Polyribonucleotides Containing Thiopurines: Synthesis and Properties of Poly(6-thioguanylic acid)†

Venkataraman Amarnath and Arthur D. Broom*

ABSTRACT: The synthesis of poly(2-amino-6-chloropurinylic acid) [poly(n^2cl^6Pu)] by the polynucleotide phosphorylase catalyzed polymerization of 2-amino-6-chloro-9-(β-D-ribofuranosyl)purine 5′-diphosphate and its chemical conversion to poly(6-thioguanylic acid) [poly(s^6G)] is described. Poly(s^6G) was found to form a relatively unstable complex with poly(C), the properties of which were incompatible with those previously reported for the same complex prepared by another method [Darlix, J. L., Fromageot, P., and Reich, E. (1973), *Biochemistry 12*, 914]. It was found that poly(s^6G) could be thermally converted to a copolymer of guanylic and 6-thioguanylic acids, the complex of which with poly(C) was strikingly similar to that reported earlier for poly(s^6G)·poly(C).

Bioisosteric substitution of sulfur for oxygen in the naturally occurring purine and pyrimidine bases has been of continuing interest since Elion and Hitchings reported (Elion et al., 1952) the synthesis of the still useful antitumor agent 6-mercaptopurine. Recent mechanism of action studies have led to the assertion that the cytotoxicity responsible for the antitumor activity of both 6-mercaptopurine and thioguanine arises from their anabolism to 2′-deoxy-6-thioguanosine 5′-triphosphate and incorporation into DNA (Nelson et al., 1975). The synthesis of a polyribonucleotide containing only 6-thioguanylic acid residues [poly(s^6G)][1] would permit a careful study of its ability to form a complex with poly(C), and a comparison with the poly(G)·poly(C) duplex (Pochon and Michelson, 1965).

The only approach to the synthesis of poly(s^6G) hitherto reported involved the polymerization of s^6GTP on a poly(C) template by RNA polymerase (Darlix et al., 1973). The homopolymer poly(s^6G) could not be separated from poly(C). The present report describes a combined enzymatic and chemical approach to the synthesis of poly(s^6G). The properties of the pure homopolymer are incompatible with those previously reported (Darlix et al., 1973). It was found, however, that partial degradation of poly(s^6G) gave poly(G, s^6G), which has properties very similar to those reported by Darlix et al. (1973).

Materials and Methods

Polynucleotide phosphorylase (*Micrococcus luteus*, EC 2.7.7.8), snake venom phosphodiesterase (*Crotalus*, EC 3.1.4.1), and alkaline phosphatase (*Escherichia coli*, EC 3.1.3.1) were obtained from P-L Biochemicals, Inc., Boehringer Mannheim Corporation, and Sigma Chemical Company, respectively. 3-(*N*-Morpholino)propanesulfonic acid (Mops), cyclohexylaminoethanesulfonic acid (Ches), poly(C), (mol wt 3.1×10^4), and poly(U) ($s_{20,w} = 6.1 \pm 0.9$) were purchased from Calbiochem. Thin-layer chromatography (TLC) was carried out on glass plates coated with SilicAR 7GF silica gel (0.25 mm thickness) in solvent systems ethyl acetate–1-propanol–water (4:1:2 upper phase), 2-propanol–ammonium hydroxide–water (7:1:2), or 1,2-dimethoxyethane–ammonium hydroxide–water (17:1.5:1.5).

Ultraviolet spectra, pK_a, T_m, and stability data were obtained using a Cary 15 spectrophotometer as previously described (Broom et al., 1976). The sedimentation of the polynucleotides was studied using 5–20% sucrose gradients on a Beckman L2-65B ultracentrifuge. The polynucleotide solutions, as well as the sucrose gradients, were prepared in 0.005 M Mops (pH 7.2)–0.0001 M EDTA.

2-Amino-6-chloro-9-(β-D-ribofuranosyl)-9*H*-purine (Gerster et al., 1963, 1968) was phosphorylated in trimethyl phosphate (2–3 ml for each mmol of the nucleoside) with 2 equiv of phosphorus oxychloride at 0–2 °C over a period of 3 h (Yoshikawa et al., 1969). The yield of the pure 5′-monophosphate was 65%. The conversion of 5′-phosphate to 5′-diphosphate was carried out in a yield of 45% according to the method of Michelson (Michelson, 1964).

The polynucleotide phosphorylase catalyzed polymerization of the diphosphate to poly(2-amino-6-chloropurinylic acid) was carried out in a reaction mixture (35 ml) containing 0.2 M Tris-HCl (pH 9.0), 5 mM magnesium chloride, 0.5 mM EDTA, polynucleotide phosphorylase (70 mg, 0.94 unit/mg protein) and 20 mM 2-amino-6-chloro-9-(β-D-ribofuranosyl)purine 5′-diphosphate at 37 °C for 7 h. After the removal of protein by extraction with 5:2 chloroform–isopentyl alcohol (8 × 40 ml), the aqueous solution was successively dialyzed against 0.3 M NaCl + 5 mM EDTA (5 l., pH 7.5), 0.1 M NaCl + 5 mM EDTA (10 l., pH 7.5) and distilled deionized water (10 l.) and lyophilized to give 120 mg of the polymer.

To prepare poly(s^6G), a solution of poly(2-amino-6-chloropurinylic acid) (15 mg) in 0.4 M Tris-HCl (15 ml, pH 8.0) in a glass tube was frozen by immersion in a dry ice–acetone bath. Hydrogen sulfide gas was condensed into the tube until 10–15 ml of liquid was collected. The tube was then transferred to a steel bomb which was also kept in a dry ice–acetone bath. The bomb was sealed and transferred to an oil bath, temperature of which was maintained at 60–62 °C for 3 days. Ethanol (150 ml) was added and the reaction mixture was cooled in ice. The Tris salt of poly(s^6G) precipitated as a white solid which was collected by centrifugation and washed with ethanol. It

† From the Department of Biopharmaceutical Sciences, College of Pharmacy, University of Utah, Salt Lake City, Utah 84112. *Received May 11, 1976.* This study was supported by Research Grant CA 11935 from the National Cancer Institute, National Institutes of Health.

[1] Abbreviations used herein are s^6G, s^6GMP, s^6GDP, and poly(s^6G) for 6-thioguanosine, its 5′-mono and diphosphates and poly(6-thioguanylic acid), respectively; n^2cl^6Pu, n^2cl^6PuMP, n^2cl^6PuDP, and poly(n^2cl^6-Pu) refer, as above, to 2-amino-6-chloro-9-(β-D-ribofuranosyl)purine and its derivatives; Mops represents the buffer made from morpholinopropanesulfonic acid and its sodium salt; Ches refers to cyclohexylaminoethanesulfonic acid; poly(U), poly(uridylic acid); poly(C), poly(cytidylic acid); TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; uv, ultraviolet.

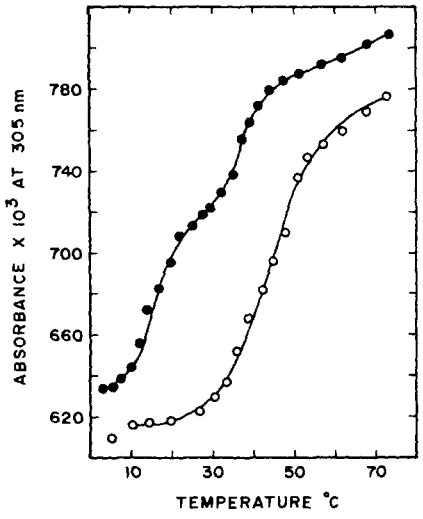


FIGURE 1: Dependence of absorbance of poly(n^2cl^6Pu) upon temperature in 0.005 M Mops buffer (pH 7.2) (●) without added salt and (○) containing 0.10 M NaCl.

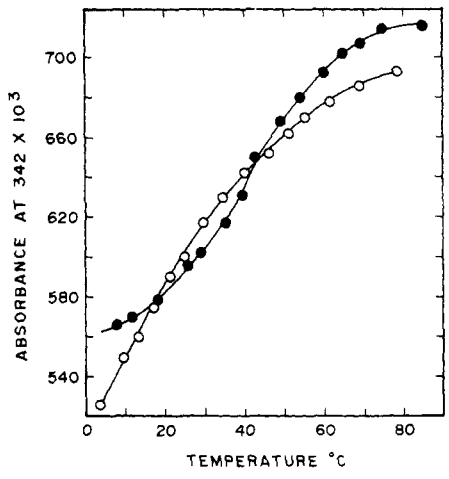


FIGURE 2: Dependence of absorbance upon temperature in 0.005 M Mops buffer–0.10 M NaCl (pH 7.2) of (○) sodium salt and (●) Tris salt of poly(s^6G).

was then dissolved in water, dialyzed against 0.1 M NaCl + 1 mM EDTA (8 l., pH 7.5) and distilled deionized water (10 l.), and freeze dried to yield 10 mg of poly(s^6G).

Enzymatic degradation of poly(2-amino-6-chloropurinylic acid) was carried out in accordance with a published procedure (Broom et al., 1976). Poly(s^6G) was degraded by incubating a mixture of 50 μl of polymer solution (1 mg/ml), 20 μl of 0.1 M Mops buffer (pH 7.2), 42 mg of urea, 10 μl of venom phosphodiesterase, and 5 μl of alkaline phosphatase at 37 °C for 16–20 h.

A complex between poly(s^6G) and poly(C) was prepared by heating a solution of the two polynucleotides in a suitable buffer at 70–75 °C for 1 h with occasional shaking and then allowing the solution to stand at room temperature for a few hours.

A solution of the Tris salt of poly(s^6G) in 0.005 M Mops (pH 7.2) was heated at about 80 °C for 20–40 h to make a copolymer of s^6G and G. Poly(C) and sodium chloride (to make the solution 0.1 M in Na^+) were added to the above solution, which was heated to 80–90 °C for a short while and cooled.

Results

The synthesis of poly(2-amino-6-chloropurinylic acid) was undertaken in order to demonstrate the utility of a combined enzymatic and chemical approach to the synthesis of the otherwise inaccessible poly(6-thioguanylic acid). It is important to note that this approach to the synthesis of poly(s^6G) is unambiguous; the homopolymer was fully characterized by uv and by enzymatic degradation to 6-thioguanosine. In contrast, the only previously reported attempt to synthesize poly(s^6G) was carried out by polymerization of the triphosphate on a poly(C) template using RNA polymerase (Darlix et al., 1973); these workers reported that the product could not be separated from poly(C) nor could the complex be degraded enzymatically.

Poly(n^2cl^6Pu) undergoes salt dependent, reasonably cooperative self-association leading to a multiple-stranded array (Figure 1). This finding emphasizes the importance of an amino group in the purine 2-position to the formation of self-association complexes; no such interaction was observed with poly(6-chloropurinylic acid) (Broom et al., 1976). No conditions were found for complex formation between poly(n^2cl^6Pu) and either poly(U) or poly(C).

The conditions required for conversion of poly(n^2cl^6Pu) to poly(s^6G) were, as expected because of the presence of an electron-releasing group in the pyrimidine ring, more vigorous than had been needed to convert poly(6-chloropurinylic acid) to poly(6-thioinosinic acid) (Broom et al., 1976). In fact, when the latter reaction was carried out under the conditions reported herein for the synthesis of poly(s^6G), a substantial amount of cross-linking (purine-S-purine) was observed which effectively prevented enzymatic degradation (A. D. Broom and M. E. Uchic, unpublished observations).

Initial attempts to degrade poly(s^6G) with a mixture of venom phosphodiesterase and alkaline phosphatase gave partial degradation or no reaction, suggesting either that cross-linking had occurred or that secondary structure was preventing the action of the phosphodiesterase. Since the temperature dependence of the uv absorption (Figure 2) revealed the probability of considerable secondary structure, the degradation was carried out in the presence of 6 M urea (Maeda et al., 1967). Under these conditions degradation proceeded smoothly and quantitatively giving only 6-thioguanosine and establishing that cross-linking did not occur.

During the course of these studies it became apparent that there were differences in the behavior of Tris salt and sodium salt of poly(s^6G). The Tris salt was less water soluble and more readily degraded enzymatically than the sodium salt. Figure 2 reveals the surprising result that, in 0.1 M NaCl–0.005 M Mops (pH 7.2) solutions, the temperature profiles of the two differ remarkably. The total hyperchromicity obtained from enzymatic degradation was about 25% for each of the polymers.

It was readily demonstrable by difference spectra (Figure 3) and mixing curves (Figure 4) that poly(s^6G) forms a 1:1 complex with poly(C). The double-stranded helical nature of the complex was supported by the dependence of T_m on salt concentration: the difference in T_m of 14 °C for a tenfold change in sodium ion concentration (Figure 5) is in accord with such a structure (Record, 1967).

A further assessment of the importance of horizontal (hydrogen bonding) interactions in the complexes of poly(s^6G) is available through pK_a determination. The pK_a of s^6GMP (base ionization) was found to be about 8.6, in agreement with the value reported previously (Darlix et al., 1973) for s^6GTP. Polymerization to poly(s^6G) led to an increase in the value to 9.60 ± 0.05 (0.005 M Ches–0.1 M NaCl); such an increase is

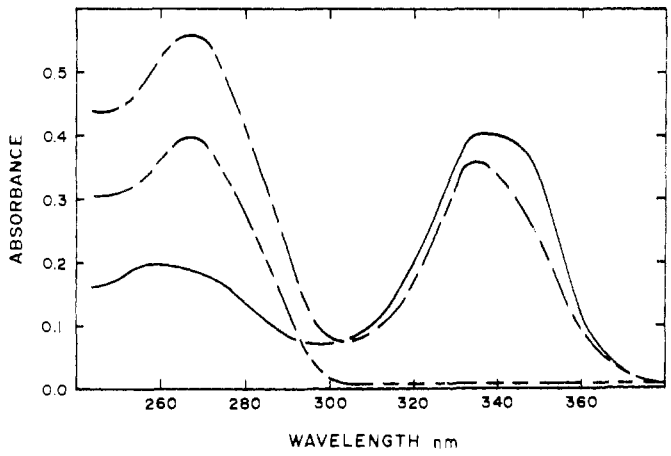


FIGURE 3: Ultraviolet absorption spectra in 0.005 M Mops buffer–0.10 M NaCl (pH 7.2) of (— · —) poly(s^{6}G)·poly(C), (—) poly(s^{6}G), and (– – –) poly(C).

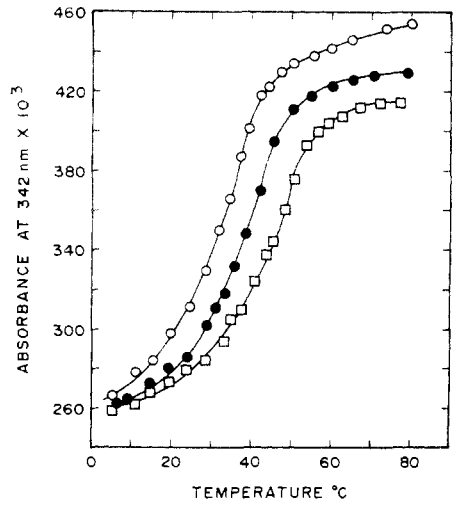


FIGURE 5: Dependence of absorbance upon temperature of the poly(s^{6}G)·poly(C) complex in 0.005 M Mops buffer, pH 7.2, containing (○) 0.10 M NaCl, (●) 0.10 M NaCl–0.001 M $MgCl_2$, or (□) 1.00 M NaCl.

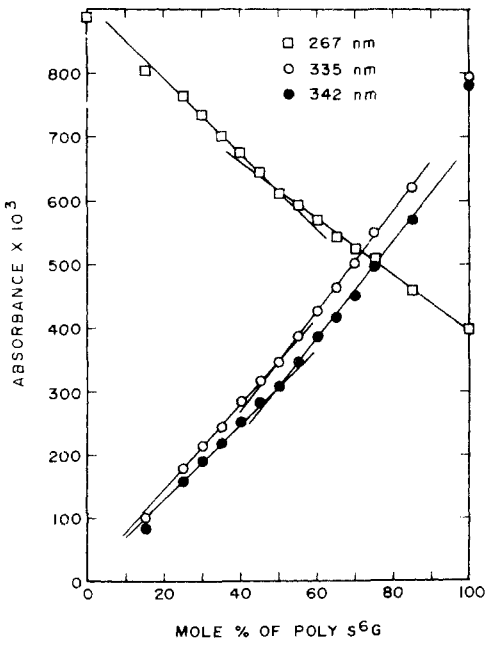


FIGURE 4: Mixing curves for poly(s^{6}G) poly(C) complex formation in 0.005 M Mops buffer–0.10 M NaCl, pH 7.2.

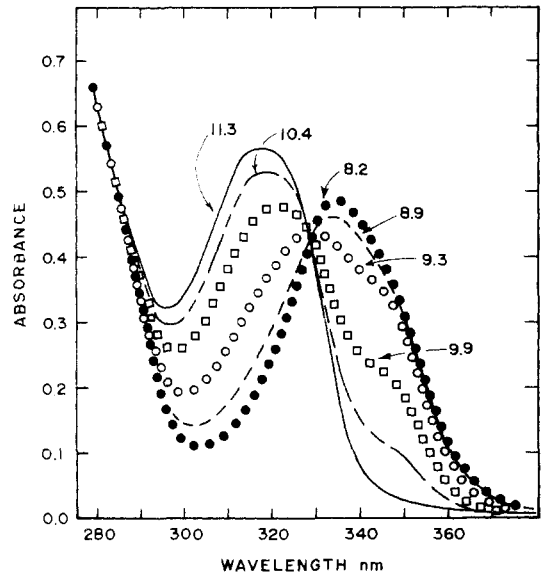


FIGURE 6: Variation of the absorption spectrum in the long wavelength region with pH of poly(s^{6}G)·poly(C).

best explained by the formation of hydrogen bonds in a poly (s^{6}G) self-association complex. As might be expected, the existence of the self-association complex prevented complementary duplex formation when poly(s^{6}G) and poly(C) were mixed at room temperature. When the solution containing the two polymers (0.005 M Mops–0.1 M NaCl, pH 7.2) was heated briefly at 70 °C and allowed to cool, the spectrum illustrated in Figure 3 was obtained. A small but definite hypochromic, hypsochromic shift in the long wavelength band provides convincing evidence for complex formation. The relatively small magnitude of these changes was expected because the poly(s^{6}G) curve used for comparison is that of the self-association complex. That the poly(s^{6}G)·poly(C) complex is only marginally more stable than the self-association complex may be seen by comparing Figures 2 and 5; this finding contrasts sharply with the previously reported value of 87 °C for the T_m of poly(s^{6}G)·poly(C) (Darlix et al., 1973). It was determined that poly(U) did not form a complex with poly(s^{6}G).

It was previously reported (Darlix et al., 1973) that no pK_a could be determined for poly(s^{6}G)·poly(C) because a spectrophotometric study revealed the absence of an isosbestic point for the set of spectra representing a titration of the complex. Examination of Figure 6, however, reveals that an isosbestic point is found in the spectra of complex reported herein, from which a pK_a of 9.75 ± 0.05 may be derived.

In their earlier study, Darlix et al. indicated that the thione moiety responsible for the long wavelength absorbance was very labile to either elevated pH or temperature, in fact, much more labile than the mononucleotide. In the present study the opposite was found to be the case. For example, heating a solution of poly(s^{6}G) sodium salt in 0.005 M Mops, pH 7.2, for 15 h at 75–77 °C resulted in a decrease in the 337-nm absorbance of less than 1%. Under the same conditions s^{6}GMP lost 53% of its thione chromophore absorption. Even at pH 11.0 in 0.005 M Ches–0.1 M NaCl only 9% of the thiolactam absorbance was lost after 2 days at room temperature. The Tris salt was found to be slightly more thermolabile than the sodium

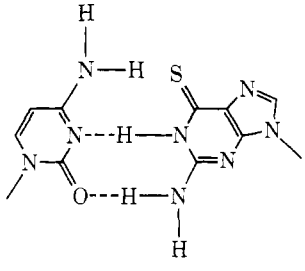
salt; it was possible to achieve significant loss of the thione chromophore by heating a solution of the polymer in 0.005 M Mops, pH 7.2, at 80 °C for several hours.

It was shown earlier (Broom et al., 1976) that poly(6-thioinosinic acid) underwent conversion to a copolymer containing inosinic acid and 6-thioinosinic acid under similar conditions. The increase in absorbance in the 250-nm region which accompanied the loss of the 337-nm absorbance for poly(s^6G) suggested that a similar conversion of s^6G to G residues was taking place. The formation of G was confirmed by enzymatic degradation of the copolymer using ribonuclease T_1 (Warrington, 1974) and alkaline phosphatase; the nucleoside product exhibited the characteristic uv spectrum and chromatographic mobility (TLC) of guanosine.

Since the structure of the partial degradation product of poly(s^6G) was firmly established as the copolymer poly(G, s^6G), it was of interest to study its complex with poly(C). The dependence of absorption spectra upon pH was essentially the same as that reported earlier as poly(s^6G)·poly(C) (Darlix et al., 1973). The absorbance–temperature profile of poly(G, s^6G) containing about a 1:1 ratio of the two bases gave a relatively noncooperative transition with a T_m of about 85 °C.

Discussion

Since both 6-mercaptopurine and 6-thioguanine apparently exert their lethal action on tumor cells by incorporation into DNA as 2′-deoxy-6-thioguanylic acid (Nelson et al., 1975), it is of considerable importance to understand the effect of such incorporation on nucleic acid structure. It has been shown (Broom et al., 1976) that poly(6-thioinosinic acid) does not form a complex with poly(C). The data described above establish that, while poly(s^6G) does form a double-stranded complex with poly(C), the interaction is a very weak one indeed. The involvement of N-1 H of s^6G in a hydrogen bond was established by the increase in pK_a for the ionization of that proton in the complex compared with the monomer. The necessity of the 2-amino group for the involvement of N-1 H in complex formation supports the idea that the interaction between s^6G and C residues occurs in the usual Watson–Crick manner as illustrated. There is no evidence available in this study bearing directly upon the issue of the H-bond accepting behavior of the 6-thione groups in poly(s^6G). The known low tendency for H-bond formation by such a system (Donahue, 1969; Zuika and Bankovskii, 1973; Broom et al., 1976) and the increased steric requirement of S vs. O both operate in the same direction to reduce the stability of the complementary hydrogen-bonding schemes.



It was conceivable, though unlikely, that the low stability of the poly(s^6G)·poly(C) complex resulted from a tendency of the thiolactam system (HN—C=S) to assume the thiolactim tautomeric modification (N=C—SH). If this were the case, one would expect that poly(s^6G) would form a complex with poly(U) in accord with the recent demonstration (Gerchman and Ludlum, 1974) that O^6-methylguanine residues code for the incorporation of U rather than C in a template directed RNA polymerase catalyzed reaction. This possibility was eliminated, however, by the demonstration that poly(s^6G) and poly(U) failed to form a complex under the conditions of poly(s^6G)·poly(C) complex formation.

The data presented above for the complex poly(G, s^6G)·poly(C) are strikingly similar to those attributed earlier (Darlix et al., 1973) to poly(s^6G)·poly(C). The complex behavior of the uv spectra upon variation of pH may be understood by the following considerations. There are probably three species undergoing ionization across the pH range 7 to 11. The initial (lowest pH) ionization may be attributed to the base ionization of those s^6G residues which are "looped out" of the helix. Very short s^6G runs in the copolymer (one or two units) ionize at somewhat higher pH, and finally, the much more stable G·C runs are broken by ionization. This interpretation accounts for both the complexity of the spectra and the marked increase in T_m as the percent of G in the poly(G, s^6G) increases.

The exact relationship of s^6G residues in DNA to delayed cytotoxicity remains obscure, but two points may be made. First, the presence of s^6G residues has a very markedly destabilizing influence on the helix and, second, s^6G has a greater (albeit weak) tendency to code for the proper complementary base (C) than for U. The role of stacking interactions may well provide most of the observed helix stabilization; this hypothesis is presently being explored.

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