STUDIES ON POLYRIBONUCLEOTIDES. SYNTHESIS OF A POLYINOSINIC: 6-THIOINOSINIC ACID COPOLYMER

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Summary

The enzymatic synthesis of a high molecular weight ($s_{20,w}$ ~10) copolymer of inosinic and 6-thioinosinic acids [poly(I:s^6I)] has been achieved. Poly (I:s^6I) forms a double stranded complex with poly C which has a Tm significantly lower than a poly I • poly C complex of equivalent molecular weight.

A great deal of interest in double-stranded RNAs has been stimulated by demonstrations of antiviral and antitumor activity of polyinosinic: polycytidylic (poly I • poly C) homopolymer duplexes.[1] Despite the intensive efforts of the past few years, however, the exact structural requirements responsible for these biological activities are still unclear.

The limited number of studies hitherto reported involving polynucleotides containing thio rather than the normal oxo functions in the base moieties are difficult to interpret in terms of the effect of such substitution on helix stability. Thus, a recent report describing incorporation of 6-thiodeoxyguanosine and 6-thiodeoxyinosine into various deoxynucleotide copolymers led the authors to conclude that a marked destabilization of the resulting complementary duplexes took place.[2] A study of complex formation between poly A and a copolymer containing uridine and 4-thiouridine, on the other hand, suggested that substitution of sulfur for oxygen in this system did not appreciably alter the stability of the poly A • poly (U:s^4U) duplex relative to poly A • poly U.[3] Finally, the complex poly (s^2C) • poly I (where s^2C = 2-thiocytidylate) was shown to form a far more stable double helix than poly I : poly C.[4]

Our initial approach to the ultimate clarification of these ambiguities lies in preparing polyribonucleotides modified by making a single specific alteration in one of the structural parameters. The enzymatic synthesis and characterization of poly I modified by the incorporation of 6-thioinosine (s^6I) residues into a copolymer (poly I : s^6I) and the preparation of a double-stranded complex between poly (I : s^6I) and poly C form the basis of this report.

In an earlier attempt to prepare poly (s^6I) by the polynucleotide phosphorylase (PNPase) catalyzed polymerization of s^6IDP, it was reported that s^6IDP was not a substrate for the enzyme even at "high concentrations" (otherwise unspecified) of the enzyme.[5] It was further reported that s^6IDP at a concentration of 3.34×10^{-4} M completely blocked the polymerization of ADP (1.67×10^{-3} M).[5] The enzyme used in the above study was that from M. luteus. A subsequent study using the E. coli enzyme gave qualitatively similar results, the primary differences being that the E. coli enzyme appeared to be somewhat less sensitive to the inhibitory effects of s^6IDP than the M. luteus enzyme and that an unspecified (but presumably small) level of incorporation of s^6IDP into poly A was observed.[6]

Preliminary evaluation of the copolymerization of IDP and s^6IDP by M. luteus PNPase revealed that, while the reaction was slower than for IDP alone, polymer was formed and some incorporation of s^6I was obtained. A typical reaction profile is illustrated in Figure 1. Although the reaction forming poly (I : s^6I) proceeds more slowly than the poly I reaction and with a lower total liberation of P_i (estimated according to Thang and Grunberg-Manago[7]), it is nonetheless clear that s^6IDP, even in proportions equimolar with IDP, does not prevent polymerization.

A number of studies were carried out to find conditions which would premit us to obtain a reasonable yield of polynucleotide using a relatively short incu-

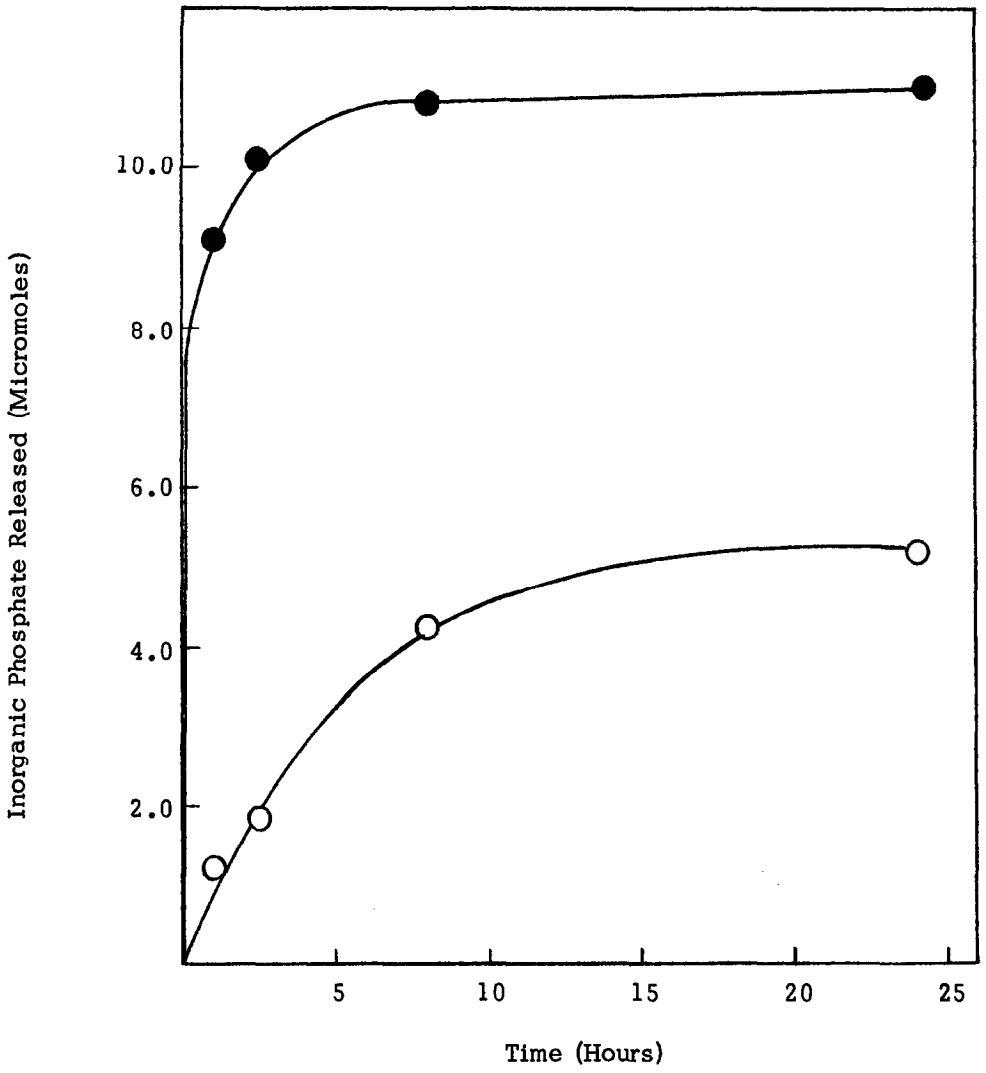

Figure 1. Polymerization of IDP (●) and IDP + s^6IDP (O). Reaction mixtures (1.0 ml) contained 0.2 M Tris (pH 9.0), 7.5 mM $MgCl_2$, 0.5 mM EDTA, 20.0 mM IDP (●) or 10.0 mN IDP + 10.0 mM s^6IDP (O), and 1.0 mg polynucleotide phosphorylase (M. luteus, specific activity = 0.94 polymerization units/mg). The pH of the solution was adjusted to 9.0 by the addition of 1.0 M NaOH (25 λ). Following incubation at 37°, 0.1 ml aliquots were removed and assayed for inorganic phosphate.

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bation time. It was found that this could be accomplished only by using a high concentration of enzyme. Thus, the final reaction conditions selected are shown in Table 1. The incubation period was 5 hours at 37°. Protein was re-

TABLE 1

Reaction Conditions for the Copolymerization of IDP and s^6IDP

	Volume Added	Final Conc.
TRIS · HCl buffer, pH 9.0 (1M)	1.400 ml	0.2 M
$MgCl_2$ (0.1M)	0.525	7.5 mM
EDTA (0.01 M)	0.350	0.5 mM
NaOH (1M)*	0.175	
H_2O	1.400	
IDP (0.2M)	0.280	8.0 mM
s^6IDP (0.2M)	0.420	12.0 mM
PNPase (20 mg/ml, 0.94 units/ml)	2.450	7.0 mg/ml
	7.000 ml	

*Added to achieve a final pH of 9.0.

moved by phenol extraction. Excess phenol was removed by extraction with ether. The resulting solution was dialyzed against 500 ml of 0.1 M NaCl and then against 20 l of distilled water at 5°C. The solution was then frozen and lyophylized. The yield from this reaction is 9.5 - 10 mg (14-15% based on total starting nucleotide). The $s_{20,w}$ value for poly (I:s^6I) was obtained from sucrose gradient ultracentrifugation (Spinco Model L) by comparison with standard samples of poly I graded according to $s_{20,w}$ by P-L Laboratories. The $s_{20,w}$ values obtained were consistently around 10 showing that a high molecular weight polymer was indeed formed. Estimation of base ratios was conveniently accomplished by uv spectrophotometry as described by Scheit[8], using ratios of the mononucleotide extinction coefficients ($\epsilon^{IMP}_{max} = 12.1 \times 10^3$; $\epsilon^{s^6IMP}_{max} = 22.6 \times 10^3$). The wavelength maxima for IMP (248 nm) and s^6IMP

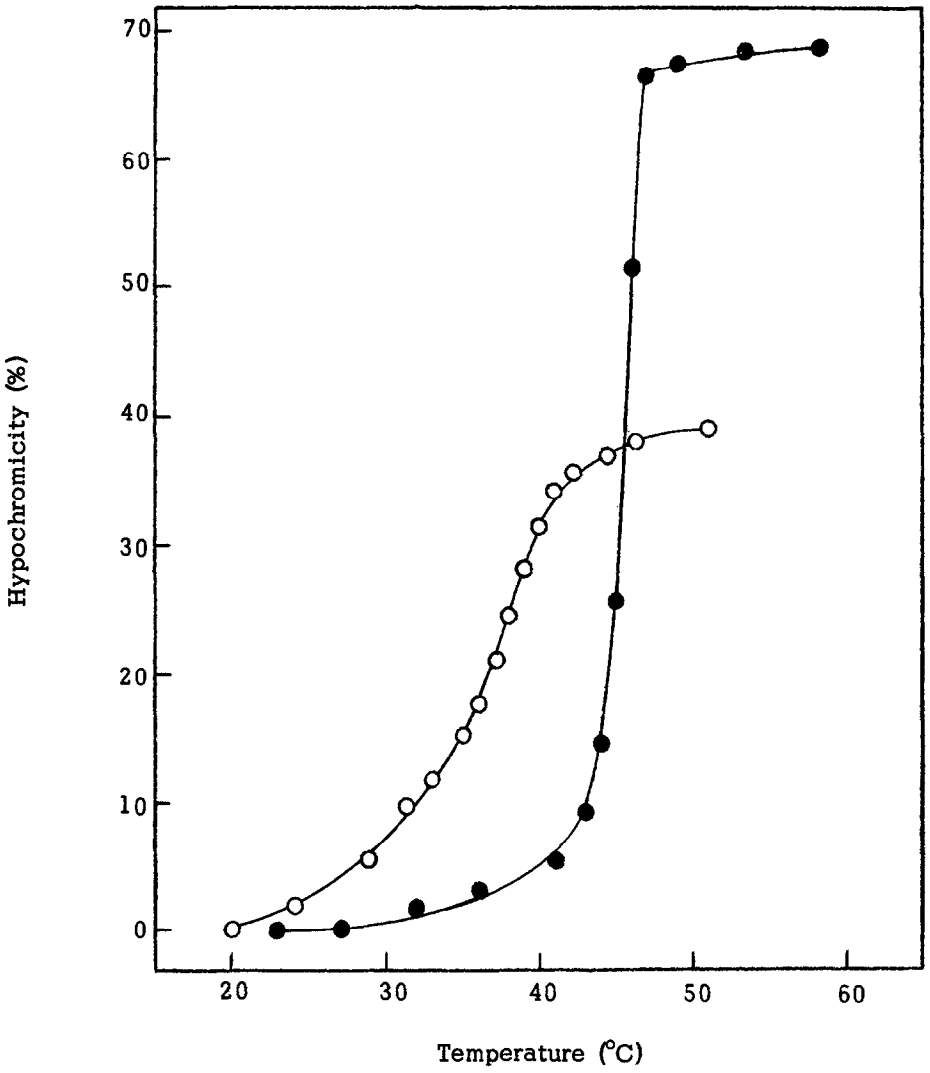

Figure 2. Thermal hypochromicity in 0.01 M sodium phosphate buffer (pH 7.4). Thermal hypochromicity was measured as $(A_{coil} - A_{helix})/A_{helix}$ for poly I · poly C (●) and poly (I:s^6I)· poly C (O) at 248 nm.

(321 nm) are completely separated and there is essentially no overlap of secondary bands in their spectra. The ratio of s^6IMP/IMP may be readily obtained from the relationship $\frac{A_{321\ nm} \cdot \epsilon_{IMP}}{A_{248\ nm} \cdot \epsilon s^6IMP}$. The experiments described in Table 1 gave the highest level of incorporation obtained with a ratio s^6IMP/IMP of 0.31 or about 24% incorporation.

Melting temperatures of complexes formed by annealing poly ($I:s^6I$) or poly I (both with $s_{20,w}$ values of about 10) with poly C (MW 3.1×10^4) were obtained by measurement of change in absorbance at 248 nm using a Cary 15 spectrophotometer and a Lauda K-2/R variable temperature circulator. Typical plots of % hypochromicity *vs.* temperature are illustrated in Figure 2 for a solution containing 0.01 M sodium phosphate buffer (pH 7.4). Similar plots having Tm values some 15° higher were obtained using solutions containing 0.15 M Na^+. However, at the higher salt concentrations and temperatures, decomposition of the 6-thiohypoxanthine chromophore began to occur, as judged by a steady decrease with time of the 321 nm absorption band. This lability does not appear to be a problem at lower temperatures. The poly ($I:s^6I$) • poly C complex has a markedly lower Tm (38°C) than the corresponding poly I • poly C complex (45.5°C), clearly demonstrating that replacement of oxygen by sulfur in the purine moiety at an obligatory hydrogen bonding site results in a considerable decrease in complex stability. This is probably due to the decreased electronegativity and increased steric requirement of >= S relative to >= 0 and is in agreement with the recent observations of Thewalt and Bugg regarding stacking and hydrogen bonding in thioguanosine crystals.[9] Although our data do not specifically exclude the possibility of "looping out" of the thiopurine bases from the helix, the relatively high Tm of the complex in which one of every four purines is a thiopurine mediates strongly against this explanation.

There is at present considerable debate regarding the importance of Tm *vs.* molecular weight in determining the interferon inducing activity of polyribonucleotide duplexes.[10] Since our poly ($I:s^6I$) has a high molecular weight, and its complex with poly C is less stable than that of poly I, evaluation of the biological activity of Poly ($I:s^6I$) • poly C will provide useful information regarding the precise parameters responsible for the antiviral and antitumor

activity of duplex polyribonucleotides. These experiments are presently in progress and will be reported elsewhere.

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