ENZYMATIC SYNTHESIS OF OLIGOGUANYLIC ACIDS CONTAINING 2'-5' PHOSPHODIESTER LINKAGES

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SUMMARY

Under certain conditions T1 ribonuclease catalyzes the synthesis of (2'-5') GpGp from G-cyclic-p. The evidence for this is that GpG (obtained from the GpGp) yields (2') Gp when specifically degraded by periodate oxidation followed by amine catalyzed β elimination of the 5'-linked nucleoside. The T1 synthesized GpG has an ORD distinctly different from (3'-5') GpG and is resistant to T1 hydrolysis.

T1 ribonuclease is well known to be capable of hydrolyzing or synthesizing the link between the 3' phosphate of guanylic acid and the 5' hydroxyl of any nucleotide (Egami, Takahashi, and Uchida, 1964). This enzyme is being used increasingly for synthesis of oligonucleotides; therefore, it is important to be sure of the specificity of the bonds formed under different conditions.

Synthesis of a series of oligoguanylic acids was done following the procedure of Lipsett (1964). The reaction mixture contained 0.15 molar G-cyclic-p and 800 units per ml of T1 ribonuclease (Calbiochem lot no. 71020) in a pH 7.4 Tris buffer. The mixture was kept at room temperature for 18 to 24 hours. It was then heated in boiling water for 5 minutes to inactivate the enzyme and diluted 1:1 by volume with 7M urea. The mixture was

brought to pH ~ 1 for 3-4 hours to open any cyclic phosphates. Finally the oligomers were separated by chain length on a DEAE-Sephadex-urea column. Measurable amounts of oligomers with chain lengths up to 5 were obtained. The chain length in each peak was determined from the ratio of nucleotide to nucleoside found after dephosphorylation and alkaline hydrolysis of the dinucleotide fraction.

The optical rotatory dispersion ORD and ultraviolet absorption of each oligomer were measured as a function of temperature. Aggregation was clearly evident; thermodynamics and kinetics of melting were studied, but will not be discussed here. The significant observation for our present purpose was that the ORD of the dinucleoside phosphate did not agree with that predicted from earlier measurements on GpGpU and GpGpC obtained from pancreatic hydrolysis of RNA (Cantor and Tinoco, 1965). An authentic sample of (3'-5') GpG was then obtained by dephosphorylation of GpGp obtained from alkaline degradation of poly G (Miles control # 11-4-314). Its ORD was found to be in reasonable agreement with the calculated values. Figure 1 compares the ORD of (3'-5') GpG and the enzymatically synthesized GpG. Both samples are in dilute ($\sim 10^{-5}$ M) solution at pH 7.3 and room temperature.

To try to further characterize the synthetic GpG it was incubated with T1 ribonuclease. There was no measurable hydrolysis. The GpG was not aggregated under the attempted hydrolysis conditions since a sedimentation equilibrium experiment gave a molecular weight within 10% of the monomer weight. Even incubation with T1 ribonuclease for 3 days at 45°C did not cause hydrolysis as measured either by increase in absorbance or chromatographic detection of guanylic acid. To test for possible inhibition

of the T1 ribonuclease, (3'-5') GpC was added to the reaction mixture. It was hydrolyzed rapidly.

A possible explanation of these results is that (2'-5') GpGp was synthesized by T1 ribonuclease from G-cyclic-p. The (2'-5') linked oligonucleotides are well known to be resistant to hydrolysis by T1 ribonuclease (Lipsett, 1964). The conditions for synthesis and hydrolysis are sufficiently different so that the (2'-5') isomer could be kinetically favored in synthesis, but the (3'-5') isomer could be preferentially hydrolyzed.

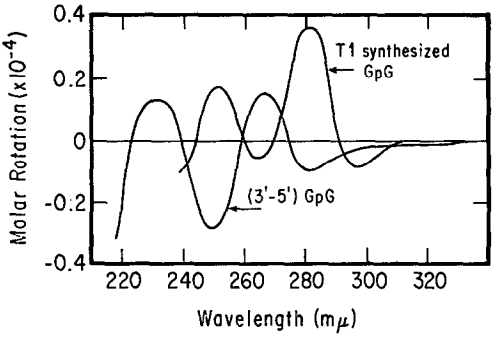
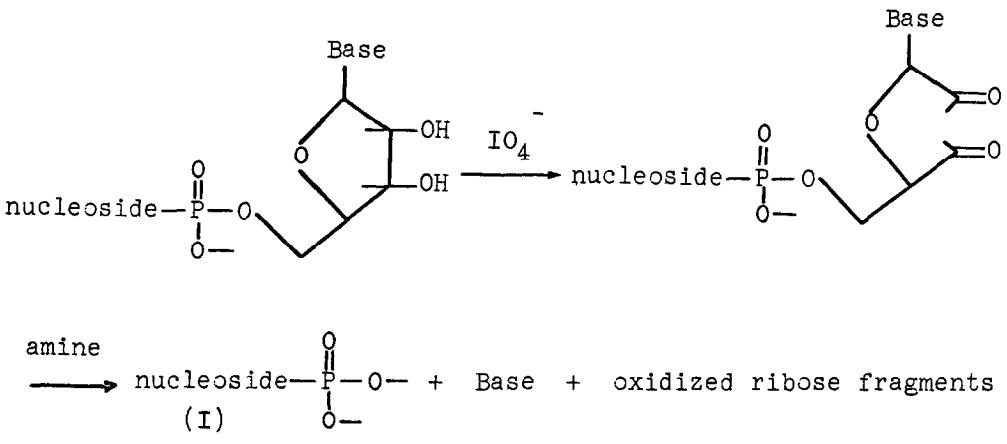


Figure 1. The optical rotatory dispersion (ORD) of (3'-5') GpG compared with the ORD of the GpG obtained from T1 ribonuclease catalyzed condensation of G-cyclic-p. The molecules are in dilute ($\sim 10^{-5}$ M) solution at pH 7.3 and room temperature.

The hypothesis that we enzymatically synthesized (2'-5') GpG was tested by specific chemical degradation of the dinucleoside phosphate. Periodate oxidation of the vicinal hydroxyl groups followed by treatment with an amine can be used to identify the type of ribose-phosphate link (Neu and Heppel, 1964). The reaction can be represented as:



Identification of the mononucleotide (I) as 2' or 3' phosphate is sufficient to identify the linkage of the parent compound. GpG from three different synthetic mixtures were combined and treated with periodate and L-lysine (pH 9) following the procedure of Neu and Heppel, 1964. A sample of commercial (3'-5') GpC was run in parallel. Each reaction mixture was incubated at 45°C for 90 minutes and then chromatographed on a Dowex AG-1 × 2 column using an HCl gradient (Wade, 1960). The elution profile of each reaction mixture showed three peaks corresponding to the base (peak 1), oxidised ribose fragments (peak 2) and the guanylic acid (peak 3). Peak 1 was identified spectrophotometrically as guanine for GpG and cytosine for GpC. Peak 2 showed no maximum at 260 mμ and was identical for GpG and GpC; therefore, it is identified as the oxidised ribose fragments. Peak 3 from each reaction mixture was co-chromatographed with an authentic sample of 3' guanylic acid. The (3') Gp was obtained from chromatography of a 2', 3' mixture on a Dowex AG-1 × 2 column with an HCl gradient (Wade, 1960). The peak 3 plus (3') Gp solutions were chromatographed under identical conditions used to separate (2', 3') Gp on the same column. The results are shown in Figure 2. The Gp from (3'-5') GpC gave one

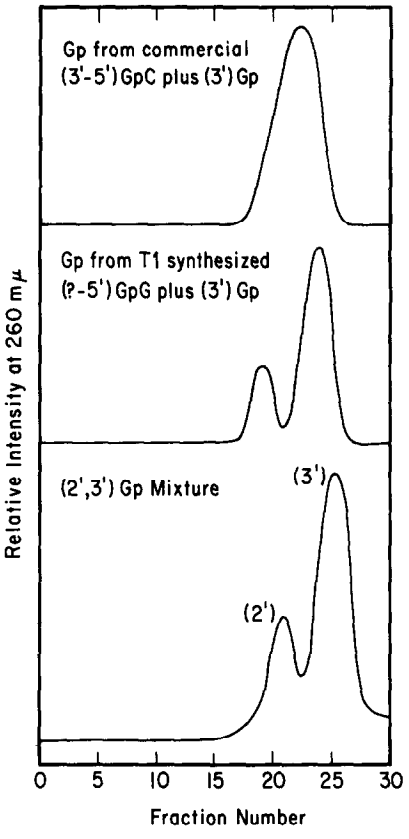


Figure 2. Chromatography on Dowex-1 resin of (2') and (3') isomers of Gp. A pH gradient from pH 2.45 to pH 2.04 was used. The bottom picture shows separation of the two isomers. The middle picture shows separation of a mixture of (3') Gp and Gp obtained from T1 ribonuclease synthesized GpG. The top picture shows no separation (as expected) of a mixture of (3') Gp and Gp obtained from (3'-5') GpC.

peak when mixed with (3') Gp; the Gp from the T1 synthesized GpG gave two peaks when mixed with (3') Gp. The first peak corresponds to the position of the 2' isomer and the other to the 3' isomer (Wade, 1960). This clearly demonstrates that the GpGp synthesized by use of T1 ribonuclease and G-cyclic-p was 2'-5' linked.

The evidence for the enzymatic synthesis of (2'-5') GpG can be summarized as follows. (1) Periodate oxidation plus amine catalyzed β elimination yields (2') Gp. (2) The GpG synthesized

is resistant to T1 ribonuclease hydrolysis. (3) The GpG has a significantly different ORD from (3'-5') GpG.

The conditions necessary to produce (2'-5') or (3'-5') G oligomers from T1 ribonuclease synthesis are not yet clear. Over 10 different synthesis experiments yielded predominantly (2'-5') GpG as measured by ORD. Further studies of different conditions are in progress.

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