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TEMPERATURE-DEPENDENCE OF THE TEMPLATE-DIRECTED SYNTHESIS OF OLIGOGUANYLATES

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Abstract—The oligomerization of (guanosine 5'-phosphor)-2-methylimidazolide on a poly(C) template proceeds efficiently at temp. as high as 37°, although the helical complex formed by the template and substrate melts at a much lower temp. At higher temp. oligomerization depends on the template-independent formation of short oligomeric initiators, which then elongate on the template. The rate of elongation is only slightly dependent on oligomer length for lengths greater than that of the initiator.

INTRODUCTION

The enzymatic synthesis of nucleic acids depends on the ordering of substrate nucleoside triphosphates on a preformed DNA or RNA strand. In a similar way the specificity of DNA ligase depends on the alignment of two oligodeoxynucleotides of a DNA molecule that has the appropriate complementary sequence. Since reactions of this kind are essential for the functioning of the genetic apparatus, it is widely believed that they were important from an early stage in the origins of life. Consequently, a good deal of effort has been devoted to attempts to produce non-enzymatic models.

The first template-directed synthesis of this kind was reported by Naylor and Gilham, who used a polyadenylic acid template to facilitate the synthesis of dodecathymidylic acid from two molecules of hexathymidylic acid.[1] More recently, many papers have described the synthesis of oligonucleotides from activated mononucleotides, and a model with some of the properties of an RNA polymerase has been described.[2] The nucleoside-5'-phosphorimidazolides have proved to be particularly useful substrates in this type of reaction.

The activated nucleotide (guanosine 5' - phosphor) - 2 - methylimidazolide (2-MeImpG) oligomerizes efficiently and regiospecifically on a polycytidylic acid (poly(C)) template at pH 8.0 to give predominantly 3'-5'-linked oligoguanylic acids (oligo(G)'s) up to at least the 40-mer.[3] Surprisingly, the efficiency remains high even at 37°, a temperature substantially above the melting point of the poly(C)-2-MeImpG helix.[4] In this paper we report a semiquantitative kinetic study designed to elucidate the nature of the initiation process that permits synthesis to proceed at these high temperatures.

RESULTS AND DISCUSSION

(a) *General.* A selection of elution profiles of reaction products obtained by the oligomerization of 2-MeImpG on a poly(C) template at various temperatures is reproduced in Figs. 1–3.† The oligomers present after relatively short incubation times are of low molecular-weight and are well separated from poly(C) in the elution profiles. Consequently, we have usually reproduced the elution profiles of samples that had not been subjected to pancreatic ribonuclease hydrolysis. At later times the oligo(G) peaks often overlap the broad poly(C) peak, so it was essential to hydrolyze the poly(C) with pancreatic ribonuclease before HPLC analysis. Since some decomposition of phosphorimidazolides always occurred during the incubation with ribonuclease, we were obliged to complete this hydrolysis by acid treatment in order to obtain reproducible results.

The half-lives for the hydrolysis of 2-Me-Imidazole derivatives to the corresponding 5'-phosphates can be determined roughly from the heights of the corresponding peaks at various times. The half-life could, of course, vary for oligomers of different lengths, but inspection of typical elution profiles shows that this effect is not usually large. The half-life of oligomers of length 4–12 at 0° is seen from Fig. 1 to be between 1 and 4 days. Inspection of elution profiles taken at intermediate times (not reproduced here), shows the half-life to be close to 2 days. A comparison of the elution profiles of non-hydrolyzed 20° samples obtained after 12 and 24 hr (not reproduced here) suggests a half-life of about 16 hr, with a slight tendency for long oligomers to hydrolyze more slowly. At 37° the half-life has fallen substantially to slightly less than 2 hr.

The progressive appearance of longer oligomers as the reaction proceeds is seen in its simplest form at 20°. The elution profile of the initial reaction mixture is shown in Fig. 2(a). The initial 2-MeImpG always contains small amounts of the pyrophosphate, GppG, and of the 2'-5'- and 3'-5'-isomers of ImpGpG and pGpG. After 4 hr, substantial amounts of the imidazolides of oligomers up to the 6–7 mer are present in addition to increased amounts of dimer and the corresponding imidazolide, ImpG$^{3'\text{-}5'}$PG. Furthermore, the peak corresponding to GppG has decreased and small peaks corresponding to pyrophosphate-terminated short oligomers up to at least the 6-mer have appeared. The progressive appearance of longer products is clearly seen in Figs. 2(c and d), corresponding to reaction times of 24 and 48 hr. Apart from the regular appearance of new peaks corresponding to longer oligomers, one also

†See Ref. 3 for identification of peaks.

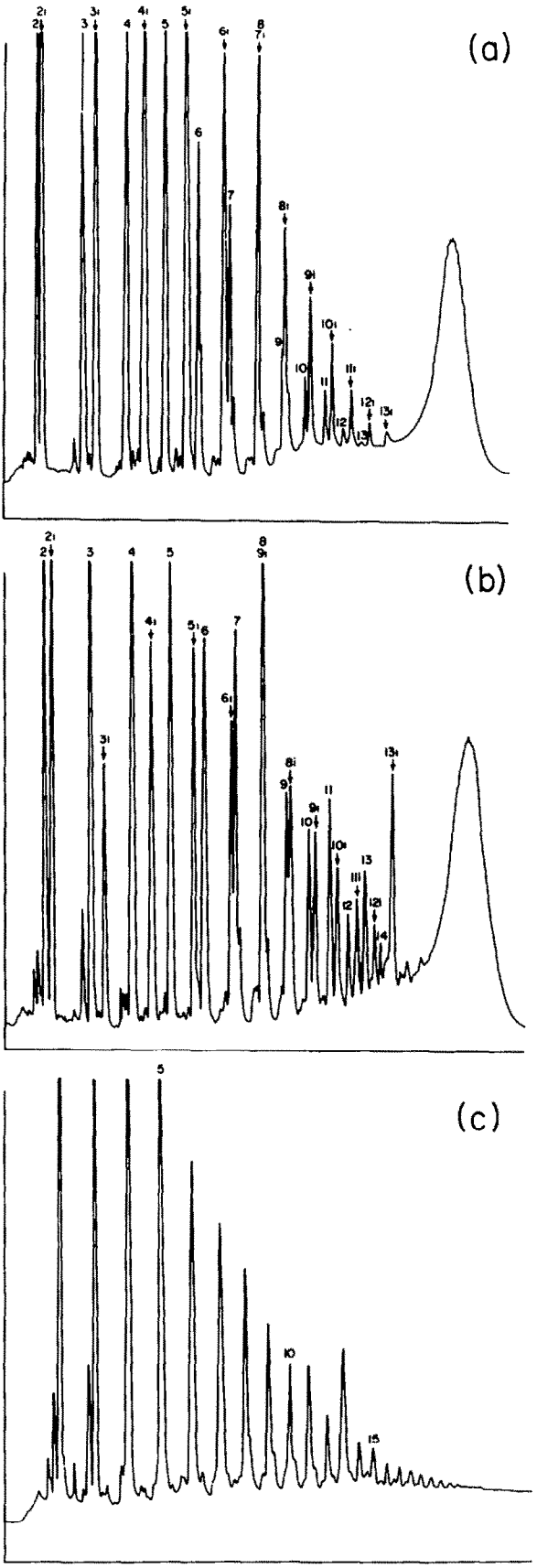

Fig. 1. Elution profiles of products from the oligomerization of 2-MeImpG on poly(C) at 0° after (a) 1 day, (b) 2 days, (c) 1 week. The sample in (c) had been subjected to acid hydrolysis. Peaks are labelled according to the length of the corresponding oligomer; phosphorimidazolides are indicated by the letter i.

notes the relative enhancement of a series of companion peaks with slightly shorter retention times than the main peaks. We believe that these correspond to oligomers with a single 2′-5′ linkage.[5]

The elution profiles of 0° samples taken at times up to 1 day (Fig. 1a) are very similar to those obtained at 20°. Of course, the reaction is slower at the lower temperatures, so corresponding oligomers appear later in the 0° reaction than in the 20° reaction. After 1 day there is a break in the steady evolution of the elution profiles. The peak corresponding to the imidazolide of the 13-mer grows rapidly (Fig. 1b) while the appearance of the 14-mer and higher oligomers is delayed. Finally, after a week or more (Fig. 1c), the yield of the 13-mer is still substantially enhanced but the yields of the larger oligomers recover to the anticipated values.

The time course of the appearance of the 13-mer and subsequently of higher oligomers is most easily explained if we suppose that the rate of conversion of 12 to 13 mer is much the same as the rate of elongation of shorter oligomers, but that the rate of elongation of the 13-mer is substantially lower. Previously published data[3] suggests that the yield of the 26-mer and to a lesser extent of the 25-mer are also enhanced at the end of the reaction. We have suggested that the preferred chain lengths of 13 and 25 or 26 correspond to one and two turns of a poly(C)-oligo(G) double-helix containing somewhat more than 12 base-pairs per turn.

The succession of elution profiles at 37° is very different from those at 0° and 20°. After 2 hr, the yield of dimer is substantial (Fig. 3a). The yields of the trimer is very much lower, but then the yields of successively higher oligomers fall off slowly. It is only after longer periods (Fig. 3b) that the yields of oligomers begin to approach those of the trimer. The reaction is essentially complete in less than 24 hr, and yields detectable amounts of oligomers up to the 20-mer.

(b) *The initiation process.* The yield of each oligomeric product was calculated from the area of the corresponding peak in the HPLC trace. Known amounts of standards were injected and the area under peaks were measured to determine a calibration constant. Then the area under each product peak was measured, a correction for hypochromicity of 38% was assumed for oligo(G)'s, and the yield calculated from the optical density units (ODU's) present in the peak and the known ODU's in the input. In every case the combined yield of tetramer and higher products estimated in this way agreed approximately with the combined yields determined previously by a completely different method.[3]

We next calculated for monomer and for each oligomer $(pG)_n$, the fraction f_n of oligomer molecules that had been converted to higher oligomers where y_i is the yield of the i-mer. These values of f_n are collected in Table 1.

If all elongation rates were equal we would expect all f_n's measured at one temperature and time to be approximately equal. If, on the other hand, monomer and one or more of the smaller oligomers elongated more slowly than longer oligomers, we would expect the f_n to be small for monomer and those shorter oligomers.

The data in Table 1 strongly suggest that, at 20°, oligomers as long as or longer than the trimer elongate at roughly the same rate. The dimer elongates somewhat more slowly than longer oligomers while the rate of initiation of chains by the combination of two monomers is substantially slower. At 0° the situation is different—all oligomers from the dimer upwards elongate at about the same rate which is greater than the initiation rate. At 37°, as expected, the change is in the opposite direction—both the monomer and the dimer elongate slowly, but all higher oligomers elongate at about the same rate.

These results are nicely consistent with the hypothesis that oligonucleotides attached to the template elongate at a rate that is almost independent of oligomer length, so that the observed rate of elongation depends on the extent of attachment to the template. At 0° we know that monomer is incompletely attached to the template.[4] Our new results suggest that the dimer and higher oligomers are

Table 1. Percentage conversion f_n of oligomers (pG)m to longer oligomers $(pG)_n$; n > m. The last entries in each row are less accurate than the earlier ones since the yields of longer oligomers are small

t_{hrs}	m= 1	2	3	4	5	6	7	8	9	10	11
0°C											
8	25.7	50.6	51.4	46.1	51.3	44.3	34.3	33.3			
20°C											
4	12.0	30.0	46.1	47.2	37.50	40.0					
8	16.8	42.5	59.7	56.8	57.8	57.1	62.5	56.0	50.0		
24	25.8	65.7	75.2	76.5	79.4	79.4	79.5	79.5	78.9	82.7	82.3
37°C											
2	14.8	16.0	51.7	55.7	41.2	57.1	50.0	50.0	50.0		
8	18.1	35.9	63.9	65.2	68.2	73.9	70.6	68.7	63.6	61.9	53.9

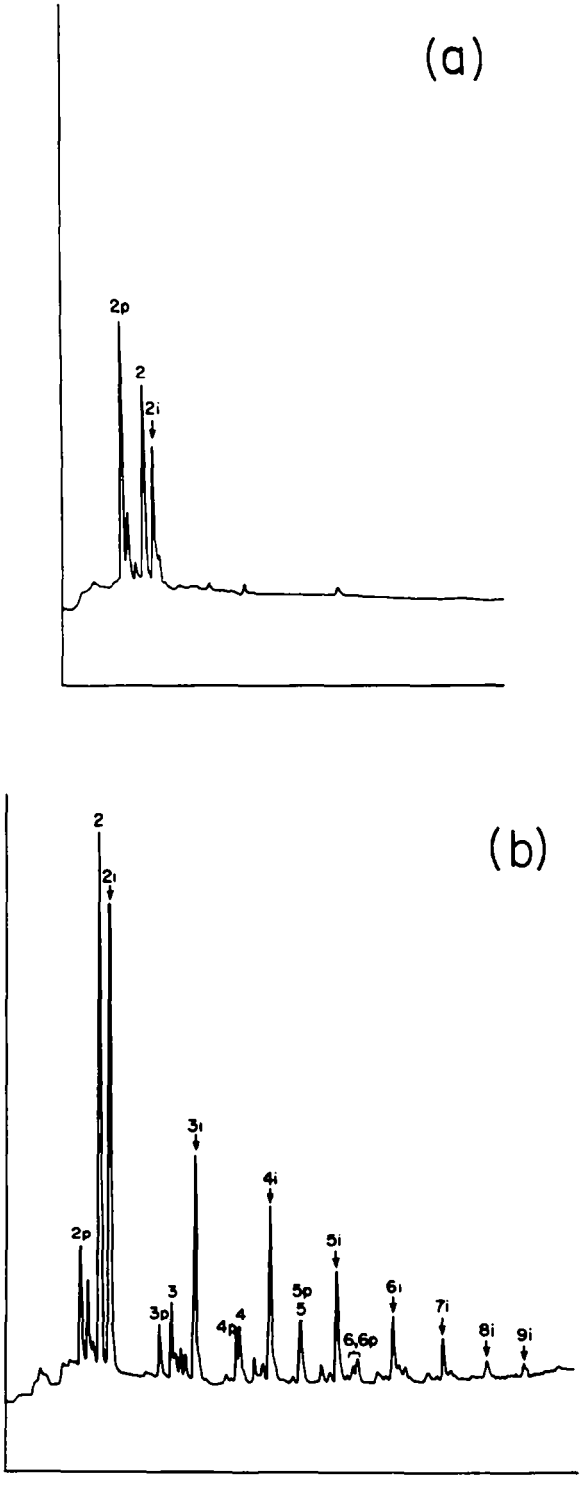

Fig. 2.

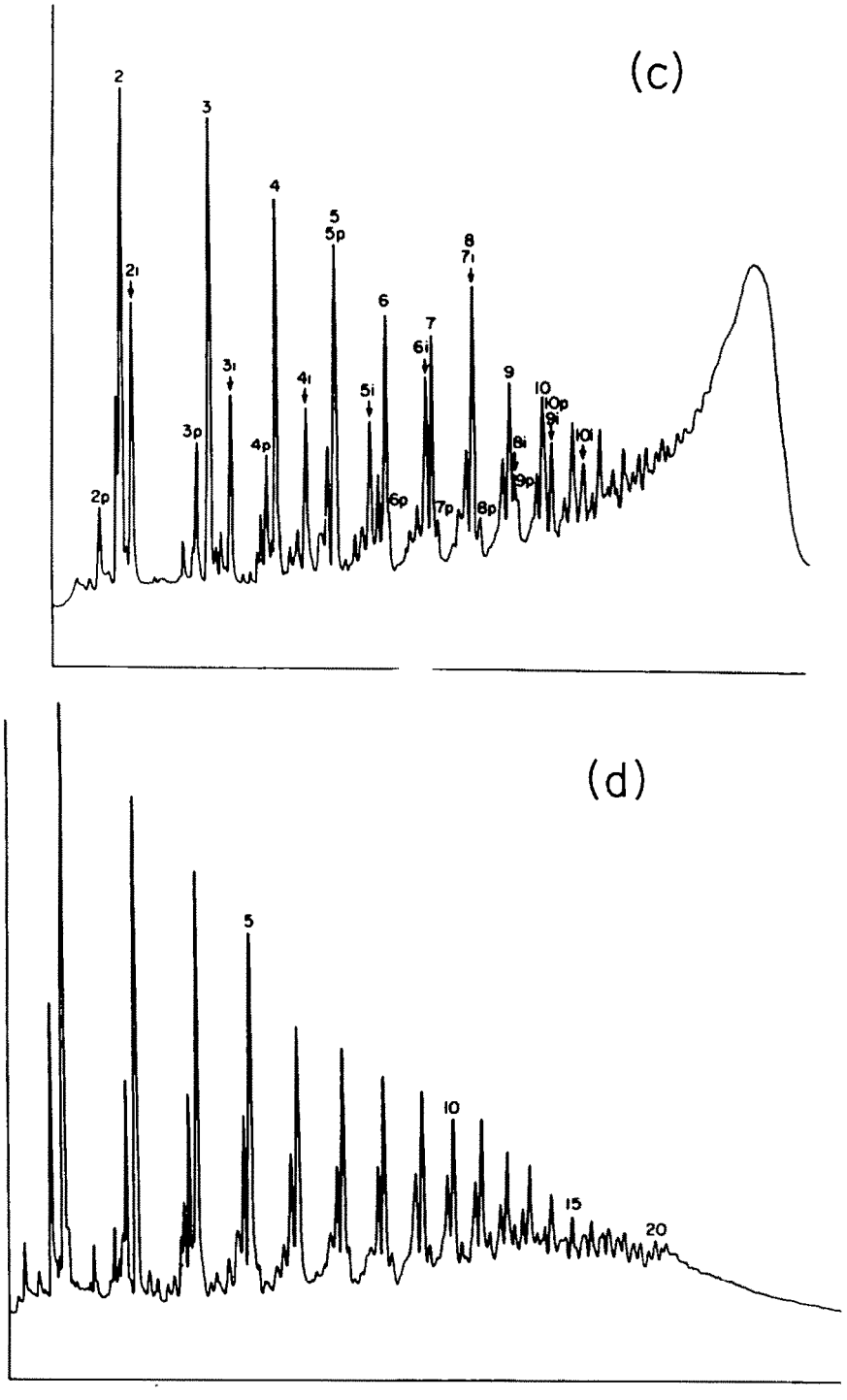

Fig. 2. Elution profiles of products from the oligomerization of 2-MeImpG on poly(C) at 20° at 0-time and after (b) 4 hr, (c) 1 day, (d) 2 days sample (d) had been subjected to acid hydrolysis. Peaks are labelled according to the length of the corresponding oligomer; phosphorimidazolides are indicated by the letter i; pyrophosphate terminated oligomers are indicated by the letter p.

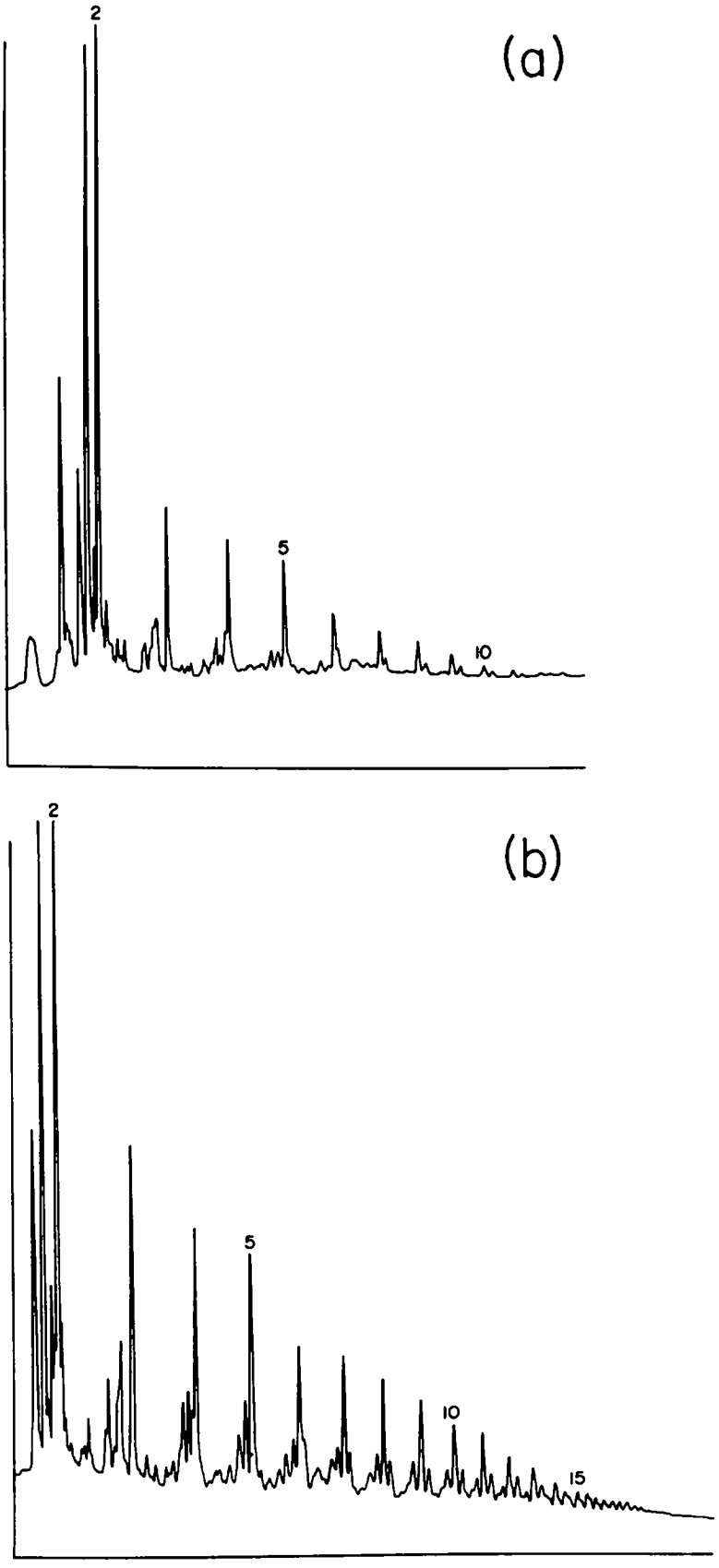

Fig. 3. Elution profiles of products from the oligomerization of 2-MeImpG on poly(C) at 37°, after (a) 2 hr, (b) 8 hr. Both samples had been subjected to acid hydrolysis. Peaks are labelled according to the length of the corresponding oligomer.

almost completely attached. At 20° the trimer and higher oligomers must be completely attached, since they elongate at the same rate, but the dimer is partially attached and the monomer is largely unattached. Finally, at 37° neither monomer nor dimer are substantially attached to the template while the trimer and higher oligomers are almost completely attached.

Thus the initiation of chain growth is a complex process. At 0° dimers may be formed on or off the template. In either case, the 3′-5′-linked dimer acts as an effective initiator for the growth of longer chains. At 20° and 37° iniators must first form off the template since the poly(C): 2-MeImpG helix is almost completely melted. At 20° the dimer is an effective initiator, but at 37° it is necessary to form a trimer before fully effective chain growth can begin.

EXPERIMENTAL

Chromatography-High performance Liquid Chromatography (HPLC) on RPC-5 at pH 12 was performed as previously described.[5]

Preparation of samples. Condensation reactions were carried out in stoppered glass tubes (0.9 cm × 7.5 cm). Aqueous soln containing NaCl, $MgCl_2$ and, if necessary, poly(C) were mixed and evaporated to dryness. 2,6-Lutidine-HCl buffer and aqueous 2-MeImpG were added at 0° to bring the total volume to 200λ for reactions at 0° or 20° or to 500λ for reactions at 37°. The tubes were thoroughly mixed with a vortex mixer and incubated at the appropriate temp. The mixtures contained: 2-MeImpG, 0.1M; $MgCl_2$, 0.2M; NaCl, 1.2M; 2,6-lutidine, 0.4M, and, when necessary, 0.1M poly(C).

The pH was adjusted so that each mixture was at pH 8.0 at the reaction temp. The pH of each mixture was measured at the beginning of the experiment using a Vanlab microelectrode and a Beckman Model 4500 digital pH meter and subsequently, when samples were taken, using pH paper. Occasionally pH's of samples were checked with the microelectrode to confirm the values determined with pH paper. The pH was found to remain essentially unchanged throughout an experiment.

Sampling procedures. At the appropriate time (reported in the result section) 3λ aliquots were taken from thoroughly mixed reaction mixtures. To these samples a 10% excess EDTA was added to complex the divalent metal ions and hence stop the reaction. The samples were then diluted with 2.6-lutidine buffer and chromatographed on RPC-5 immediately. The remainder of the sample, or the complete sample when chromatography could not be carried out immediately, was frozen in dry ice acetone and transferred to a −80° freezer. The sample was thawed on ice just before use. This procedure proved adequate for preserving the 2-Me-imidazolides of the oligo(G) products.

Acid hydrolysis of phosphorimidazolides. The hydrolysis of 2-methyl-imidazolides of the oligo(G) products to the corresponding oligo(G)'s was carried out by incubating at 37° and pH 3 for 16 hr.

Pancreatic ribonuclease treatment. Aliquots of samples that had been acidified to hydrolyze phosphorimidazolides were neutralized to pH 7. Tris-HCl buffer (pH 7.0) and pancreatic ribonuclease were added to the neutralized solution to give final concentrations of 0.1M tris-HCl and 1 unit of ribonuclease per 5 ODU of total nucleotides. Pancreatic ribonuclease treatment did not hydrolyze oligo(G) products.

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