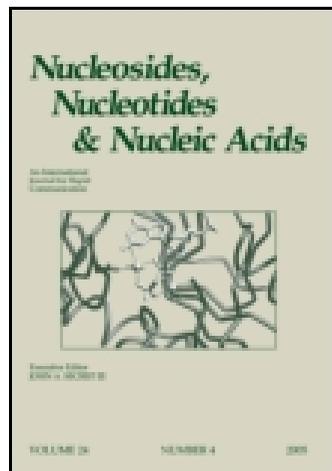


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The Cu^{2+} -Promoted Cleavage of mRNA 5'-cap Analogs: A Kinetic Study with P^1 -(7-Methylguanosin-5'-yl) P^3 -(Nucleosid-5'-yl) Triphosphates and P^1 -(7-Methylguanosin-5'-yl) P^4 -(Guanosin-5'-yl) Tetrphosphate

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THE Cu^{2+} -PROMOTED CLEAVAGE OF mRNA 5'-*cap* ANALOGS: A KINETIC STUDY WITH P^1 -(7-METHYLGUANOSIN-5'-YL) P^3 -(NUCLEOSID-5'-YL) TRIPHOSPHATES AND P^1 -(7-METHYLGUANOSIN-5'-YL) P^4 -(GUANOSIN-5'-YL) TETRAPHOSPHATE

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ABSTRACT: A kinetic study on the cleavage of a number of mRNA 5'-*cap* analogs, m^7GpppN and m^7GppppG , with Cu^{2+} aquo ion has been performed. Time-dependent product distributions at various pH and metal ion concentrations have been determined by capillary zone electrophoresis, and these data have been used to calculate the rate constants for various parallel reactions of the breakdown of the *cap* analogs.

The 5'-terminus of eukaryotic mRNA consists of 7-methylguanosine linked *via* a 5',5'-triphosphate bridge to the next nucleoside, which often is a 2'-*O*-alkylated purine nucleoside.¹⁻³ This *cap*-structure enhances pre-mRNA splicing⁴ and transport from the nucleus to cytoplasm,⁵ protects mRNA against exonucleases,⁶ and facilitates the attachment of mRNA to ribosomes.¹⁻³ Accordingly, expression of a certain gene may be expected to be effectively inhibited by cleavage of the 5'-*cap* from the transcribed mRNA. The *cap* structure hence offers an attractive target for artificial nucleases designed to recognize the 5'-terminal sequence of mRNA and cleave the triphosphate bridge. As a first indication of the feasibility of such of an approach, a 23 nucleosides long oligodeoxyribonucleotide bearing close to its 3'-end two Cu^{2+} /mercapto-

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acetylglutamate complexes tethered to N^2 of two adjacent guanine bases has been shown to decapitate the complementary mRNA.⁷ This kind of oligonucleotide conjugates are hoped to find applications in chemotherapy as chemically reactive antisense oligonucleotides. It has also been shown that the europium (III) complex of 1,4,7,10-tetrakis-(2-hydroxyethyl)-1,4,7,10-tetraazacyclo-dodecane rather rapidly decapitated a 5'-capped oligoribonucleotide hybridized with a complementary DNA sequence.⁸

To obtain useful background information for the development of metal-ion-dependent artificial nucleases for sequence selective decapitation of RNA, we now report a kinetic analysis on the Cu^{2+} promoted cleavage of a variety of 7-methylguanosine ($m^7\text{G}$) *cap* analogs having a general structure of $m^7\text{GpppN}$ or $m^7\text{GppppG}$. The time-dependent concentrations of the reaction products have been determined by capillary zone electrophoresis, and these data have been used to calculate the rate constants for the parallel reactions of the breakdown of the *cap* analogs at various pH and metal ion concentrations. Cu^{2+} was selected as the cleaving agent since the Cu^{2+} complex of *o*-phenantroline has earlier⁹ been reported to decapitate a 5'-capped RNA. It is also well known that Cu^{2+} exceptionally readily cleaves the 5'-triphosphates of nucleosides.¹⁰ To the best of our knowledge, this is the first quantitative description of metal-ion-induced cleavage of *cap* structures.

RESULTS AND DISCUSSION

The cleavage of the *cap* analog $m^7\text{GpppG}$ (**1a**) was followed in 0.25 - 3 mM solutions of copper(II) nitrate at pH 4-5 and 60 °C. The composition of the aliquots withdrawn at appropriate intervals from the reaction solution was determined by capillary zone electrophoresis. The products were identified by spiking with authentic samples, and their concentrations were determined by comparing the ratios of the signal areas with those obtained with mixtures of authentic reference compounds at known concentrations. FIG. 1 shows as an illustrative example the time-dependent product mixture obtained with $m^7\text{GpppG}$ (**1a**) at pH 5.0 and $[\mathbf{1a}] = [\text{Cu}^{2+}] = 1.0$ mM. Six products are initially formed: $m^7\text{GDP}$, $m^7\text{GMP}$, GDP, GMP, 7-methylguanine ($m^7\text{Gua}$) and a product tentatively assigned as P^1 -(ribofuranos-5'-yl) P^3 -(guanosin-5'-yl)

triphosphate (pppG, **2**). During early stages of the reaction, *i. e.* when less than 10 % of the starting material has reacted, the concentration of m⁷GMP remains equal to that of GDP, and the concentration of m⁷GDP equal to that of GMP. The concentration of m⁷Gua also equals to that of **2**, assuming that the chromophore of this product really is a guanine base. No hydrolysis of m⁷GMP or GMP to the corresponding nucleosides was detected.

1a: R=H; B=guanine

1b: R=H; B=7-methylguanine

1c: R=Me; B=guanine

1d: R=H; B=N⁶-methyladenine

1e: R=H; B=adenine

1f: R=H; B=cytosine

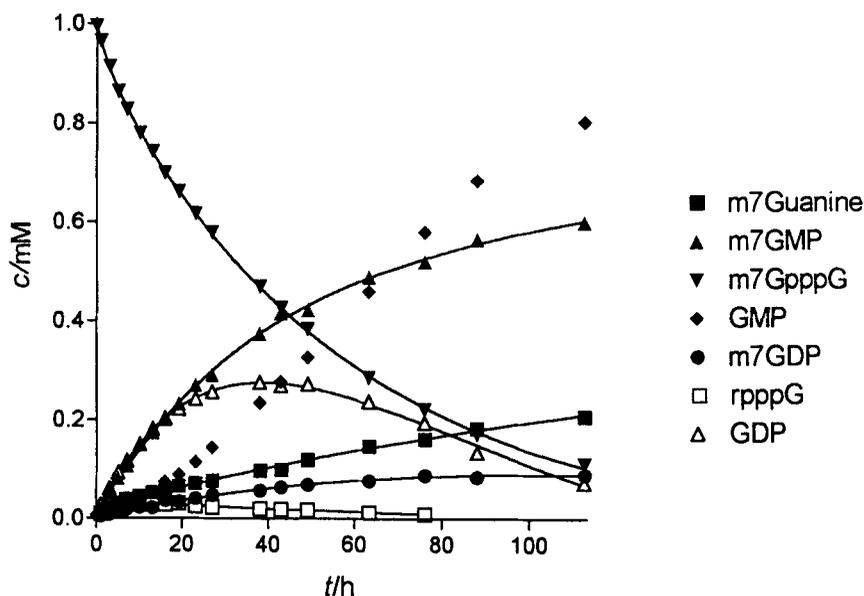
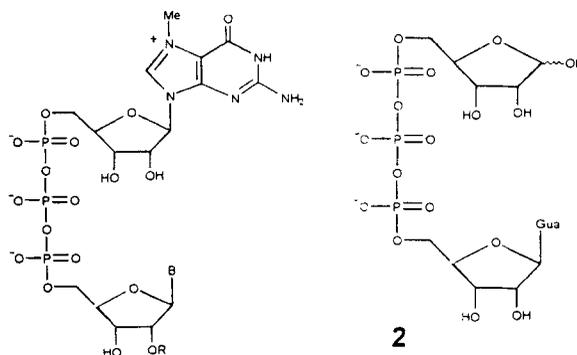
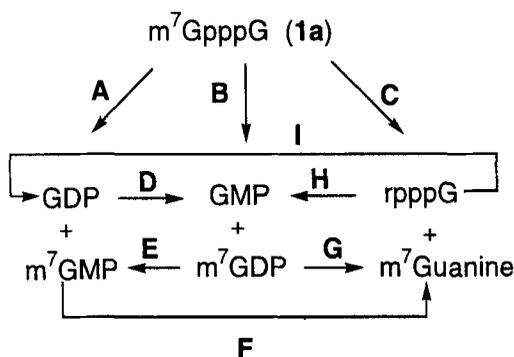


FIG. 1: Time-dependent product distribution for the Cu²⁺-promoted cleavage of m⁷GpppG (**1a**) at pH 5.0 and 60 °C ([Cu²⁺] = [1a] = 1.0 mM).



Scheme 1

Accordingly, three initial reactions, indicated in *Scheme 1* by A, B and C, appear to take place. Independent measurements with authentic samples of the initial products, viz. m^7GDP , m^7GMP , m^7Gua , GDP and GMP , revealed that the following subsequent reactions take place: (i) dephosphorylation of GDP to GMP (Reaction D in *Scheme 1*), (ii) dephosphorylation of m^7GDP to m^7GMP (E), (iii) *N*-glycosidic hydrolysis of m^7GMP to m^7Gua (F), and (iv) *N*-glycosidic hydrolysis of m^7GDP to m^7Gua (G). Moreover, the time-dependent product distribution of the cleavage of m^7GpppG during the later stages of the reaction strongly suggests that $rpppG$ (2) undergoes hydrolysis to GDP (H) and GMP (I). GMP and GDP were not dephosphorylated, and m^7GMP and GMP were not dephosphorylated under the experimental conditions.

In the absence of Cu^{2+} , m^7GpppG (1a) underwent in the pH range 4-5 only depurination to m^7Gua and $rpppG$ (2), and even this reaction was slower than in the presence of Cu^{2+} . No sign of the cleavage of the triphosphate bridge was detected. In other words, Reactions A-C (*Scheme 1*) are all promoted by the Cu^{2+} ion, but the triphosphate cleavage (A and B) much more efficiently than the depurination (C).

TABLE 1 records the half-lives for the partial reactions A-I at 1mM initial concentration of Cu^{2+} and the starting material (pH 5.0, 60 °C). All these reactions, except the dephosphorylation of GDP to GMP (D) obeyed first-order kinetics under these conditions. For an unknown reason the latter reaction exhibited a clearly sigmoid dependence of $[GDP]$ (and $[GMP]$) on time. The observed first-order kinetics for the

TABLE 1. Half-lives for the partial reactions of the Cu^{2+} -promoted breakdown of m^7GpppG (**1a**; Scheme 1) at pH 5.0 and 60 °C when the initial concentration of Cu^{2+} and the starting materials were 1.0 mM.

| Reaction | Starting material | Products | $\tau_{1/2} / \text{h}^{-1}$ ^a |
|------------|--------------------------|---|---|
| A | m^7GpppG | $\text{m}^7\text{GMP} + \text{GDP}$ | 46 |
| B | m^7GpppG | $\text{m}^7\text{GDP} + \text{GMP}$ | 210 |
| C | m^7GpppG | $\text{m}^7\text{Guanine} + \text{rpppG}$ | 210 |
| D | GDP | GMP | 18 ^b |
| E | m^7GDP | m^7GMP | 270 |
| F | m^7GMP | $\text{m}^7\text{Guanine}$ | 420 |
| G | m^7GDP | $\text{m}^7\text{Guanine}$ | 300 |
| H+I | rpppG | GDP + GMP | 10 |

^aFor the evaluation of the half-lives, see the experimental.

^bReaction **D** did not obey the first-order kinetics.

disappearance of **1a** is somewhat unexpected. Under the experimental conditions, *i. e.* at 1 mM concentration of Cu^{2+} and **1a**, Cu^{2+} is not present as a free aquo ion but in all likelihood is to a significant extent complexed with the starting material and reaction products (and also with the acetate ion used as a buffer constituent). The stability constants for the Cu^{2+} complexes of NMP^{2-} , NDP^{3-} and NTPH^{3-} (NTPH^{3-} stands for the trianion NTP and is assumed to mimic the complexing ability of **1a**) have been reported to be of the order of 10^3 , 10^5 and 10^4 M, respectively.^{11,12} Accordingly, it appears quite clear that under the experimental conditions (pH 4-5, $[\text{Cu}^{2+}]$ 0.25-4 mM, **[1a]** 1-3 mM), the speciation of Cu^{2+} is continuously changed with the progress of the cleavage of **1a**. In spite of this, the disappearance of **1a** rather strictly obeyed the first-order kinetics, the correlation coefficients ranging from 0.997 to 0.9995.

TABLE 2 records the first-order rate constants obtained for the initial reactions **A-C** of **1a** at different pH and initial concentrations of Cu^{2+} and the starting material. Under all the conditions employed, the fastest reaction is the cleavage of **1a** to m^7GMP and GDP, representing from 55 to 80% of the total disappearance of **1a**. The rate of this reaction experiences a 4-fold increase on going from pH 4.0 to 5.0 and keeping **[1a]** = $[\text{Cu}^{2+}]$ = 1.0 mM. At a fixed pH (pH 5.0) and concentration of **1a** (1.0 mM), the reaction is

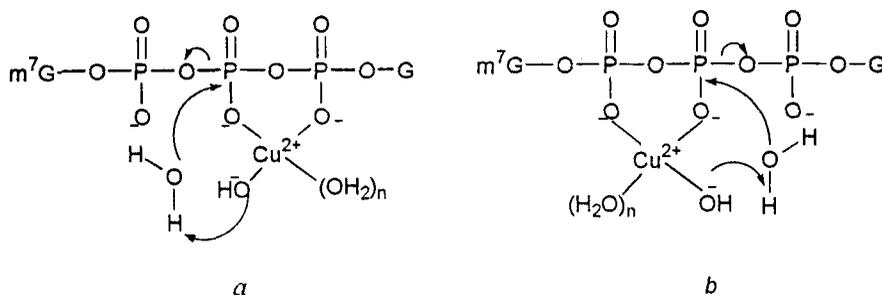
TABLE 2. First-order rate constants for the concurrent breakdown of m^7GpppG (**1a**) to m^7GMP and GDP (k_A), to m^7GDP and GMP (k_B), and to m^7Gua and $rpppG$ (k_C) at various pH and initial concentrations of Cu^{2+} and m^7GpppG at $60\text{ }^\circ\text{C}$.^a

| pH | $[Cu^{2+}]/mM$ | $[m^7GpppG]/mM$ | $k_A/10^{-6}\text{ s}^{-1}$ | $k_B/10^{-6}\text{ s}^{-1}$ | $k_C/10^{-6}\text{ s}^{-1}$ |
|-----|----------------|-----------------|-----------------------------|-----------------------------|-----------------------------|
| 5.0 | 0.25 | 1.0 | 0.65 | 0.15 | 0.44 |
| 5.0 | 0.5 | 1.0 | 1.4 | 0.29 | 0.60 |
| 5.0 | 1.0 | 1.0 | 4.2 | 0.90 | 0.90 |
| 5.0 | 2.0 | 1.0 | 16 | 2.5 | 2.1 |
| 5.0 | 4.0 | 1.0 | 33 | 6.6 | 4.2 |
| 5.0 | 3.0 | 3.0 | 9.3 | 1.7 | 1.7 |
| 4.5 | 1.0 | 1.0 | 2.2 | 0.44 | 0.46 |
| 4.0 | 1.0 | 1.0 | 1.0 | 0.26 | 0.49 |
| 5.0 | - | 1.0 | - | - | 0.44 |
| 4.5 | - | 1.0 | - | - | 0.41 |
| 4.0 | - | 1.0 | - | - | 0.45 |

^aThe pH was adjusted with 10 mM acetate buffer. The ionic strength was adjusted to 0.1 M with sodium nitrate.

markedly accelerated with the increasing Cu^{2+} concentration, the apparent reaction order in $[Cu^{2+}]$ being actually higher than unity. By contrast, when the metal ion concentration is increased from 1.0 to 3.0 mM keeping the $[1a]/[Cu^{2+}]$ ratio unity, only a 2-fold rate acceleration is observed. In all likelihood this kind of intermediary reaction orders largely result from changes in the speciation of Cu^{2+} , and hence they do not allow straightforward mechanistic conclusions.

In addition to the cleavage of **1a** to m^7GMP and GDP (**A**), a parallel reaction giving m^7GDP and GMP (**B**) takes place in the presence of Cu^{2+} . In fact, the rates of both of these reactions depend in a very similar manner on the reaction conditions, the ratio of the rate constants, k_B/k_A , being approximately 0.2. The preference of reaction **A** may tentatively be explained as depicted in *Scheme 2*. Cu^{2+} ion is assumed to undergo bidentate coordination to the anionic phosphoryl oxygen ligands of two adjacent phosphate groups. The electron density of the phosphate groups engaged in the metal ion binding is reduced, and hence nucleophilic attack of a water molecule on phosphorus is facilitated. The hydroxo ligand of the coordinated metal ion may even facilitate this



Scheme 2

process by deprotonating the attacking water molecule (as indicated in *Scheme 2*), or the hydroxo ligand itself may serve as an intracomplex nucleophile. As a result of the nucleophilic attack, the phosphate group which is not engaged in the metal ion binding is displaced. Since the base moiety of 7-methylguanosine is positively charged at pH 4-5, Cu^{2+} may be expected to prefer binding to the phosphate groups at the opposite end of the triphosphate bridge. In other words, the binding mode *a* in *Scheme 2* is the favored one, and hence reaction **A** occurs more readily than **B**. This proposal receives some additional support from the fact that the Cu^{2+} -promoted dephosphorylation of GDP to GMP is more than one order of magnitude faster process than the corresponding reaction of m^7GDP (cf. Reactions **D** and **E** in TABLE 1).

As mentioned above, cleavage of the 7-methylguanine base (**C**) from **1a** competes with the hydrolysis of the triphosphate bridge. This depurination reaction takes place at a measurable pH-independent rate even in the absence of Cu^{2+} , but it also is metal ion promoted, exhibiting approximately first-order dependence of rate on $[\text{Cu}^{2+}]$.

TABLE 3 summarizes the first-order rate constants obtained for partial reactions **A-C** of various *cap*-analogs (**1a-f**). In all these analogs one of the nucleosides is 7-methylguanosine, while the other is 7-methylguanosine (**1b**), 2'-*O*-methylguanosine (**1c**), *N*⁶-methyladenosine (**1d**), adenosine (**1e**), or cytidine (**1f**). As seen, the identity of this second nucleoside moiety has a marked influence on the hydrolytic stability of the triphosphate bridge. The rates of reaction **A**, giving m^7GMP and NDP as initial products, differ by more than one order of magnitude, $\text{m}^7\text{Gpppm}^{2'}\text{G}$ (**1c**) being the most reactive

TABLE 3. First-order rate constants for the concurrent breakdown of various *cap* analogs, m^7GpppN , to m^7GMP and NDP (k_A), to m^7GDP and NMP (k_B), and to m^7Gua and $rpppN$ (k_C) in 1.0 mM $Cu(NO_3)_2$ solutions at pH 5.0 and 60 °C.^a

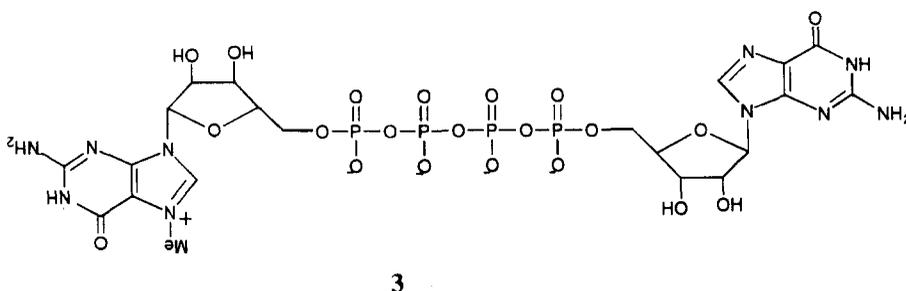
| Compound | $k_A/10^{-6} s^{-1}$ | $k_B/10^{-6} s^{-1}$ | $k_C/10^{-6} s^{-1}$ |
|-----------------------------|----------------------|----------------------|----------------------|
| m^7GpppG (1a) | 4.2 | 0.90 | 0.90 |
| m^7Gpppm^7G (1b) | 0.73 | - | 1.1 |
| m^7Gpppm^2G (1c) | 6.6 | 0.97 | 1.0 |
| m^7Gpppm^6A (1d) | 0.90 | 0.70 | 0.80 |
| m^7GpppA (1e) | 1.2 | 0.76 | 0.76 |
| m^7GpppC (1f) | 0.43 | 0.18 | 0.61 |

^aThe pH was adjusted with 10 mM acetate buffer. The ionic strength was adjusted to 0.1 M with sodium nitrate. The initial concentration of the starting material was 1.0 mM.

and m^7GpppC (**1f**) the least reactive analog. The cleavage of **1a-f** to m^7GDP and NMP (**B**) is less sensitive to the structure of the starting material, in particular as long as N is a purine nucleoside. These observations are consistent with the mechanistic suggestion of *Scheme 2*. Reaction **A** is initiated by coordination of Cu^{2+} to the phosphate groups adjacent to the nucleoside the structure of which is altered, while reaction **B** proceeds by Cu^{2+} -binding close to the unaltered 7-methylguanosine. Accordingly, the pre-equilibrium step of reaction **A** may well be more susceptible than that of reaction **B** to nucleoside N in m^7GpppN . As seen from TABLE 3, the cleavage of 7-methylguanine from **1a-f** is rather insensitive to the structure of the starting material.

The cleavage of the tetraphosphate *cap* analog, $m^7GppppG$ (**3**), was studied at pH 5.0 and 60 °C, the initial concentration of Cu^{2+} and the starting material being 1.0 mM. The overall rate of the breakdown of this compound was about 35 % smaller than that of the corresponding triphosphate (**1a**). Four reactions took initially place, *viz.* cleavage to (i) m^7GMP and GTP , (ii) m^7GDP and GDP , (iii) m^7GTP and GMP , and (iv) m^7Gua and $rppppG$. These reactions represented 16, 45, 6, and 33 % of the total disappearance of the starting material, respectively. These observations are again consistent with the mechanistic hypothesis presented above. The preferred formation of m^7GDP and GDP may be explained by initial binding of Cu^{2+} to γ - and δ -phosphates, *i. e.* as far from 7-

methylguanosine as possible. The other two reactions, *viz.* formation of m⁷GMP/GTP and m⁷GTP/GMP, may be expected to proceed by coordination to β - and γ -phosphates. Of these two binding modes, the β/γ -binding is probably less favourable, owing to shorter distance to the cationic 7-methylguanine base. The depurination of **3** is approximately as fast as that of **1a**.



EXPERIMENTAL SECTION

Materials. The preparation of the *cap* analogs^{13,14} (**1a-f**; **2**) and the 7-methylguanosine nucleotides¹⁵ (m⁷GMP, m⁷GDP, m⁷GTP) has been described previously. The other nucleotides used as reference materials were commercial products of Sigma. Copper nitrate and the buffer constituents were of reagent grade.

Kinetic measurements. The reactions were carried out in stoppered bottles immersed in a water bath, the temperature of which was kept at 60.0 ± 0.1 °C. The total volume of the reaction solution was 5 mL. Aliquots of 20 μ L were withdrawn at appropriate intervals, and the reaction was stopped by complexing the Cu(II) ion with EDTA. The composition of the aliquots was determined by capillary zone electrophoresis (HP^{3D} CE equipment). A fused silica capillary (50 μ m i.d., 77 cm) was employed. The separation was carried out in a 50 mM phosphate buffer, pH 7.0, applying a voltage of 20 kV and UV detection at 260, 279 and 282 nm. The signal areas were converted to concentrations with the aid of mixtures of authentic reference materials at known concentrations.

Calculation of the rate constants. The first-order rate constants for the disappearance of **1a-f** and **2** were obtained by applying the integrated first-order rate equation to the

time-dependent concentration of the starting material. The rate constants (k_A , k_B and k_C) for the three parallel partial reactions **A-C** (see *Scheme 1*) of the decomposition of **1a-f** were then obtained by breakdown of this overall rate constant to contributions of the partial reactions with the aid of the concentration ratio of m⁷GMP (**A**), m⁷GDP (**B**), and 7-methylguanine (**C**) at very early stage of the reaction, *i. e.* when less than 10 % of the starting material had decomposed. During this period the concentration ratio of these three initial products remained constant within the limits of experimental error. In the case of the tetraphosphate analog, m⁷GppppG (**3**), the breakdown of the experimentally observed overall rate constant was based on formation of m⁷GMP, m⁷GDP, m⁷GTP and 7-methylguanine. The half-lives reported in Table 1 for reactions **D-G** were determined separately using either GDP (**D**), m⁷GDP (**E** and **G**), or m⁷GMP (**F**) as a starting material. The half-life for the disappearance of rpppG (**2**; **H+I**) was estimated from the time-dependent product distribution of **1a** by applying the kinetics of parallel consecutive first-order reactions¹⁶ to the time-dependent concentration of **2**.

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REFERENCES

1. Shatkin, A.J., *Cell* **1985**, *40*, 223-223.
2. Rhoads, R.E. *Progress in Molecular and Subcellular Biology*, Hahn, H.F., Kopecho, D.J. and Muller W.E., Eds., Vol 9, Sringer, Berlin 1985, pp. 104-155.
3. Sonenberg, N. *Progr. Nucleic Acids Res. Mol. Biol.* **1988**, *35*, 173-207.
4. Konarska, M.M.; Padgett, R.A.; Sharp, P.A. *Cell* **1984**, *38*, 731-736.
5. Hamm, J.; Mattaj, I.W. *Cell* **1990**, *63*, 109-118.
6. Murthy, K.G.K., Park, P.; Manley, J.L. *Nucleic Acids Res.* **1991**, *19*, 2685-2692.
7. Baker, B.F.; Ramsamy, K.; Kiely, J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1647-1652.
8. Barber, B.F.; Khalili, H.; Wei, N.; Morrow, J.R.; *J. Am. Chem. Soc.* **1997**, *119*, 8749-8755.
9. Barker, B.F.; *J. Am. Chem. Soc.* **1993**, *115*, 3378-3379.
10. Sigel, S., Hofstetter, F., Martin, R.B.; Milburn, R.M.; Scheller-Krattiger, V., Scheller, K.H. *J. Am. Chem. Soc.* **1984**, *106*, 7935-7946.
11. Smith, R.M.; Martell, A.E. *Pure Appl. Chem.* **1991**, *63*, 1015-1080.
12. Sigel, H. *Eur. J. Biochem.* **1987**, *165*, 65-72.

13. Stepinski, J.; Bretner, M.; Jankowska, M.; Felczak, K.; Stolarski, R.; Wieczorek, Z.; Cai, A.-L.; Rhoads, R.E.; Temeriusz, A.; Haber, D.; Darzynkiewicz, E. *Nucleosides, Nucleotides* **1995**, *14*, 771-775.
14. Jankowska, M.; Stepinski, J.; Stolarski, R.; Wieczorek, Z.; Temeriusz, A.; Haber, D.; Darzynkiewicz, E. *Collect. Czech. Chem. Commun.* **1996**, *61* (Special issue), S197-S202.
15. Darzynkiewicz, E.; Ekiel, I.; Tahara, S.M., Seliger, L.S.; Shatkin, A.J. *Biochemistry* **1985**, *24*, 1701-1707.
16. Rodiguin, N.M., Rodiguina, E.N. in *Consecutive Chemical Reactions-Mathematical Analysis and Development*; Schneider, R.F., Ed.; van Nostrand Inc., Princeton, **1964**, pp.49-69.

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