SYNTHESIS OF 3'-5' DINUCLEOTIDES WITH RNase T_1*

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Summary: The synthetic reaction of Ribonuclease T_1 has been used in the synthesis of dinucleoside monophosphates. Under conditions of maximum yield (greater than 50%) 3'-5' phosphodiester linkages were the exclusive product.

Studies on the mechanism of protein synthesis have demonstrated the need for oligonucleotides of predetermined sequence. The synthetic reactions of several nucleases have been used in the synthesis of such oligonucleotides (1-4). Ribonuclease T_1 (EC 2.7.7.26) has been used to form guanosine containing oligonucleotides (5-11). However Podder and Tinoco (12) demonstrated that the reverse reaction of RNase T_1 also catalyzes the formation of unnatural 2'-5' phosphodiester bonds. The usefulness of RNase T_1 in the synthesis of oligonucleotides of predetermined base sequence therefore becomes questionable, especially if they are to be used as model compounds in studying the mechanism of protein synthesis, since oligoribonucleotides containing 2'-5' bonds fail to stimulate the binding of tRNA to ribosomes (13). It would therefore be beneficial to outline conditions in which exclusively 3'-5' phosphodiester linkages are formed. Data in this communication indicate that GpC containing only 3'-5' phosphodiester bonds can be synthesized in the RNase T_1 catalyzed reaction using G:p and C as substrates.

MATERIALS AND METHODS

Guanosine 2',3' cyclic phosphate (G:p) and all nucleosides were obtained from Schwarz Bioresearch. DEAE-cellulose (DE 23, 1.0 meq/g) was purchased from Whatman.

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The solvent system used for descending paper chromatography was isopropanol/1% $(NH_4)_2SO_4$ in a ratio of 2/1 (v/v).

Column chromatography was carried out on DEAE-cellulose columns (1.5 mm X 25 cm) using an exponential gradient of NH_4HCO_3 (0-0.15 M, pH 8.6) and a 125 ml mixer. Four columns were eluted simultaneously using a single gradient producing device. The flow rate was 460 ml/cm^2/hr. Column effluents were monitored continuously at 260 mμ with a Gilford multiple sample recording spectrophotometer.

The synthetic activity of RNase T_1 was assayed by the following method. Aliquots of reaction mixtures were spotted on 1" X 7" strips of Whatman #1 paper. These were subsequently dipped in a 0.05 M ammonium bicarbonate buffer (pH 8.6), excess moisture removed by blotting, and placed in a Gelman electrophoresis chamber containing the same buffer. A potential of 20 V/cm was applied. Ultraviolet absorbing regions subsequently obtained were cut out and eluted overnight in 1.2 ml of 0.01 M NH_4OH. The absorbance of each sample was measured at 260 mμ. The molar extinction coefficient for GpC was assumed to be identical to that of GpCp (14).

RESULTS AND DISCUSSION

CONDITIONS FOR OPTIMUM SYNTHESIS WITH RNase T_1:

The nature of the phosphodiester linkage formed under conditions of maximal dinucleotide synthesis offers a starting point from which conditions favoring 3'-5' diester linkages may be found. Maximum yields are important since substrates are expensive and time consuming to prepare. Since the kinetics of the reaction are complex, it was necessary to control time, temperature, and enzyme concentration carefully. The influence of each of these factors was determined by varying each separately.

RNase T_1 concentration: As shown in Figure 1, the amount of GpC formed in two hours goes through a maximum at a RNase T_1 concentration of 0.35 μg/ml. At lower concentrations a typical "enzyme verses time" relationship is obtained. At higher enzyme concentrations the depolymerization reaction dominates the

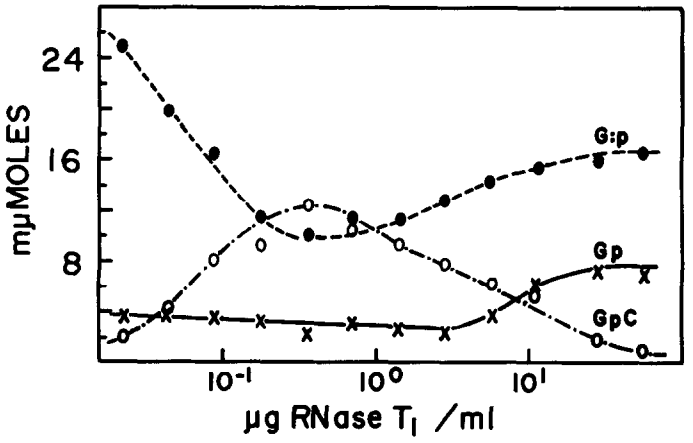

Figure 1: Influence of RNase T_1 concentration on the synthesis of GpC. Reaction mixtures (17 μl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 2.75 mM G:p. RNase T_1 was varied from less than 0.1 to 100 μg 1 ml. Reactions were carried out at 0° C for two hours.

addition reaction. In concentrations greater than about 59 μg/ml no GpC is synthesized, and the formation of 3' GMP (G:p→3'GMP) is increased. GMP seen at low RNase T_1 concentrations is a contaminant in the G:p (about 10%). On the basis of G:p incorporation into dinucleotide, the yield of GpC (Figure 1) is about 55%.

Influence of temperature: Figure 2 shows the effect of temperature on the synthesis of GpC. The yield decreased almost linearly with increasing temperature, from 0 to 37°C. At the higher temperatures depolymerization is favored, resulting in the breakdown of GpC. At 37°C about a 20% incorporation of G:p into GpC was obtained. The yield of GpC at 37°C goes through a maximum at 30 minutes but never reaches a value comparable to that obtained at lower temperatures.

Reaction kinetics: Figure 3 shows the distribution of substrates and products at intervals over a 48 hour period, at both 0 and 37°C. At 0° the yield of GpC was maximal at six hours, and 58% of the cyclic phosphate was incorporated into dinucleoside monophosphate. At 37° a maximum yield of GpC occurred in 30 minutes and was three fold less than at 0°C. Although considerably less hydrolysis

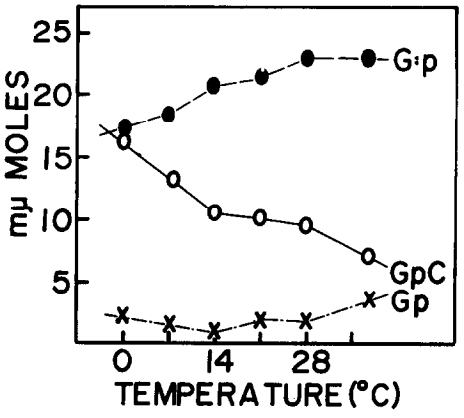

Figure 2: The effect of temperature on the synthesis of GpC from G:p and C. The reaction mixtures (17 μl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 2.75 mM G:p and 0.30 μg ml of RNase T_1. Reactions were carried out at temperatures ranging from 0 to 37°C for six hours.

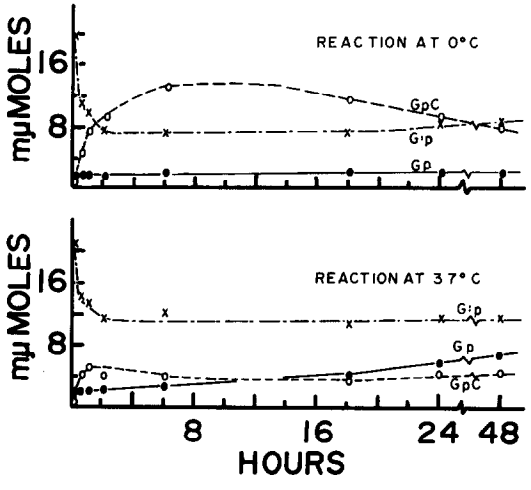

Figure 3: Synthesis of GpC over a 48 hour period at 0 and 37°C. The reaction mixtures (102 μl) contained 59 mM Tris-HCl buffer (pH 7.3), 0.35 M cytidine, 2.75 mM G:p and 0.30 μg ml of RNase T_1. Ten μl aliquots were removed at the times indicated.

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occurred at 0°,3'GMP was produced at a constant rate at both temperatures.

CHARACTERIZATION OF GpC:

Dinucleoside monophosphate (GpC) synthesized at 0 and 37°, was characterized with regard to the nucleosides present, their sequence, and the nature of the

phosphodiester linkage, i.e. 3'-5' or 2'-5'. The 37°C product was characterized because formation of 2'-5' diester linkages was shown to occur at temperatures greater than 0°C (12).

Alkaline hydrolysis: Alkaline hydrolysis was used to identify the component nucleosides or nucleotides and their sequence. This was carried out in 0.5 M KOH for 25 minutes at 100°C. GpC synthesized at both 0 and 37° was converted to equal amounts of cytidine and 2' (3') GMP as shown in Table I. The products of the hydrolysis were separated by column and paper chromatography and identified using authentic markers.

Sensitivity of GpC to RNase T_1: The 2'-5' phosphodiester bond in oligonucleotides is known to be resistant to RNase T_1 (15). This provides a tool for characterization of the diester linkage in GpC. Aliquots of GpC formed at both 0 and 37° were incubated for 16 hours at 37° with 32 units of RNase T_1 in 1 mM Tris-HCl (pH 7.3). Products were separated by column chromatography. As shown in Table I GpC was quantitatively hydrolyzed to 3' GMP and C. This gave convincing

TABLE I

Characterization of RNase T_1 synthesized GpC.

Treatment	Initial mμ moles GpC	Products (mμ moles) 2'GMP	3'GMP	C	C/G	Recovery
0.5 M KOH	(0°) 154	75	75	158	1.05	97%
	(37°)158	76	76	164	1.08	96%
RNase T_1	(0°) 155	0	154	155	1.01	99%
	(37°) 71	0	63	71	1.12	94%
IO_4^-, Lysine	(0°) 170	0	166	---	----	97%
	(37°)168	0	165	---	----	98%

Analysis of the products was done by column chromatography as described above. The recovery is based on the mμ moles of GMP recovered from the initial GpC.

evidence that 3'-5' GpC was the exclusive product, at 0 and 37°C.

Periodate oxidation of GpC: Additional evidence of the nature of the phosphodiester linkage was obtained by oxidation of the 3'-cytidine portion of GpC, followed by amine catalyzed β elimination of the 5'-linked nucleoside (16).

This was accomplished with periodate followed by cleavage with lysine. Products from such reactions were analyzed by column chromatography as described above. 3'GMP was quantitatively recovered from GpC synthesized at both 0° and 37° (Table I), and was shown to cochromatograph with authentic 3'-GMP. No 2'-GMP was detected.

These results are extremely important since they demonstrate that under conditions of maximum polymerization RNase T_1 can be catalytically used in the synthesis of oligonucleotides containing entirely 3'-5' phosphodiester linkages. Although Podder and Tinoco have been successful in demonstrating the formation of 2'-5' oligoguanylic acid using high concentrations of RNase T_1 (12), the synthesis of GpC can be carried out under conditions such that entirely 3'-5' linkages are obtained. Preliminary experiments carried out in this laboratory under our conditions indicate that pG:p and cytidine are also converted to 3'-5' pGpC in high yields. The apparent discrepancy might be a consequence of pecularities observed in the absence of added nucleosides, or the combination of higher RNase T_1 concentrations and temperatures, used in the formation of oligoguanylic acid. Since we have not extended our studies beyond those conditions supporting maximal conversion of G:p into dinucleotide we are unable to clarify the question further.

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