Kinetic and Mechanistic Analysis of Dinucleotide and Oligonucleotide Formation from the 5′-Phosphorimidazolide of Adenosine on Na^+-Montmorillonite

Kunio Kawamura and James P. Ferris*

Contribution from the Department of Chemistry, Rensselaer Polytechnic Institute, Troy, New York 12180-3590

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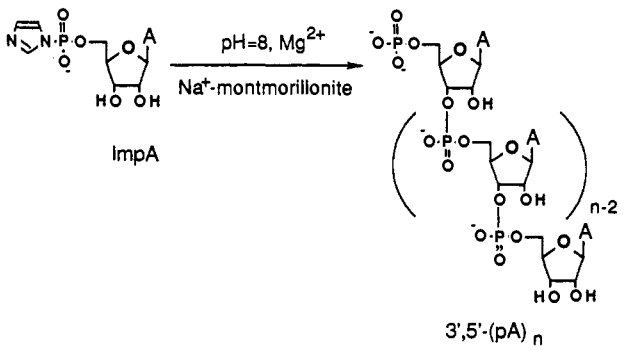
Abstract: The rate constants for the condensation reaction of the 5′-phosphorimidazolide of adenosine (ImpA) to form dinucleotides and oligonucleotides have been measured in the presence of Na^+-volclay (a Na^+-montmorillonite) in pH 8 aqueous solution at 25 °C. The rates of the reaction of ImpA with an excess of adenosine 5′-monophosphoramidate (NH_2pA), P_1,P_2-diadenosine 5′,5′-pyrophosphate (A^5′ppA), or adenosine 5′-monophosphate (5′-AMP or pA) in the presence of the montmorillonite to form NH_2pA^3′pA, A^5′ppA^3′pA, and pA^3′pA, respectively, were measured. Only 3′,5′-linked products were observed. The magnitude of the rate constants decrease in the order NH_2pA^3′pA > A^5′-ppA^3′pA > pA^3′pA. The binding of ImpA to montmorillonite was measured, and the adsorption isotherm was determined. The binding of ImpA to montmorillonite and the formation of higher oligonucleotides is not observed in the absence of salts. Mg^{2+} enhances binding and oligonucleotide formation more than Ca^{2+} and Na^+. The rate constants for the oligonucleotide formation were determined from the reaction products formed from 10 to 40 mM ImpA in the presence of Na^+-montmorillonite using the computer program SIMFIT. The magnitudes of the rate constants for the formation of oligonucleotides increased in the order 2-mer < 3-mer < 4-mer ··· 7-mer. The rate constants for dinucleotide and trinucleotide formation are more than 1000 times larger than those measured in the absence of montmorillonite. The rate constants for the formation of dinucleotide, trinucleotide, and tetranucleotide are 41, 2.6, and 3.7 times larger than those for the formation of oligo(G)s with a poly(C) template. The hydrolysis of ImpA was accelerated 35 times in the presence of the montmorillonite. The catalytic ability of montmorillonite to form dinucleotides and oligonucleotides is quantitatively evaluated and possible pathways for oligo(A) formation are proposed.

Introduction

The observation of the catalytic role of RNA in processing RNA transcripts[1,2] suggests that RNA-like molecules[3,4] had a central role in the first life on earth. If this hypothesis is correct then RNA-like molecules formed spontaneously under primitive earth reaction conditions. There have been a number of reports of successful studies[5–7] of the condensation of activated nucleotides to form RNA oligonucleotides in the presence and absence of RNA templates. Examples include the condensation of the 2-methylimidazolide of 5′-GMP on a poly(C) template to form oligonucleotides of guanylic acid,[5] the condensation of the imidazolide derivatives of some ribonucleotide diphosphates in the absence of a template in aqueous solution to give oligonucleotides linked by pyrophosphate bonds,[8,9] and the condensation of the 5′-phosphorimidazolides of nucleosides to oligonucleotides in the presence of uranyl ion.[10] Recently, several kinetic investigations of the template-directed synthesis of oligomers[11–17] and the hydrolysis of activated nucleotides[18,19] have been carried out to provide insight into the mechanism of oligonucleotide formation on RNA templates.

The possible role of clays and other minerals in the process leading to the origin of life was first proposed by Bernal in 1949.[20] Experimental studies of the possible role of clay minerals in the origins of life until 1985 have been reviewed.[21] We have been investigating the possibility that montmorillonite clay minerals served to concentrate and catalyze the oligomerization of nucleotides.[22–25] Recently, we have found that the condensation of the 5′-phosphorimidazolide of adenosine (ImpA) occurs on montmorillonite clays, leading to the preferential formation of 3′,5′-$(pA)_n$.[26–29] These oligonucleotides also contain 2′,5′-links and pyrophosphate (A^5′ppA) groupings.



In this report, we have investigated the rates of the condensation of ImpA to form dinucleotides and oligonucleotides in the presence of montmorillonite. First, the formation of dinucleotides was studied by a kinetic analysis of the reaction of ImpA with an excess of NH_2pA, A^5′ppA, or pA to form NH_2pA^3′A, A^5′ppA^3′-pA, or pA^3′pA. Second, the formation of RNA oligonucleotides

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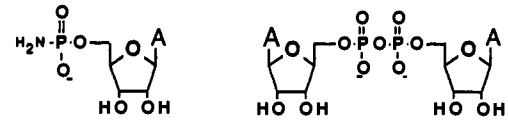

NH_2pA A^5ppA

by the reaction of ImpA on montmorillonite was investigated and the rate constants were obtained for formation of the 2-mer to 9-mer using the computer program SIMFIT.[30] In the course of this study, the binding behavior of ImpA on montmorillonite was investigated. The results of these studies led to proposals for pathways by which oligonucleotides are formed on montmorillonite.

Experimental Section

Materials. ImpA was synthesized using a modification of the procedure of Joyce et al.[27,31] Montmorillonite volclay (Vol) was a gift from American Colloid Compound, Arlington Heights, IL. Japan montmorillonite, extra pure grade (Jpn), was a gift from Dr. Seiji Yuasa of Osaka University, who obtained it from Nakarai Tesque Ltd. Wyoming (Wy) and Otay (Ot) montmorillonites were obtained from the Clay Minerals Society, Source Clay Minerals, Department of Geology, University of Missouri, Columbia, MO 65211. Homoionic montmorillonites were prepared by the titration method.[32] The Vol, Wy, and Ot montmorillonites contain quartz and other particulates which form a discernable bottom layer in the final centrifugation step. Careful removal of the top layer yields 2–3 g of Na^+-montmorillonite from the initial 6 g of crude montmorillonite. Adenosine 5′-monophosphoramidate (NH_2pA), P_1,P_2-diadenosine 5′,5′-pyrophosphate (A^5ppA), adenosine 5′-monophosphate (5′-AMP or pA), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), ribonuclease T_2 (RNase T_2), and alkaline phosphatase (APH) were purchased from Sigma or Aldrich.

HPLC analyses were performed on a Waters μBondapak C-18 column or a Whatman EQC (4.6 × 216 mm) C-18 column using a gradient of 0.005 M NaH_2PO_4 in 5% CH_3OH at pH 3.5 mixed with 0.01 M NaH_2PO_4 in 40% CH_3OH at pH 4.0[23] and on a HEMA-IEC BIO Q anion-exchange column from Alltech using a gradient of 0.008–0.4 M $NaClO_4$ at pH 8 with 2 mM Tris buffer.[26] No Tris was used when samples were collected for further analysis.

Dialyses were performed for 12 h at 2 °C using Spectrum Spectra/Pro MWCO 1000 tubing.

Reaction of the ImpA in the Presence and Absence of Montmorillonite. Unless noted otherwise, reaction solutions were prepared by dissolving 6 mg of ImpA in a 1-mL electrolyte solution (the standard buffer solution) which contains 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES (pH 8.0). The 15 mM solution of ImpA was added to 50 mg of Na^+-montmorillonite and mixed by vortexing. At the end of the reaction, the mixture was centrifuged and the supernatant was removed. To the supernatant was added 1 mL of 0.1 M ammonium acetate solution, 1 mL of 1 M ammonium hydroxide solution (pH = 12), or 1 mL of 0.1 M EDTA solution (pH = 8), and the solution was allowed to stand for 24 h to desorb the oligo(A)s from the montmorillonite. The montmorillonite suspension was centrifuged, and the supernatant was removed. Both the supernatants were analyzed separately by anion-exchange HPLC. Control reactions were performed under exactly the same conditions in the absence of montmorillonite.

Measurement of the Partition Coefficient of ImpA on Na^+-Montmorillonite. ImpA was dissolved in 1 mL of the standard buffer solution at 2 °C, and the mixture was added to 50 mg of Na^+-montmorillonite and vortexed. The montmorillonite suspension was allowed to stand at 2 °C in order to slow the oligomerization of ImpA. The binding was measured by centrifuging, withdrawing a 10-μL aliquot, and then mixing again by vortexing. The supernatant was diluted 1000-fold, and the absorbance at 260 nm was measured to determine the extent of binding. The binding at 2 °C was monitored over a 32-h time period.

Kinetic Measurements. Partition Model for the Kinetic Analysis of ImpA Condensation in the Presence of Montmorillonite. It was necessary to measure the partitioning of ImpA, pA, and oligo(A)s between the aqueous and clay phases in order to analyze the kinetics of ImpA condensation. The partition coefficient of the chemical species, X, is given in eq 1, and the concentration in the clay phase is defined for X in eq 2, where $[X]_{total}$ is the original concentration of X in the solution prior to addition to the clay. The coefficient for partition of each species to the clay is expressed by eq 3, where the solution phase is 1 mL and 50 mg of montmorillonite is used.

$$K = (\text{mol of X in the clay phase})/(\text{mol of X in the aqueous phase}) \quad (1)$$

$$[X]_{clay} = [X]_{total} - [X]_{aq} \quad (2)$$

$$K = [X]_{clay}/[X]_{aq} = ([X]_{total} - [X]_{aq})/[X]_{aq} \quad (3)$$

The amounts of ImpA and oligo(A)s in aqueous and clay phases were determined by anion-exchange HPLC, and the partition coefficients were calculated from the extent of binding.

Hydrolysis of ImpA in the Presence of Na^+-Vol. A 1-mL solution of 4.6×10^{-2} mM ImpA was prepared in the standard buffer solution, and the mixture was added to 50 mg of the Na^+-Vol. The rate of hydrolysis was determined by periodic centrifugation of the reaction mixture and removing a 10-μL aliquot of the supernatant for anion-exchange HPLC analysis. The hydrolysis reaction was monitored for 24 h, and at the end of that time, the montmorillonite suspension was centrifuged and the supernatant was removed. To the montmorillonite was added 1 mL of 0.1 M ammonium acetate, and the mixture was allowed to stand for 24 h at 25 °C. The mixture was then centrifuged and the supernatant was removed. Both the supernatants were analyzed separately by anion-exchange HPLC.

Reaction of NH_2pA, A^5ppA, or pA with ImpA. The rates of the reactions of 2.5 mM ImpA with 50 mM NH_2pA, A^5ppA, or pA in the presence of 50 mg of Na^+-Vol in 1 mL of the standard buffer solution were measured. Product formation was monitored for 24 h by anion-exchange HPLC analysis of the supernatant obtained by centrifugation of the reaction mixture. At the end of 24 h, the suspension was centrifuged and the supernatant was removed. To the precipitate was added 1 mL of 1 M ammonium hydroxide (pH = 12) solution, and the mixture was allowed to stand for 15 min at 2 °C to desorb the oligo(A)s from the montmorillonite. The mixture was centrifuged, and the supernatant was removed. Both the supernatants were analyzed by anion-exchange and reversed-phase HPLC.

The structures of the dinucleotides were determined by selective enzymatic hydrolysis with RNase T_2 and APH. RNase T_2 hydrolysis was carried out in 0.1 mL of the reaction solution for 3 h at 37 °C at pH 5.0 using 0.1 unit of enzyme, and APH hydrolysis was performed on 0.1 mL for 12 h at 37 °C at pH 8.0 with 0.6 unit of enzyme.

Oligomerization of ImpA on Na^+-Vol. ImpA solutions (10, 15, 20, or 40 mM) in 1 mL of the standard buffer solution were added to 50 mg of the Na^+-Vol, and the mixture was vortexed. The suspension was allowed to stand at 25 °C, and 10-μL aliquots were taken at regular intervals over 168 h after centrifugation. When monitoring was completed the reaction mixture was centrifuged and the supernatant was removed. To the precipitate was added 1 mL of 0.1 M ammonium acetate solution, and the mixture was allowed to stand for 24 h to desorb the oligo(A)s from the montmorillonite. The mixture was centrifuged, and the supernatant was removed. Both the supernatants were analyzed on the anion-exchange HPLC, and the partition coefficients of the oligo(A)s were calculated from the concentrations of oligonucleotides in the aqueous and clay phases.

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Table 1. Yields of Oligo(A) on Different Na^+-Montmorillonites and in the Presence of Mg^{2+}, Ca^{2+}, and Na^+

	oligomers (%)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
				Effect of Clays[a]						
Vol	52	27	11	5.9	2.0	1.5	0.56	0.26	0.17	0.07
Wy	44	31	13	7.3	2.9	1.3	0.66	0.29	0.11	
Jpn	48	33	11	6.4	1.5	0.79	0.32	0.08	0.04	
Ot	93	6.5	0.15	0.05						
none	97	2.8	0.19							
				Effect of Salts[b]						
Mg^{2+}	53	27	9.8	6.8	2.0	1.2	0.53	0.26	0.18	0.10
Ca^{2+} [c]	40	47	7.5	3.3	0.97	0.35	0.12	0.03	0.01	
Na^+	26	65	6.5	2.3	0.43	0.13	0.04	0.01		
none	43	53	3.3	0.75	0.04					

[a] Reaction for 7 days in the presence of Na^+-Vol in pH 8 aqueous solution containing 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES at 25 °C (anion-exchange HPLC analysis). The percentages are obtained by dividing the percentage obtained from the HPLC absorbances by oligonucleotide length. [b] 0.075 M Mg^{2+} or 0.2 M Na^+ added to 0.1 M HEPES (pH 8) and Na^+-Vol. [c] 0.075 M Ca^{2+} added to 0.2 M NaCl, 0.1 M HEPES (pH 8), and Na^+-Vol.

Kinetics of the Elongation of Tetramer Fraction by Reaction with ImpA in the Presence of Na^+-Vol. The tetranucleotide fraction was collected from the anion-exchange HPLC column and was dialyzed for 12 h and lyophilized. The fraction was redissolved in 300 μL of distilled water and was shown to be 95% pure by anion-exchange HPLC. A 280-μL aliquot of the tetranucleotide solution (ca. 10^{-2} mM) was added to 280 μL of a buffer solution containing 0.4 M NaCl, 0.15 M $MgCl_2$, and 0.2 M HEPES (pH = 8.0). To 500 μL of the tetranucleotide electrolyte mixture was added 50 μL of 10 mM ImpA to give a 0.9 mM solution, and this mixture was added to 25 mg of Na^+-Vol. The montmorillonite suspension was vortexed and allowed to stand at 25 °C and the course of the reaction followed for 24 h by anion-exchange HPLC. The montmorillonite mixture was vortexed and kept for further monitoring. At the end of 24 h, the mixture was centrifuged and supernatant I was removed. The montmorillonite was mixed for 30 min with 500 μL of 1 M ammonium hydroxide solution (pH = 12, 2 °C) to stop the reaction and desorb the oligo(A)s. The mixture was centrifuged, and supernatant II was removed. Then 500 μL of 0.1 M EDTA solution (pH 8) was added to the montmorillonite and the mixture was allowed to stand for 2 h at 2 °C. The mixture was centrifuged and supernatant III was removed. Supernatants I, II, and III were analyzed by the anion-exchange HPLC.

Determination of Rate Constants. Computer programs FITSIM[33,34] and SIMFIT[30] were used for the evaluation of the rate constants. The rate constants for the reactions of NH_2pA, A5′ppA, and pA with ImpA were determined by FITSIM.[33,34] All other kinetic analyses for oligomerization and elongation reactions were evaluated by SIMFIT.[30]

Results

Effect of the Montmorillonite Source on Oligomer Formation. The reaction of ImpA at pH 8 in the presence of the Na^+-Vol yields oligo(A)s of up to 10 nucleotides in length.[26] In order to investigate the effect of mineral source on oligonucleotide formation, the ImpA reaction was investigated using three additional montmorillonites, Na^+-Wy, Na^+-Jpn, and Na^+-Ot montmorillonites. In addition, the effects of Mg^{2+}, Ca^{2+}, and Na^+ on oligonucleotide formation (Table 1) were investigated.

The Vol and Wy montmorillonites catalyzed the formation of oligo(A)s with comparable efficiencies, and the Jpn montmorillonite was only slightly less efficient. However, oligonucleotide formation did not proceed in the presence of the Ot montmorillonite.

When calcium chloride was used instead of magnesium chloride in the standard buffer solution, oligonucleotides were formed but the yields of the oligo(A)s were smaller than those formed in the presence of Mg^{2+}. When the reaction was done in the absence of Mg^{2+}, with only Na^+ present, the oligonucleotide yield was less than that in the presence of Mg^{2+} or Ca^{2+} (Table 1).

Table 2. Binding of ImpA on Na^+-Montmorillonite[a]

	binding (%)	K[b]
ImpA concn (mM)[c]		
5	68.8	2.21
10	67.3	2.06
15	64.1	1.78
20	59.3	1.46
40	44.9	0.813
60	32.3	0.478
80	26.0	0.351
120	19.0	0.234
15[c]	59.8	1.49
15[d]	60.2	1.513
15[e]	17.1	0.206
effect of salts[f]		
no $MgCl_2$[g]	9.28	0.102
$CaCl_2$[h]	66.3	1.97
effect of clays[f]		
Wy	54.5	1.20
Jpn	42.3	0.732
Ot	1.27	0.0129

[a] Binding measurement in the presence of Na^+-Vol in pH 8 aqueous solution containing 15 mM ImpA, 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES at 2 °C for 32 h except where noted. [b] Partition coefficient K = (mol amount in clay phase)/(mol amount in aqueous phase). [c] Binding measurement at 25 °C instead of 2 °C. [d] Binding measurement at pH 7. [e] Binding measurement at pH 9. [f] ImpA concentration, 15 mM. [g] Binding measurement in the presence of 0.2 M NaCl and 0.1 M HEPES. [h] Binding measurement in the presence of 0.075 M $CaCl_2$ instead of $MgCl_2$.

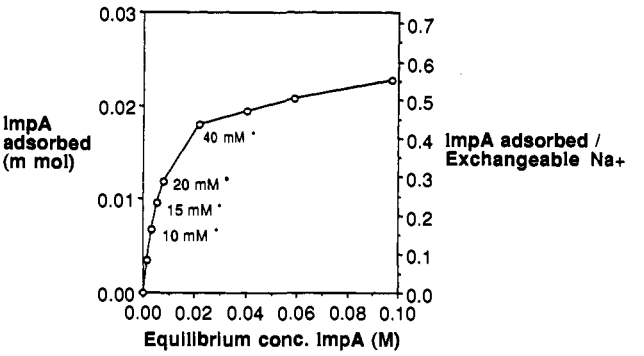

Figure 1. Adsorption isotherm curve for binding of ImpA to the Na^+-Vol clay: [NaCl] = 0.2 M, [$MgCl_2$] = 0.075 M, [HEPES] = 0.1 M, pH = 8, 2 °C. (*) The initial concentrations (mM) of ImpA which were used for the kinetic analysis of ImpA oligomerization.

Binding of ImpA on Na^+-Montmorillonite. Since the oligomerization of ImpA proceeds on montmorillonite it was important to determine the effect of ImpA concentration on binding (the adsorption isotherm). The binding of ImpA was measured at 2 °C instead of 25 °C to inhibit oligomerization over a period of 32 h. Previous studies demonstrated little or no temperature effect in the binding of nucleotides to montmorillonite.[22] The absorbance decreased rapidly in 4 h and more slowly in 8–32 h. The linear part of the absorbance change from 8 to 32 h was extrapolated to zero time. The rapid absorbance change (ΔA), that is, the difference between the initial absorbance (A_0) and the intercept of the line (A_t), was due to ImpA binding. The slow change of absorbance after 8 h is due to oligo(A) formation. The partition coefficients were calculated by eq 4 (Table 2), and the adsorption isotherm for ImpA on Na^+-montmorillonite is given in Figure 1.

$$K = \Delta A / A_0 \qquad (4)$$

The data were analyzed using the Langmuir adsorption

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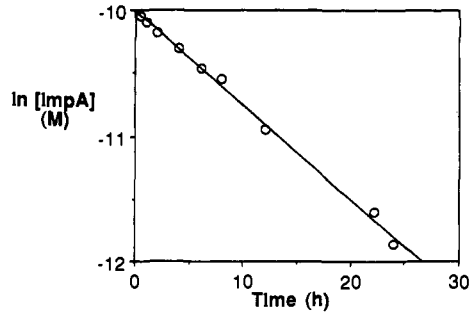


Figure 2. Pseudo-first-order plot of the hydrolysis of ImpA in the presence Na⁺-Vol: $[ImpA]_t = 4.6 \times 10^{-2}$ mM, [NaCl] = 0.2 M, $[MgCl_2]$ = 0.075 M, [HEPES] = 0.1 M, pH = 8, 25 °C.

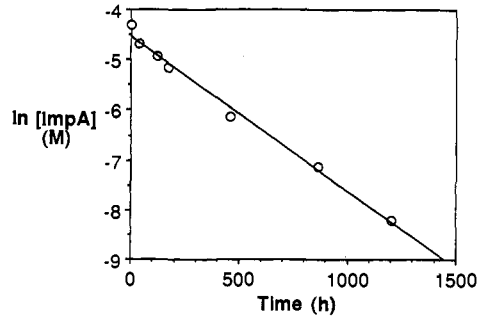


Figure 3. Pseudo-first-order plot of the hydrolysis of ImpA in the absence Na⁺-Vol: $[ImpA]_t$ = 15 mM, [NaCl] = 0.2 M, $[MgCl_2]$ = 0.075 M, [HEPES] = 0.1 M, pH = 8, 25 °C.

equation[35] where [ImpA] is given in eq 5, where a = moles of

$$[ImpA]/a = [ImpA]/a_s + 1/(a_s K_L) \quad (5)$$

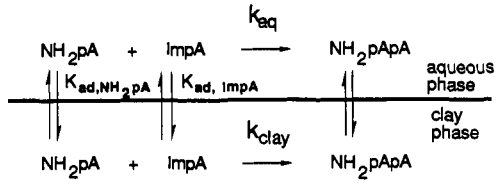
ImpA adsorption/mole of exchangeable cation, a_s = moles of ImpA adsorbed/mole of exchangeable cation at saturation, and K_L = Langumuir adsorption coefficient. The adsorption parameters a_s and K_L were calculated from the (slope)$^{-1}$ and the slope/intercept obtained from a plot of $[ImpA]/a$ vs [ImpA]. The values are K_L = 111 M^{-1} and a_s = 0.60. The exchangeable Na⁺ was calculated theoretically on the basis of the composition of Na⁺-Vol. The value is 82.4 mequiv/100 g, and it corresponds to 0.0412 mmol/50 mg of the clay.

The effect of different montmorillonites and metal ions on the binding of 15 mM ImpA was also investigated. ImpA binding decreases in the order Na⁺-Vol > Na⁺-Wy > Na⁺-Jpn > Na⁺-Ot (Table 2). The catalytic ability of the montmorillonite decreases in approximately the same order. The binding of ImpA to Na⁺-Vol decreases in the order $Mg^{2+} > Ca^{2+} > Na^+$. The degree of oligonucleotide formation decreases in the same order although the differences in yield between Mg^{2+} and Ca^{2+} are small (Table 1). There is essentially no binding in the absence of divalent ions, yet significant, albeit decreased, oligonucleotide formation is observed (Table 1).

Order of the ImpA Hydrolysis in the Absence and Presence of the Na⁺-Vol. The hydrolysis of ImpA was investigated in the presence and absence of Na⁺-Vol to determine if it followed pseudo-first- or second-order kinetics. The hydrolysis of adenosine 5′-triphosphate obeys pseudo-second-order kinetics (the hydrolysis rate is proportional to the square of the ATP concentration in aqueous solution) in the presence of Mg^{2+} ion, and thus, it is possible that pseudo-second-order hydrolysis occurs in ImpA hydrolysis.[36] The rate constants for the hydrolysis of ImpA were required for the determination of the rates of oligonucleotide formation described below. Hydrolysis studies were carried out with 4.6×10^{-2} mM ImpA, a concentration where oligonucleotide formation was minimal. (Oligonucleotide formation was still observed when 1 mM ImpA was used.) The rate of hydrolysis of ImpA in the absence of Na⁺-Vol was also measured to determine the extent of montmorillonite catalysis of ImpA hydrolysis. First-order plots of the data (Figures 2 and 3) are consistent with pseudo-first-order processes with rate constants of 10.7×10^{-2} and 3.10×10^{-3} h^{-1}, respectively.

Kinetic Analyses of the Reaction of NH_2pA, A⁵′ppA, and pA with ImpA in the Presence of Na⁺-Vol. The rate of dinucleotide formation from ImpA in the presence of the montmorillonite cannot be measured directly because the dinucleotide is being converted to oligonucleotides. However, the rate of dinucleotide formation can be estimated independently by measuring the rate of reaction of ImpA in the presence of an excess of a monomer which does not form oligonucleotides. Thus the reactions of ImpA with excess NH_2pA, A⁵′ppA, and 5′-AMP to form NH_2pApA, A⁵′ppApA, and pApA, respectively were investigated.

Scheme 1



Only one product was formed in each of the reactions as determined by ion-exchange and reversed-phase HPLC. The product of the reaction with NH_2pA had a retention time consistent with the presence of two negative charges as would be expected for NH_2pApA, and the products from A⁵′ppA and pA had retention times consistent with the presence of three negative charges as expected for A⁵′ppApA and pApA. Only one main HPLC peak was observed when each reaction was analyzed by reversed-phase HPLC consistent with the presence of only one isomer.

The dinucleotide products were shown to have 3′,5′-linkages by selective enzymatic hydrolysis with RNase T_2 and APH. The product from the condensation of ImpA and NH_2pA was completely cleaved by RNase T_2 into equal amounts of two compounds, one of which was shown to be adenosine by reversed-phase HPLC. This finding is consistent with the presence of a 3′,5′-phosphodiester bond. The reaction product was not changed on treatment with APH, indicative of the absence of a terminal phosphate. These data are consistent with $NH_2pA^{3'}pA$ as the structure. The product from the reaction of ImpA with A⁵′ppA was cleaved with RNase T_2 to two compounds which were identified as adenosine and A⁵′ppAp by comparison of their retention times with those of authentic samples on an anion-exchange HPLC.[27] The reaction product was not changed on treatment with APH. It was possible to assign structure $A^{5'}ppA^{3'}pA$ to the reaction product on the basis of these data. The pApA was shown to have a 3′,5′-linkage by cleavage with RNase T_2 to pAp and A. These hydrolysis products were identified by their retention times on anion-exchange HPLC.[27] In addition, it was cleaved to $A^{3'}pA$ by APH as shown by coinjection with an authentic sample. The formation of exclusively 3′,5′-linked dinucleotides is in marked contrast to previous prebiotic simulations where 60–90% of the phosphodiester bonds are 2′,5′-linked.[37]

The rate constants for the formation of 3′,5′-linked dinucleotides were determined using FITSIM[33,34] and the reactions are shown in Scheme 1 and equations (I-1)–(I-12). The rate of the formation of $NH_2pA^{3'}pA$ is given by eq I-1, where k_{clay,NH_2pApA} and k_{aq,NH_2pApA} are the rate constants for the formation of $3',5'\text{-}NH_2pA^{3'}pA$ in the clay phase and aqueous phase, respectively. Since the rate of reaction of NH_2pA with ImpA in the presence of

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$$d[NH_2pApA]_{total}/dt = k_{clay,NH_2pApA}[NH_2pA]_{clay}[ImpA]_{clay} + k_{aq,NH_2pApA}[NH_2pA]_{aq}[ImpA]_{aq} \quad (I-1)$$

$$d[NH_2pApA]_{total}/dt = k_{clay,NH_2pApA}[NH_2pA]_{clay}[ImpA]_{clay} \quad (I-2)$$

$$d[NH_2pApA]_{aq}/dt = k_{obs,NH_2pApA}[NH_2pA]_{aq}[ImpA]_{aq} \quad (I-3)$$

$$d[NH_2pApA]_{aq}/dt = k'_{obs,NH_2pApA}[ImpA]_{aq} \quad (I-4)$$

$$[ImpA]_{total} = [ImpA]_{clay} + [ImpA]_{aq} \quad (I-5)$$

$$[NH_2pA]_{total} = [NH_2pA]_{clay} + [NH_2pA]_{aq} \quad (I-6)$$

$$[NH_2pApA]_{total} = [NH_2pApA]_{clay} + [NH_2pApA]_{aq} \quad (I-7)$$

$$K_{ImpA} = [ImpA]_{clay}/[ImpA]_{aq} \quad (I-8)$$

$$K_{NH_2pA} = [NH_2pA]_{clay}/[NH_2pA]_{aq} \quad (I-9)$$

$$K_{NH_2pApA} = [NH_2pApA]_{clay}/[NH_2pApA]_{aq} \quad (I-10)$$

$$d[NH_2pApA]_{total}/dt = -(K_{NH_2pApA} + 1)d[NH_2pApA]_{aq}/dt \quad (I-11)$$

$$d[NH_2pApA]_{total}/dt = k_{clay,NH_2pApA}[NH_2pA]_{clay}K_{ImpA}[ImpA]_{aq} = -(K_{NH_2pApA} + 1)k_{obs,NH_2pApA}[ImpA]_{aq} \quad (I-12)$$

montmorillonite is about 100 times faster than that in the absence of montmorillonite (see below), the rate in the aqueous solution can be neglected and eq I-1 is reduced to eq I-2. The observed rate constant for the formation of NH_2pApA in aqueous solution in the presence of montmorillonite is given in eq I-3. The concentration of NH_2pA in aqueous solution can be regarded as constant since the amount of NH_2pA is 20 times greater than ImpA. Thus, the observed rate constant k'_{obs,NH_2pApA}, which was determined by computer calculation, is given in eq I-4. The material balance of ImpA, NH_2pA, and $NH_2pA^{3'}pA$ between the clay and aqueous phases and the partition coefficients are given in (I-5)–(I-10), where K_{ImpA}, K_{NH_2pA}, and K_{NH_2pApA} are the partition coefficients for ImpA, NH_2pA, and NH_2pApA. Thus the rate of the formation of $NH_2pA^{3'}pA$ is given in eq I-11. The rate is expressed by eq I-12 from the difference of eqs I-2 and I-10. Equation I-13 is obtained by reorganizing eq I-12. The

$$k_{clay,NH_2pApA} = (1/K_{ImpA})(1 + K_{NH_2pApA})(1 + K_{NH_2pA})/(K_{NH_2pA}[NH_2pA]_{total})k_{obs,NH_2pApA} \quad (I-13)$$

observed rate constant, k'_{obs,NH_2pApA}, for the formation of 3′,5′-linked isomers was measured, and the rate constant on the clay phase was calculated by eq I-13. The reaction curves for the reaction of NH_2pA with ImpA on montmorillonite are shown in Figure 4, and the partition coefficients, K_{ImpA}, K_{NH_2pA}, and K_{NH_2pApA}, were calculated from binding studies (Table 3). These data were input to the FITSIM program used to calculate the second-order rate constant for $NH_2pA^{3'}pA$ formation. The reactions of $A^{5'}ppA$ and pA with ImpA were analyzed in a similar fashion, and the rate constants are given in Table 4.

Kinetics of the Oligomerization of ImpA on Na^+-Vol. The formation of oligo(A)s on montmorillonite was investigated using

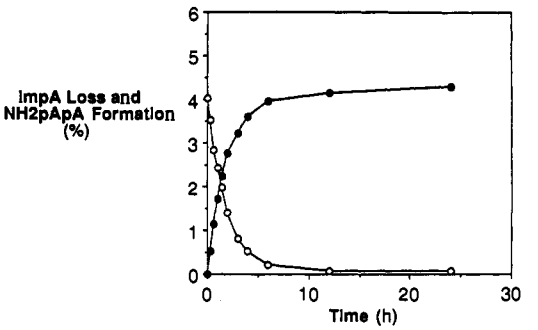

Figure 4. Reaction curves for the reaction of NH_2pA with ImpA under the pseudo-first-order reaction conditions: $[NH_2pA]_t$ = 50 mM, $[ImpA]_t$ = 2.5 mM, [NaCl] = 0.2 M, $[MgCl_2]$ = 0.075 M, [HEPES] = 0.1 M, pH = 8, 25 °C. (Open circles, loss of ImpA; solid circles, formation of $NH_2pA^{3'}pA$). Percentages are the uncorrected HPLC absorbance readings.

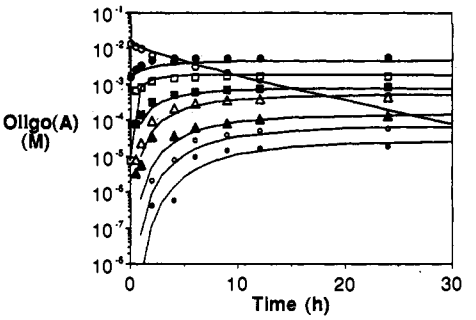

Figure 5. Reaction curve for the loss of 15 mM ImpA and the formation of oligo(A)s in the presence of Na^+-Vol. Total concentrations of the oligomer in the aqueous phase and bound to the Na^+-Vol are plotted. The lines drawn through the experimental points were fit by SIMFIT: ○, ImpA; ●, pA; □, 2-mer; ■, 3-mer; △, 4-mer; ▲, 5-mer; ∘, 6-mer; ·, 7-mer.

Table 3. Partition Coefficients for Dinucleotide Formation[a]

reactions	K_{ImpA}	$K_{nucleotide}$[b]	$K_{product}$
NH_2pA, ImpA	0.14	0.23	0.31
$A^{5'}ppA$, ImpA	0.20	0.27	0.42
pA, ImpA	0.33	0.31	0.34

[a] The coefficients for partition between the aqueous phase and Na^+-Vol were obtained under the same conditions of the reaction shown in Table 4. [b] Partition coefficients for NH_2pA, $A^{5'}ppA$, and pA, respectively.

Table 4. Rate Constants for Dinucleotide Formation[a] (h^{-1} M^{-1})

reaction	k[b]	error
NH_2pA + ImpA → 3′,5′-NH_2pApA	257	±3.2
$A^{5'}ppA$ + ImpA → 3′,5′-$A^{5'}ppApA$	75.5	±0.45
pA + ImpA → 3′,5′-pApA	14.7	±0.084

[a] Reaction in the presence of Na^+-Vol in pH 8 aqueous solution containing 2.5 mM ImpA, 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES at 25 °C. Reaction was monitored using an excess amount of NH_2pA, $A^{5'}ppA$, or pA (0.05 M, 20-fold excess to ImpA) under the pseudo-first-order conditions. [b] The rate constant on the clay reaction was determined.

10, 15, 20, and 40 mM ImpA. These concentrations were chosen to span the concentration range where the amounts of ImpA in the solution phase and bound to the Na^+-Vol were changing rapidly (Figure 1) and where the percentages of moles of ImpA adsorbed/mole of ImpA adsorbed at saturation are 27.4, 39.1, 48.3, and 72.9, respectively. The oligonucleotides formed were separated and analyzed by anion-exchange HPLC. This method separates fractions on the basis of the number of negative charges on each oligonucleotide and does not distinguish between 2′,5′- or 3′,5′-linked isomers or between oligonucleotides with and without incorporated $A^{5'}ppA$ groupings. The partition coefficient of each oligonucleotide fraction was determined from the ratio of the amount of that oligonucleotide in the reaction supernatant to the

Scheme 2

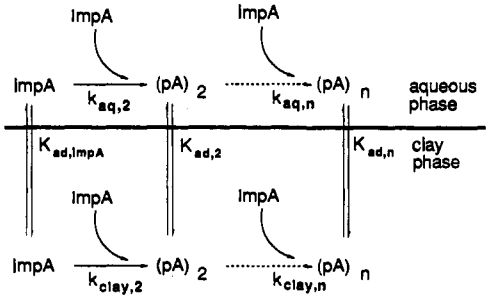

Table 5. Oligomerization Rate Constants[a] (h^{-1} M^{-1})

n[b]	k_n[c]	error[d]
2	1.36E1	±0.61E1
3	6.59E1	±2.90E1
4	1.63E2	±0.39E2
5	1.04E2	±0.37E2
6	2.03E2	±0.65E2
7	1.76E2	±0.36E2
ImpA hydrolysis	7.33E-2/h^{-1}	±5.96E-2/h^{-1}

[a] Reaction in the presence of Na^+-Vol in pH 8 aqueous solution containing 10, 15, 20, or 40 mM ImpA, 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES at 25 °C. The rate constants were determined by SIMFIT. [b] Reaction step is to form an oligo(A) of length n. [c] Rate constants were determined for four different concentrations of ImpA, and their averages were taken. [d] The value of standard deviation for the rate constants among four different concentrations of ImpA.

amount eluted from Na^+-Vol by washing with 0.1 M ammonium acetate after the oligomerization reaction was terminated.

The reaction curves for the loss of ImpA and the formation of oligonucleotides for 15 mM ImpA (Figure 5) were fitted by SIMFIT using the reaction pathway in Scheme 2 by varying the second-order rate constants. Similar reaction curves were obtained by starting from 10, 20, and 40 mM ImpA. The mixture of oligonucleotides present in each fraction in Scheme 2 was designated collectively as $(pA)_n$. For example, ImpApA is the initial product in eq II-3 but is rapidly hydrolyzed to $(pA)_2$. Reactions II-2 to II-5 describe the oligomerization process in aqueous solution (aq) and on the surface of Na^+-Vol (clay). The equations which describe these reactions are given in (II-6)–(II-8), where the subscripts "hy" and "oligo" designate the

$$\mathrm{ImpA} \rightarrow \mathrm{pA} \quad \text{or} \quad 2\mathrm{ImpA} \rightarrow \mathrm{pA} + \mathrm{ImpA} \qquad \text{(II-1a,b)}$$

$$\mathrm{ImpA} + \mathrm{pA} \rightarrow (\mathrm{pA})_2 \qquad \text{(II-2)}$$

$$2\mathrm{ImpA} \rightarrow (\mathrm{pA})_2 \qquad \text{(II-3)}$$

$$\mathrm{ImpA} + (\mathrm{pA})_2 \rightarrow (\mathrm{pA})_3 \qquad \text{(II-4)}$$

$$\vdots$$

$$\mathrm{ImpA} + (\mathrm{pA})_8 \rightarrow (\mathrm{pA})_9 \qquad \text{(II-5)}$$

$$\mathrm{d[ImpA]}/\mathrm{d}t = \mathrm{d[ImpA]_{hy}}/\mathrm{d}t + \mathrm{d[ImpA]_{oligo}}/\mathrm{d}t \qquad \text{(II-6)}$$

$$-\mathrm{d[ImpA]_{hy}}/\mathrm{d}t = k_{hy,aq}[\mathrm{ImpA}]_{aq} + k_{hy,clay}[\mathrm{ImpA}]_{clay} \qquad \text{(II-7)}$$

hydrolysis and oligomerization of ImpA. The material balances in the aqueous and clay phases are given in (II-9)–(II-11), and the partition coefficients for ImpA on Na^+-Vol are given in (II-11) and (II-12). The rate constants in (II-13)–(II-17) were determined by continuous iteration to a convergent result using SIMFIT.[30] The value of k_2 was calculated from the experimentally determined rate constant for pA3′pA formation (Table 6) and the partition coefficients of K_{ImpA} and K_{pA} (Table 3).

It was possible to obtain convergent rate constants for initial concentrations of ImpA of 10–40 mM (Table 5) using a weighting factor of 2^{n-1}, where n = number of monomer units in the oligonucleotide fraction was used to enhance the significance of experimentally determined concentrations of oligonucleotides up to $(pA)_7$. The weighting factor of 64 used for $(pA)_7$ was also used for $(pA)_8$ and $(pA)_9$. Rate constants were calculated up to $(pA)_9$; however, the values of the rate constants did not give a good fit to the reaction curves for $(pA)_8$ and $(pA)_9$. This may be because of the errors in determining the small amounts of $(pA)_8$ and $(pA)_9$ formed. A good fit to the experimental data was obtained for oligonucleotides as long as $(pA)_7$ (Figure 5). The rate constants for oligonucleotide formation in the absence of Na^+-Vol were determined by the procedures used for the montmorillonite-catalyzed oligomerization (Table 6). Mainly 2′,5′-linked oligonucleotides are formed under these reaction conditions.[37]

$$-\mathrm{d[ImpA]_{oligo}}/\mathrm{d}t = k_{2,aq}[\mathrm{ImpA}]_{aq}[\mathrm{pA}]_{aq} + k_{2,clay}[\mathrm{ImpA}]_{clay}[\mathrm{pA}]_{clay} + k_{2',aq}[\mathrm{ImpA}]^2_{aq} + k_{2',clay}[\mathrm{ImpA}]^2_{clay} + \sum(k_{n,aq}[\mathrm{ImpA}]_{aq}[(\mathrm{pA})_{n-1}]_{aq}) + \sum(k_{n,clay}[\mathrm{ImpA}]_{clay}[(\mathrm{pA})_{n-1}]_{clay}) \quad (n = 3\text{–}9) \qquad \text{(II-8)}$$

$$[\mathrm{ImpA}]_{total} = [\mathrm{ImpA}]_{aq} + [\mathrm{ImpA}]_{clay} \qquad \text{(II-9)}$$

$$[(\mathrm{pA})_n]_{total} = [(\mathrm{pA})_n]_{aq} + [(\mathrm{pA})_n]_{clay} \quad (n = 1\text{–}9) \qquad \text{(II-10)}$$

$$K_{ImpA} = [\mathrm{ImpA}]_{clay}/[\mathrm{ImpA}]_{aq} \qquad \text{(II-11)}$$

$$K_{(pA)_n} = [(\mathrm{pA})_n]_{clay}/[(\mathrm{pA})_n]_{aq} \quad (n = 1\text{–}9) \qquad \text{(II-12)}$$

$$-\mathrm{d[ImpA]_{total}}/\mathrm{d}t = k_{hy}[\mathrm{ImpA}]_{total} + k_2[\mathrm{ImpA}]_{total}[\mathrm{pA}]_{total} + k_{2'}[\mathrm{ImpA}]^2_{total} + \sum k_n[\mathrm{ImpA}]_{total}[(\mathrm{pA})_{n-1}]_{total} \quad (n = 3\text{–}9) \qquad \text{(II-13)}$$

$$k_{hy} = \{k_{hy,aq} + k_{hy,clay}K_{ImpA}\}/\{1 + K_{ImpA}\} \qquad \text{(II-14)}$$

$$k_2 = \{k_{2,aq} + k_{2,clay}K_{ImpA}K_{pA}\}/\{\{1 + K_{ImpA}\}\{1 + K_{pA}\}\} \qquad \text{(II-15)}$$

$$k_{2'} = \{k_{2',aq} + k_{2',clay}K_{ImpA}{}^2\}/\{1 + K_{ImpA}\}^2 \qquad \text{(II-16)}$$

$$k_n = \{k_{n,aq} + k_{n,clay}K_{ImpA}K_{n-1}\}/\{1 + K_{ImpA}\}\{1 + K_{n-1}\} \qquad \text{(II-17)}$$

The rate constants for the elongation of tetramers to pentamers and hexamers were determined to check the values of the rate constants in Table 5. The tetranucleotide fraction was isolated by anion-exchange HPLC, added to a 0.9 mM solution of ImpA, and allowed to react in the presence of Na^+-Vol. Control experiments established that very low yields of pentamers and hexamers are obtained from 0.9 mM ImpA under these reaction conditions. The partition coefficients for the $(pA)_n$ oligonucleotides were determined under these reaction conditions and the rate constants for pentamer and hexamer formation were calculated by SIMFIT. In this calculation the rate constants calculated previously for the formation of pA to $(pA)_4$ were fixed and the rate constants for pentamer and hexamer formation were determined (Table 7). The rate constant for pentamer formation is the same, within experimental error, as the one obtained by SIMFIT fitting of the reaction curves (e.g., Figure 5) (Table 5). There is large uncertainty in the rate constant for hexamer formation (Table 7) because of the low yield of hexamer. It is of the same order of magnitude as the value reported in Table 5.

Discussion

Effect of Montmorillonite on ImpA Oligomerization. Oligomerization of ImpA is catalyzed by a number of montmorillonites

Table 6. Oligomerization Rate Constants in the Absence of Na^+-Vol[a] (h^{-1} M^{-1})

reaction	k_n[b]	error[c]
ImpA + pA → $(pA)_2$	1.04E-2	±0.18E-2
2ImpA → $(pA)_2$	1.11E-2	±0.18E-2
ImpA + $(pA)_2$ → $(pA)_3$	3.24E-2	±0.68E-2
ImpA → pA	4.22E-3	±0.04E-5

[a] Reaction in the absence of Na^+-Vol in pH 8 aqueous solution containing 15 mM ImpA, 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES at 25 °C. Anion-Exchange HPLC analysis. The rate constants were determined by FITSIM. The oligonucleotides have mainly 2′,5′-phosphodiester bonds. [b] Rate constants were determined by SIMFIT. [c] Error is estimated by SIMFIT.

Table 7. Elongation Rate Constants[a] (h^{-1} M^{-1})

n[b]	k_n[c]	error[d]
5	1.60E2	±0.34E2
6	2.22E2	±2.85E2

[a] Reaction in the presence of Na^+-Vol in pH 8 aqueous solution containing 0.9 mM ImpA, 5 × 10^{-2} mM tetramer, 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M HEPES at 25 °C (anion-exchange HPLC analysis). The rate constants were determined by SIMFIT. A tetramer was isolated with anion-exchange HPLC and was dialyzed and lyophilized before use. [b] Reaction step is to form an oligo(A) of length n. [c] Rate constants were determined by SIMFIT. [d] Error is estimated by SIMFIT.

Table 8. Composition of Na^+-Montmorillonites[a]

origin	abbreviation	composition
Volclay SPV-200 American Colloid Co.	Vol	$(Si_{3.89}Al_{0.11})(Al_{1.57}Fe_{0.17}Fe_{0.02}Mg_{0.27})O_{10}(OH)_2$
Crook County, WY Clay Mineral Sources SWy-1	Wy	$(Si_{3.84}Al_{0.16})(Al_{1.53}Fe_{0.20}Mg_{0.32})O_{10}(OH)_2$
Japan Nakarai Tesque Ltd.	Jpn	composition not available[b]
Otay, San Diego County, CA Clay Mineral Sources SCa-3	Ot	$(Si_{3.99}Al_{0.01})(Al_{1.43}Fe_{0.03}Mg_{0.64})O_{10}(OH)_2$

[a] Composition is based on Table 1 in ref 25. [b] Elements found are Si (64.6%), Al (11.5), Ca (4.2), Fe (3.9), Mg (1.7), and Na (1.0) by Nakarai Tesque Ltd. It was not possible to formulate a montmorillonite structure on the basis of these analytical data.

(Table 1). Previously, it was shown that Na^+-Vol and Na^+-22A[29] are catalysts so Na^+-Wy and Na^+-Jpn were investigated in the present study. The order of reactivity of these montmorillonites for the formation of longer oligonucleotides is Na^+-Vol, Na^+-Wy > Na^+-Jpn > Na^+-22A > Na^+-Ot, where Na^+-Ot exhibited almost no catalytic activity. A different order of reactivity (Na^+-22A > Na^+-Wy > Na^+-Vol > Na^+-Ot) was observed for the conversion of dpA to dpApA with excess 1-ethyl-3-(3-(dimethylamino)propyl)carbodimide (EDAC), but Na^+-Ot was shown to have no activity in that study as well.[25] There appears to be a correlation between ImpA binding and the extent of oligonucleotide formation. There is also some correlation between the extent of iron incorporation (Table 8) in the montmorillonite and the binding and catalytic abilities,[25] but this correlation depends on the reactants since we observed that Na^+-22A, instead of Na^+-Vol, was the best catalyst in the reactions where EDAC was used as the condensing agent.[27] Iron content or the structural defects resulting from iron incorporation appear to be important since Na^+-Ot, Na^+-Az, and Na^+-Tx all have low iron content and exhibit little or no catalytic ability.[27]

The effect of salt concentration on catalytic ability was investigated briefly. Virtually no catalysis was observed in the absence of added salts (Table 1), while the yield of oligonucleotides increased in the order 0.1 M HEPES < 0.1 M HEPES, 0.2 M NaCl < 0.075 M $MgCl_2$, 0.1 M HEPES ≈ 0.075 M $MgCl_2$, 0.1 M HEPES, 0.2 M NaCl. Ca^{2+} was less effective than Mg^{2+} in enhancing the yield of higher molecular weight oligonucleotides. The effect of HEPES and NaCl on the yield is probably due to the higher ionic strength of the medium.[22,27] The enhancement in the yield of higher oligonucleotides due to 0.075 M $MgCl_2$ alone is comparable to that of 0.1 M HEPES, 0.2 M NaCl, and 0.075 M $MgCl_2$. This finding suggests that there is a specific interaction of Mg^{2+} with reactants and/or products and not simply an ionic strength effect.

Calculation of Rate Constants for ImpA Oligomerization by SIMFIT. The ImpA binding isotherm, hydrolysis data, and the rates of formation of $(pA)_2$ were determined as inputs into SIMFIT using the reaction model in Scheme 2. The binding isotherm of ImpA is similar to the one determined for binding 5′-AMP to Na^+-montmorillonite 22A.[22] ImpA binding to Na^+-Vol increases with increasing ionic strength (Table 2) as does the formation of longer oligonucleotides (Table 1). The effect of Mg^{2+} on binding is dramatic: addition of 0.075 M $MgCl_2$ to 0.1 M HEPES and 0.2 M PIPES results in a 7-fold increase in the percentage of ImpA bound (Table 2). This finding suggests that there is a specific interaction between ImpA and Mg^{2+} which enhances its binding to the Na^+-Vol. There is kinetic evidence for formation of a complex between the phosphate oxygens of ImpA and Mg^{2+},[19] and formation constants for the binding of Mg^{2+} to adenosine nucleotides have been measured.[38-43] The shielding of the negative charge on the phosphate group of ImpA by Mg^{2+} may enhance the binding of ImpA to the negative sites on the clay laminae.

Substitution of Ca^{2+} for Mg^{2+} has little effect on the binding of ImpA to Na^+-Vol (Table 2). The Ca^{2+} probably forms a complex with ImpA which is similar to that formed with Mg^{2+}.[40,41] The Ca^{2+} complex of ImpA probably binds to Na^+-Vol by a mechanism that is similar to that of the Mg^{2+} complex.

There is little difference in the binding of ImpA to Na^+-Vol at pH 7 and pH 8,[22] but the binding drops off markedly at pH 9. Some of the acidic sites on the Na^+-Vol may be neutralized at the higher pH, so they are not available to protonate the adenine ring and the imidazolide group (pK_a = 6[19]) or bind the negative phosphate grouping. The cations formed by protonation of ImpA are believed to have a role in the binding of adenosine nucleotides to the negative surface of montmorillonite.[22]

Hydrolysis of ImpA to pA and imidazole is a pseudo-first-order process in solution and on the montmorillonite surface. The experimentally determined rate constant in the presence of the Vol is 10.7 × 10^{-2} h^{-1}, while the value obtained from the reaction curve by SIMFIT is 7.33 × 10^{-2} h^{-1}. The hydrolysis rate constant in solution determined from the slope of first-order plots is 3.10 × 10^{-3} h^{-1}, while the value obtained from SIMFIT is 4.22 × 10^{-3} h^{-1}. The rate constant in the solution is 3.10 × 10^{-3} h^{-1}, and this rate constant increases 35-fold to 10.7 × 10^{-2} h^{-1} in the presence of montmorillonite. Thus Na^+-Vol enhances the rate of the hydrolysis reaction as well as the rate of oligomerization.

The second-order rate constants for the elongations of pA, A5′ppA, and NH_2pA by one nucleotide to form dinucleotides increase in the ratio of 1:5:17.5 (Table 4). Previous studies[26,28,29] suggest that the reaction of ImpA with A5′ppA on montmorillonite proceeds more rapidly than the self-condensation of ImpA. The reason for the 17.5-fold greater rate of condensation of NH_2pA with ImpA as compared to pA is not understood. The NH_2pA does not stimulate the formation of longer oligonucleotides, as does A5′ppA when a 9:1 ImpA/NH_2pA mixture reacts in the presence of montmorillonite.

The exclusive formation of 3′,5′-linked dinucleotides under the conditions used for the kinetic studies was an unexpected finding. Previous studies showed that the regioselectivity of 3′,5′-phosphodiester bond formation on montmorillonite was in the 80% range when oligomerization reactions were performed with

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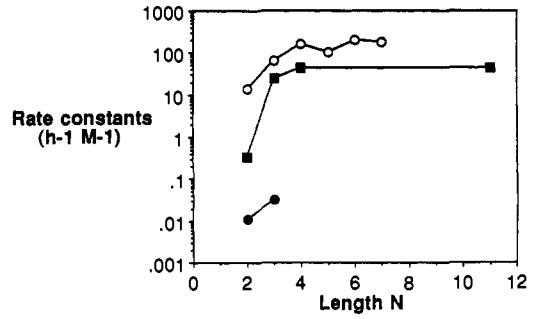

Figure 6. Relationships between logarithmic values of k_n and length n for the oligomerization of ImpA in the presence and absence of Na^+-Vol for template-directed synthesis of oligo(G)s on poly(C): lines, open circles, oligomerization of ImpA on Na^+-Vol; solid circles, in aqueous solution; square, template-directed synthesis. The rate constants for the formation of oligo(A)s are determined by the present study. The rate constants for the template-directed synthesis were determined under the conditions of 1.2 M NaCl, 0.2 M $MgCl_2$, 0.05 M HEPES, 50 mM poly(C), 5–45 mM MeImpG, pH 7.95, and 37 °C, where the rate constants for the formation of the 4-mer to 11-mer were indistinguishable and an averaged value was reported.[12]

9:1 ImpA/A⁵'ppA mixtures, but the regioselectivity was about 67% for the self-condensation of ImpA. This high regioselectivity may reflect the reaction conditions in which the amount of ImpA used is 20 times less than that of NH_2pA, A⁵'ppA, or pA. If monomer is present in excess on the montmorillonite surface, 3',5'-phosphodiester bonds are formed, while if ImpA is bound to the montmorillonite, then both 2',5'- and 3',5'-links are formed. This explanation is supported by the observation that the percentage of 3',5'-links increases with the increasing amounts of A⁵'ppA in ImpA/A⁵'ppA reaction mixtures.[28]

The rate constants for the oligomerization of ImpA were calculated using SIMFIT (Table 5).[30] Initially, studies were carried out with FITSIM,[33,34] but it was only possible to use one reaction curve while the rate constants for product formation or reactant loss were varied. SIMFIT has the capability of using all the reaction curves to obtain the best fit of the rate constants.

The rate constants were determined for all the oligonucleotides present in each fraction (monomer, dinucleotide, ···) eluted from the ion-exchange column. We do not have sufficient data at the present time to derive the rate constants for the elongation of the different structure types in each oligonucleotide fraction. For example, it is known that elongation of a nucleotide containing a 3',5'-linked nucleotide on its 3'-terminus proceeds much faster than if it were 2',5'-linked and the formation of a 3',5'-link on A⁵'ppA proceeds faster than on pA.[29] A quantitative analysis of these data requires new methodology for the separation of the isomeric products formed in these condensation reactions.

The rate constants for oligonucleotide formation on montmorillonite increase in the order 13.6, 66, and 160 h^{-1} M^{-1} for the formation of dinucleotides, trinucleotides, and tetranucleotides, respectively (Table 5). The value of 13.6 h^{-1} M^{-1} for dinucleotide formation from ImpA is very close to the value of 14.7 h^{-1} M^{-1} determined for the reaction of ImpA with pA. The rate constants for the formations of pentamers up to heptamers are the same within experimental error as those for tetranucleotides (Table 5, Figure 6). The rate constants for dinucleotide and trinucleotide formation in the absence of montmorillonite are more than a factor of 10^3 less than those observed in the presence of montmorillonite (Tables 5 and 6). This 10^3 enhancement of the rate of oligomerization results in the net conversion of ImpA to oligonucleotides even though the montmorillonite increases the rate of hydrolysis of ImpA by a factor of 35.

The trend in rate constants for oligo(A) chain elongation on montmorillonite is comparable to the trend in the rate constants for oligo(G) chain elongation on the poly(C) template (Figure 6).[12] In both cases the rate constants increase from dinucleotides

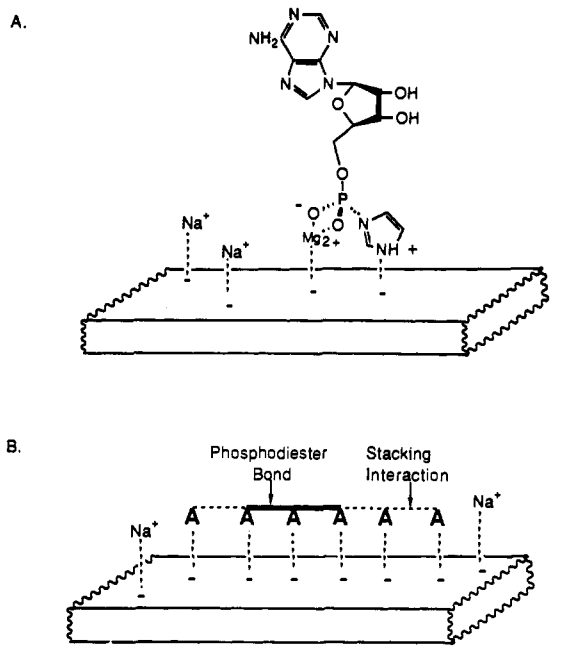

Figure 7. (A) Proposed binding of ImpA to negative sites on the surface of Na^+-Vol. (B) Proposed model for elongation of $(pA)_3$ by reaction with ImpA at the negative sites on the surface of Na^+-Vol: $(pA)_3$ = A A A and ImpA = A.

through tetranucleotides and then level off. It is of interest that the extents of catalytic acceleration due to montmorillonite for the formations of dinucleotides, trinucleotides, and tetranucleotides are greater than that of poly(C) for the formation of oligo(G)s by factors of 41, 2.6, and 3.7, respectively.

The increasing rates of oligomerization with chain length are indicative of preassociation of the ImpA monomer with the growing oligonucleotide chain. A similar effect noted with template-directed synthesis of oligo(G)s on poly(C) is proposed to be due to the stacking interaction of unreacted monomers which are H-bonded to the poly(C) template.[12] A similar model for the reaction on montmorillonite requires that the adenine rings of the growing oligonucleotide chains not be bound to the surface of the montmorillonite but instead are held away from the clay surface and are accessible for stacking interactions with ImpA. This model implies that the growing oligonucleotide chain is bound to the montmorillonite by interaction with the phosphate groups.

Interaction of the oligonucleotide with montmorillonite could occur at the face or edges of the clay laminae. The Mg^{2+} complex of the phosphate group could bind to the negative sites on the montmorillonite faces (Figure 7A). If a Mg^{2+} is bound to each phosphate group, the net charge would be +1. This model is consistent with the enhanced nucleotide and oligonucleotide[22,44] binding observed in the presence of Mg^{2+}. ImpA binding to montmorillonite near the ends of the growing oligo(A) chain would be favored by stacking interactions (Figure 7B). Since the magnitude of the stacking interactions do not increase appreciably above the trinucleotide, there is no corresponding increase in the magnitude of the rate constants.[14]

Chain elongation can also occur at the edges if there is a direct interaction between the negative phosphate groups and the positive edge sites. This model, which is similar to the one given in Figure 7, does not contradict the proposed interactions of ImpA or the other nucelotides at the clay faces but rather proposes that only the binding of ImpA at or near the edge sites results in chain elongation. ImpA binding to the montmorillonite at the terminus of the growing oligonucleotide chain would also be enhanced by

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stacking interactions in this model for oligo(A) synthesis. Studies to differentiate between these two models are in progress.

Conclusions

Kinetic analysis of oligonucleotide formation has provided insight into the mechanism of reaction on montmorillonite and has suggested approaches to the regiospecific formation of oligonucleotides. Rate constants for the formation of oligonucleotides up to the 7-mer were determined by fitting the experimental data by the SIMFIT method.[30] The rate constants increase with increasing chain length up to the tetranucleotide and then remain constant. The magnitude of and trend in the rate constants is similar to the magnitude and trend observed in the template-directed synthesis of oligo(G)s on poly(C) templates.[12] These findings suggest that oligonucleotide formation involves not only binding of the activated monomers to montmorillonite but also association of the activated monomer with the growing oligonucleotide before phosphodiester bond formation occurs. These constraints make it possible to propose the mechanism in Figure 7B for the oligomerization process.

The rate constants for the formation of dinucleotides were determined as input parameters for the FITSIM analysis by measurement of the rates of reaction of ImpA with excess NH_2-pA, $A^{5'}$ppA, and pA. Structure analysis of the reaction products revealed that only the 3′,5′-linked dinucleotide was formed in each reaction. The observation of the regiospecific formation of the corresponding 3′,5′-linked dinucleotide was unexpected. This observation suggests that it will be possible to synthesize 3′,5′-linked oligonucleotides on montmorillonite if the activated monomer, in this case ImpA, is the limiting reagent. This is also a plausible prebiotic scenario for regiospecific oligonucleotide synthesis since it is likely that the concentrations of activated mononucleotides were quite low on the primitive earth.

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