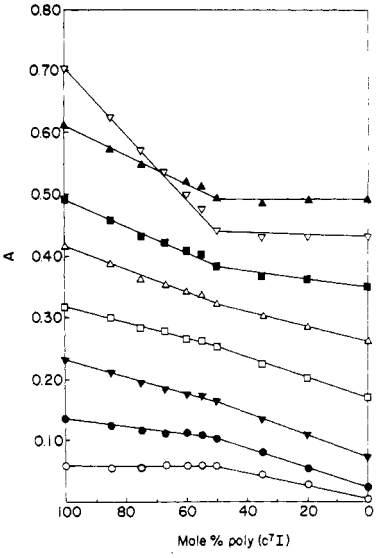

FIGURE 2: Mixing curves as obtained at representative wavelengths for the interaction of poly(C) with poly(c^7I) in 0.2 M NaCl-0.01 M sodium cacodylate (pH 7.0), 22°. The wavelengths illustrated are: 305 nm (○), 300 nm (●), 295 nm (▼), 290 nm (□), 285 nm (△), 280 nm (■), 270 nm (▲), 260 nm (▽).

NH_4OH-water (60:30:10) as developing solvent (Howard *et al.*, 1969). For the determination of concentration, the ϵ_{max} value of 5510 (0.2 M sodium cacodylate, pH 7) determined previously (Howard *et al.*, 1969) was employed. Other polynucleotides were obtained from P-L Biochemicals (Milwaukee, Wis.). Poly(C) had s_{20} = 8.8 S and poly(I) had s_{20} = 9.4 S.

Results

The Action of KOH and Degradative Enzymes on Poly(c^7I). Poly(c^7I) was degraded to mononucleotide by 0.3 N KOH, snake venom phosphodiesterase, bovine spleen phosphodiesterase, T_1 ribonuclease, and micrococcal nuclease but, under the experimental conditions investigated, was totally resistant to pancreatic ribonuclease A. Thus, in at least a qualitative sense, poly(c^7I) exhibits the same substrate behavior toward these enzymes as does poly(I).

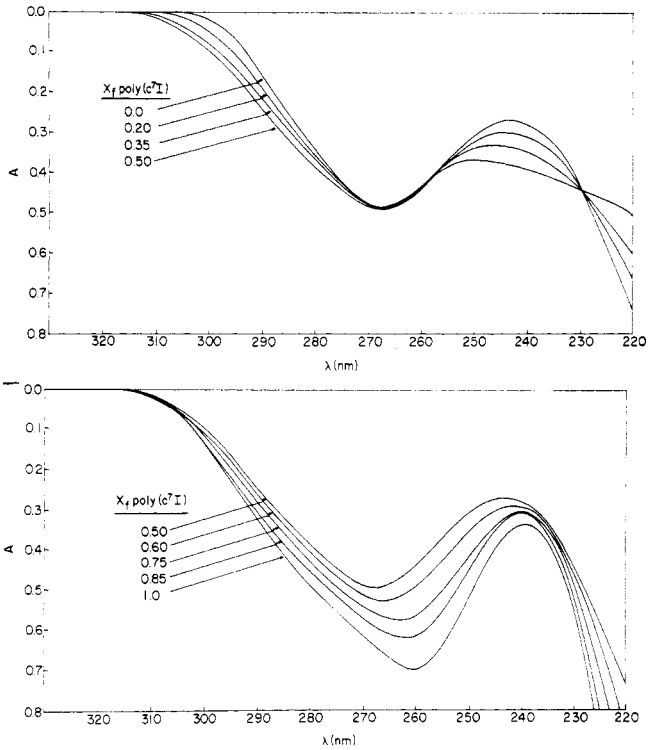

FIGURE 3: Spectra generated from the various solutions used to construct the mixing curve in Figure 2. Upper spectra, 0.0–50 X_f (mole fraction) poly(c^7I); lower spectra, 0.50–1.00 X_f poly(c^7I).

The Homopolymer, Poly(c^7I). While poly(I) forms an ordered structure at relatively low salt concentration (Hinz *et al.*, 1970), there was no evidence for any temperature-dependent ultraviolet spectrum of poly(c^7I) at 0.5 M NaCl. At [Na^+] of 1.0 M, however, a small reversible, yet reproducible, change in absorbance could be seen (Figure 1). The "T_m" for this noncooperative transition was 20–25°. This may represent a salt-induced aggregation since some preparations of poly(c^7I) formed a visible precipitate when aqueous aliquots were introduced into 1 M NaCl solution. The melting behavior of poly(c^7I) contrasts distinctly with that observed for poly(I) under the same conditions; thus, poly(I) melted cooperatively with a T_m of 39°, in reasonable agreement with the literature (Hinz *et al.*, 1970).

Interaction of Poly(c^7I) with Poly(C). Mixing curves were constructed for the interaction of poly(C) and poly(c^7I) by the method of continuous variation (Felsenfeld and Rich, 1957) and techniques outlined previously (Howard *et al.*, 1971; Torrence *et al.*, 1973a). The absorbance (at 290, 280, 270, and 260 nm) of a 50 mol % mixture was determined at 1 hr after mixing and again 23 hr later. Since there was no significant change in absorbance during this time period, it was assumed that equilibrium was achieved in less than 1 hr. When the entire mixing curve was constructed, the solutions were allowed 72 hr after mixing to achieve equilibrium before their spectra were recorded. The results are given in Figure 2 and clearly indicate only one break occurring at 50 mol % poly(^{7}I), corresponding to the formation of a 1:1 poly(C) · poly(c^7I) complex. This conclusion, based on the wavelength dependency of the mixing curve, was confirmed by the existence of isosbestic points arising from the superimposed spectra of solutions derived from the 0–50 mol % poly(c^7I) arm of the mixing curve (Figure 3). This series of spectra possessed three isosbestic points indicative of the presence of but two components,

TABLE I: Sedimentation Velocity Analysis of the Poly(c^7I)–Poly(C) Interaction.[a]

Polynucleotide(s)	Median Sedimentation Velocity[b]
Poly(C)	8.8 S
Poly(c^7I)	4.3 S
Poly(C) + poly(c^7I)[c]	12.8 S
Poly(C) + poly(c^7I)[d]	8.5 S

[a] Determined on a Beckman Model E analytical ultracentrifuge equipped with an AN-G titanium six-hole rotor at 40,000 rpm and a temperature of 20° with 0.20 M NaCl–0.01 M sodium cacodylate (pH 7) buffer. [b] Expressed as Svedbergs. [c] 50 mol % poly(c^7I). [d] 67 mol % poly(c^7I).

namely, poly(C) · poly(c^7I) and free poly(C). The spectra of solutions from the 50–100 mol % poly(c^7I) arm of the mixing curve did not intersect at any wavelength. They cannot, therefore, be employed as evidence regarding the number of species present; nonetheless, those spectra are not inconsistent with the existence of but one complex.

Sedimentation velocity data were also gathered in order to determine the nature of interaction between poly(C) and poly(c^7I). The results of such experiments are summarized in Table I. Typically, the sedimentation profiles indicated the polynucleotide homopolymers were polydisperse; however, the data clearly demonstrate that interaction occurs between poly(C) and poly(c^7I). Significantly, these data also indicated that of a 67 mol % poly(c^7I) mixture (corresponding to a hypothetical poly(C) · 2poly(c^7I) complex) sedimented much slower than the 50 mol % poly(c^7I) mixture. In addition the sedimentation profile for the 67 mol % poly(c^7I) mixture was much more disperse than that of the 50 mol % poly(c^7I) composition. These latter observations were consistent with the conclusion that the 67 mol % poly(c^7I) mixture consisted of poly(C) · poly(c^7I) complex plus free poly(c^7I).

The temperature-absorbance profile for poly(C) · poly(c^7I) (in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7)) is represented in Figure 4. The duplex underwent a comparatively noncooperative, monophasic transition (T_m = 49°) at all wavelengths monitored in reasonable agreement with the T_m reported by Ikehara *et al.* (1974). The monophasic nature of the transition was consistent with the assigned 1:1 stoichiometry, indicating that no rearrangements or disproportionations occurred during melting. For comparison the melting profile of poly(I) · poly(C) was determined under the same conditions and found to be 67°.

Interaction of Poly(c^7I) with Poly(br^5C). Mixing curves, constructed in the same manner as above, revealed that poly(br^5C) also forms a 1:1 stoichiometric complex with poly(c^7I) (Figure 5) with no evidence of any other complex. Furthermore, spectra of the 0–50 mol % poly(c^7I) solutions could be superimposed to give two isosbestic points (281 and 256 nm) indicative of presence of but two components (poly(br^5C) and poly(br^5C) · poly(I)) in that series of mixtures. The spectra of the 50–100 mol % poly(c^7I) solutions also intersected to give two different isosbestic points (285.7 and 240 nm) pointing to the existence of only two components (poly(c^7I) and poly(c^7I) · poly(br^5C)) in that range of composition (data not illustrated).

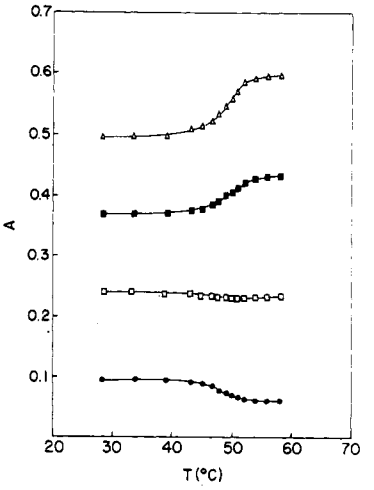

FIGURE 4: Ultraviolet absorbance-temperature profile for poly(c^7I) · poly(C) (50 mol % poly(c^7I)) in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0). Wavelengths represented are: 300 nm (●), 290 nm (□), 280 nm (■), 267 nm (△).

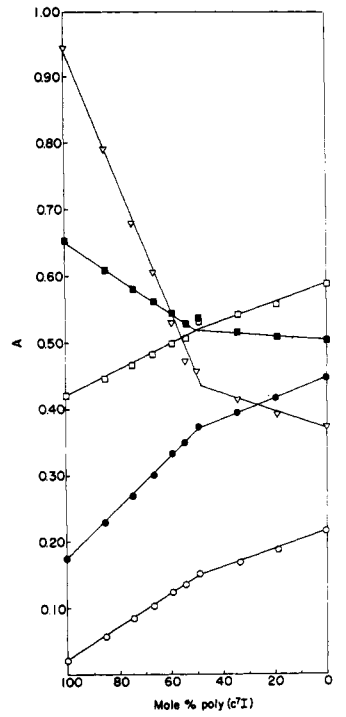

FIGURE 5: Mixing curves (as a function of wavelength) for the interaction of poly(br^5C) with poly(c^7I) in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0), 22°. Representative wavelengths are as follows: 310 nm (○), 300 nm (●), 290 nm (□), 280 nm (■), 260 nm (▽).

Figure 6 presents the melting profile of the 50 mol % poly(c^7I) mixture (0.2 M NaCl–0.01 M sodium cacodylate (pH 7)). Again, at all wavelengths, only one transition with a T_m of 86° can be seen. Thus no rearrangements or disproportionations are involved; the poly(c^7I) · poly(br^5C) duplex melts directly to constituent homopolymers. The melting behavior of poly(I) · poly(br^5C) (T_m = 89°, 0.2 M NaCl–0.01 M sodium cacodylate (pH 7)) was determined for comparison (see also Howard *et al.*, 1969).

Antiviral Activity and Interferon Inducing Ability of Poly(c^7I), Poly(br^5C), and Derived Complexes. POLY(7-DEAZAINOSINIC ACID). The homopolymer poly(c^7I) conferred some antiviral resistance upon cells when those cells

TABLE II: Antiviral Activity and Interferon Inducing Ability of Polynucleotides.[a]

Complex or Homopolymer	Induction of Cellular Resistance to Virus Infection Minimum Inhibitory Concn[c] Experiment			Interferon Production[b]				
				Normal PRK Cells	Interferon-Primed PRK Cells	"Superinduced" PRK Cells Polynucleotide dose[c]		
	I	II	III			0.1	1.0	10
Poly(c-I)	3	10	1–10					<3
Poly(I)	>10	>10	>10					<3
Poly(br^{5}C)	0.3	0.3		15	45			300
Poly(C)	1	3	>10					<3
Poly(c^{7}I)·poly(C)[d]	0.3	0.3	0.3	30	300	30	300	600
Poly(c^{7}I)·poly(br^{5}C)	0.0003	0.0003		300	2000	1000	2000	3000
Poly(I)·poly(br^{5}C)	0.0003	0.0003		150	1500	300	800	3000
Poly(I)·poly(C)[e]	0.003	0.006		100	300	30	300	3000
Poly(I)·poly(C)[f]	0.003	0.003	0.003	200	1000	100	1000	3000
Poly(I)·poly(C)[g]			0.003					

[a] Procedures as described previously (De Clercq *et al.*, 1974). [b] In units/ml. [c] In μg/ml. [d] When a 2:1 mixture of poly(c^{7}I) to poly(C) was evaluated, there was no significant change in either MIC's or interferon titer. [e] Source: constructed from the homopolymers used to prepare the above complexes. [f] Source, P. L. Biochemicals. [g] Source, Miles Laboratories.

were preincubated with the polynucleotide for 20 hr (Table II). Comparable concentrations of poly(c^{7}I), however, failed to induce interferon production in superinduced PRK cell cultures (Table II). No changes in cell morphology were observed with the concentration levels of poly(c^{7}I) that conferred direct antiviral resistance.

POLY(5-BROMOCYTIDYLIC ACID). Poly(br^{5}C) was able to induce antiviral resistance at a concentration (0.3 μg/ml) even lower than that witnessed with poly(c^{7}I) (Table II). In addition, poly(br^{5}C) induced relatively high amounts of interferon both in interferon primed PRK cells (Table IV) and superinduced PRK cells (Table II).

POLY(CYTIDYLIC ACID)·POLY(7-DEAZAINOSINIC ACID). When evaluated by the criterion of induction of cellular resistance to virus infection (Table II), poly(C)·poly(c^{7}I) proved to be 100-fold less active than poly(I)·poly(C). When the components of the complex were administered sequentially, the MIC for the poly(C)·poly(c^{7}I) system remained the same when the components were administered in the order poly(C) followed by poly(c^{7}I) but the MIC decreased by an order of magnitude if the addition was reversed (Table III). When the purine component of poly(I)·poly(C) was added first in these experiments, there was a tenfold decrease in MIC (of the second polymer added) as compared to experiments in which the complex is added as such (Tables II and III, see also De Clercq and De Somer, 1972). A similar increase in activity (decrease of MIC) was noted with the poly(c^{7}I)·poly(C) system upon sequential administration of the homopolymers in the order poly(c^{7}I) followed by poly(C) (Tables II and III).

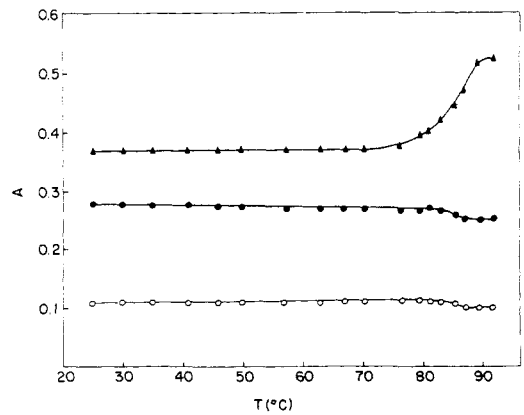

FIGURE 6: Melting profile of poly(br^{5}C)·poly(c^{7}I) in 0.2 M NaCl–0.01 M sodium cacodylate (pH 7.0). Wavelengths monitored are: 310 nm (○), 300 nm (●), 270 nm (▲).

TABLE III: Induction of Cellular Resistance to Virus Infection Complementary Homopolynucleotides Added Sequentially.

Sequence of Addition		Minimum Inhibitory Concn[a] (μg/ml)
First	Second	
Poly(I)	Poly(C)	0.0003 (0.0001)[b]
Poly(I)	Poly(br^{5}C)	0.003 (0.001)[b]
Poly(c^{7}I)	Poly(C)	0.01
Poly(c^{7}I)	Poly(br^{5}C)	0.001
Poly(C)	Poly(I)	0.01 (0.001)[b]
Poly(C)	Poly(c^{7}I)	>0.1 (0.3)[b]
Poly(br^{5}C)	Poly(I)	0.1
Poly(br^{5}C)	Poly(c^{7}I)	>0.1

[a] Refers to the homopolymer that was added second to PRK cell cultures in tubes. The first homopolymer was added at either 1 μg/ml [poly(c^{7}I) and poly(I)] or 0.1 μg/ml [poly(C) and poly(br^{5}C)] in MEM (1 ml/tube) for 1 hr at 37°. The cells were then washed (3 × with MEM) and further incubated with varying concentrations of the second homopolymer (1, 0.1, 0.01 μg/ml) for 20 hr at 37°. The cells were then processed as described in the footnote to Table II. [b] Values in parentheses refer to experiments in which the MIC was determined by adding the first homopolymer at a concentration of 10 μg/ml. This procedure could not be executed with poly(br^{5}C) and poly(c^{7}I) as first polymer becase of their inherent activity (MIC of 0.3 and 3 μg/ml, respectively) (see Table II).

Poly(C) · poly(c^7I) was also evaluated by its ability to induce interferon production in normal, interferon primed, and superinduced PRK cell cultures (Table II). In all three assay systems, poly(C) · poly(c^7I) led to the production of three to six times less interferon than did poly(I) · poly(C) (source: P. L. Biochemicals). In certain experiments, however, the modified polymer was as effective as the poly(I) · poly(C) preparation originating from the homopolymers used in the preparation of the modified complexes.

POLY(5-BROMOCYTIDYLIC ACID) · POLY(7-DEAZAINOSINIC ACID). In all assay systems (Table II), save that of the sequential administration experiments (Table III), poly(br^5C) · poly(c^7I) proved to be more active than poly(I) · poly(C). When the complexes were administered as such (Table II), poly(br^5C) · poly(c^7I) had a MIC of 0.0003 μg/ml as compared to a MIC of 0.003 μg/ml for poly(I) · poly(C). At concentrations of 10 μg/ml, poly(br^5C) · poly(c^7I) gave rise to an interferon titer of 2000 units/ml compared to 1000 and 300 units/ml for poly(I) · poly(C) in interferon primed PRK cells (Table II). In superinduced PRK cell cultures (Table II), poly(br^5C) · poly(c^7I) produced 10–30-fold more interferon at 0.1 μg/ml than did poly(I) · poly(C). As the concentration of inducing polymer was increased, this difference was reduced until at 10 μg/ml, both poly(br^5C) · poly(c^7I) and poly(I) · poly(C) led to equivalent amounts of interferon.

Different behavior is obvious in the sequential addition experiments (Table III). In contrast to the decreased MIC witnessed when poly(I) is given followed by poly(C), administration of poly(c^7I) followed by poly(br^5C) led to a small increase in MIC. Even though a tenfold increase in MIC was usually seen when poly(C) was given followed by poly(I), addition of poly(br^5C) followed by poly(c^7I) led to a much greater increase in MIC ($\geq$1000 ×) compared to that seen when the complex is administered as such.

POLY(5-BROMOCYTIDYLIC ACID · POLY(INOSINIC ACID). In most experiments (Tables II and III), poly(br^5C) · poly(I) exhibited a behavior intermediary between poly(I) · poly(C) and poly(br^5C) · poly(c^7I). In ability to induce antiviral resistance (Table II), it was equally effective as poly(c^7I) · poly(br^5C) and ten times more effective than poly(I) · poly(C). In interferon primed PRK cells, it led to 1500 units/ml of interferon compared to 2000 units/ml for poly(br^5C) · poly(c^7I) and 300–1000 units/ml for poly(I) · poly(C) (Table II). In superinduced PRK cells, at low concentration (0.1 μg/ml), poly(I) · poly(br^5C) gave rise to 3–10 times more interferon than did poly(I) · poly(C) but significantly less than did poly(c^7I) · poly(br^5C) (Table II). As in the case of poly(c^7I) · poly(br^5C), the differences disappeared at higher concentration (10 μg/ml) of inducer.

The behavior of poly(br^5C) · poly(I) in the sequential addition experiments (Table III) was similar to that of the poly(br^5C) · poly(c^7I) system; thus, poly(I) followed by poly(br^5C) led to an increase in MIC (relative to administration of the intact complex) as did the addition of poly(br^5C) followed by poly(I).

Competition Experiments. While the homopolymers, poly(I) and poly(C), did not lead to a significant reduction in interferon titer deriving from poly(I) · poly(C), both poly(c^7I) and its complex with poly(C) brought about an equally small reduction (Table IV).

TABLE IV: Competition of Polynucleotides with Poly(I)·Poly(C) As Monitored by Interferon Production in PRK Cell Cultures Superinduced with Cycloheximide and Actinomycin D.[a]

Inactive Polynucleotide	Active Polynucleotide	Interferon Titer (units/ml)	Decrease in Interferon Titer
Poly(I)		$\leq$3	
Poly(C)		$\leq$3	
Poly(c^7I)		$\leq$3	
	Poly(I)·poly(C)	6000	
Poly(c^7I)·poly(C)		600	
Poly(I)	Poly(I)·poly(C)	6000	×1.0
Poly(C)	Poly(I)·poly(C)	4800	×1.2
Poly(c^7I)	Poly(I)·poly(C)	2000	×3
Poly(c^7I)·poly(C)	Poly(I)·poly(C)	2000	×3

[a] PRK cell cultures were exposed to 10 μg/ml of the inactive polynucleotide in MEM (1 ml/petri dish) for 1 hr at 37°, washed (three times with MEM) and immediately thereafter exposed to 10 μg/ml of the active polynucleotide in MEM (1 ml/petri dish) for another hr at 37°, washed (three times with MEM) and then processed as explained in the footnote to Table II.

Discussion

While in a qualitative sense, poly(c^7I) paralleled poly(I) with respect to its ability to act as a substrate for a number of degradative enzymes, its behavior became radically different from that of poly(I) in its ability to form an ordered secondary structure. Poly(c^7I) in 1 M sodium chloride does not form an ordered structure similar to poly(I) (Figure 1). This conclusion is based on the assumption that ultraviolet hyperchromicity is, in this instance, an accurate measure of the extent of ordered structure. Such an assumption may not be justified in those cases in which a chromophore from a significantly altered heterocyclic base is involved [although poly(c^7A) exhibits uv spectroscopic behavior more nearly related to poly(A) (Ikehara and Fukui, 1968; P. F. Torrence and B. Witkop, unpublished observations)]. It appears that replacement of N-7 by CH in poly(I) leads to a polynucleotide which has some ordered structure only at temperatures below 10° and which melts out very noncooperatively. This ordered structure, formed by poly(c^7I), seems significantly different from that formed by poly(I). This behavior is consistent with the notion that poly(I) in 1.0 M sodium chloride may exist as a four-stranded left-handed helix with hydrogen bonding between N^1 and N^7 of neighboring hypoxanthine bases (Thiele and Guschlbauer, 1973). Ikehara *et al.* (1974) reached a contrary conclusion that N^7 of the hypoxanthine ring is not required for the hydrogen bonding of the ordered structure of poly(I) since they observed that a poly(c^7I) underwent cooperative hyperchromic transition at 10°. We have already commented upon the tendency of poly(c^7I) to form a precipitate at low temperature and high ionic strength (*cf.* Results).

Initial mixing experiments led us to believe that poly(c^7I) interacted in a 2:1 stoichiometric ratio with poly(C) (Waters *et al.*, 1973); however, there is now no doubt that poly(c^7I) forms only a 1:1 complex with poly(C). The evidence for this conclusion is summarized as follows: (i) Mixing curves (Figure 2) constructed at over a dozen widely spaced wavelengths reveal but one break at 50 mol % poly(c^7I). (ii)

Isosbestic points (Figure 3) are consistent with the existence of but one complex in mixtures of poly(c^7I) and poly(C). (iii) Sedimentation velocity data (Table I) show that a 67 mol % poly(c^7I) mixture sediments more slowly and is more polydisperse than a 50 mol % poly(c^7I) mixture. (iv) The melting profile (Figure 4) of the 50 mol % poly(c^7I) mixture is monophasic and shows no evidence for rearrangements. Furthermore, the 67 mol % poly(c^7I) mixture gives the same T_m as the 50 mol % mixture with no evidence for rearrangement. (v) When poly(c^7I) · poly(C) was evaluated as an interferon inducer in the presence of an additional mole of poly(c^7I), there was no apparent change in the MIC or interferon titer compared to the 1:1 complex (Tables II–IV). Since triple strands are always less effective than their double-stranded counterparts (De Clercq *et al.*, 1974), a significant drop in activity would have occurred if poly(C) · 2 poly(c^7I) were formed.

Similarly, mixing curves (Figure 5), isosbestic points, and melting profile (Figure 6) are consistent with the existence of but one complex in the interaction of poly(c^7I) with poly(br^5C); namely, poly(br^5C) · poly(c^7I). Ikehara *et al.* (1974) also concluded on the basis of a cursory examination of the mixing curve that a 1:1 complex between poly(C) and poly(c^7I) was formed. Their experimental methodology did not, however, permit any conclusions to be drawn concerning the existence of other stoichiometric complexes.

Although triple-stranded structures have been found in other hypoxanthine–cytosine derived interactions [*e.g.*, the interaction of poly(dI) with poly(dC), poly(rC), poly(dbr^5C), or poly(dm^{5}C) (Inman, 1964; Chamberlin and Patterson, 1965; Zmudzka *et al.*, 1969)], as is the case with poly(I) · poly(C) (Michelson *et al.*, 1967) and poly(I) · poly(br^5C) (Howard *et al.*, 1969), no triple-stranded structures are formed in the interaction of poly(c^7I) with poly(C) or poly(br^5C). Thus, interpretation of the biological activity of such complexes is not complicated by the occurrence of a possible rearrangement from two- to three-stranded structures (De Clercq *et al.*, 1974b).

The homopolymers and derived complexes described herein were evaluated for antiviral activity by four different assays in PRK *vs.* VSV: (a) inhibition of viral CPE in cells exposed to the homopolynucleotides or the complexes thereof; (b) inhibition of viral CPE in cells exposed to components of the complexes in sequential order (De Clercq and De Somer, 1972); (c) interferon production in normal cells and in cells treated with homologous interferon prior to exposure of the cells to the complexes (priming) (Stewart *et al.*, 1971, 1972; Margolis *et al.*, 1972; De Clercq *et al.*, 1973); (d) interferon production in cells treated with cycloheximide and actinomycin D after exposure of the cells to the polynucleotide complex (superinduction) (Vilcek, 1970; Tan *et al.*, 1970, 1971; Vilcek and Ng, 1971).

According to the results obtained in assays a, c, and d (Tables II–IV), the complexes evaluated can be arranged as follows (in order of decreasing activity): poly(c^7I) · poly(br^5C) > poly(I) · poly(br^5C) > poly(I) · poly(C) > poly(c^7I) · poly(C). It is clear that the brominated polymers are consistently more active than their unhalogenated counterparts although the difference is greatest in the case in which poly(c^7I) is the complementary homopolymer. These results contrast sharply with those obtained in the analogous poly(7-deazaadenylic acid) system (De Clercq *et al.*, 1974b). In this instance, the order of activity (again with the use of assays a, c, and d) was: poly(I) · poly(C) > poly(A) · poly(rT) > poly(A) · poly(U) > poly(c^7A) · poly(U), poly(c^7A) · poly(rT), poly(c^7A) · poly(br^5U). Compared to the results obtained herein, the poly(c^7A) complexes are 10^3–10^5 times less active than the poly(c^7I) complexes in inducing interferon.

What factors are responsible for (i) the significant differences in activity among the poly(I) · poly(C) analogs themselves and (ii) the profound differences in activity between the poly(c^7I) derived complexes and those derived from poly(c^7A)? In some aspects, the differences in activity among the poly(I) · poly(C) analogs could be related to differences in thermal stability: the analog with the lowest T_m, poly(C) · poly(c^7I) (T_m = 49°), was also the least effective as demonstrated in all assay systems (Tables II and III). The greater activity of poly(br^5C) · poly(c^7I) and poly(br^5C) · poly(I) as compared to poly(I) · poly(C) in assays a, c, and d may also be related to their high T_m values. However, melting temperature cannot be held responsible for the differences in activity between the poly(c^7I) derived and the poly(c^7A) derived complexes since it has been previously demonstrated that the poly(c^7A) · poly(rT) and poly(c^7A) · poly(br^5U) systems have T_m's sufficiently high to classify them as potentially active inducers (De Clercq *et al.*, 1974b). In addition, any relation of T_m and interferon inducing ability may be due to the more facile degradation of the lower melting duplexes by cellular nucleases (which may be exuded into the extracellular medium). In support of this, pancreatic ribonuclease was found to degrade the poly(c^7A) · poly(U) complex (T_m = 37°, 0.15 M salt) at a much faster rate than the poly(c^7A) · poly(br^5U) complex (T_m = 72°, 0.15 M salt) (P. F. Torrence and B. Witkop, unpublished observations). In this view, poly(c^7I) · poly(C) (T_m = 49°) is less active than poly(c^7I) · poly(br^5C) (T_m = 86°) because it may not be delivered to the cellular receptor site as efficiently as the latter complex.

Degradation by nucleases cannot, however, be the sole explanation for the relative biological behavior of such complexes. In point of fact, poly(c^7A) · poly(br^5U), inactive as an interferon inducer (De Clercq *et al.*, 1974b), is significantly more resistant to degradation by ribonuclease than are the active inducers, poly(I) · poly(C) and poly(A) · poly(U) (P. F. Torrence and B. Witkop, unpublished observations). That degradation by nucleases may not even be a satisfactory explanation is indicated by the behavior of the poly(I) · poly(C) analogs at low dosages in the superinduction experiments (Table II). At a concentration at which nuclease degradation would be relatively more important (0.1 μg/ml), poly(C) · poly(c^7I) induces interferon production at levels comparable to that induced by the same dose of poly(I) · poly(C).

An alternate, more satisfactory, explanation may lie in the conformations possessed (or achieved) by the various analogs. We suggested (De Clercq *et al.*, 1974b) that the lack of activity of poly(c^7A) duplexes may be due to the existence of a cellular receptor site for N-7 of the purine or, more likely, that introduction of CH in place of N-7 in such poly(c^7A) duplexes led to a significant conformational change which, although allowing binding to the receptor site for interferon inducers, did not permit the optimum interaction required to trigger the message for interferon production. The results presented in this paper show that no such receptor site for purine N-7 exists, since the poly(c^7I) complexes are both quite active inducers. Circular dichroism studies of the poly(c^7A) complexes and their poly(A) counterparts indicate that significant conformational differences probably do exist (A. M. Bobst, P. F. Tor-

rence and B. Witkop, unpublished observations). It is quite possible, therefore, that much of the observed differences in activity of the poly(I) · poly(C) analogs themselves and also between the poly(c^7I) duplexes and poly(c^7A) duplexes may be interpretable in terms of conformational properties which allow for varying degrees of interaction with the receptor site (both in the sense of binding and of triggering).

In this respect, it is of interest that while the T_m of the poly(c^7I) · poly(c) complex is destabilized by about 20° compared to its parent poly(I) · poly(C), the T_m of poly(c^7I) · poly(br^5C) is nearly (±2°) the same as that of poly(I) · poly(br^5C). To the extent that T_m may reflect conformation, introduction of bromine may overcome the conformational restraints imposed by substitution of CH for N-7 of the hypoxanthine ring.

Within the tenor of this discussion, the data presented in Table IV became of interest. As in previous studies (De Clercq *et al.*, 1974a,b), single-stranded polymers failed to reduce significantly the interferon titer of an active interferon inducer (poly(I) · poly(C)); however, in contrast to other double-stranded complexes, poly(C) · poly(c^7I) did not significantly reduce the interferon titer of poly(I) · poly(C), an implication that the modified complex could not effectively compete with the binding of poly(I) · poly(C) to the receptor site for interferon induction. These competition studies were carried out with poly(I) · poly(C) as the active inducer whereas the previous studies employed other active inducers. If poly(I) · poly(C) does not possess a much higher affinity for the receptor site than do the inducers previously employed (De Clercq *et al.*, 1974b), then it is possible that some separation of two of the variables (De Clercq *et al.*, 1974b) (binding and triggering) involved in determining the relative activity of interferon inducers is being witnessed. Compared to poly(I) · poly(C), poly(c^7I) · poly(C) may possess a lower affinity for the receptor site and thus be easily displaced. At the same time poly(c^7I) · poly(C) may possess (or be able to achieve) a conformational (or structural) property which results in the triggering of the message for interferon production. The poly(c^7A) duplexes (De Clercq *et al.*, 1974b), on the other hand, may bind more effectively to the receptor site but fail to trigger the necessary message because of a lack of (or inability to achieve) the required conformational (or structural) property.

The results of the sequential administration experiments (Table III) (De Clercq and De Somer, 1972) are in contrast to experiments in which the complexes were evaluated as such. When the complexes are listed in order of decreasing activity, if either poly(I) or poly(c^7I) were added first, the following sequence emerges: poly(I) · poly(C) > poly(c^7I) · poly(br^5C) > poly(I) · poly(br^5C) > poly(c^7I) · poly(C). If poly(C) or poly(br^5C) were added first (again in order of decreasing activity): poly(I) · poly(C) > poly(I) · poly(br^5C) > poly(c^7I) · poly(br^5C) > poly(c^7I) · poly(C). Thus, poly(c^7I) · poly(br^5C) > poly(I) · poly(C) when the complexes are added as such but poly(I) · poly(C) > poly(c^7I) · poly(br^5C) when the homopolymer components are added sequentially. It appears, therefore, that the activity of poly(c^7I) · poly(br^5C) is only partially reconstituted if the homopolymers are added separately, in contrast with the activity of poly(I) · poly(C) which can be surpassed when the homopolymers are added separately (Table III, see also De Clercq and De Somer, 1972). No simple explanation is forthcoming for these effects. Yet it is quite likely that the factors that govern the activity of a polynucleotide duplex administered to the cells as such or by sequential administration of its components are profoundly different. There is little doubt that degradation by nucleases plays a more important role in the sequential administration experiments. Furthermore, it is certain that the parameters involved in binding of the polymer to the cell are different in the two instances since in the sequential administration experiments, only a single-stranded polynucleotide is involved. It is even possible that these single strands may be bound to sites differing from those of their double-stranded counterparts. Such homopolymers may, for instance, bind nonspecifically to the cell and later, upon addition of a complementary homopolymer, migrate to the site to which interferon inducers are bound.

A final point regards the antiviral activity of the poly(c^7I) and poly(br^5C) homopolymers. While some marginal antiviral activity and/or interferon inducing activity has been observed with certain preparations of single homopolymers (De Clercq and Merigan, 1969; Baron *et al.*, 1969; Black *et al.*, 1972), some polymers employed in this and a previous study (De Clercq *et al.*, 1974b) [poly(c^7A), poly(c^7I), poly(br^5C)] proved remarkably active in inducing interferon and/or cellular resistance to virus infection. Poly(c^7A) and poly(c^7I) induced cellular resistance to virus infection at relatively low doses [0.1–1 and 1–10 μg/ml, respectively [De Clercq *et al.*, 1974b, Table IV, and this report (Table II)]; they failed to induce interferon, even in superinduced PRK cells. Thus, their antiviral activity cannot be ascribed to the interferon system. In fact, recent studies (E. De Clercq, P. F. Torrence, J. A. Waters, and B. Witkop, unpublished observations) have demonstrated that these homopolymers (poly(c^7A) and poly(c^7I)) inhibit host cell RNA synthesis at concentrations comparable to those at which they exhibit antiviral activity. On the other hand, poly(br^5C) does induce resistance to virus infection (Table II) and interferon as well (Table II). It remains to be determined whether or not this is the sole mechanism underlying its antiviral effectiveness.

Conclusions

The basis for our investigations and those of others on the polynucleotide structural parameters influencing interferon induction has resided in the concept that if a cellular receptor site for interferon inducers exists, then definition of the factors determining interaction of a nucleic acid with this site should permit the design of molecules with enhanced affinity for this hypothetical site, resulting in a highly active inducer. Although a number of originally promising polynucleotide modifications have been executed, poly(I) · poly(C) remained the *primus inter pares*. The results presented in this paper, however, at least partially reinforce the validity of the above concept since two modified polynucleotides (poly(I) · poly(br^5C) and poly(c^7I) · poly(br^5C)) proved clearly more active in providing antiviral resistance and inducing interferon in PRK cells than is poly(I) · poly(C). While this result stems in part from a semiempirical approach to the problem and while we cannot yet state unequivocally what structural or conformational parameters are required for maximum interaction with the receptor site, a continued application of this approach should result in a better definition of both the polynucleotide features and the cellular processes involved in the induction of interferon.

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