Structural Characterization of Partially Thiolated Poly(cytidylic acid)†

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ABSTRACT: Partially thiolated poly(cytidylic acid) (MPC), a biologically active synthetic polynucleotide, was obtained by introducing 5-mercapto groups into some of the bases of poly(C). By use of the previously described thiolation procedure, about 10% of the cytidylate units can be converted to 5-mercaptocytidylates, as determined by neutron activation analysis or indirectly, calculated from ultraviolet spectral data by an empirical formula. 5-Mercaptocytidylic and cytidylic acids were identified, after alkaline hydrolysis, as the only nucleotide components of MPC. Micrococcal nuclease digestion of ^{35}S-labeled MPC, followed by column chromatography, led to the recovery of most of the radioactivity in the pentameric and higher oligonucleotide fractions, each of which contained one 5-mercaptocytidylate unit per molecule of oligomer, indicating a random distribution of the thiolated base in the polymer chain. Gel filtration, sedimentation, and light-scattering measurements on various MPC preparations yielded molecular weights in range of 10^5–10^6; addition of dithiothreitol (DTT) reduced the molecular weights by 30–70%, to equal those of the corresponding poly(C) starting materials. These results, together with the ultraviolet spectral measurements in the presence and absence of DTT, indicate that MPC contains both inter- and intramolecular disulfide bonds which are readily reduced by DTT to the free thiols. The circular dichroism spectra of MPC at both pH 4.0 and pH 7.5, and the melting profile of the double-helical conformation of MPC at pH 4.0, either in the presence or in the absence of DTT, are similar to the corresponding characteristics of oligo(cytidylic acids) rather than to those of poly(C). It is clear that the 5-mercapto substituent alters the normal base-pairing properties of cytosine and that it has a disruptive effect on the organized conformation of MPC.

Previous reports from this laboratory described the preparation and biological activities of partially thiolated polynucleotides, a class of macromolecular antimetabolites designed to act as inhibitory analogues of the functional templates (i.e., as "antitemplates") of nucleic acid polymerizing enzymes (Bardos et al., 1972; Mikulski et al., 1973; Srivastava & Bardos, 1973; Bardos, 1974; Chandra et al., 1975; Bardos & Ho, 1978). Among the various modified polynucleotides tested, the partially thiolated poly(cytidylic acid) (MPC)[1] strongly inhibited the reverse transcriptases of oncorna viruses (Chandra & Bardos, 1972; Srivastava, 1973) as well as the mammalian DNA polymerase α from regenerating rat liver (Kung et al., 1976; Ho et al., 1976). Double-helical complexes of MPC with poly(I) were found to be effective interferon inducers (O'Malley et al., 1975) and, in other studies, inhibited DNA synthesis in tumor cells (Le et al., 1978). MPC alone, in vitro and in vivo, significantly decreased the colony-forming ability of murine leukemic bone marrow and spleen cells (Ho et al., 1979). Preliminary clinical results indicated that MPC may have therapeutic potential as an antileukemic agent in man (Chandra et al., 1977a,b). Our chemical procedure for the synthesis of MPC was recently published in detail (Bardos et al., 1978). The present paper describes our studies aimed at the structural characterization of this biologically active macromolecule.

Materials and Methods

Poly(cytidylic acid) and poly(inosinic acid) were obtained from Miles Laboratories, Elkhart, IN. Hexadecyltrimethylammonium bromide was purchased from Eastman Kodak Co., Rochester, NY, and was recrystallized before use. Sodium hydrosulfide hydrate ($NaSH \cdot XH_2O$) was a product of Aldrich Chemical Co., Milwaukee, WI, $Na^{35}SH$ was purchased from Amersham/Searle, and micrococcal nuclease from *Staphylococcus aureus* and alkaline phosphatase from *Escherichia coli* were purchased from Worthington Biochemical Corp., Freehold, NJ.

Partially Thiolated Poly(cytidylic acid). The procedure for the preparation of MPC has been reported (Bardos et al., 1978). Briefly, potassium polycytidylate in water was precipitated as hexadecyltrimethylammonium polycytidylate [CTA poly(C)] with dropwise addition of hexadecyltrimethylammonium bromide solution. The CTA poly(C) in dry methanol was treated with methyl hypobromite followed by sodium sulfhydrate (NaSH) at 0 °C. The modified CTA poly(C) was converted to the sodium form by dropwise addition of NaCl solution. The MPC was purified by column chromatography on Sephadex G-100 gel or Bio-Gel A-5M. The ^{35}S-labeled MPC was prepared in a similar manner but by using $Na^{35}SH$ in the thiolation step.

Alkaline Hydrolysis of MPC. MPC (2.0 μmol) in potassium hydroxide (0.43 mL of 0.3 N solution) was incubated at 37 °C for 16 h. Perchloric acid was added until the hydrolysate was 0.05 N in hydrogen ion (H^+) concentration (0.16 mL of 1 N $HClO_4$ solution). After 2 h at 25 °C, potassium hydroxide (0.03 mL of 1 N solution) was added to neutralize the hydrolysate which was then cooled to 0 °C for 1 h and centrifuged to remove the potassium perchlorate. The clear solution was dephosphorylated by using the alkaline phosphatase from *E. coli*. The mixture, containing the above hydrolysate (1.2 μmol of nucleotides), $MgCl_2$ (1 μmol), NH_4HCO_3, pH 8.0, buffer (50 μmol), and the enzyme (10 μg), in 1 mL was incubated at 35 °C for 16 h. It was then lyophilized, dissolved in 50 μL of H_2O, and analyzed by thin-layer chromatography.

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[1] Abbreviations used: MPC, partially thiolated poly(cytidylic acid); poly(C), poly(cytidylic acid); poly(I), poly(inosinic acid); DTT, dithiothreitol; HMB, *p*-(hydroxymercuri)benzoate; CTAB, hexadecyltrimethylammonium bromide; hs^5CMP, 5-mercapto-5′-cytidylic acid.

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Enzymatic Hydrolysis. The ^{35}S-labeled MPC was hydrolyzed with the micrococcal nuclease from *S. aureus*. The reaction mixture containing, in 6.2 mL, MPC (15 μmol), Tris buffer, pH 8.5 (310 μmol), $CaCl_2$ (41 μmol), the enzyme (93 μg), and mercaptoethanol (19 μmol) was incubated at 37 °C for 8 h. After hydrolysis, the reaction mixture was diluted to 102 mL with buffer A (Tris-HCl, 0.01 M, pH 7.5, containing 5 mM NaCl and 1 mM mercaptoethanol) and applied to a DEAE-cellulose column (1.2 × 26 cm) previously equilibrated with buffer A. After the column was washed with 145 mL of buffer A, the column was eluted with Tris buffer (0.01 M, pH 7.5, containing 1 mM mercaptoethanol) and a linear gradient of NaCl, 0.005–0.35 M (1 L), followed by a steeper gradient of 0.35–1 M NaCl (500 mL). Fractions, 7.7-mL each, were collected at a flow rate of 114 mL/h.

Quantitative Determination of Thiol Groups by the Neutron Activation Method. MPC (3.6 μmol containing approximately 0.4 μmol of thiols) in 7.2 mL of potassium phosphate buffer (56 mM, pH 7.2) containing either 0.9 μmol or no dithiothreitol was incubated with *p*-(hydroxymercuri)benzoate (HMB, 2.4 μmol) at 37 °C for 30 min. The reaction mixture was passed through a column of Bio-Gel P-2 (1.8 × 43.5 cm) to separate the MPC from excess HMB or DTT. Deionized water was used to elute the column and fractions of 2 mL were collected. Aliquots of the MPC solution were irradiated with thermal neutron flux as described by Bruce & Malchman (1965). The unmodified poly(C) treated in the same manner was employed as a control for the background radioactivity. HMB was irradiated together with the samples as a standard. The quantity of thiol groups was calculated according to the formula

$$\% \text{ thiolation} = \frac{[\text{hs}^5\text{CMP}]}{[\text{MPC}]} \times 100 = \frac{[\text{dpm(sample)}]\epsilon_{270} b}{[\text{dpm/(mol of Hg)}]A_{270}} \times 100$$

where [hs^{5}CMP] = concentration of 5-mercaptocytidylate in the sample, [MPC] = concentration of MPC in the sample, dpm = disintegrations per minute, A_{270} = absorbance at 270 nm, ϵ_{270} = molar extinction coefficient of MPC in water at 270 nm, and b = length of UV light path (centimeters).

Sedimentation Coefficients. The sedimentation velocity of MPC (2.6 mg/mL in Tris-HCl, 0.01 M, pH 7.5) was measured in a Spinco Model E analytical ultracentrifuge by using the Schlieren optical system. Sedimentation coefficients were calculated from the slopes of lines relating the logarithm of the distance between the axis of rotation and the position of the sedimenting boundary corresponding to half of the total polynucleotide concentration at each exposure to time.

Light Scattering. Measurements were performed with a Brice-Phoenix Model 2000 (Virtis Co., Inc.) photometer using blue light (436 nm) as previously described (Fiel et al., 1970). Polynucleotide solutions and buffers (1 M NaCl plus 0.1 M K_2HPO_4, pH 7.5 or 4.0) were filtered at least twice through a 0.2-μm nucleopore filter. The final filtration was made directly into the scattering cell. The concentration of the polynucleotide was generally between 180 and 200 μg/mL except in the case of one low molecular weight MPC sample in which case concentrations up to 880 μg/mL were used. Loss of polynucleotide on filtration was found to be approximately 10% in all cases reported here. Apparent molecular weights and radii of gyration were calculated as previously described (Fiel et al., 1970).

Refractive index increment dn/dc measurements were performed with a Phoenix differential refractometer (Virtis Co., Inc.). A value of 0.147 mL/g was obtained for poly(C) in 1 M NaCl plus 0.01 M K_2HPO_4 at pH 4.0 over a concentration range of 1–5 mg/mL. This value was used for all polynucleotide samples.

Circular Dichroism. Ultraviolet CD measurements were determined on a Cary Model 6001 CD attached to a Cary 60 recording spectropolarimeter equipped with a thermostatable cylindrical cell holder. Results are reported as $\Delta\epsilon$ ($\epsilon_L - \epsilon_R$) on the basis of the molar concentration of nucleotide residues.

Ultraviolet Spectroscopic Measurements. Ultraviolet spectra were determined on a Gilford Model 2400-S spectrophotometer. Absorbance–temperature profiles were procured by using the same spectrophotometer equipped with a thermostated cell compartment, so that the absorbance measurements could be made at the ambient temperature.

Results

(*A*) *Thiolation of Poly(cytidylic acid).* Although the alkali metal or ammonium salts of poly(C) can be dissolved with some difficulty in the organic solvents required for the thiolation procedure with the addition of a small amount of water, it was found that even the presence of a trace amount of water in the reaction mixture results in substantial degradation of the polymeric structure. Therefore, the cetyltrimethylammonium salt of the polynucleotide was prepared, and this was dissolved in dry methanol for the reaction with methyl hypobromite. This reaction proceeds at 0 °C within 30 min to completion, as indicated by the disappearance of the ultraviolet absorption maximum at 270 nm; the polymeric adducts formed show a maximum absorption at 237 nm. After the addition of dimethylacetamide as the solvent and the sodium hydrosulfide reagent, the reaction mixture was stirred at 0 °C under nitrogen atmosphere. The modified polynucleotides were reconverted to the water-soluble sodium salts and purified in the previously described manner (Bardos et al., 1978).

As in the case of the mononucleotides (Ho et al., 1978), only a portion of the pyrimidine–methyl hypobromite adducts in the polynucleotide molecules is converted to the 5-mercapto derivatives, and the remaining portion is reduced to the original 5-unsubstituted base components. The maximum ratio of 5-mercaptocytidylate units in these modified polymers that could be obtained by the above procedure was 10–15%; lower levels of thiolation could be obtained by reducing the amounts of methyl hypobromite and sodium hydrosulfide reagents.

(*B*) *Determination of the Percent of Thiolated Bases in the Modified Polynucleotides.* The ultraviolet spectrum of the partially thiolated polycytidylate at neutral pH showed a similar absorption maximum to that of the unmodified poly(cytidylic acid); however, upon the addition of DTT, a second absorption maximum appeared in the 330–335-nm region, indicating the presence of the ionized 5-mercapto groups (Bardos et al., 1975). Thus, it appears that in the process of conversion to the sodium salt and purification of MPC in aqueous solution most of the SH groups were oxidized to disulfides and the addition of DTT is required to reduce them to the free thiols. The absorbance in the 330–335-nm region (in the presence of DTT) depends on the amount of 5-mercaptocytidylate units in the polymer, and its ratio to the absorbance at 270 nm (in the absence of DTT, corresponding to the total nucleotides) may be used in estimating, for comparative purposes, the "percent thiolation" (i.e., the percent of nucleotides thiolated). However, at lower than 1–2% thiolation, the appearance of the second absorption maximum

at 334 nm upon the addition of DTT is not clearly distinguishable in the spectrum. Therefore, for accurate determination of the total percent thiolation and of the relative amounts of oxidized and reduced thiol groups in the polymer, the highly sensitive neutron activation analysis method has been employed, after reacting the modified polynucleotides with *p*-(hydroxymercuri)benzoate both before and after reduction with DTT (see Materials and Methods).

In the case of MPC, the values for the total percent thiolation as determined by the neutron activation analysis have been found to be lower by a factor of 0.6 than those calculated from the ultraviolet spectra based on the ratio of molar absorbances of analytically pure monomeric cytidylic and 5-mercaptocytidylic acids. This discrepancy can be largely accounted for by the hypochromicity of MPC.[2] Thus, the correction factor of 0.6 has to be used when the percent thiolation is calculated from the spectral data. For this, the empirical formula

$$\% \text{ thiolation} = 0.6\left(\frac{\epsilon_c}{\epsilon_s}\right)\left(\frac{\Delta A_{334}}{A_{270}}\right) \times 100 = 1.28\left(\frac{\Delta A_{334}}{A_{270}}\right) \times 100$$

is used, where ϵ_c = extinction coefficient of 5′-CMP at 270 nm = 9.12×10^3, ϵ_s = extinction coefficient of hs^{5}CMP at 334 nm = 4.28×10^3, ΔA_{334} = differential absorbance of MPC at 334 nm, in the presence and in the absence of DTT, and A_{270} = absorbance of MPC at 270 nm.

(*C*) *Identification of 5-Mercaptocytidylate (Disulfide) in the Alkaline Hydrolysate of MPC.* Exhaustive digestion of MPC with potassium hydroxide at 37 °C, followed by enzymatic dephosphorylation with alkaline phosphatase as described under Materials and Methods, yielded a mixture of nucleosides which gave only two spots by thin-layer chromatography on cellulose plates (Eastman 6065 cellulose) using two different solvent systems. The R_f values were compared with those of authentic samples of cytidine and 5-mercaptocytidine (Ryu and Bardos, unpublished experiments) and the results were as follows: (1) in 95% ethanol–1 M ammonium acetate (7:3 v/v) for hydrolysate, R_f = 0.75 and 0.53; for cytidine, R_f = 0.75; for 5-mercaptocytidine (disulfide), R_f = 0.56; (2) in 2-propanol–1% aqueous ammonium sulfate (2:1 v/v) for hydrolysate, R_f = 0.64 and 0.42; for cytidine, R_f = 0.62; for 5-mercaptocytidine (disulfide), R_f = 0.42. The thin-layer chromatography spot of the hydrolysate with the R_f corresponding to that of 5-mercaptocytidine (disulfide) was eluted and further identified by its UV spectra (λ_{max} 276 nm at pH 8.0; upon addition of DTT, λ_{max} 334 nm).

(*D*) *Distribution of Thiolated Bases in the Polymer Chain.* It was of interest to determine the positions of the sulfhydryl groups in the polynucleotides when introduced by the thiolation procedure. Exhaustive digestion of ^{35}S-labeled MPC (in the presence of mercaptoethanol) with micrococcal nuclease, followed by column chromatography on DEAE-cellulose (Figure 1), yielded about 37% monocytidylate and 11% dicytidylate fractions, both of which contained only small amounts of ^{35}S-labeled 5-mercaptocytidylate (Table I).

Essentially all the radioactivity was recovered in the trinucleotide and higher oligonucleotide fractions. The specific

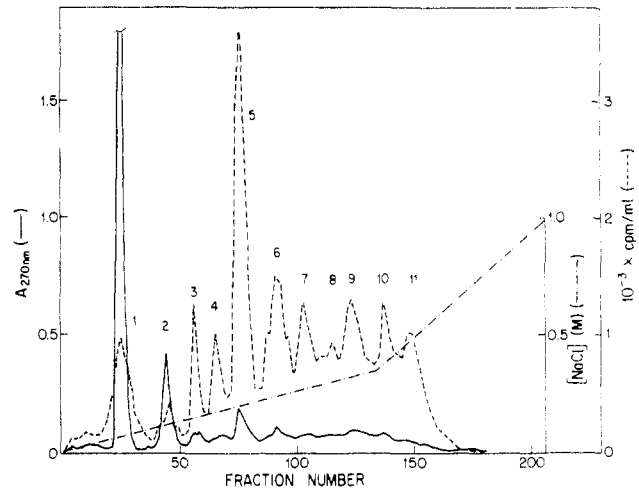

FIGURE 1: Chromatography of ^{35}S-labeled MPC hydrolysate. ^{35}S-Labeled MPC was hydrolyzed by micrococcal nuclease as described under Materials and Methods. The hydrolysate was chromatographed on a DEAE-cellulose column. Aliquots of the fractions were withdrawn for UV measurements (—); samples of 200 μL of various fractions were assayed for radioactivity (- - -).

Table I: Analysis of Chromatographic Fractions[a] Derived from the Micrococcal Nuclease Hydrolysate of MPC

peak[a] no.	chain length	total nucleotides concn[d]	total nucleotides % [e]	10^{-4} × cpm/μmol	% hs^{5}CMP found	% hs^{5}CMP calcd[g]
MPC			100	7.36	10.1[f]	
1	0.98[b]	730	36.8	0.91	1.24	
2	1.89[b]	220	11.1	1.22	1.64	
3	3[c]	81.1	4.1	6.75	9.19	
4	4	88.3	4.5	7.48	10.3	
5	5	150	7.6	13.83	19.0	20.0
6	6	129	6.6	11.30	15.5	16.6
7	7	78.5	3.9	11.30	15.5	14.3
8	8	91.6	4.6	9.04	12.4	12.5
9	9	168	8.5	8.61	11.8	11.1
10	10	129	8.6	8.06	11.0	10.0
11	11	114	5.8	8.70	11.9	

[a] See Figure 1 for an explanation. [b] Determined by the ratio of total phosphate to acid-labile phosphate. [c] The chain lengths of this and the following peaks were assigned according to their peak positions in the elution profile (Figure 1). [d] Concentration of nucleotides in pooled fractions of each peak, as determined on the basis of the ε values reported for oligocytidylates (Brahms et al., 1967). [e] Percent of total hydrolysate. [f] Percent of thiolation determined by the neutron activation analysis. [g] Calculated for *one* 5-mercaptocytidylate per molecule of oligonucleotide.

activity reached a maximum in the pentanucleotide fraction, corresponding to 19% 5-mercaptocytidylate, i.e., close to *one* modified base for each molecule of pentanucleotide. As the chain length increased, the percent of 5-mercaptocytidylate decreased correspondingly, to maintain an average of *one* SH group per oligonucleotide. This result is consistent with a random distribution of the modified bases in the polymeric MPC. It also shows that the micrococcal nuclease is unable to hydrolyze the phosphodiester linkage on either side of a 5-mercaptocytidylate, since the inhibitory effect of the 5-mercapto group[3] appears to extend to a certain degree over 5–11 nucleotides, as indicated by the relative accumulation of radioactivity in the pentanucleotide and higher fractions.

If the mercapto group had been inserted nonrandomly (i.e.,

[2] Hypochromicity is defined (Janik, 1971) as [1 − ε(polymer)/ε(monomer)] × 100. For poly(C), this value is 33% in accordance with literature data, and the hypochromicity of MPC with respect to the mixture of its monomeric components (i.e., 90% CMP plus 10% hs^{5}CMP) was found to be nearly the same, 31%.

[3] The molar concentration of mercaptoethanol used in the enzymatic reaction mixture (3.06 mM) was 12.5 times higher than that of the 5-mercaptocytidylate units (10.1% of 2.42 mM MPC); thus, all 5-mercapto groups were present during enzymatic hydrolysis in the reduced and ionized form.

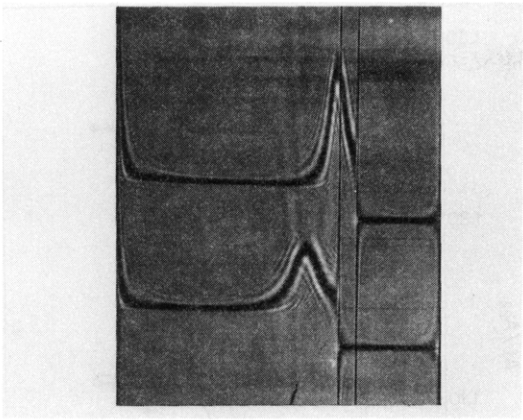

FIGURE 2: Sedimentation of MPC. MPC (2.6 mg/mL in Tris-HCl, 0.01 M, pH 7.5) was centrifuged in a Spinco Model E ultracentrifuge at 42 000 rpm with an AnD 1380 rotor at 6 °C. Sedimentation was from right to left. The lower pattern was MPC in the absence of DTT, and the upper pattern was MPC in the presence of DTT. The patterns shown were obtained 56 min after reaching the speed.

in "clusters"), larger oligonucleotides would have been obtained; however, virtually all of the UV absorbance as well as radioactivity of the starting material was recovered in the 11 peak fractions. In contrast to MPC, the unmodified poly(C) is hydrolyzed by micrococcal nuclease almost completely to mono- and dinucleotides in a characteristic 5:1 molar ratio (Sulkowski et al., 1970).

(*E*) *Gel Chromatography and Sedimentation Rate.* The poly(C) used as starting material for the preparation of MPC was obtained from commercial sources, and the various lots showed considerable variations in molecular weight. The sedimentation coefficients of the poly(C) ranged from 4 to 8 S, corresponding to estimated molecular weights of approximately 10^5–10^6. After partial thiolation, the modified polycytidylates were recovered without any apparent degradation as indicated by analytic column chromatography on Bio-Gel A-15M: in the presence of mercaptoethanol or DTT, each MPC preparation gave a peak eluate fraction containing the bulk of the polynucleotides at approximately the same position as the corresponding starting material.

Figure 2 shows the ultracentrifugation profile of an MPC sample containing 9.1% 5-mercaptocytidylate which was prepared from a relatively low molecular weight (4.5 S) poly(C). It is evident that the presence of DTT in the buffer has a significant effect on the sedimentation rate of MPC. The s_{20} value of the MPC sample was 6.5, and it decreased to 4.4 upon the addition of DTT to the buffer. These results indicate that intermolecular disulfide linkages had been formed between individual MPC chains which were reduced in the presence of DTT. Cleavage of the disulfide bonds by the reducing agent resulted in a decrease of the molecular weight of the modified polynucleotide to equal that of the corresponding unmodified poly(C).

(*F*) *Light Scattering.* The results of light-scattering measurements conducted on buffered solutions of two different preparations of MPC and the corresponding unmodified poly(C) starting materials are shown in Table II. In both pairs (no. 48 and 49), the partially thiolated polycytidylate (containing 10 and 10.5% 5-mercaptocytidylate units, respectively) had a significantly higher weight-average molecular weight and, at the same time, a smaller radius of gyration than the corresponding unmodified polynucleotide. These data indicate that in the thiolated polymer substantial conformational changes have occurred, in addition to some intermolecular association between the individual polynucleotide strands.

Table II: Light-Scattering Parameters of Polynucleotides

sample	$M_r \times 10^{-6}$	R_g (A)[a]
no. 48, poly(C)	0.614	429
no. 48, MPC	1.02	352
no. 49, poly(C)	0.829	538
no. 49, MPC	2.69	519
no. 48, MPC	1.02	352
no. 48, MPC, plus 0.001 M mercaptoethanol	0.726	334
no. 25, MPC	0.192	318
no. 25, MPC, plus 0.01 M DTT		
10 min	0.113	388
30 min	0.107	361
60 min	0.108	370

[a] R_g, radius of gyration.

Upon 30-min incubation with 1 mM mercaptoethanol, the average molecular weight of no. 48 MPC was reduced by approximately 30% without significant change in the radius of gyration.

A time study of the effect of DTT (1 mM) on the light-scattering parameters of a low molecular weight MPC sample (no. 25) is also included in Table II. Immediately upon addition of DTT the molecular weight of MPC was reduced to nearly half of its original value, and at the same time the radius of gyration *increased* by approximately 20%. These values then remained essentially constant during 60 min of further incubation with DTT.

These results are consistent with the interpretation that, in the absence of a reducing agent, both intra- and intermolecular disulfide linkages are formed between the 5-mercapto groups of MPC. Addition of either mercaptoethanol or DTT causes reductive cleavage of the disulfide linkages and restores the molecular weight but not necessarily the conformation of MPC to match those of the original unmodified poly(C).

(*G*) *Circular Dichroism.* The CD spectra of MPC (9.1% thiolated) at pH 7.5 and pH 4.0, both in the presence and in the absence of DTT, are shown in parts B and C of Figure 3, and those of the unmodified poly(C) are shown in Figure 3A. A comparison of these spectra reveals that the general shapes of the CD curves of MPC and poly(C) at both pH 7.5 and pH 4.0 are similar, i.e., they have the same maxima and minima, but the intensity of the bands are different.

It has been reported that in a series of oligo(cytidylic acids) (Brahms et al., 1967) the intensity (rotational strength) of both the positive and negative CD bands increases with the chain length. The negative band (at pH 4.0), the rotational strength of which is a measure of the double-stranded conformation, was not detectable in the CD curves of oligonucleotides that were smaller than a heptamer. Comparison of the intensities of the CD bands of MPC with those reported for the series of oligocytidylates reveals that (1) at pH 7.5 MPC has a single-stranded "stacked-base" helical conformation similar to that reported for oligo(cytidylic acids) with a chain length of 10–12 bases and (2) at pH 4.0 MPC forms a double-stranded helix which again resembles that of an oligocytidylate having a length of 10–12 base pairs. Addition of DTT has very little effect upon the CD curves of MPC (Figure 3B,C).

(*H*) *Ultraviolet Absorbance–Temperature Profile.* As shown in Figure 4, at pH 4.0 in 0.1 M NaCl and 0.05 M sodium acetate, poly(C) manifested a cooperative change of ultraviolet absorbance–temperature profile with a T_m at 81 °C, in agreement with the literature values (Akinrimisi et al., 1963; Fasman et al., 1964; Brahms et al., 1967), whereas MPC (containing 9.1% of 5-mercaptocytidylic acid) showed a lower T_m (50 °C) and a much smaller hyperchromicity typical of

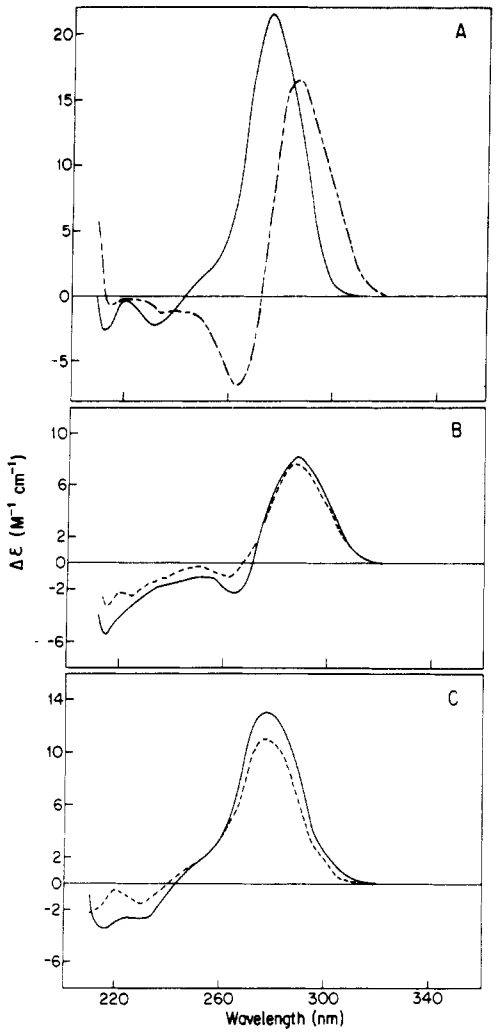

FIGURE 3: Circular dichroic spectra of MPC and poly(C) at 25 °C. (A) Poly(C) (0.1 mM): in 0.1 M NaCl and 0.05 M sodium acetate, pH 4.0 (---); in 0.1 M NaCl and 0.01 M Tris-HCl, pH 7.5 (—). (B) MPC (0.1 mM): in 0.1 M NaCl and 0.05 M sodium acetate, pH 4.0, without DTT (—); in the same buffer with 1 mM DTT (---). (C) MPC (0.1 mM): in 0.1 M NaCl and 0.01 M Tris-HCl, pH 7.5, without DTT (—); in the same buffer with 1 mM DTT (---).

an oligo(cytidylic acid). Addition of DTT to the system increased the T_m (59 °C) but did not significantly affect the hyperchromicity. In comparison with the T_m values reported for oligo(cytidylic acids) under identical experimental conditions (Brahms et al., 1967), the MPC preparation appeared to demonstrate the property of an oligomer of 10–12 bases in length. The effects of the 5-mercaptocytidylate contents of the MPC preparations on their T_m and on the hyperchromicity of the melting profiles are summarized in Table III. In general, the change of the hyperchromicity appeared to be more dependent than the T_m value on the percent of 5-mercaptocytidylate content.

Discussion

The results of the gel filtration, ultracentrifugation, and light-scattering studies are in agreement that MPC is a polynucleotide with molecular weight greater than 100 000. However, the circular dichroism spectra (both at pH 4.0 and at pH 7.5) and the melting profiles of MPC (at pH 4.0), either in the presence or in the absence of DTT, are similar to those of oligo(cytidylic acids) consisting of only 10–12 nucleotide units rather than to those of a polynucleotide.

This apparent discrepancy between molecular weight and conformational properties is readily understandable in the case

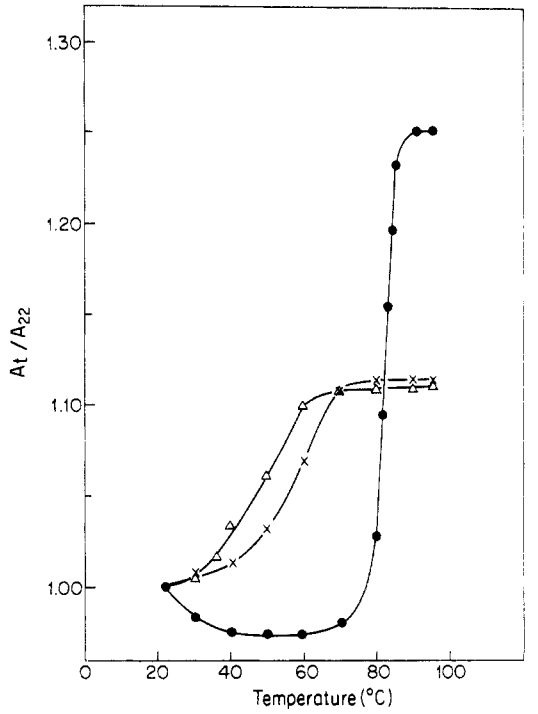

FIGURE 4: Ultraviolet absorbance–temperature profile of MPC and poly(C). The concentration of the polynucleotides in 0.1 M NaCl and 0.05 M sodium acetate, pH 4.05, was as follows: poly(C), 0.1 mM (●); MPC, 0.13 mM (△); MPC, 0.13 mM, with 0.5 mM DTT (×). The ordinate A_t/A_{22} is the ratio of absorbance at 275 nm at temperature t to that at 22 °C.

Table III: Effect of 5-Mercaptocytidylate Content on the T_m and Thermal Hyperchromicity of MPC

polynucleotide	% hs⁵CMP	pH 4.0 − DTT T_m	pH 4.0 − DTT % H^a	pH 4.0 + DTT T_m	pH 4.0 + DTT % H^a
poly(C)	0	81	27.6	81	27.6
MPC no. 18	1.2	55	21	59.5	20.6
MPC no. 2	2.0	54	17	56.5	17
MPC no. 4	4.0				
MPC no. 5	6.0	53	16	57	16
MPC no. 10	8.1	51	11	56	10.8
MPC no. 26	9.1	50	11	59	11.4

[a] % H, the percent thermal hyperchromicity accompanying the transition of ordered form ⇌ melted form of the polymer, is defined as $[A_m(\lambda)/A_o(\lambda) - 1] \times 100$ where A_m and A_o are the absorbances at the wavelength λ of the melted and ordered forms, respectively.

of the oxidized form of MPC where the geometry of the inter- and intramolecular disulfide bonds can be expected to restrict the formation of ordered helical structures to short oligocytidylate segments which are separated and disoriented with respect to each other by the cross-linking disulfide bonds. Accordingly, the average length of these oligocytidylate segments is determined by the random distribution of the 5-mercaptocytidylate units in the polymer chain. The rotational strength and conformational stability of each of these segments (which form single-stranded helices at pH 7.5 and double-stranded helices at pH 4.0) appear to be independent and noncooperative with respect to each other, presumably due to their different orientations.

It is more surprising that the addition of DTT, which demonstrably reduces essentially all the disulfide bonds of MPC, has a much smaller effect on the CD spectra and melting profiles than one might have expected. It is evident that the 5-mercaptocytidylate units even in their free thiol form are capable of preventing to a large extent the reconstitution of

a homogeneous, cooperative conformation throughout the polymer chain. The reasons for this are not quite clear but it is possible to offer some hypothetical explanations. At pH 7.5, poly(C) exists as a single-stranded helix stabilized by the hydrophobic interaction between the stacked bases (Fasman et al., 1964; Arnott et al., 1976). In the case of MPC, in the presence of DTT at pH 7.5, the mercapto groups are completely ionized (Bardos et al., 1975). This anionic character of the 5-mercaptocytidylate residues may disturb the continuity of base stacking of the polynucleotide chain presumably due to the polarity of the thiolate ions randomly distributed throughout the polymer and to the clustering of water molecules around them which would derange the hydrophobic forces. At pH 4.0, the double-stranded helix of poly(C) is stabilized by the hydrogen bonds involving the carbonyl and amino groups of each pair of cytosine residues as well as by the sharing of an additional proton between the N_3 nitrogens of the two bases (Akinrimisi et al., 1963; Langridge & Rich, 1962). In the case of MPC, zwitterion formation would occur at pH 4.3 between the 4-amino and 5-thiol groups of the 5-mercaptocytosine moieties (Bardos et al., 1975). This would prevent protonation at N_3 and thus weaken the hydrogen bonding with the "complementary" cytosine of the other strand. Additionally, the zwitterions (or even the un-ionized sulfhydryl groups) would bind the surrounding water molecules, and the "hydrated" 5-mercaptocytosine moieties would disrupt the base-stacking interactions required for maintaining the stability of the helical configuration.

The effects of various other substituents at the 5 position of the cytosine ring on the stability of helical conformations of the substituted homopolymers have been reported. The neutral and acid forms of poly(5-methylcytidylic acid) have properties similar to those of the corresponding forms of poly(C) (Szer & Shugar, 1966). Poly(5-halogenocytidylic acids) also form organized helical structures which are in some cases even more stable than poly(C) (Michelson & Monny, 1967; Folayan & Hutchinson, 1974). In contrast to the aforementioned 5-substituted poly(cytidylic acids), poly(5-hydroxycytidylic acid) appears to have little secondary structure in either neutral or acid solution (Eaton & Hutchinson, 1973). Although no explanation was given in the original article for this unique behavior of the 5-hydroxyl-substituted poly(cytidylic acid), the analogy between the effects of the 5-hydroxyl and 5-thiol groups, vs. those of the 5-methyl and 5-halogens, supports our hypothesis that the stability of the helical configurations in the 5-substituted poly(cytidylic acids) depends on the hydrophobic or hydrophilic character of the substituent in the 5 position of the cytosines, i.e., on the "outside face" in the case of the double-stranded acidic forms.

The significance of these conformational characteristics of MPC with respect to its biological activities is still unknown. Comparative physicochemical and biological studies with various S-substituted derivatives of MPC (Bardos & Ho, 1978) as well as with partially thiolated oligocytidylates (Ho et al., 1976) may answer this question. Such studies are currently in progress.

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