OLIGONUCLEOTIDES CONTAINING MODIFIED BASES.

I. INHIBITION OF DNA AND RNA POLYMERASES BY PARTIALLY THIOLATED OLIGOCYTIDYLIC ACIDS.

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SUMMARY

A series of oligomers of cytidylic acid were prepared and partially (6-9% of the bases) thiolated in the 5 positions. The modified oligomers showed increasing inhibition with increasing chain length of both the DNA polymerase-α from regenerating rat liver and the DNA-dependent RNA polymerase of E. coli, but the minimum chain length for observable inhibitory activity was 5 nucleotide units for the DNA polymerase-α and 16 units for the RNA polymerase.

INTRODUCTION

Recent development of the antitemplate concept (1,2) has been focused on the chemical and enzymatic synthesis of partially thiolated polynucleotides (3,4) and on their biological activities such as cellular uptake (5,6), antiviral activity (7), and inhibition of various nucleic acid polymerases including DNA-dependent RNA polymerases (3,8), DNA-dependent DNA polymerases (9,10) and RNA-dependent DNA polymerases (9,11-14). The results suggest that the antitemplate activities depend on the percent of thiolated nucleotide units, the configuration, base composition and other specific structural properties of the modified polymers. In order to investigate the effect of molecular size (chain-length) of the modified nucleic acids on their inhibitory activities, a series of partially thiolated oligocytidylic acids were prepared and their inhibitory actions on the DNA polymerase-α from regenerating rat liver and the DNA-dependent RNA polymerase of E. coli were examined.

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EXPERIMENTAL

Materials: The unlabeled ribo- and deoxyribonucleoside triphosphates were purchased from Calbiochem, San Diego, Calif. [^{3}H]GTP (8.12 Ci/mmole) and [^{3}H]dGTP (8.0 Ci/mmole) were purchased from New England Nuclear Corp. Boston, Mass, and from Schwartz/Mann, Orangeburg, New York, respectively. Poly(C) was obtained from Miles Laboratories, Elkhart, Indiana. Partially (9.1%) thiolated polycytidylic acid (MPC)[2] was prepared according to the previously described procedure (3). Calf thymus DNA, and E. coli alkaline phosphatase (EC 3.1.3.1., 34 units/mg) were purchased from Worthington Co., Freehold, N.J. DNA polymerase-α from regenerating rat liver was isolated and purified as described previously (10). The DNA-dependent RNA polymerase was isolated from E. coli cells (purchased from Miles Laboratories, Elkhart, Ind.) and purified to fraction 5 (900 units/mg protein) according to the method of Burgess (15).

Partially Thiolated Oligocytidylic Acids: Poly(C) was hydrolyzed with 0.1N NaOH at 37° for 20 min, then adjusted to pH 1 with 1N HCl. The precipitate formed was further hydrolyzed with 0.1N NaOH at 37° for 30 min, then adjusted to pH 1 as above. The combined acidified hydrolysates were left standing at room temperature for 4 hr to cleave the 2',3'-cyclic phosphate at the 3' end of the oligonucleotides, then adjusted to pH 8.0 with 1N NH_4OH and incubated with alkaline phosphatase at 37° for 16 hr to remove the terminal phosphate groups. The oligonucleotides were separated by chromatography on a DEAE-cellulose column using a linear gradient of 0.01-1M triethylammonium acetate buffer (pH 6.7) in 7M urea according to the method of Ts'o (16) which, in the case of oligocytidylates, gave an elution profile with clearly distinguishable uv absorbance peaks for each oligo(C) up to C_{21}; beyond this point, the "peak positions" were arbitrarily chosen for separation of the higher oligomers. Each oligonucleotide fraction was subsequently purified (16) and the chain length in several of the fractions was determined on the basis of the ratio of the total organic phosphate (P_t) to the terminal phosphate (P_i). Total phosphate was assayed by the method of Chen et al (17). Terminal phosphate was liberated using the alkaline phosphatase and assayed as above. Before hydrolysis with the alkaline phosphatase, the free 3'-OH cytidine was removed by treatment with $NaIO_4$ and L-lysine (18).

The oligocytidylic acids thus obtained and characterized (19) were partially thiolated using the same procedure as described previously for the partial thiolation of polynucleotides (3). The oligomers, after conversion to their cetyltrimethylammonium salts, were dissolved in dry methanol and treated with freshly prepared methyl hypobromite (CH_3OBr) followed by sodium sulfhydrate (NaSH). In some cases (when the chain length was less than 10), the oligomers being soluble in methanol as the triethylammonium salts, were thiolated directly after separation on the DEAE-cellulose column (see above) and subsequent lyophilization of the fractions. The "percent of thiolation" data given in Table 1, were determined on the basis of the uv absorbancies at λ_{max} 335 nm (upon the addition of dithiothreitol) and 270 nm (in the absence of dithiothreitol) (3).

Enzyme Assays:

DNA Polymerase-α: The reaction mixture, in a final volume of 0.5 ml, contained 0.04 mM each of dATP, dCTP, dTTP, and [^{3}H]dGTP (21 mCi/mmole), 40 mM of glycine buffer (pH 8.0), 16 mM of $MgCl_2$, 1 mM of 2-mercaptoethanol, 50 µg of "activated" calf thymus DNA, 0.4 units of the α-polymerase, and 0.04 mM of the inhibitor. The mixture was incubated at 37° for 1 hr and processed for radioactivity analysis as described previously (10).

[2] Abbreviations: MPC, partially thiolated polycytidylic acid; $MP_{\bar{n}}$, partially thiolated oligocytidylic acid containing n nucleotide units (a bar over n indicates average); C_n, oligomer of n cytidylates.

Table 1. Oligocytidylic Acids from the DEAE-cellulose Column Fractions and Their Partial Thiolation.

Fraction No.[a]	Chain length by P_t/P_i	Chain length Assigned[b]	Percent of thiolation
3		3	7.0
4	3.56	4	-
5	5.09	5	7.4
6	6.04	6	8.5
9		9	6.6
11		11	8.7
13		13	7.3
15		15	6.0
16		16	6.4
21		21	6.0
24	25.5	$\overline{26}$	7.7
28	40.2	$\overline{40}$	9.1

[a] Combined chromatographic fractions according to the "peak positions" as explained in the Experimental section.

[b] Assignments of chain lengths for fractions No 1 to 21 were based on the positions of the corresponding uv absorbance peaks in the elution profile. The higher oligomer fractions were combined in fractions No.22 to 28, and their average chain lengths were determined on the basis of the P_t/P_i ratios (see Experimental).

DNA-Dependent RNA Polymerase: The assay was essentially the same as described in the literature(15). The reaction mixture, 0.25 ml, contained 0.04M Tris, pH 7.9, 0.01M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.15 mM KCl, 0.4 mM phosphate buffer (pH 7.5), 0.15 mM each of ATP, CTP, UTP and [^{3}H]GTP (4.4 mCi/mmole), 0.4 mg/ml of bovine serum albumin, 50 µg of native calf thymus DNA, 6.2 µg of enzyme, and 0.04 mM of the inhibitor. The mixture was incubated at 37° for 20 min.

RESULTS

The DEAE-cellulose column chromatography of the hydrolysate of poly(C) yielded a series of oligomers, from the monomer(C_1) up to an average chain length of higher than 40 ($C_{\overline{40}}$). Oligomers up to C_{12} were well separated, and their "peak positions" corresponded to their chain lengths as shown by the data in Table 1. The thiolations were so conducted that each of the oligonucleotide

Table 2. Inhibition of DNA Polymerase-α from Regenerating Rat Liver by Partially Thiolated Oligocytidylic Acids.

Inhibitor[a] (40 μM)	[^{3}]dGTP incorporated[b] (nmoles)	% inhibition
None	0.414	0
MC_3	0.415	0
MC_5	0.350	13
MC_9	0.290	30
MC_{11}	0.273	34
MC_{13}	0.236	44
MC_{21}	0.178	57
$MC_{\overline{40}}$	0.124	70
MPC	0.046	89

[a] Abbreviations: see text (footnote 2).

[b] Assay conditions: see Experimental.

fractions contained nearly the same ratio (6-9%) of 5-mercaptocytidylate moieties (see Table 1).

The effects of the partially thiolated oligocytidylic acids on the DNA polymerase-α are shown in Table 2. It is clear that the trimer did not inhibit the enzymic reaction at the standard concentration used in all experiments, while the pentamer caused 13% inhibitions. The extent of inhibition increased rapidly with the size of the modified oligomers up to a chain length of 13 (44% inhibition) and then more slowly to 70% as the oligomer size increased to an average chain length of 40. However, the 9.1%-MPC was still somewhat more potent inhibitor of the DNA polymerase-α than the 9.1% $MC_{\overline{40}}$.

The inhibition of the DNA-dependent RNA polymerase of *E. coli* by the modified oligocytidylates showed a much larger minimum size requirement. It is evident from the results in Table 3 that the partially thiolated oligocytidylates with a chain length of less than 15 nucleotide units did not inhibit the RNA polymerase. Only larger modified oligonucleotides inhibited the enzyme reaction

Table 3. Inhibition of the DNA-dependent RNA Polymerase of E. coli by Partially Thiolated Oligocytidylic Acids.

Inhibitor[a] (40 μM)	[^{3}H]GMP incorporated[b] (nmoles)	% inhibition
None	1.12	0
MC_3	1.13	0
MC_5	1.13	0
MC_9	1.13	0
MC_{13}	1.12	0
MC_{15}	1.13	0
MC_{16}	0.96	14.0
MC_{21}	0.78	30.3
$MC_{\overline{26}}$	0.73	35.0
$MC_{\overline{40}}$	0.30	73.1
MPC	0.20	81.5

[a] Abbreviations: see text (footnote 2).

[b] Assay conditions: see Experimental.

and the inhibition steeply increased with the size of the oligomers from 16 to 40 nucleotide units. As in the case of DNA polymerase-α, the modified polynucleotide (MPC) showed the highest activity. Increasing the concentrations of the modified oligomers above the standard 40 μM used had no effect on the assay results (see Figure 1) and the observed minimum size requirement remained the same. Unmodified poly(C) as well as several of the unmodified oligo(C) fractions were also tested and were found to have no effect on the enzyme reaction.

DISCUSSION

Previous studies of the partial thiolation of poly(C) by the procedure used in these experiments have shown 1. that the phosphodiester bonds remain intact and there is no change in molecular weight during thiolation, and 2. that the 5-mercaptocytidylate units are randomly distributed in the molecule,

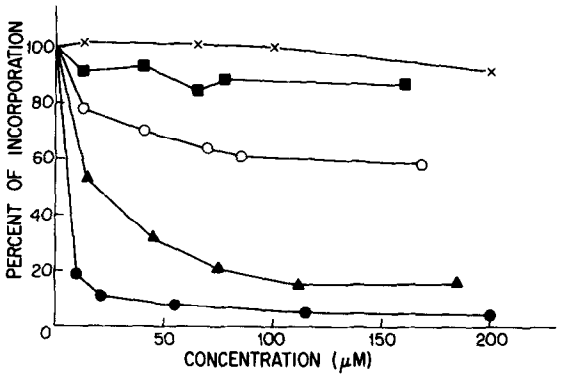

Figure 1. Effect of Increasing Concentration of the Partially Thiolated Oligocytidylic Acids on the DNA-dependent RNA Polymerase of E. coli. The assay conditions were the same as described in the Experimental section, except that the concentration of the inhibitors was varied. ×—× MC_9; ■——■ MC_{15}; ○——○ MC_{16}; ▲——▲ MC_{40}; ●——● MPC.

i.e., there are no "clusters" of thiolated bases (7). Furthermore, in most of the polymerase assays, the inhibitory potencies of various MPC preparations were directly related to their "percent of thiolation" (9,10). For this reason, to investigate the effect of molecular size on the inhibitory activities, we chose to compare in the present studies a series of partially thiolated oligo(C) fractions of different chain lengths but containing similar percent of 5-mercaptocytidylate units per total nucleotides. Thus, using a standard concentration of the modified oligo(C) fractions, the concentration of 5-mercaptocytidylate units in the assay was kept nearly constant, and the differences in activity were attributed to the differences in chain length.

It has been demonstrated that partially thiolated polynucleotides inhibit RNA and DNA polymerases by competing with the functional templates for their binding sites on the enzyme (3,9,10). It is reasonable to assume that the active site of the enzyme can accomodate only a relatively short segment of the template (or, of the antitemplate) at any given time. Since an antitemplate is characterized by its strong binding and by being "non-functional" (2), it is presumably not required to move along the enzyme surface; therefore, a partially

thiolated oligonucleotide of a certain minimal length would be expected to occupy the active site and to show full inhibitory activity. The observation that MPC is still more potent than the largest modified oligonucleotide fraction tested may be due to the presumably more facile interaction of the polymerases with polynucleotides as compared to smaller molecules.

The most interesting result of the present study is the observation that the minimal chain length requirement for inhibitory activity is significantly different in the case of the two polymerases. One might speculate that the inhibition of the DNA polymerase-α by the modified oligomers in the range of MC_5-MC_{15} could be due to interference with the primer (rather than the template) function of the "activated" DNA. It is known that the DNA polymerases can utilize short-chain oligonucleotides as primers (20,21) while the RNA polymerases have no primer requirement. Thus, the MC_5-MC_{15} oligomers may inhibit the DNA polymerase-α as "antiprimers", but do not interact with the RNA polymerase.

The observed difference in the minimal chain length requirement for inhibitory activity between these two enzymes suggests that the use of partially thiolated oligonucleotides offers additional possibilities for the selective inhibition of various polymerases. It is probable that differences in base sequence will have more significant and more readily demonstrable effects on the inhibitory activities and selectivities of partially thiolated oligonucleotides as compared to polynucleotides.

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