Enzymatic synthesis of polyuridylic acid containing modified bases

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ABSTRACT

5-Mercaptouridine-5'-diphosphate (hs^5UDP) has been synthesized and investigated as a substrate of the polynucleotide phosphorylase of *Micrococcus luteus*. While hs^5UDP is not utilized alone, it can be copolymerized with UDP; however, unusually for this enzyme, the ratio of 5-mercaptouridylylate vs. uridylylate residues in the polynucleotide product (MPU) is always lower than the ratio of hs^5UDP vs. UDP in the substrate mixture. Furthermore, hs^5UDP decreases the rate of the enzymic polymerization reaction. The MPU product forms two-stranded and three-stranded complexes with poly(A). The circular dichroic spectra of these complexes are similar to those formed between poly(U) and poly(A), but their melting profiles indicate somewhat lower stability. The physicochemical and biochemical properties of the enzymic product are qualitatively similar to those of MPU prepared by chemical modification; both are potent inhibitors of a DNA-dependent RNA polymerase.

INTRODUCTION

An approach to the design of "antitemplates", i.e., inhibitory structural analogs of the templates of DNA and RNA polymerases, involves the chemical modification of polynucleotides.^{1,2} Based on this approach, the preparation of partially thiolated polynucleotides,³ and their effects on various template-dependent nucleic acid polymerases from bacteria,⁴⁻⁶ RNA tumor viruses⁷⁻¹⁰ and mammalian cells^{9,11-13} have been reported. Partially thiolated polycytidylic acid (MPC) decreases the colony forming ability of murine leukemia bone marrow and spleen cells.¹⁴ The double-helical complex $poly(I) \cdot MPC$ is readily taken up by tumor cells¹⁵ and elicits potent antiviral activity.¹⁶ Preliminary clinical results indicate that MPC is active against acute lymphocytic leukemia in children.^{17,18} It was of interest to investigate the possibility of synthesizing partially thiolated polynucleotides via enzymatic incorporation of the monomeric 5-mercaptanucleotide analog during polymerization, rather than by chemical modification of the preformed polynucleotides. The two types of approaches¹⁹ may complement each other in their (a) range of applicability,

(b) control of specificity of the sites of "thiolation" (position of 5-mercaptopyrimidine bases) of the resulting modified polynucleotides, and (c) predictability, and/or ease of confirmation, of the exact structural changes obtained.

It is well documented that various modified nucleoside diphosphates are utilized as substrates by polynucleotide phosphorylases.²⁰ Recently, the interesting biological and biochemical activities of some enzymatically synthesized polynucleotide analogs have been reviewed.^{21,22} Among the pyrimidine nucleoside diphosphate analogs with modification on the base, the polymerization of 5-halogeno-,²³⁻²⁸ 5-hydroxy-,^{24,29} 5-alkyl-,³⁰ 2-thio-, 4-thio- and 2,4-dithio-³¹⁻³⁵ pyrimidine nucleoside diphosphates have been reported. While the 5-halogeno derivatives are readily polymerized, some of the other analogs, notably 5-hydroxycytidine diphosphate (ho⁵CDP)²⁹ which is substituted with an ionizable hydroxyl group, and 4-thiopyrimidine nucleoside diphosphates^{33,34} are polymerized with some difficulty. Our preliminary results indicated that also the 5-mercaptopyrimidine nucleoside diphosphates in which the free thiol groups are essentially ionized at physiological pH, behaved as rather poor substrates for the polynucleotide phosphorylase of *M. luteus*, while their S-methylated derivatives were utilized much more effectively by this enzyme.^{19,36} In this report we wish to describe our studies of the enzymatic synthesis of partially thiolated polyuridylic acid (enzMPU)³⁷ and to compare the physico-chemical and biochemical properties of the enzymatically synthesized product with those of the chemically synthesized MPU obtained via the chemical modification of preformed poly(U).

MATERIALS AND METHODS

Uridine 5'-phosphate (UMP) was purchased from Sigma Chemical Co. Uridine-¹⁴C-5'-diphosphate was purchased from Amersham/Searle Corp. Polynucleotide phosphorylase of *M. luteus* (1 unit/mg) and of *E. coli* (10 units/mg) were purchased from Worthington Biochemical Corporation and General Biochemicals, respectively.

Synthesis of Substrates

5-Mercaptouridine 5'-phosphate (hs⁵UMP). Uridine 5'-phosphate was treated with MeOBr in methanol, followed by sodium disulfide (Na₂S₂) in dimethylacetamide, according to the method reported previously.³⁸ The product, obtained in 80% yield, was purified by column chromatography on DEAE cellulose.

5-Mercaptouridine-5'-diphosphate (hs⁵UDP). To a refluxing solution of the triethylammonium salt of hs⁵UMP (280 mg., 0.5 mmol) and morpholine

(0.17 ml, 2 mmol) in tert-BuOH-H₂O (1:1), a solution of DCC (412 mg., 2 mmol) in tert-BuOH was added dropwise.³⁹ The phosphoromorpholidate obtained was reacted with the mono-(tri-n-butylammonium) dihydrogen phosphate (1.5 mmol) in anhydrous pyridine at room temperature for 48 hr.³⁹ The hs⁵UDP was isolated and purified by DEAE cellulose column chromatography using a linear gradient of triethylammonium bicarbonate buffer (pH 7.5) with mercaptoethanol (2 mM). The diphosphate, obtained in 60% yield, was further characterized by the ratio of the acid labile phosphate⁴⁰ to the total phosphate which was determined by the method of Chen.⁴¹

Preparation of Partially Thiolated Polyuridylic Acid

Mixtures of hs⁵UDP and UDP were polymerized with polynucleotide phosphor-ylase according to the established general procedure.⁴² The reaction mixture, incubated at 48° for 8 hr, contained in a final volume of 0.2 ml: 15 mM total nucleotides (hs⁵UDP and UDP), 10 units/ml of enzyme, 100 mM Tris (pH 8.5), 5 mM MgCl₂, and 10 mM DTT. Variations of the composition of the reaction mixture are given in the legends of the appropriate tables and figures describing various experiments. The percent of polymerization was followed by assaying for inorganic phosphate⁴¹; appropriate blank (reaction mixture without enzyme) was subtracted from the readings. After incubation, the solution was depro-tenized by repeated extraction with chloroform-isoamyl alcohol (5:3). The polynucleotide was purified by chromatography through Sephadex G-100 in the presence of mercaptoethanol.

The relative amounts of thiolated base incorporated into the polymeric products (expressed as percent of total pyrimidine bases) were determined spectrophotometrically based on the uv absorbances of the 5-mercaptopyrimi-dine derivative at 334 nm in the presence of dithiothreitol (DTT) at pH 8.0.^{43,44} For MPU (or enzMPU):

$$\% \text{ thiolation} = h \times \frac{\Delta A_{334}}{A_{260}} \times \frac{\epsilon_u}{\epsilon_s} \times 100 = 1.8 \times \frac{\Delta A_{334}}{A_{260}} \times 100$$

Where h = hypochromicity of poly(U) at 260 nm = 0.9

ϵ_u = extinction coefficient of 5'UMP at 260 nm = 10×10^3

ϵ_s = extinction coefficient of hs⁵Urd at 334 nm = 5.0×10^3

ΔA_{334} = differential absorbance of MPU at 334 nm, in the presence or absence of DTT, and A_{260} = absorbance of MPU at 260 nm

Mixing curves and ultraviolet spectroscopic measurements

Ultraviolet spectra and mixing curves were determined on a Gilford Model 2400 spectrophotometer. The data for the mixing curves were obtained by the method of continuous variation.⁴⁵ Annealing of polynucleotides was carried out as previously described using a 0.01M Tris buffer, pH 7.5, containing 0.1M NaCl or 1 mM MgCl₂.¹⁶ Absorbance-temperature profiles were procured using the same spectrophotometer equipped with a thermostated cell compartment, so that the absorbance measurements could be made at the ambient temperatures.

Circular Dichroism

Ultraviolet CD measurements were determined on a JASCO Model attached to a J-41C recording spectropolarimeter equipped with a thermostated cylindrical cell holder. Results are reported as $\Delta\epsilon(\epsilon_L - \epsilon_R)$ on the basis of the molar concentration of nucleotide residues of the polymers.

Effects of hs⁵UDP and poly(U, hs⁵U) on the Polymerization of UDP by Polynucleotide Phosphorylase.

Uridine-4-¹⁴C-5'-diphosphate was polymerized by the enzyme in the presence of the thiolated nucleotides as specified in the legends of Fig. 2 and Table 3. The polynucleotide formed upon incubation was precipitated in the form of the cetyltrimethylammonium salt, using 0.1 ml of 2.5% aqueous cetyltrimethylammonium bromide (CTAB) solution containing 0.05M of sodium pyrophosphate. The precipitate was collected by centrifugation, washed 3 times with 0.5 ml solution of 0.25% CTAB and 0.05M sodium pyrophosphate, dissolved in 1.5 ml of methanol and analyzed with a liquid scintillation spectrometer (Nuclear Chicago, Model 6850).

DNA-dependent RNA Polymerase Assay.

The enzyme assay was carried out as previously described.⁶ The assay mixture contained, in 0.25 ml, 0.04M Tris, pH 7.9, 0.01M MgCl₂, 0.1 mM EDTA, 0.1 mM DTT, 0.15M KCl, 0.4 mM phosphate buffer (pH 7.5), 0.4 mg per ml of bovine serum albumin, 0.15 mM each of ATP, CTP, UTP and [³H]GTP (4 mCi/mmoles), 0.15 mg/ml of calf thymus DNA, 6 µg of enzyme and various concentrations of the inhibitors. Assays were incubated at 37° for 30 minutes.

RESULTS

Preliminary experiments indicated that the polynucleotide phosphorylase of *M. luteus* cannot utilize hs⁵UDP in the absence of other substrates. However, our goal was the enzymatic synthesis of partially thiolated polynucleotides, therefore, we investigated the feasibility of copolymerizing the

5-mercaptopyrimidine nucleotide with the natural substrate UDP.

Figure 1 shows that both the rate and extent of polymerization was significantly lower if the UDP substrate was replaced with a mixture of hs^5UDP and UDP in a molar ratio of 1:1. However, the polymeric product obtained in the latter case was characterized as $poly(U_{95}, hs^5U_5)$, *i.e.*, it was found to contain 5% of 5-mercaptouridylate units. This indicated that hs^5UDP was being utilized as a substrate under these conditions by the polynucleotide phosphorylase, although much less efficiently, than the UDP present in the reaction mixture.

In order to optimize the conditions for the enzymatic synthesis of partially thiolated polynucleotides, particularly with respect to increasing the percent of 5-mercaptopyrimidine nucleotides in the polymeric product, the effects of temperature, pH, divalent cation, enzyme concentration and substrate ratio (hs^5UDP to UDP) upon the yield and composition (% hs^5U) of the copolymerization product were investigated. On the basis of the kinetic study shown in Fig. 1, an incubation time of 8-10 hr was used.

Fig. 1. Kinetics of copolymerization of sh^5UDP and UDP, and polymerization of UDP. Aliquots were withdrawn from the reaction mixture (2.0 ml) at different time intervals for P_i determination. Substrate: $\bigcirc$ — $\bigcirc$, UDP; $\bullet$ — $\bullet$, sh^5UDP and UDP, 1:1.

The results obtained with some variations of enzyme and cation concentrations, temperatures, and pH, are summarized in Table 1. The extent of polymerization as well as the percent of 5-mercapto nucleotide content is independent of the temperature within the range of 37° and 48°. While the percent of modified nucleotides incorporated into the polymer is not affected by the change of pH from 7.5 to 8.5, the extent of polymerization increases as the pH increases. This is consistent with the finding that the pH optimum of the enzyme is 8.8⁴² and that the 5-mercapto group is essentially ionized already at pH 7.5.³⁶ Both Mg^{++} and Mn^{++} were found to be about equally good cationic activators. Increase of either the cation or the enzyme concentration had no effect on the ratio of the modified nucleotide incorporated into the polynucleotide, although they did increase the overall yield of polymerization to a certain extent. (Optimal yields were obtained with 5 mM $MgCl_2$ and 10 units/ml enzyme concentration).

In contrast, the ratio of 5-mercapto nucleoside diphosphate present in the substrate mixture had a profound effect on both the extent of polymerization and the ratio of thiolated bases incorporated into the polymeric product. As the relative amount of hs^5UDP was increased from 4.5% to 94% in the substrate mixture, the percent of hs^5U in the polynucleotide obtained increased from 1.0% to 18.3% while at the same time the extent of polymerization de-

Table 1

Polymerization of hs^5UDP and UDP Mixture by the Polynucleotide Phosphorylase of *M. luteus*

The reaction mixture contained in a final volume of 0.2 ml: 15 mM substrates (5 mM UDP, 10 mM hs^5UDP), and the indicated concentration of other components.

Mg^{++} mM	pH	Enzyme units/ml	Temp.	% Polymer- ization	% 5-mercapto- uridylate in polymer
5	8.5	7.5	37°	35.2	4.9
5	8.5	7.5	48°	35.1	4.8
1	8.5	5	48°	9.1	6.5
5	8.5	5	48°	22.4	5.8
10	8.5	5	48°	24.4	-
5	7.5	10	48°	25.6	6.9
5	8.0	10	48°	29.7	5.6
5	8.5	10	48°	35.0	6.7

creased from 42.8% to 3.7% (Table 2). These data clearly show that, under the conditions employed, the polynucleotide phosphorylase utilizes hs^5UDP as a substrate much less efficiently than it does UDP. In addition, the manner in which the extent of polymerization decreased as the percent of hs^5UDP was increased in the substrate mixture, indicates that hs^5UDP also acts as an inhibitor of the overall enzymic reaction. This was directly confirmed by the results of an experiment shown in Fig. 2, in which the concentration of UDP was kept constant while increasing amounts of UMP, hs^5UMP , or hs^5UDP , respectively, were added to the enzymic reaction mixture. Whereas UMP had no effect, both hs^5UMP and hs^5UDP strongly inhibited the overall extent of polymerization; the latter, itself a substrate, was the more potent inhibitor.

Table 3 shows the effect of the product of an enzymatic copolymerization reaction, poly(U_{95} , hs^5U_5), on the polymerization of UDP, in comparison with the effect of poly(U). While poly(U) increased the extent of polymerization in accordance with previous literature results,⁴² poly(U_{95} , hs^5U_5) also stimulated the polymerization of UDP.

The products isolated from all polymerization experiments were high molecular weight polymers with an average S_{20} value of 4.2 in 0.05M PO_4 buffer, 0.1M NaCl, pH 7.0. Using the gel filtration method (Bio-Gel A-15M) and the calibration graph of Miller *et al.*,⁴⁶ the molecular weight of a typical partially thiolated poly(U) preparation was estimated to be 4×10^5 dalton. The ultraviolet absorption spectra of the substrate 5-mercaptouridine diphosphate

Table 2

Effect of 5-Mercaptouridine Diphosphate (hs^5UDP) on the Yield and Composition of the Polymeric Product Obtained with Polynucleotide Phosphorylase.

UDP	Substrates (%) <u>hs^5UDP</u>	% Polymerization	% 5-Mercaptouridylate in Polynucleotide
100	0	52.8	0
95.5	4.5	42.8	1.0
80	20	39.9	2.5
52.5	47.5	35.5	4.2
50	50	35.0	4.8
26.5	73.5	20.0	7.0
6	94	3.7	18.3
0	100	0	-

Fig. 2. Inhibition of the polymerization of UDP by nucleotide analogs. The reaction mixture, incubated at 37° for 1 hr, contained in a final volume of 0.1 ml: 10 mM UDP, 0.53 unit/ml of polynucleotide phosphorylase, 10 mM Tris (pH 8.5), 5 mM MgCl₂, 10 mM DTT, and the indicated concentration of nucleotides; O — O, UMP; ▲ — ▲, sh⁵UMP; ■ — ■, sh⁵UDP.

(hs⁵UDP) and of the enzymic product, partially thiolated poly(U), are shown in Figure 3. In the presence of dithiothreitol, at pH 8.0, the characteristic absorption peak at 334 nm, similar to that of the monomer hs⁵UDP, was evident in the spectrum of the enzMPU which contained 6.6% hs⁵UMP residues.

Table 3
Effects of Polyuridyate and Partially Thiolated Polyuridyate
on the Polymerization of UDP

The reaction mixture, incubated at 37° for 1 hr, contained in a final volume of 0.1 ml: 15 mM uridine-4-¹⁴C-5'-diphosphate (0.23 mCi/mmol), 0.1 units/ml of polynucleotide phosphorylase, and the indicated concentration of polynucleotide.

Poly(U), mM	0	0.25	0.50	0.75	1.05
Radioactivity of precipitate cpm x 10 ⁻⁴	1.085	1.231	1.315	1.315	1.392
Poly(U ₉₅ ,sh ⁵ U ₅), mM	0	0.25	0.50	0.75	1.05
Radioactivity of precipitate* cpm x 10 ⁻⁴	1.085	1.285	1.345	1.456	1.622

* see Materials and Methods

Fig. 3. Ultraviolet spectra of nucleotides and polynucleotides, $\times$ — $\times$, UDP; $\bullet$ — $\bullet$, hs^5 UDP; $\circ$ — $\circ$, hs^5 UDP in the presence of DTT; —, enzMPU; - - - -, enzMPU in the presence of DTT.

The uv spectrum of a chemically synthesized MPU containing more hs^5 UMP residues (11.5%) showed a higher absorption peak at 334 nm upon addition of DTT. The increase in absorbance at 334 nm after addition of DTT was employed for the calculation of the "% thiolation" (i.e., % of 5-mercaptopuridylate residues) of MPU using the appropriate ϵ values (See Materials and Methods).

As shown by the mixing curves (Figure 4a,b), MPU formed a 1:1 double helical complex as well as a 2:1 triple stranded complex with poly(A). The CD spectra of both of these complexes were similar to those of the corresponding complexes between poly(A) and poly(U) with respect to the positions and intensities of the absorption maxima and minima, respectively (Fig. 5).

Ultraviolet absorbance-temperature profiles (Fig. 6a,b) show that in the case of the 1:1 complexes, both the enzymatically and chemically modified polymers manifested lower T_m values and hyperchromicities than did the unmodified poly(A)·poly(U). (Table 4). Addition of DTT did not significantly affect

Fig. 4. Mixing curve of poly(A) and MPU. (a) Buffer in which the polynucleotides were dissolved was Tris-HCl, 0.01M, pH 7.9 containing NaCl, 0.1M and DTT, 1 mM. (b) Same as (a) except that MgCl_2 , 1 mM was presented in the buffer. O — O, poly(U); ● — ●, MPU.

the T_m values of the modified polymers, but increased the hyperchromicity of the complexes to only slightly lower values than those of the unmodified poly(U)-poly(A) complexes. The characteristic transition of a three-stranded to a two-stranded complex, which is clearly seen in the melting profile of

Fig. 5. Circular dichroic spectra of the complexes formed between poly(A) and MPU. (a). 1:2 complexes: O — O, poly(A)·poly(U); Δ — Δ, poly(A)·enzMPU in the absence or presence of DTT; × — ×, poly(A)·MPU in the absence or presence of DTT. (b) 1:1 complexes: × — ×, poly(A)·poly(U); Δ — Δ, poly(A)·enzMPU in the absence or presence of DTT; O — O, poly(A)·MPU in the absence or presence of DTT.

Fig. 6. Uv absorbance temperature profile of poly(A)·MPU complexes. The buffer used was Tris, 0.01M, pH 7.5 containing NaCl, 0.1M. (a) 1:1 complexes: $\square$ — $\square$, poly(A)·poly(U); $\bullet$ — $\bullet$, poly(A)·enzMPU; $\blacktriangle$ — $\blacktriangle$, poly(A)·enzMPU + DTT; $\circ$ — $\circ$, poly(A)·MPU; $\triangle$ — $\triangle$, poly(A)·MPU + DTT. (b) 1:2 complexes: $\blacktriangle$ — $\blacktriangle$, poly(A)·poly(U); $\bullet$ — $\bullet$, poly(A)·enzMPU; $\blacksquare$ — $\blacksquare$, poly(A)·enzMPU + DTT; $\times$ — $\times$, poly(A)·MPU; $\circ$ — $\circ$, poly(A)·MPU + DTT.

poly(A)·poly(U),⁴⁷ was not observed in the case of the modified three-stranded complexes whose melting profile showed only one transition. That the T_m value of the chemically modified MPU·poly(A) complex was lower than that of its enzymatically synthesized counterpart, was due to the fact that the former contained a higher percent of thiolated bases. All complexes, including those of the unmodified poly(U), showed in the presence of 1mM Mg^{++} ion in the buffer, a one-transition melting profile with approximately 4° higher T_m values (data not shown) than those represented in Fig. 6a,b which were determined in

Table 4

Melting Temperature of poly(A)·MPU complexes

Complex	DTT	T _m °C
Poly(A)·poly(U) 1:1	-	55.2
Poly(A)·enzMPU 1:1	-	52
Poly(A)·enzMPU 1:1	+	49.5
Poly(A)·MPU 1:1	-	50.2
Poly(A)·MPU 1:1	+	51.5
Poly(A)·poly(U) 1:2	-	40.8, 53.8
Poly(A)·enzMPU 1:2	-	52.8
Poly(A)·enzMPU 1:2	+	52.5
Poly(A)·MPU 1:2	-	49.6
Poly(A)·MPU 1:2	+	49.5

the absence of Mg salt.

The biochemical activity of the enzymatically synthesized MPU (6.6% thiolation) was investigated using the DNA-dependent RNA polymerase of *E. coli* as a model. Figure 7 shows that it was a potent inhibitor with an $I_{50} = 2.7 \times 10^{-5} M$.

Fig. 7. Effect of partially thiolated poly(U) on the DNA-dependent RNA polymerase of *E. coli*. □ — □, poly(U); ○ — ○, MPU; Δ — Δ, enzMPU. Control = 6,098 cpm = 100%.

The chemically modified MPU at 11.5% thiolation is a more potent inhibitor ($I_{50} = 8 \times 10^{-6} M$) consistent with the previous finding^{7,12} that the potency of enzymic inhibition increases with the % of thiolation.

DISCUSSION

The most unusual feature of the copolymerization of 5-mercaptouridine diphosphate with uridine diphosphate is that the polynucleotide product was found to contain a substantially lower ratio of the base analogue than the relative amount of hs^5UDP employed in the reaction mixture. In all other copolymerization reactions catalyzed by polynucleotide phosphorylase which have been reported in the literature, the base composition of the polynucleotide product was identical with that of the substrate mixture. This was true even in those cases when a relatively "poor substrate", such as s^2CDP or s^4UDP , was copolymerized with the corresponding natural substrate, CDP or UDP, respectively.^{34,48} Although the rates and yields of polymerization decreased with increasing ratio of the analogue diphosphate, the base ratio in the resulting polynucleotide was always the same as the input ratio.⁴⁸ Furthermore, in the case of $poly(s^4U, U)$ it was demonstrated that the inhibition of the overall rate of polymerization was due to the effect of the $poly(U, s^4U)$ product formed in the reaction, rather than to a direct inhibitory effect of the substrate analogue s^4UDP ³⁴. This, too, is in striking contrast with the mode of action of hs^5UDP . Not only is the latter substrate-analogue per se also an inhibitor of the enzymic reaction, but conversely, its copolymerization product $poly(U, hs^5U)$ stimulates rather than inhibits the polymerization of UDP (see Table 3). This observation is particularly interesting in view of the fact that polynucleotides containing 5-mercapto-pyrimidine bases were found to be potent inhibitors of other, template-dependent RNA (and DNA) polymerases by competing with the functional templates for the "template-sites" of these enzymes.⁴⁻¹³ There is no such competition, of course, in the case of the template-independent polynucleotide phosphorylase. In this enzyme, the stimulating effect of $poly(U)$ has been attributed to its action as a "primer";⁴² $poly(U, sh^5U)$ may act in the same manner. On the other hand, the reported³⁴ inhibitory effect of the $poly(U, s^4U)$ is difficult to explain on the basis of available information; no explanation was offered in the original paper.

The pK_a of the SH group of the 5-mercapto-2'-deoxyuridine is 5.0⁴³. In the alkaline pH range required by the polynucleotide phosphorylase of M. luteus⁴⁹, the 5-mercaptonucleotide diphosphate, carrying an ionized sulphhydryl

group, would have vastly different electronic properties than the natural substrate or its readily polymerizable analogues which are substituted with neutral groups. However, in addition to being a rather poor substrate, hs^5UDP was also shown to be an effective inhibitor of the polymerization of UDP by the polynucleotide phosphorylase. The fact that hs^5UMP , which is, at best, a distant structural analogue of the natural substrate, is also inhibitory, in contrast to UMP which is not (Fig. 2), seems to indicate that the 5-mercapto group of hs^5UDP had a role in the binding to the enzyme either via ionic interaction or the formation of a mixed disulfide bond. The diverse effects of hs^5UDP as both substrate and inhibitor of the overall enzymic reaction can be best understood if one assumes that the reaction of the analogue with the 3'-OH terminal nucleotide is mediated by the polynucleotide phosphorylase but after this reaction the mixed disulfide bond formed may hinder the further movement of the growing polynucleotide chain along the catalytic site of the enzyme surface. This may lead to premature release of oligonucleotides and to the relative accumulation of the analogue in the acid soluble fraction.

It has been reported³³ that the polynucleotide phosphorylase of E. coli was much more effective than the M. luteus enzyme in the utilization of s^4UDP , and that it was capable of polymerizing this analogue even in the absence of other substrates. For this reason, we conducted some preliminary experiments on the polymerization of hs^5UDP with the E. coli B enzyme. However, the results were essentially similar to those obtained with the polynucleotide phosphorylase of M. luteus, in that hs^5UDP could be polymerized only in the presence of UDP, and in that the analogue was far less efficiently incorporated than UDP from the substrate mixture into the polynucleotide product. Thus, these experiments only served for further demonstration of the essential difference in the modes of action of the two sulfur-substituted substrate-analogues of polynucleotide phosphorylase, hs^5UDP and s^4UDP .

Although the circular dichroism spectra of the two-stranded and three-stranded complexes of MPU with poly(A) are similar to those of poly(U) with poly(A), the melting profiles of these complexes are different. The complexes formed between MPU and poly(A) are less stable than their unmodified counterparts. As in the case of MPC⁴⁴, the instability of the complexes could be attributed to the effect of the 5-mercapto groups present in the modified poly(U). In the oxidized form, the inter- and intramolecular disulfide bonds would destabilize the helical configuration of the MPU and poly(A) complexes due to geometric restriction. In the presence of DTT, at pH 7.5, the anionic character of the 5-mercaptouridylylate residues would derange the hydrophobic

forces between the stacked bases. That the effect of the 5-SH groups on the helical conformations of MPU is significantly less than in the case of MPC, may be due to the fact that in the case of MPU there is no zwitterion formation which was believed to be a possible contributing factor to the derangement of ordered conformations in the case of MPC⁴⁴.

Comparison of the results for enzMPU with those for the MPU obtained by chemical modification, confirms that these two types of MPU products show similar properties with respect to their uv spectra, their complex formation with poly(A), the CD spectra and the melting profiles of the resulting complexes, and their inhibitory activity against the DNA-dependent RNA polymerase of *E. coli*. These findings indicate that the enzymatic and chemical methods for synthesis of modified polynucleotides may lead to qualitatively similar but not necessarily identical products. Due to the poor utilization of hs^5UDP by the polynucleotide phosphorylase, the enzymatic method does not offer any advantages over the chemical method in the preparation of MPU. However, higher percentages of the modified nucleotide may be incorporated when the free SH group of hs^5UDP is blocked (e.g., with methyl¹⁹); therefore, the enzymic method might be preferable for the synthesis of S-substituted MPU (or, S-substituted MPC). Thus, the two methods can complement each other in the synthesis of a variety of modified polynucleotides.

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37. Abbreviations: MPU, partially thiolated polyuridylic acid; enzMPU, enzymatically synthesized partially thiolated polyuridylic acid; hs⁵UMP, 5-mercaptopuridine-5'-phosphate; hs⁵UDP, 5-mercaptopuridine-5'-diphosphate; s²CDP, 2-thiocytidine-5'-diphosphate, s⁴UDP, 4-thiouridine-5'-diphosphate.
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