DIMERIZATION IN HIGHLY CONCENTRATED SOLUTIONS OF PHOSPHOIMIDAZOLIDE ACTIVATED MONONUCLEOTIDES

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Abstract. Phosphoimidazolide activated ribomononucleotides (*pN) are useful substrates for the non-enzymatic synthesis of polynucleotides. However, dilute neutral aqueous solutions of *pN typically yield small amounts of dimers and traces of polymers; most of *pN hydrolyzes to yield nucleoside 5′-monophosphate. Here we report the self-condensation of nucleoside 5′-phosphate 2-methylimidazolide (2-MeImpN with N = cytidine, uridine or guanosine) in the presence of Mg^{2+} in concentrated solutions, such as might have been found in an evaporating lagoon on prebiotic Earth. The product distribution indicates that oligomerization is favored at the expense of hydrolysis. At 1.0 M, 2-MeImpU and 2-MeImpC produce about 65% of oligomers including 4% of the 3′,5′-linked dimer. Examination of the product distribution of the three isomeric dimers in a self-condensation allows identification of reaction pathways that lead to dimer formation. Condensations in a concentrated mixture of all three nucleotides (U,C,G mixtures) is made possible by the enhanced solubility of 2-MeImpG in such mixtures. Although percent yield of internucleotide linked dimers is enhanced as a function of initial monomer concentration, pyrophosphate dimer yields remain practically unchanged at about 20% for 2-MeImpU, 16% for 2-MeImpC and 25% of the total pyrophosphate in the U,C,G mixtures. The efficiency by which oligomers are produced in these concentrated solutions makes the evaporating lagoon scenario a potentially interesting medium for the prebiotic synthesis of dimers and short RNAs.

1. Introduction

Non-enzymatic template-directed (TD) synthesis of polynucleotides offers a mechanism by which a non-random population of informational molecules (RNAs, DNAs, etc.) could have formed on prebiotic Earth (Ferris, 1994; Kanavarioti, 1994; James and Ellington, 1995), possibly giving rise to the RNA world (Gilbert, 1986). A large number of TD experiments performed with DNA or RNA molecules as the template and with *pN as building blocks established the potential as well as the limitations of TD chemistry with respect to polynucleotide synthesis (Joyce, 1987; Orgel, 1995; Joyce and Orgel, 1993). Other mechanisms for polymerization, involving metal ion (Sawai and Orgel, 1975; Sawai, 1976; Lohrmann, 1982; Sawai, 1988; Sawai *et al.*, 1992) and mineral (Acevedo and Orgel, 1986; Ferris *et al.*, 1989; Ferris, 1993; Ferris and Ertem, 1993; Ferris *et al.*, 1996) catalysis, have proven efficient, but the major advantage of TD over non-TD reactions lies in the fact that TD reactions lead to substantial information transfer. However, despite the intrinsic appeal of TD chemistry, there is consensus that using monomers as building blocks is inefficient and does not produce copies of a template for most RNAs (Joyce and Orgel, 1988; Wu and Orgel, 1992).

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To circumvent the difficulties in the incorporation of monomers in the TD syntheses, the use of activated *dimers* was proposed and tested more than 15 years ago (Lohrmann and Orgel, 1979; Ninio and Orgel, 1979; Lohrmann *et al.*, 1981). Dimers bind more strongly than the two corresponding monomers to the template and therefore the use of dimers as building blocks should lead to more efficient and more regioselective TD syntheses. Because of this potential, we have become interested in the prebiotic syntheses of dimers and especially of the 3′,5′ internucleotide linked ones. Prebiotically plausible syntheses that produce 3′,5′-linked dimers in good yield are scarce. Efficient methods of condensation, such as phosphoramidite chemistry, require protection/deprotection steps in a non-aqueous medium (Caruthers, 1987; Lehman *et al.*, 1989; Ferris and Peyser, 1994), which are prebiotically implausible processes. In the absence of protecting groups the number of possible isomeric dimers is six. These include the pyrophosphate-, 2′,5′- and 3′,5′-dimers and three cyclic dimers (Figure 1). The yield decreases in the order pyrophosphate- to 2′,5′- to 3′,5′-dimer, whereas cyclic dimers in substantial yield form with the pyrimidines only (Sawai *et al.*, 1992; Ertem and Ferris, 1996).

Although it is indisputable that metal ions catalyze internucleotide bond formation, the yields of 3′,5′-linked dimers and longer oligomers are unsatisfactory as shown by the examples listed below. In one study where the 5′-methylesters of the nucleotides, MepN, were used as the nucleophiles and ImpU as the electrophile, the ratio of the 2′,5′ to the 3′,5′ dimer (abbreviated 2′/3′) ranged from 6 to 9 and the yield in 3′,5′ dimer did not exceed 1% (Lohrmann and Orgel, 1978). In the presence of Mg^{2+}, ImpC self-condensation yields less than 1% and ImpG less than 0.3% of oligomers excluding the corresponding pyrophosphate dimers (Sawai, 1988); under similar conditions ImpA condensation yields 0.5% of $pA^{3'}pA$ (Sawai, 1976). In the presence of Pb^{2+} and Zn^{2+}, which are much better catalysts than Mg^{2+}, the yield in dimers and short oligomers reaches 35–55% and 10–20%, respectively, but 2′/3′ rises to 10 and the 3′,5′-dimer yield is less than 3% (Sawai and Orgel, 1975; Sawai, 1988). In the presence of Zn^{2+}, ImpU forms up to 11.5% of oligomers including 1.4% of $pU^{3'}pU$ (Sawai and Orgel, 1975). Condensations performed in the presence of minerals, especially montmorillonite, are promising because they yield up to 67% of products and because there is an inversion of the ratio 2′/3′ to 0.5 (Ferris and Ertem, 1993). For example, in the presence of Na^{+}-montmorillonite the self-condensation of ImpA yields 12% of $pA^{3'}pA$ (Ferris and Ertem, 1993) and the condensation of 5′-AMP with water-soluble carbodiimide EDAC yields 11% of $pA^{3'}pA$ (Ferris *et al.*, 1989).

Dimer yield undoubtedly suffers because hydrolysis competes with dimerization. Typical conditions, as used in the above described experiments are 0.025 to 0.05 M of *pN with an equivalent amount of a divalent metal ion. This concentration range is too low to favor dimerization unless a very efficient catalyst is present that selectively enhances dimerization but not hydrolysis. Thus, it seemed reasonable to investigate the effect of increasing concentration of *pN on the distribution of the hydrolysis vs. dimerization products. Solutions with high concentrations of organic

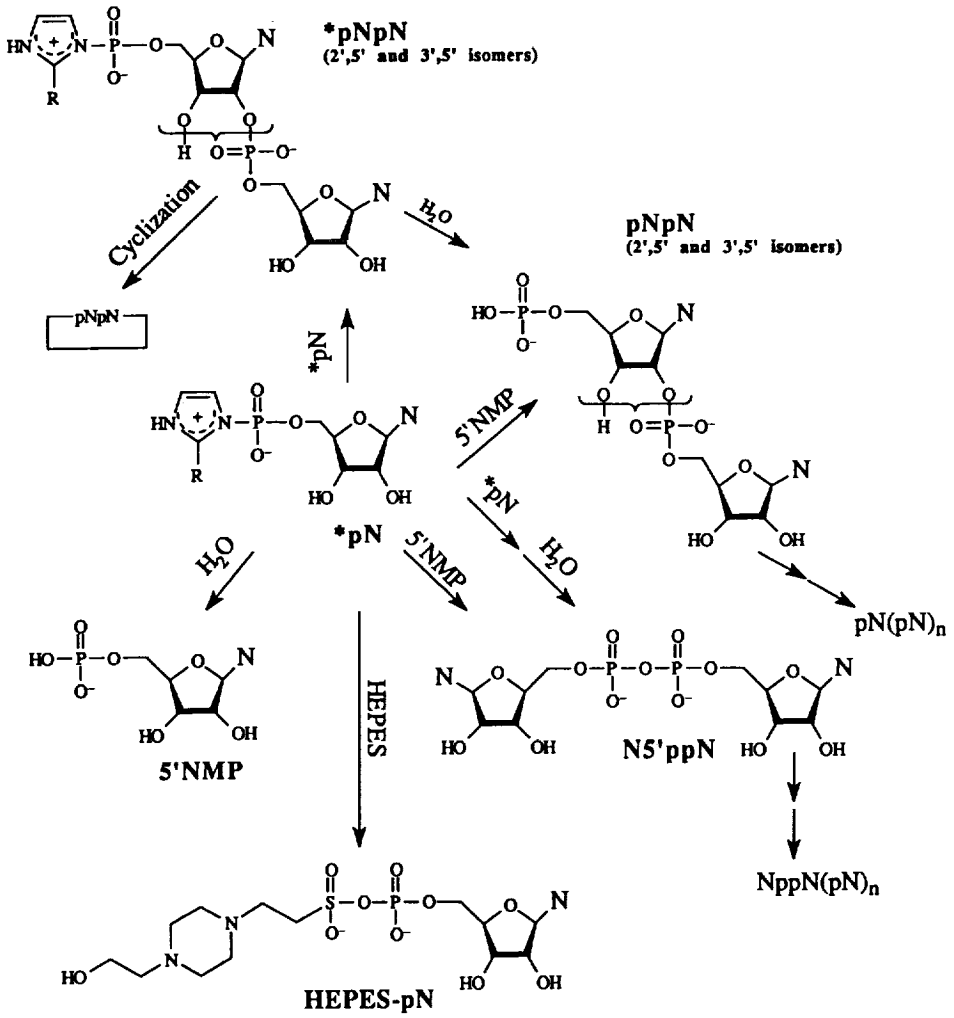

Figure 1. Major pathways in the self-condensation of a nucleoside 5′-phosphoimidazolide (N = Cytosine (C), Guanine (G), Uracil (U), R = Me. There are three plausible cyclic dimers: the all 3′,5′-, the all 2′,5′-linked and one with mixed linkages. Elongation of the linear dimers results in oligomers. The plausible oligomers are of two types: $(pN)_n$ or $Np(pN)_n$, i.e. pyrophosphate capped. In the self-condensation only homodimers are formed, in the reaction in mixtures both homo- as well as heterodimers are produced.

molecules are plausible in an evaporating lagoon or in pools on drying beaches on the early Earth. This scenario was recently implemented for the efficient synthesis of cytosine from the reaction of cyanoacetaldehyde in concentrated urea solutions (Robertson and Miller, 1995).

It is experimentally difficult to genuinely simulate the effect of a drying lagoon, because both substrate concentration and temperature should vary simultaneously

in a controlled way. In this preliminary report, we focused only on varying the concentration of *pN at constant temperature. We envision a lagoon with a small volume of water most of which can be lost to evaporation within a few hours at ambient temperature concentrating the water-soluble compounds by a factor of 20 or more. As expected, we found that with increasing concentration of *pN dimerization is favored over hydrolysis. However, at the highest monomer concentrations dimer formation reaches a plateau, most likely because the more dimer is formed the more is being consumed by further oligomerization. In conclusion, dimer formation is not only in competition with hydrolysis, but also with oligomerization and it is not clear that general conditions can be found to favor dimerization selectively. Several other observations were made, some of which being quite unexpected.

2. Results and Discussion

2.1. General

Experiments were performed at 20 °C in the presence of 1.0 M NaCl, 0.2 M $MgCl_2$ in a 0.5 M HEPES buffer at pH 7.5. 2-MeImpU and 2-MeImpC are soluble at concentrations up to 1.0 M. However, 2-MeImpG is not soluble at concentrations $\geq$0.1 M and such experiments were done in a suspension. Interestingly, in U,C,G mixtures, 2-MeImpG becomes soluble at concentrations up to 0.4 M. This solubility enhancement is reminiscent of the observation that aqueous solutions of [poly(cytidylate)] $\geq$0.02 M can be made with great ease if 2-MeImpG is present (Kanavarioti *et al.*, 1995) and suggests the presence of interactions not only among polynucleotide with its complementary mononucleotide but also among the mononucleotides. Moreover, the increased solubility of 2-MeImpG made the study of reactions with *pN mixtures in concentrated solutions possible.

2.2. Reaction pathways of *pN

The *pN undergo a variety of reactions in water (Figure 1). They include loss of activation, reaction with the buffer N-2-hydroxyethylpiperazine-N′-2-ethanesulfonate, HEPES, and other second-order processes that lead to dimers. The obvious pathway for formation of the phosphoimidazolide activated dimers (*pNpN, both 2′,5′ and 3′,5′) is the reaction of two molecules of *pN. Another pathway that leads to deactivated dimers, pNpN, is the reaction of the hydrolysis product, 5′-NMP, with *pN. This pathway also leads to pyrophosphate dimer by nucleophilic attack of the free phosphate group on the P-N bond of *pN (Chu and Orgel, 1984; Kanavarioti *et al.*, 1992). However, evidence will be presented below to support the notion that the pyrophosphate dimer is formed primarily by the reaction of two molecules of *pN, just like the internucleotide linked dimers. The activated dimers can elongate as well as hydrolyze or cyclize. The phosphoimidazolide derivatives of the pyrophosphate dimers (not shown in Figure 1) have not been detected, perhaps because

they hydrolyze much faster than the *pNpN. Other detectable pathways are the decomposition to the corresponding nucleoside and to the nucleoside cyclic 3′:5′-monophosphate, with yields less than 1.5%. Successive addition of monomer to the dimers leads to oligomers of the type $(pN)_n$ or $Np(pN)_n$, i.e. pyrophosphate capped. The difference between reactions with one substrate only (self-condensation) and reactions in a mixture is that in the former there is a relatively small number of dimeric products (homodimers), whereas in the latter there is a much larger number of products which include both the homo- and the heterodimers. Similar logic applies to the oligomers.

2.3. Concentration Effect on the Product Distribution of the Self-Condensations

In one set of experiments the concentration of 2-MeImpU or 2-MeImpC was varied from about 0.1 to 1.0 M which resulted in the expected increase in dimer and oligomer yield at the expense of hydrolysis. Specifically, at pH 7.45 reaction with 0.106 M 2-MeImpC yields after 16 days 56.9% of the hydrolysis product, 5′-CMP, and 33.3% of the condensation products (Table I). With 1.0 M 2-MeImpC the order is reversed; the yields are 26.1% in 5′-CMP and 65.5% in condensation products (Table I). Reactions with increasing concentrations of 2-MeImpU and 2-MeImpG also show an increase in the oligomer products at the expense of the hydrolysis products (Table I). While the yield of pyrophosphate dimer stays essentially constant at 20% with increasing initial monomer concentration in the reactions with 2-MeImpC and 2-MeImpU, with 2-MeImpG it increases from 11.9% to 50% for a change from 30 to 327 mM 2-MeImpG. The increasing yield of $G^{5'}$ppG may be related to the precipitation of $G^{5'}$ppG in the presence of Mg^{2+}.

The independence of the pyrophosphate dimer yield in the self-condensations of 2-MeImpU and 2-MeImpC is in sharp contrast to the increase of the internucleotide linked dimers. The very last column of Table I reports the sum of the internucleotide linked dimers, including the imidazolides, as a function of initial monomer concentration. For 2-MeImpU the increase is 2-fold, for 2-MeImpC 1.7-fold and for 2-MeImpG 4-fold in going from the lowest to the highest concentration. The sum of the product yield in dimers and longer oligomers excluding the pyrophosphate dimer in the 2-MeImpU self-condensation varies from 17.6 to 42.1% depending on initial monomer concentration. To the best of our knowledge, this is one out of three most efficient oligomerizations obtained with a uridine derivative. Of comparable efficiency, but more prebiotically plausible because of the low initial monomer concentration employed, is the montmorillonite-catalyzed ImpU oligomerization which yields 24.5% of dimers and longer oligomers in the absence and 40.8% in the presence of $A^{5'}$ppA acting as primer (Ding *et al.*, 1996). The other efficient ImpU oligomerization is the one observed in the presence of UO_2^{2+} which at high concentrations of the catalyst (1 mM) favors almost complete conversion of the substrate to oligomers, but with 2′,5′-linkages being predominant (Sawai *et al.*,

Table I

Percent yield of major products in the self-condensations of 5′-phosphate nucleoside 2-methylimidazolide (U = 2-MeImpU, C = 2-MeImpC, G = 2-MeImpG) at pH 7.45±0.05 after 16 days of incubation at 20 °C

Substrate	Conc. mM	PrD+[a]	Hydrolysis products	Unreacted substrates	HEPESpN	$N^{5'}$ ppN	pNpN[b] 2′&3′
U[c]	180	10.0	53.9	3.6	5.6	19.3	7.6
	311	15.1	41.9	4.7	5.3	23.4	9.6
	500	17.0	40.2	4.1	4.2	22.5	12.0
	546	17.4	36.2	6.5	4.2	22.0	13.7
	1000	26.0	27.3	6.8	3.6	20.2	16.1
C[d]	106	5.0	56.9	3.9	5.9	18.1	10.2
	258	15.2	41.2	4.4	4.9	21.6	12.7
	500	19.4	37.3	5.1	3.4	20.6	14.2
	746	25.5	31.5	6.2	3.0	18.3	15.5
	776	26.7	30.0	6.5	3.1	18.2	15.5
	982	32.6	26.0	5.8	2.9	16.6	16.1
	1000	31.6	26.1	5.7	2.7	16.5	17.4
G[e]	30	1.3	76.3	5.4	3.4	11.9	1.7
	61	2.0	64.6	4.2	4.2	21.1	3.9
	98	3.0	55.2	3.4	4.3	28.1	6.0
	235	3.2	37.2	2.2	3.1	47.0	7.3
	327	4.0	34.7	2.1	2.4	50.0	6.8

Percent yield is expressed in monomer equivalents and is not corrected for dimer hypochromicity, therefore dimer yield is underestimated by about 10%.

[a] PrD+ is the percent yield of unaccounted for products and represents the sum of the cyclic dimers and elongation products such as trimers or longer oligomers including pyrophosphate capped ones.

[b] Sum of internucleotide linked dimers including the unhydrolyzed imidazolides of the dimers.

[c] 1000 mM solution at pH 7.83, 18 days incubation.

[d] 746 mM at pH 7.65, 776 mM at pH 7.71, 982 mM at pH 7.74, 1000 mM solution at pH 7.98 incubated for 18 days.

[e] Experiments at 98, 235 and 327 mM were suspensions.

1989). Interestingly, at lower concentrations of UO_2^{2+} (0.01 mM) oligomerization is somewhat less efficient, but more than 30% of the products are 3′,5′-linked (Sawai *et al.*, 1989).

2.4. Concentration Effect on the Product Distribution of the Reactions with Mixtures

Table II reports the major product distribution in U,C,G mixtures as a function of incubation time and total initial monomer concentration. Samples were quenched and analyzed after 30 h, 70 h and 10 days. After 10 days about 85% of the imidazolides have reacted. Oligomer yield is seen to increase with monomer con-

Table II

Percent yield of major products in the reactions of mixtures with 2-MeImpC, 2-MeImpU and 2-MeImpG as a function of time and initial total substrate concentration at pH 7.50±0.1 and 20 °C

Total conc.	Reaction	PrD+[a]	Unreacted substrates		Hydrolyzed products			Composition
mM	time, h		U+C[b]	G	5′-UMP	5′-CMP	5′-GMP	mM U-C-G
174	30 h	15.7	33.5	13.5	19.1	7.0	11.2	87–44–43
	70 h	27.0	21.0	7.5	23.0	8.3	13.2	
	10 d	36.2	9.1	2.5	25.7	8.8	17.7	
350	30 h	23.9	36.3	10.5	10.3	11.8	7.2	106–181–63
	70 h	35.2	23.8	6.6	12.3	13.8	8.3	
	10 d	51.3	8.3	2.6	14.5	13.2	10.1	
595	30 h	26.2	32.9	13.7	10.9	6.4	9.9	219–191–185
	70 h	37.6	25.3	8.7	11.6	6.7	10.1	
	10 d	50.1	14.7	3.5	12.8	7.2	11.7	
654	30 h	28.7	32.6	15.7	8.2	6.4	8.4	213–260–181
	70 h	35.8	26.0	12.0	9.7	7.3	9.2	
	10 d	56.9	11.4	4.1	10.6	7.8	9.2	
731	30 h	30.2	32.7	15.8	7.4	5.3	8.6	248–258–225
	70 h	48.7	25.4	10.1	0.1	6.3	9.4	
	10 d	57.1	12.0	4.3	10.5	7.0	9.1	
885	30 h	29.0	36.4	13.9	6.4	7.6	6.7	243–432–210
	70 h	40.8	26.5	9.0	7.6	8.7	7.4	
	10 d	60.4	10.5	4.0	8.0	8.3	8.8	

Absorbance was monitored at 262 nm which underestimates C compared to U and g derivatives.
[a] PrD+ is the percent yield of unaccounted for products and represents the sum of all dimers and elongation products such as trimers and longer oligomers.
[b] 2-MeImpC and 2-MeImpU coelute with the TFA chromatography used for the HPLC analysis.

centration at the expense of the hydrolysis products, just as was observed with the self-condensations. With the lowest concentration of total monomer tested, i.e. 174 mM, oligomer yield amounts to 36.2% after 10 days, whereas with the highest concentration, i.e. 885 mM, the corresponding yield is 60.4% (Table II). The increase in condensation products is not proportional to the increase in total monomer concentration, but levels off at high concentrations. The reasons for this leveling off are unclear. Another interesting comparison between the low and high concentration data is that with 174 mM monomer the percent yield in hydrolysis products increases somewhat with incubation time, e.g. for 5′-UMP it amounts to 19.1% at 30 h, 23% at 70 h and 25.7% at 10 days, whereas with high initial monomer concentration there is no significant increase, e.g. with 885 mM monomer 5′-CMP amounts to 7.6% at 30 h, 8.7% at 70 h and 8.3% after 10 days of incubation. Hence, it appears as if hydrolysis occurs only at the onset of the reaction, up to 30 h, and soon after practically ceases. This behavior could be explained

by early accumulation of the dimers, followed by consumption of the monomer mainly by dimer elongation which is much faster than hydrolysis and dimerization. The high yields of oligomer products produced in the presence of Mg^{2+} under the conditions of this study (up to 60%, Tables I and II) are to be compared with yields of 1% or less obtained in dilute solutions of *pN (see in the Introduction). This comparison suggests that the evaporating lagoon scenario represents a potentially interesting model for polynucleotide synthesis, especially if the lagoon is cool (close to 20 °C) and small so that concentration by evaporation occurs relatively fast. This is because concentration of materials need to occur before the hydrolysis process consumes the activated nucleotides. The half-life of 2-MeImpN hydrolysis at 23 °C in the range $7 \leq$ pH ≤ 8 is 4.5 to 6 days in the presence of 0.2 M Mg^{2+} (T. B. Hurley thesis, UCSC 1993), and increases dramatically with decreasing metal ion concentration (Kanavarioti *et al.*, 1989).

2.5. 2-MeImpC SELF-CONDENSATION: PATHWAYS THAT LEAD TO THE THREE ISOMERIC DIMERS

Table III reports the results of the self-condensation of 2-MeImpC as a function of time for two different initial concentrations. The yield of all unidentified, presumably, oligomer products is reported under PrD+. A few comments are called for: The increase in monomer concentration from A = 746 to B = 982 mM has a small, but significant effect on the yield of 5′CMP and of the oligomerization products PrD+ which in this data set represent the cyclic dimers and oligomers longer than the dimers: 5′-CMP yield decreases from 31.5 to 26%, PrD+ increases from 25.6 to 32.7% after 385 hours of incubation. The variation in $pC^{3'}pC$, 2-MeImpCpC and $C^{5'}ppC$ yield with increasing monomer concentration from 746 to 982 mm are negligible. However, the almost identical *dimer* distribution between these two samples (A and B) is deceiving because the increase in the percent yield of oligomers must be the result of an equivalent decrease in dimers. This means that more dimer products were produced with the higher initial concentration (B) but these dimers were consumed by further elongation. Whether or not these oligomers are elongation products of the pyrophosphate or of the internucleotide linked dimers will have to await identification of the oligomers. Sawai and coworkers (Sawai *et al.*, 1992) investigated the oligomerization of ImpC in the presence of UO_2^{2+} and concluded that the primary route for consumption of the 2′,5′-linked dimer is elongation whereas the primary route for further reaction of the 3′,5′-linked dimer is cyclization.

The ratio between $pC^{2'}pC$ and $pC^{3'}pC$, abbreviated 2′/3′, is given in the last column of Table III. It varies between 3.2 and 2.8 in favor of the 2′ isomer (seen also in Figure 2). Analyses of the experiments done with 2-MeImpU (see Table IV and Figure 3) and 2-MeImpG (Figure 4) also indicate a ratio 2′/3′ of about 3. This is in contrast with a number or earlier studies in similar systems which reported that the ratio of 2′/3′ varied in the range of 6–9 (Sawai and Orgel, 1975; Sawai,

Table III

Percent yield of major products in the self-condensation of 2-MeImpC as a function of time and initial substrate concentration at 20 °C; A = 0.746 M at pH 7.65; B = 0.982 M at pH 7.74

Reaction time, h	PrD+[a]	5′-CMP	HEPES-pC	$C^{5'}$ppC	$pC^{2'}$pC	$pC^{3'}$pC	2-MeImpC	2-MeIm--$(pC)_2$	p/2′[b]	2′/3′[b]
A										
0	2.8	13.7	0.2	1.5	0.1	–	81.7	–		
20	5.5	22.2	1.2	8.6	2.6	1.3	49.6	9.0	0.9	3.2
30	7.1	23.7	1.5	10.1	3.3	1.6	43.3	9.3	0.9	3.2
117	17.2	28.2	2.4	15.5	6.8	3.0	20.1	6.8	1.2	3.0
193	21.1	29.8	3.0	16.9	8.1	3.4	13.1	4.6	1.4	3.0
385	25.6	31.5	3.0	18.3	9.5	3.9	6.2	2.0	1.6	2.8
B										
0	1.4	13.6	0.2	1.2	–	–	83.0	0.6		
20	6.7	19.6	1.3	8.5	2.7	1.4	49.3	10.5	0.8	3.1
30	9.7	20.3	1.5	10.3	3.4	1.7	41.8	11.3	0.8	3.0
117	22.2	23.5	2.4	14.6	6.9	3.2	19.2	8.0	1.1	2.7
193	27.3	24.9	2.7	15.7	8.1	3.7	12.4	5.2	1.3	2.7
385	32.7	26.0	2.9	16.6	9.3	4.2	5.8	2.5	1.4	2.6

[a] PrD+ is the percent yield of unaccounted for products and represents the sum of the cyclic dimers and elongation products such as trimers or longer oligomers including pyrophosphate capped ones. [b] p/2′ refers to the ratio of the $C^{5'}$ppC/$pC^{2'}$pC where $pC^{2'}$pC is the sum of the imidazolide activated and the hydrolyzed dimer; 2′/3′ refers to the ratio of $pC^{2'}$pC/$pC^{3'}$pC.

Table IV

Percent yield of major products in the self-condensation of 2-MeImpU as a function of time and initial substrate concentration at 20 °C; A = 0.765 M at pH 8.11; B = 0.990 M at pH 8.20

Reaction time, h	PrD+[a]	5′-UMP	HEPES-pU	$U^{5'}$ppU	$pU^{2'}$pU	$pU^{3'}$pU + unknown[b]	2-MeImpU	2-MeIm-$-(pU)_2$	p/2′[c]	2′/3′[d]
A										
0	1.5	11.5	–	2.9	0.1	–	82.1	2.0		
20	1.4	25.7	0.1	3.3	0.8	0.6	62.7	5.4	0.8	
30	1.3	27.7	–	3.2	1.0	0.9	60.4	5.5	0.7	
117	2.6	44.7	0.4	5.0	3.9	3.0	33.4	7.0	0.6	
193	3.1	50.7	0.5	6.1	5.6	4.1	23.9	6.0	0.7	
385	4.0	59.0	1.0	7.5	8.2	5.6	11.2	3.5	0.8	
d				8.6	10.0	3.3			0.9	3.0
B										
0	1.3	12.2	–	3.2	0.1	0.2	80.9	2.1		
20	1.8	28.1	–	3.7	0.9	0.7	58.8	6.0	0.8	
30	1.7	28.2	–	3.5	1.1	0.9	58.6	6.0	0.7	
117	2.9	44.1	0.4	5.8	4.2	3.2	32.1	7.3	0.7	
193	3.1	52.8	0.5	6.6	5.3	3.8	22.5	5.4	0.7	
385	4.1	60.8	0.7	8.6	7.8	5.1	9.9	3.0	0.9	

[a] PrD+ is the percent yield of unaccounted for products and represents the sum of the cyclic dimers and elongation products such as trimers or longer oligomers including pyrophosphate capped ones.

[b] Unknown product that coelutes with $pU^{3'}$pU.

[c] p/2′ refers to the ratio of the $U^{5'}$ppU/$pU^{2'}$pU where $pU^{2'}$pU is the sum of the imidazolide activated and the hydrolyzed dimer;

[d] Product distribution of dimers as obtained after alkaline phosphatase (APH) digestion of the 385 h sample; 2′/3′ refers to the ratio of $U^{2'}$pU/$U^{3'}$pU.

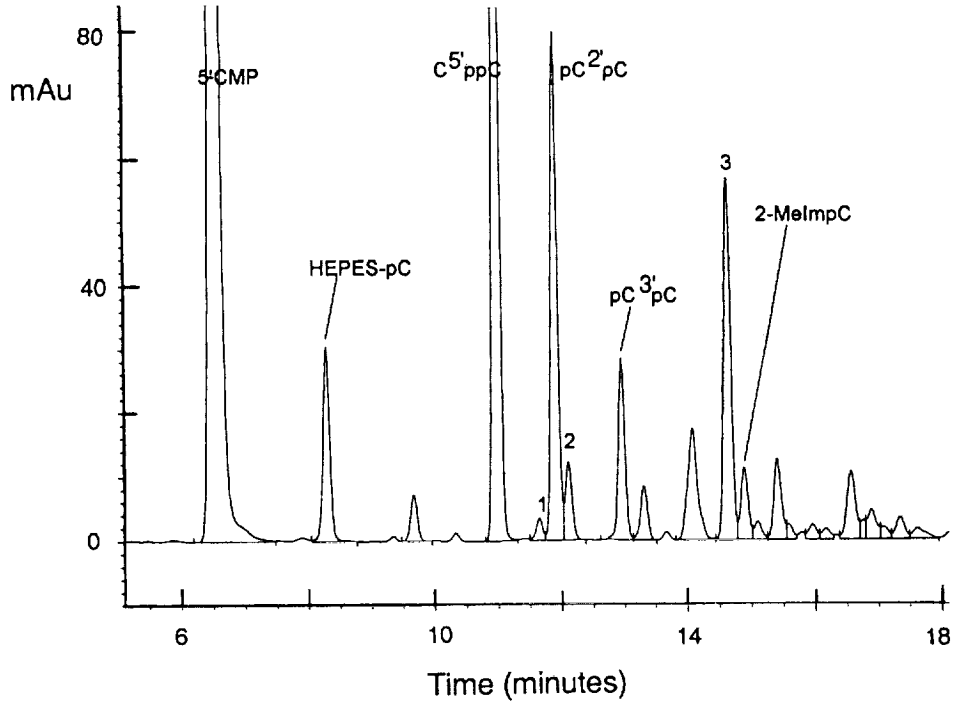

Figure 2. HPLC profile from the reaction of 0.5 M 2-MeImpC at pH 7.50 in a 0.5 M HEPES buffer in the presence of 0.2 M Mg^{2+} and 1.0 M NaCl incubated at 20 °C for 27 days. y axis: mAu, milliabsorbance units. Analysis was done with TFA chromatography (see Experimental). Peaks positively identified with standards are indicated on the figure. In addition: 1, cytidine; 2, cytidine 3′:5′-cyclic monophosphate at about 0.5% and 1.5% yield, respectively; 3, thought to be a cyclic dimer because it does not get degraded by digestion with alkaline phosphatase (APH).

1988; Sawai, 1976; Lohrmann and Orgel, 1978). The main differences between the earlier experiments and the ones reported here are the lower *pN concentration and the use of imidazole instead of 2-methylimidazole as activating group in the earlier studies. It is unlikely that the 2′/3′ ratio is concentration dependent, in view of the fact that a similar ratio 2′/3′ ≈ 3 is obtained with a 0.1 M and a 1.0 M 2-MeImpC solution. We are currently investigating the reactions of the imidazolide activated substrates in order to determine the 2′/3′ ratios.

Another interesting comparison is the ratio between $C^{5'}$ppC and $pC^{2'}$pC, abbreviated p/2′, given in second to the last column in Table III. The ratio p/2′ = 0.9±0.1 stays constant for the first 30 hours and then increases with incubation time and reaches 1.5±0.1 after 385 hours. It is important to notice that the constancy of 2′/3′ and p/2′ at the onset of the reaction provides strong evidence that the pyrophosphate dimer is formed via the same reactive intermediate as the internucleotide linked dimers. To the best of our knowledge these are the first data that support the postulate of pyrophosphate synthesis from two *pN molecules, abbreviated 2X*pN

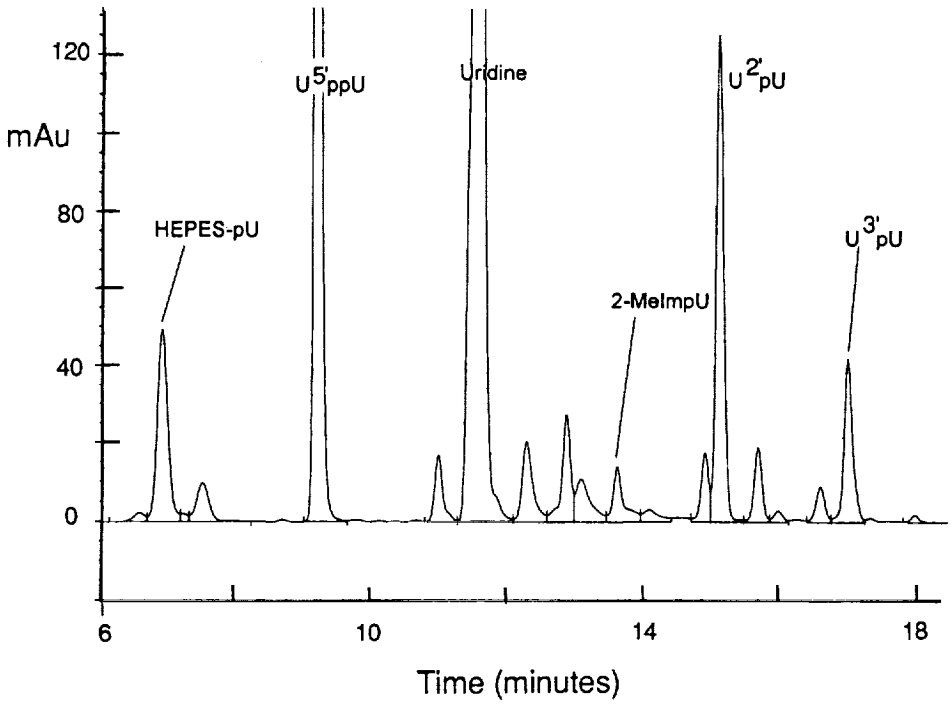

Figure 3. HPLC profile of an alkaline phosphatase (APH) digest from the reaction of 0.5 M 2-MeImpU at pH 7.45 in a 0.5 M HEPES buffer in the presence of 0.2 M Mg^{2+} and 1.0 M NaCl incubated at 20 °C for 27 days. y axis: mAu, milliabsorbance units. Analysis was done with TFA chromatography (see Experimental). Peaks positively identified with standards are indicated on the figure. Uridine is formed by APH digestion of 5′-UMP; during incubation it is formed at less than 1% yield. The peak eluting at 12.9 min is identified as uridine 3′:5′-cyclic monophosphate.

pathway. The conceptual difficulty with this proposition is that the nucleophile for pyrophosphate synthesis is a O^- with a pK_a of about 1.5 (Kanavarioti, 1986), i.e. a very poor nucleophile. However, the data reported in Table IV (see discussion of Table IV below) are consistent with the postulate that the 2X*pN pathway is the main pathway for formation of all three isomeric dimers.

The ratios p/2′ and 2′/3′ in Table III exhibit a trend of increasing and decreasing value, respectively, with incubation time; the p/3′ ratio (not shown) increases. These trends, seen in all the experiments with 2-MeImpC, could be attributed to different rates of further reaction among the three dimers as well as to an additional pathway that leads to their formation besides 2X*pN. This additional pathway represented by reaction of 5′-NMP with *pN, abbreviated 5′-NMP pathway, becomes important only after hydrolysis product has accumulated. Synthesis of pyrophosphate dimer by the 5′-NMP pathway has been documented for a number of *pN substrates in the absence of Mg^{2+} (Kanavarioti *et al.*, 1992) and could be available in the presence of Mg^{2+}, too. The increases in p/2′ and p/3′ can be explained by postulating that

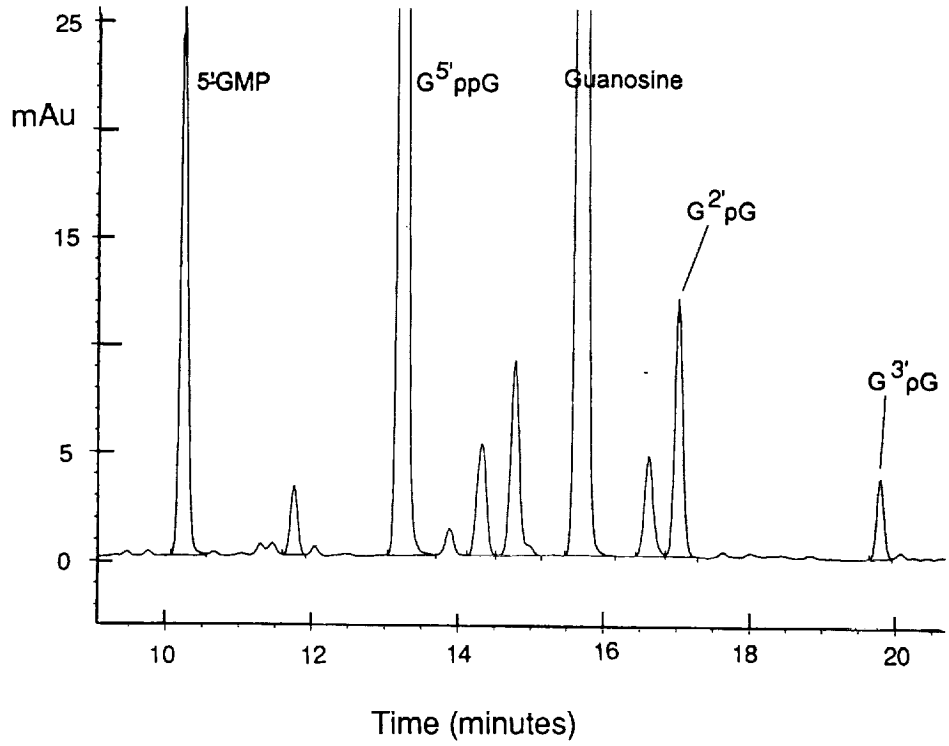

Figure 4. HPLC profile of an alkaline phosphatase (APH) digest from the reaction of 0.1 M 2-MeImpG at pH 7.30 in a 0.5 M HEPES buffer in the presence of 0.2 M Mg^{2+} and 1.0 M NaCl incubated for 17 days at 20 °C. y axis: mAu, milliabsorbance units. Analysis was done with TFA chromatography (see Experimental). Peaks positively identified with standards are indicated on the figure. Also the peak at 11.75 min is identified as HEPES-pG. Some or all of the unidentified peaks could be cyclic dimers because they remain unaltered by APH digestion.

the 5′-NMP pathway is important for the synthesis of the pyrophosphate dimer and not as important for the synthesis of the internucleotide linked dimers.

2.6. Self-condensation of 2-MeImpU

Similar observations to the ones made with 2-MeImpC were made with 2-MeImpU at pH $\leq$7.85 (Table I). For contrast, Table IV presents the data from two reactions with 2-MeImpU at higher pH, i.e. pH 8.11 and 8.20. Here oligomerization efficiency is diminished including $U^{5'}$ppU yield which is at pH $\geq$8.11 about 2.5 times less than at pH $\leq$7.85. This inhibition could be attributed to uridine (N3) ionization (pK_a of 9.3 see Saenger, 1984) that leads to reduced reactivity due to electrostatic repulsion between uridine molecules. Experiments with 2-MeImpC in the pH range 7.4 to 8.0 do not indicate a pH effect on the relative product distribution, consistent with the fact that cytidine does not ionize in the slightly basic medium. The reaction of 2-MeImpU at pH >8, where pyrophosphate and oligomer synthesis are inhibited,

provides a testing ground for the postulate that the 2X*pN pathway produces all three isomeric dimers. Indeed, p/2′ and 2′/3′ ratios in Table IV stay constant with incubation time at p/2′ = 0.75±0.15 and 2′/3′ = 3.0±0.2, suggesting that 2X*pN is the major pathway for the synthesis of the three isomeric dimers; other pathways for formation as well as consumption of these dimers would alter the above ratios. The fact that the p/2′ and 2′/3′ values ratios have similar values for 2-MeImpC (Table III, onset of reaction) and for 2-MeImpU (Table IV) indicates that, at least with the 2X*pN pathway, the product distribution is independent of the base, i.e. C or U.

2.7. Independence of Pyrophosphate Yield with Monomer Concentration

The conclusion reached above that dimers are produced with a ratio of p/2′≈0.75 ±0.15 and 2′/3′≈3.0±0.2 seems to contradict an important observation. This observation, described earlier, states that the yield of internucleotide linked dimers increases with initial concentration of 2-MeImpC and 2-MeImpU, whereas the yield of pyrophosphate dimer is concentration independent (Table I). Since the pyrophosphate dimer is produced by the same pathway as the internucleotide linked dimers, its yield should increase parallel to the others. Moreover, the additional pathway available for pyrophosphate synthesis should result in a relatively larger increase for the pyrophosphate as compared to the yield of the internucleotide linked dimers. This seeming inconsistency can be explained by taking into consideration that the 5′-NMP pathway depends linearly on 5′-NMP concentration (Kanavarioti *et al.*, 1992), and therefore it is more effective with the lower concentrations of substrate that provide higher yields of hydrolysis product, and less important with the higher concentrations of substrate that produce much lower yields of the hydrolysis products. In other words, the 5′-NMP pathway results in a higher yield of pyrophosphate with decreasing initial monomer concentration, whereas the inverse dependence occurs with the 2X*pN pathway. The net effect is an apparent independence of the pyrophosphate yield on monomer concentration. Supporting evidence for this conclusion is that the yield of $C^{5'}$ppC and $U^{5'}$ppU actually shows a trend of initially increasing and then decreasing as a function of initial monomer concentration (see Table I). We believe that this trend is real because pyrophosphates are very stable compounds and yields are reproducible to ±0.3%.

The self-condensation of 2-MeImpG was also monitored as a function of time (data not shown). Figure 4 illustrates the HPLC profile of a 0.1 M 2-MeImpG sample incubated for 17 days at 20 °C and later digested with alkaline phosphatase (APH) that cleaves free phosphate monoesters yielding $G^{2'}$pG and $G^{3'}$pG. The ratio $G^{2'}$pG to $G^{3'}$pG is 3, in accordance with the 2′/3′ ratio obtained with 2-MeImpC and 2-MeImpU. As seen in Table I under PrD+, oligomerization of 2-MeImpG is much less efficient than that of 2-MeImpC and 2-MeImpU, most likely because most of the substrate is consumed by pyrophosphate formation. It

Table V

Percent yield of pyrophosphate dimers in the reaction of the ternary mixture with 2-MeImpU, 2-MeImpC and 2-MeImpG as a function of total monomer concentration after 384 hr of incubation at 20 °C. Total concen. of 765 mM (composition: U-C-G = 333-281-256) at pH 7.67; with total concent. of 1350 mM (composition: U-C-G = 453-460-439) at pH 7.78

Conc. mM	$U^{5'}$ppU	$U^{5'}$ppC	$C^{5'}$ppC[a]	$U^{5'}$ppG	$C^{5'}$ppG[b]	$G^{5'}$ppG[c]
765	3.6	5.3	4.7	4.2	<5.2	<3.5
1350	2.6	4.4	3.9	4.9	5.1	4.0

Absorbance was monitored at 267 nm where all three nucleotides have similar extinction coefficients and therefore HPLC units determined under different product peaks are directly comparable. Yield of a certain pyrophosphate that coelutes with one or more compounds was determined by comparing the HPLC profiles of the samples quenched after 384 h of incubation before and after APH digest. In TFA chromatography (see Experimental).

[a] 5'-GMP and $C^{5'}$ppC coelute;

[b] $C^{5'}$ppG, $pU^{3'}$pC and $pC^{3'}$pC coelute;

[c] 2-MeImpC, 2-MeImpU, $G^{5'}$ppG and $pG^{3'}$pC coelute.

is remarkable that 2-MeImpG which oligomerizes so efficiently in the presence of the template poly(cytidylate) (Inoue and Orgel, 1982), is the poorest substrate in a self-condensation reaction.

2.8. Reactions in U,C,G mixtures

The major products of reactions performed with U,C,G mixtures, i.e. approximately equimolar mixtures of 2-MeImpU, 2-MeImpC and 2-MeImpG, have been identified and quantitatively estimated; these results will be reported elsewhere. To the best of our knowledge, this is the first time that analysis of the majority of the products in a reaction with a mixture of three *pNs was performed. Table V reports the percent yield of the six pyrophosphate dimers in two selected U,C,G mixtures. The yields range from the low 2.6% for $U^{5'}$ppU to the high 5.1% for $G^{5'}$ppC. The yield of $G^{5'}$ppG is <3.5% with 765 mM and 4.0% with 1350 mM of total monomer concentration (Table V). The possibility that $G^{5'}$ppG was produced initially in higher yield and was consumed by elongation has been discounted based on the following reasoning. Presumably products of the type $G^{5'}$ppG$(pN)_n$ with $n \geq 1$ elute later than $G^{5'}$ppG in the HPLC profiles. However, all the major products that elute later than $G^{5'}$ppG have been positively identified with standards and are dimers of the type pNpN. The $G^{5'}$ppG yields, calculated with respect to total monomer concentration, amount to $3.5 \times 765/256 = 10.5\%$ and $4 \times 1350/439 = 12.3\%$ with respect to the 2-MeImpG amount present in the mixtures for the 765 mM and the 1350 mM mixture, respectively; they are much lower than the 50% yield observed in the self-condensation of 327 mM 2-MeImpG. This difference in percent yield

supports the claim made earlier that intermolecular interactions play a role in the nucleotide mixtures. These interactions have such a dramatic influence on the product distribution as to change a major product to a minor one ($G^{5'}$ppG). It is conceivable that a certain stacking conformation assumed by 2-MeImpG alone is disrupted in the presence of the other two nucleotides resulting in a diminished yield of $G^{5'}$ppG in the mixtures.

3. Conclusions

In summary, the experiments performed in this study permit the following conclusions:

(i) With increasing initial monomer concentration from 0.1 to 1.0 M, dimer yield increases for the 2′,5′-, 3′,5′-, but not for the pyrophosphate-linked dimer in the self-condensation of 2-MeImpC and 2-MeImpU.

(ii) Self-condensations of 2-MeImpC and 2-MeImpU and 2-MeImpG produce linear internucleotide linked dimers in a ratio of about 3 favoring the 2′,5′-linked isomer. At 1.0 M, 2-MeImpU yields 42% oligomers including 4% of $pU^{3'}$pU, while 2-MeImpC yields 49% of oligomers including 4.3% of $pC^{3'}$pC. These yields exclude the pyrophosphate (≈20%).

(iii) Reaction of two 2-MeImpN (N = C or U) molecules is the major pathway for the synthesis of the three isomeric dimers in ratios p/2′≈0.75±0.15 and 2′/3′≈3.0±0.2 in the pH range 7.65 to 8.20; other pathways for formation as well as consumption of these dimers alter the above ratios.

(iv) 2-MeImpG solubility is enhanced by 4-fold in the presence of 2-MeImpU and 2-MeImpC, suggesting the presence of strong intermolecular interactions.

(v) Reaction of 2-MeImpG in the presence of 2-MeImpC and 2-MeImpU forms $G^{5'}$ppG up to 12% with respect to the amount of 2-MeImpG present in the mixtures, much lower than the 50% in the self-condensation of 2-MeImpG, most likely a result of interactions mentioned under (iv).

4. Experimental Section

4.1. Materials

Reagent grade chemicals were used throughout. Solvents were HPLC quality. The sodium salts of 2-MeImpG, 2-MeImpC and 2-MeImpU at 98% or higher purity were synthesized as described (Kanavarioti *et al.*, 1995B); they contain about 1% each of 5′NMP and of the pyrophosphate homodimer as impurities. HEPES and tris(hydroxylmethyl)aminomethane hydrochloride, Tris, buffer were purchased from Aldrich. Dimers NpN 2′,5′- and 3′,5′-linked (both homo- and heterodimers of all combinations between cytidine, uridine and guanosine) were purchased from Sigma. Enzymes were purchased from Boeringer Mannheim: alkaline phosphatase

from calf intestine (APH) and polynucleotide kinase from T4 phage infected E.C. (PNK).

4.2. ENZYMATIC DEGRADATIONS

Samples were subjected to APH degradation in order to dephosphorylate pNpN for identification purposes. Both $pN^{2'}pN$ and $pN^{3'}pN$ were dephosphorylated to form the corresponding $N^{2'}pN$ and $N^{3'}pN$ and controls showed that dephosphorylation of these dimers occurred quantitatively and without cleavage of the internucleotide bond. Typically, 10 to 50 micromoles of nucleotide were incubated in 25 μL of 0.1 M tris.HCl buffer with 1 mM Mg^{2+} at pH 8.0 with 1 unit of APH enzyme for 1 to 2 hours at 37 °C. Aliquots of 1 or 2 μL that were to be digested with APH were directly taken from the reaction mixtures. Aqueous solutions of $N^{3'}pN$ standards were digested with PNK following the instructions of the manufacturer. $N^{3'}pN$ were semiquantitatively phosphorylated at the 5′-end, whereas $N^{2'}pN$ were left intact. Typically, a total volume of 10 μL with 1 mM of nucleotide and 5 mM of ATP.tris solution brought to pH 7.5 was incubated with a fresh enzyme/buffer solution (1 unit of enzyme) overnight at 37 °C. These solutions were used as standards for the identification of $pN^{3'}pN$ in the reaction mixtures.

4.3. PREPARATION OF REACTION MIXTURES AND ANALYSIS

Substrates were weighed into the test tubes and the solid was dissolved with half the volume of water; buffer containing the salts was added next. Final buffer concentration was 0.5 M HEPES with 0.2 M $MgCl_2$ and 1.0 M NaCl at pH 7.45±0.05 unless otherwise noted. The pH was measured directly in the solutions using a microelectrode probe (Microelectrodes MI 410). Despite the relatively weak buffering capability of the buffer (0.5 M with substrates of comparable concentration) the pH of the solutions did not vary during incubation even for the highly concentrated ones. Solutions (0.04 mL) were incubated at 20 °C. Solutions, excluding the ones with 2-MeImpG $\geq$0.1 M, were clear and remained clear, although some of the more concentrated ones were viscous. Once diluted and quenched with EDTA, the 2-MeImpG samples became homogeneous solutions and were analyzed in the same way as the others. Reactions were run either with one substrate, self-condensation, or in a mixture of approximately equimolar concentration of 2-MeImpC, 2-MeImpU and 2-MeImpG, so called U,C,G mixture. The composition of nucleotide in the mixtures is given in Table II.

The reactions were monitored by obtaining aliquots (1 or 2 μL), which were diluted and quenched with EDTA before high performance liquid chromatography (HPLC) analysis. HPLC was run on a 1090 LC from Hewlett Packard equipped with a diode array detector and an Alltima C18 column 4X250 mm 5 μ from Alltech. Most of the analyses were performed with a chromatography, abbreviated TFA, which is: Solvent A: 0.02 M KH_2PO4 with 0.2% w/v trifluoroacetic

acid (TFA) pH 2.5; solvent B: 30% CH_3CN in water v/v with 0.2% w/v TFA. 0 to 15% B in 10 min; isocratic at 15% B for 4 min and then 15 to 30% B in 15 min. The other chromatography, abbreviated phosphate, is: Solvent A: 0.05 M KH_2PO_4 pH 6.5; solvent B: 30% CH_3CN in water. 0 to 15% B in 35 min. Among the two chromatographies the TFA is the best in resolving the components and producing sharp peaks. Typically with the TFA chromatography compounds elute later than with the phosphate chromatography. The two chromatographies differ enough from each other so that the order of elution is reversed in some cases. For example, $pU^{2'}pU$ elutes after $U^{5'}ppU$ with TFA but ahead of $U^{5'}ppU$ with phosphate chromatography. Also $pU^{3'}pU$ coelutes with an unknown compound in TFA chromatography, but pure with phosphate chromatography. This allowed us to calculate the ratio of $pU^{2'}pU$ to $pU^{3'}pU$ in selected samples. The order of elution of nucleotide derivatives with TFA chromatography can be seen in Figure 2 for cytidine, Figure 3 for uridine and Figure 4 for guanosine.

In the experiments reported in Table V the wavelength of 267 nm was chosen where all three nucleotides have very similar extinction coefficients $e \approx 9430 \pm 130$ in the acidic medium of the chromatography employed. Yield of a given product is calculated as the percentage of total initial material that corresponds to the HPLC area under the specific peak. Yields are expressed in monomer equivalents; no corrections were made for the hypochromicity of dimers ($\approx$10%). Experimental uncertainty is typically at $\pm$1% but rises to $\pm$5% for peaks with relatively small HPLC areas.

4.4. IDENTIFICATION OF PRODUCTS

The nucleoside 5′-monophosphates (5′-GMP, 5′-CMP and 5′-UMP) as well as the corresponding nucleosides that result from APH digestion of the above were identified by coelution with standards purchased from Sigma. The six pyrophosphates ($U^{5'}ppU$, $C^{5'}ppC$, $G^{5'}ppG$, $U^{5'}ppC$, $U^{5'}ppG$ and $C^{5'}ppG$) were identified by coelution with a standard synthesized by a method developed earlier (Kanavarioti *et al.*, 1991). The reaction product of HEPES with the nucleoside (HEPES-pG, HEPES-pC and HEPES-pU) were characterized by running the self-condensation of each substrate at 0.001 M monomer concentration in a 0.5 M and in a 1.0 M HEPES buffer and identifying the only product peak that doubles in size by doubling the concentration of the buffer. It should be noted that the yield of HEPES-pN product under these conditions is 15 to 30% whereas at the high concentrations of *pN that the typical reaction mixtures were run the HEPES-pN product was about 4%. The 2-methylimidazolides of the dimers accumulate during the first 30 hours; they remain practically unchanged for a couple of days and then their% yield decreases slowly because of P-N bond hydrolysis. For example, 2-MeImpCpC 2′,5′ and 3′,5′, although resolved with TFA chromatography, were isolated as one fraction; it was shown by hydrolysis in a pH 1.5 solution at 40 °C that they form quantitatively $pC^{2'}pC$ and $pC^{3'}pC$ in a ratio of about 3. $N^{2'}pN$ and $N^{3'}pN$ isomers that result from

APH digestion were identified by standards purchased from Sigma. NpN elutes much later than the corresponding pNpN. $pN^{3'}pN$ were also directly identified by standards that were made by enzymatic phosphorylation of $N^{3'}pN$ standards with PNK. The enzymatic phosphorylation of dimers $N^{2'}pN$ was not successful and therefore the corresponding $pN^{2'}pN$ standards are missing.

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