

EFFECT OF INHIBITORS ON THE MONTMORILLONITE CLAY-CATALYZED FORMATION OF RNA: STUDIES ON THE REACTION PATHWAY

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Abstract. The Langmuir adsorption isotherms of the phosphoroimidazolides of adenosine (ImpA) and uridine (ImpU), dA^{5'}ppdA and N⁶, N⁶-dimethyladenine binding on montmorillonite are consistent with their forming a monolayer on the clay surface. This suggests the condensation of ImpA and ImpU to oligomers proceeds on the surface of the clay and not in groups of monomers stacked on the clay surfaces. The binding and reactions of ImpU and ImpA on montmorillonite are blocked by N⁶, N⁶-dimethyladenine and dA^{5'}ppdA. dA^{5'}ppdA is a better inhibitor of oligomer formation than N⁶, N⁶-dimethyladenine because both adenine rings of dA^{5'}ppdA bind to the clay surface and block adjacent catalytic sites. An upper limit of 5–10 × 10¹⁵ catalytic sites on 50 mg of clay was estimated from the binding of ImpU and the inhibition of oligomer formation by dA^{5'}ppdA.

Keywords: catalysis, montmorillonite, prebiotic catalysis, RNA binding to clay, RNA formation, RNA inhibition, RNA oligomers

1. Introduction

The role of catalysis in prebiotic chemistry is the central theme of our studies on the origins of life (Ferris, 1993). We feel that mineral and/or metal ion catalysis was essential for the formation of the biopolymers which initiated the first life. Catalysis resulted in the efficient sequence selective formation of polymers long enough to have had the catalytic activity and genetic complexity which would have been necessary.

The conversion of RNA monomers to RNA polymers and their role in the initiation of the RNA world (Gilbert, 1986) is the focus of our studies on the catalysis of prebiotic reactions. Montmorillonite catalysis resulted in the formation of 6–14 mers of RNA from the 5'-phosphoroimidazolides of mononucleotides (ImpN) (Figure 1) in pH 8 aqueous solution (Ferris and Ertem, 1993a; Ding *et al.*, 1996; Ertem and Ferris, 1997; Kawamura and Ferris, 1999). Daily addition of the activated monomer to a 10 mer of A for a period of fourteen days resulted in its elongation to a 50 mer (Ferris *et al.*, 1996). Montmorillonite catalysis has also been shown to control the sequence selectivity in the first two nucleotides on the 5'-end of a growing RNA chain (Ertem and Ferris, 2000).



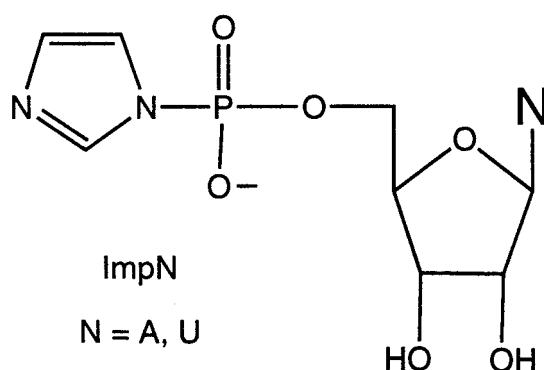


Figure 1. The structures of the activated mononucleotides used in this study.

TABLE I

Langmuir adsorption coefficients (K_L) and moles of nucleotide adsorbed per mole of exchangeable Na^+ (a_s)^a

	N^6, N^6 - dimethyl- adenine	$\text{dA}^{5'}$ ppdA	ImpA	ImpU	CH_3pA	CH_3ppA
K_L	1000	340	56	15	46	88
a_s	0.96	0.37	0.20	0.11	0.17	0.03

^a Data derived from the Langmuir adsorption isotherms in Figures 2–4.

The pathway by which montmorillonite catalyzes RNA synthesis has been investigated. It has been shown that the reaction proceeds in the interlayer of the montmorillonite and not at the edges (Ertem and Ferris, 1998). Kinetic analysis established that the rate constants for the formation of oligomers longer than 3 mers are greater than the rate constants for dimer formation (Kawamura and Ferris, 1994, 1999).

Binding studies, which may provide insight into the reaction pathways, established that activated purine nucleotides bind more strongly to montmorillonite than do the corresponding pyrimidine derivatives (Kawamura and Ferris, 1999). It was recently concluded that Van der Waals forces between the purine nucleotides and the silicate surface of the montmorillonite were responsible for the enhanced binding of the activated purine nucleotides (Kawamura and Ferris, 1999). The previous proposal that the binding was due to the protonation of the purine rings by acidic site on the montmorillonite was replaced by the hydrophobic binding scenario when it was observed that the activated nucleotide of inosine, which has a non-basic hypoxanthine purine ring, binds to montmorillonite almost as strongly as nucleotides of containing the basic adenine ring (Ferris *et al.*, 1989).

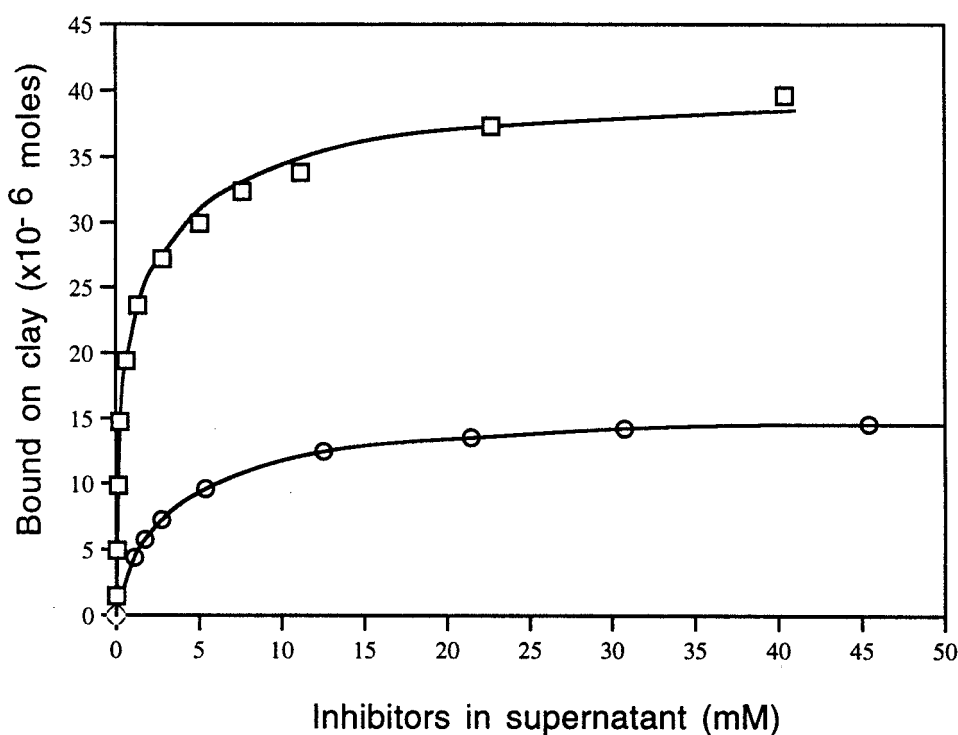


Figure 2. The adsorption isotherms of and N^6, N^6 -dimethyladenine (\square) and $dA^{5'}$ ppdA (\circ) measured at 25 °C.

In the course of another study it was observed that the oligomerization of ImpU is inhibited by P^1, P^2 -dideoxyadenosine-5'-diphosphate ($dA^{5'}$ ppdA) (Ding *et al.*, 1996). 2'-Deoxynucleotides do not react with ImpN so ImpNs do not add to $dA^{5'}$ ppdA (Ding *et al.*, 1996). The observation of inhibition prompted this more detailed investigation of the inhibition of RNA synthesis with the goal of gaining additional insight into the reaction pathway on the montmorillonite surface.

2. Materials and Methods

2.1. GENERAL

Most of the materials and experimental methods used in this study were described previously (Ferris and Ertem, 1993a). N^6, N^6 -dimethyladenine, HEPES, sodium 3-(trimethylsilyl)-1-propanesulfonate and dicyclohexylcarbodiimide were from Sigma and morpholine was from Aldrich. Homoionic Na^+ -montmorillonite was prepared from Volclay (American Colloid Co., SPV-200) by titration method (Banin *et al.*, 1985). Unless specified otherwise all the binding and reactions were carried out in the standard buffer mixture of 0.1 M HEPES, 0.2 M NaCl and 0.075 M

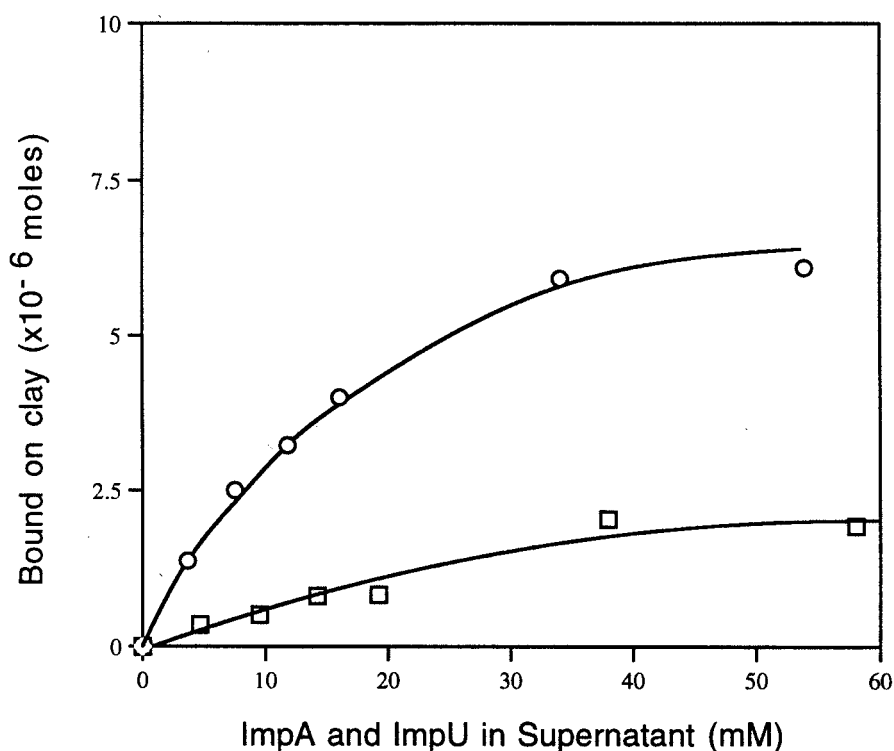


Figure 3. The adsorption isotherms of ImpU (\square) and ImpA (\circ) measured at 4 °C.

MgCl₂, pH 8.0. The phosphoroimidazolides of adenosine and uridine were prepared by the literature procedure (Joyce *et al.*, 1984). Anion exchange HPLC was performed on a HEMA IEC BIO Q anion exchange column (now called Biospher GMB 100Q from Melcor Technologies) using the same conditions described previously (Ferris and Ertem, 1993a). Reverse phase chromatography was performed on Water's μ Bondapak C-18 column using conditions described previously unless otherwise noted (Ferris and Ertem, 1992). NMR spectra were determined on a Varian Unity XL-500 spectrometer in D₂O solution using sodium 3-(trimethylsilyl)-1-propanesulfonate as the internal standard.

2.2. BINDING

Stock solutions of the compounds were prepared in standard buffer mixture and their concentrations were determined by the UV absorption at their absorption maximum. The molar extinction coefficients of ImpA, CH₃ppA and CH₃pA were assumed to be the same as 5'-AMP. ImpU was assumed to have the same extinction coefficient as 5'-UMP. A hyperchromicity of 1.17 (Ferris and Ertem, 1993b) was used to determine the concentration of dA^{5'}ppdA. The extent of binding of dA^{5'}ppdA, CH₃ppA and CH₃pA did not change after an incubation times with

TABLE II
Oligomerization of 15 mM ImpU in the presence of increasing amounts of N⁶, N⁶-dimethyladenine^a

N ⁶ , N ⁶ - dimethyl- adenine (mM)	0	1	Cyc ^b	2	3	4	5	6	7	8	9	10	11	12
+0.0	8.05	26.5	29.8	11.3	10.7	4.96	2.84	1.62	0.84	0.49	0.31	0.17	+	-
+0.15	7.63	25.8	29.3	11.1	10.6	5.09	3.04	1.78	1.05	0.69	0.51	0.39	+	-
+0.75	8.33	26.1	29.3	12.4	9.66	4.43	2.96	1.62	0.95	0.61	0.48	0.30	+	-
+1.5	9.70	27.4	29.6	10.9	9.16	4.07	2.36	1.29	0.72	0.38	+	+	-	-
+3	11.9	27.0	30.1	10.5	9.63	4.36	2.03	0.93	0.51	0.18	+	+	-	-
+5	15.0	27.5	29.4	10.8	9.56	3.85	1.67	0.72	0.30	0.12	+	-	-	-
+8	19.0	27.8	28.4	9.16	8.49	2.67	1.05	0.38	0.14	+	-	-	-	-
+11	23.7	28.8	26.2	8.24	7.08	2.38	0.73	0.29	0.03	-	-	-	-	-
+15	31.1	28.6	22.6	6.75	6.28	1.35	0.41	0.09	-	-	-	-	-	-
+20	42.5	35.8	13.5	3.99	1.51	+	-	-	-	-	-	-	-	-
+40	63.9	25.5	5.60	2.46	+	-	-	-	-	-	-	-	-	-
+60	67.8	23.4	7.49	1.34	-	-	-	-	-	-	-	-	-	-

^a The reaction of 15 mM activated nucleotide was carried out for 4 days at 25 °C, the montmorillonite was washed with 0.1 M ammonium acetate and the combined extract was analyzed by anion exchange HPLC. The peak for N⁶, N⁶-dimethyladenine was eluted before that of ImpU (0 mer) and its area was not included in the calculation of the percentages. The percentages are not corrected for oligomer chain length or for hyperchromicity.

^b Cyc = Cyclic oligomers.

TABLE III
Oligomerization of 15 mM ImpA in the presence of increasing amounts of N⁶, N⁶-dimethyladenine^a

N ⁶ , N ⁶ - dimethyl- adenine (mM)	0	1	Cyc ^a	2	3	4	5	6	7	8	9	10	11	12
+0.0	8.47	24.2	2.03	24.4	15.3	10.9	4.35	2.57	1.40	0.73	0.13	+	-	-
+0.15	7.67	22.7	2.09	24.5	15.6	11.6	4.77	2.81	1.54	0.68	0.52	+	-	-
+0.75	8.27	23.0	2.37	24.6	16.4	11.1	4.62	2.47	1.41	0.67	0.56	+	-	-
+1.5	8.91	24.8	2.08	25.6	15.3	10.8	4.24	2.32	1.24	0.61	0.34	+	-	-
+3	11.8	28.0	1.82	26.8	14.0	8.95	3.38	1.67	0.83	0.46	0.14	+	-	-
+5	13.4	25.9	1.70	27.2	13.4	8.23	2.98	1.38	0.63	0.22	0.09	-	-	-
+8	18.8	36.9	0.47	25.1	9.50	4.22	1.18	0.31	0.08	0.02	-	-	-	-
+11	19.1	31.3	1.17	28.1	9.08	4.18	1.20	0.40	0.12	+	-	-	-	-
+15	22.3	38.2	0.79	26.0	5.39	1.94	0.38	0.12	0.04	-	-	-	-	-
+20	25.4	35.2	1.54	25.3	4.24	1.53	0.39	0.14	+	-	-	-	-	-
+40	56.2	32.6	-	7.58	0.71	-	-	-	-	-	-	-	-	-
+60	62.9	33.1	-	1.38	-	-	-	-	-	-	-	-	-	-

^a See footnotes to Table II.

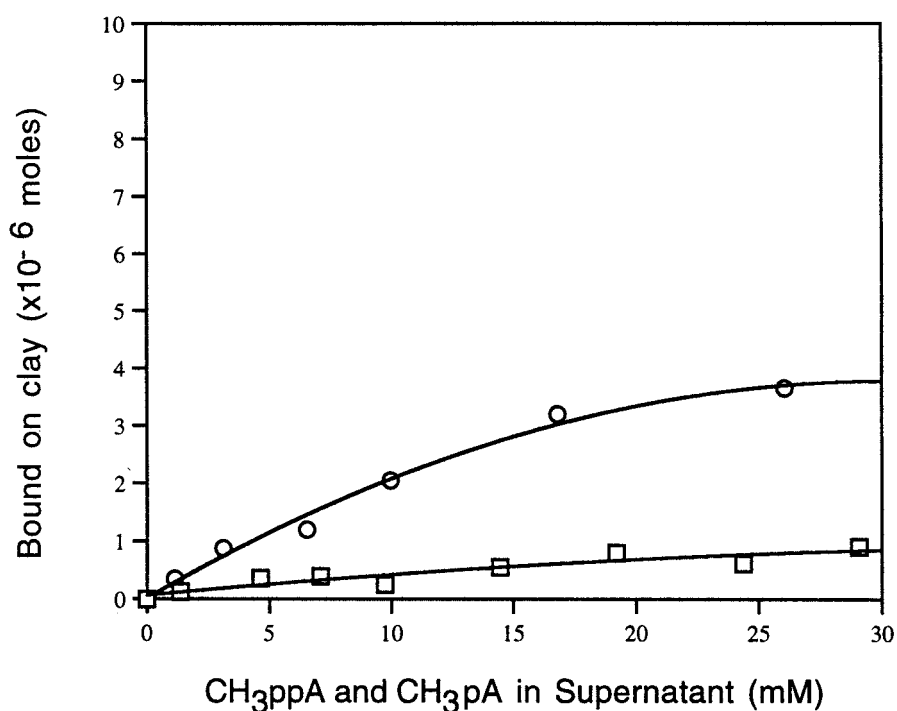


Figure 4. The adsorption isotherms of CH₃ppA (□) and CH₃pA (○) measured at 25 °C.

montmorillonite of 10 min to 24 hr, so adsorption isotherm measurements were recorded after 30 min incubation. An incubation of time of 1 hr was used for N⁶, N⁶-dimethyladenine.

Previously, the extent of binding was based on the UV adsorption difference in the supernatant before and after standing the substrates with clay (Ferris *et al.*, 1989; Ertem and Ferris, 1997; Kawamura and Ferris, 1999). This approach is not sufficiently sensitive for weakly binding compounds like ImpU or applicable for the measurement of the simultaneous binding of two compounds. Consequently, HPLC was used to determine the extent of binding. In studies on the binding of one compound a solution of known concentration was prepared in the standard buffer mixture. One mL of this solution was added to 50 mg of montmorillonite, vortexed and allowed to stand for the prescribed time. It was then centrifuged and the supernatant was chromatographed. The solution that had not been mixed with montmorillonite was also chromatographed and the difference in the peak areas was used to measure the extent of binding. For the studies of binding of two compounds on montmorillonite both were first dissolved in standard buffer and 1 mL of this mixture was added immediately to 50 mg of montmorillonite and the binding was carried out at 2–4 °C. After incubating the samples, the solution was centrifuged and the concentration of an aliquot of the original solution and that

TABLE IV
Oligomerization of 15 mM ImpU in the presence of increasing amounts of dA^{5'} ppdA^a

dA ^{5'} ppdA (mM)	0	1	Cyc ^b	2	3	4	5	6	7	8	9	10	11	12
+0.0	2.76	31.0	30.2	11.9	10.8	4.92	2.66	1.47	0.80	0.40	0.22	0.11	0.07	-
+0.01	3.06	28.9	30.0	12.2	10.7	5.26	2.91	1.62	0.94	0.48	0.31	0.17	0.08	-
+0.05	2.99	28.5	33.7	11.7	9.69	4.66	2.45	1.31	0.70	0.32	0.17	0.06	+	-
+0.1	2.86	29.9	32.2	11.9	10.3	4.94	2.67	1.46	0.85	0.31	0.12	+	-	-
+0.5	3.30	34.3	28.1	12.2	9.50	3.97	2.00	1.17	0.58	0.29	0.14	+	-	-
+1	3.77	35.9	25.6	12.4	9.68	4.15	2.13	1.16	0.65	0.21	0.12	-	-	-
+1.5	3.69	40.0	26.3	11.4	7.88	2.91	1.34	0.60	0.25	0.09	+	-	-	-
+3	5.88	50.8	18.9	10.4	5.27	1.73	0.61	0.23	0.07	+	-	-	-	-
+5	7.00	61.0	10.2	8.62	2.59	0.91	0.38	0.14	0.09	-	-	-	-	-
+8	6.69	70.8	7.33	4.59	1.62	0.37	0.09	+	-	-	-	-	-	-
+12	6.29	76.0	2.50	5.76	0.80	0.13	+	-	-	-	-	-	-	-
+20	11.0	59.9	13.0	3.69	0.43	+	-	-	-	-	-	-	-	-
+25	11.9	62.0	17.3	3.26	0.34	-	-	-	-	-	-	-	-	-
+30	9.74	71.1	12.5	2.25	+	-	-	-	-	-	-	-	-	-
+40	9.91	70.4	14.0	2.17	+	-	-	-	-	-	-	-	-	-

^a The reaction of 15 mM activated nucleotide was carried out for 4 days at 25 °C, the montmorillonite was washed with 0.1 M ammonium acetate and the combined extract was analyzed by anion exchange HPLC. dA^{5'} ppdA coelutes with 5'-AMP (1 mer) so its area is included in the calculation of the percentages. The percentages are not corrected for oligomer chain length or for hyperchromicity.

^b Cyc = Cyclic oligomers.

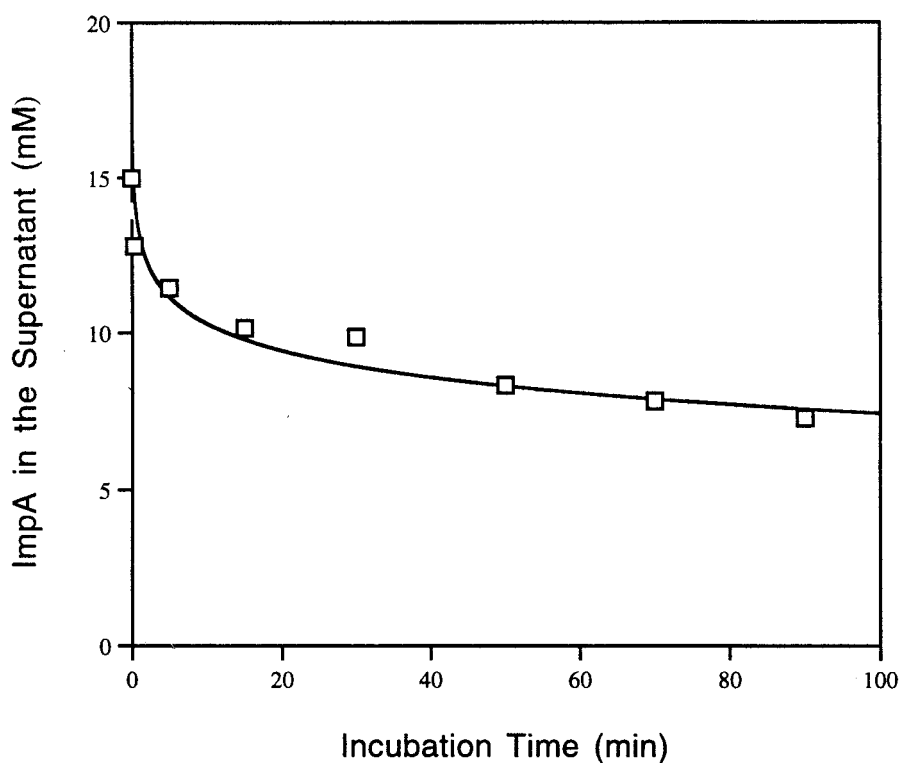


Figure 5. The binding of 15 mM ImpA as a function of time measured at 4 °C.

TABLE V

Oligomerization of 15 mM ImpA in the presence of increasing amounts of dA^{5'}ppdA^a

dA ^{5'} ppdA (mM)	0	1	Cyc ^a	2	3	4	5	6	7	8	9	10	11
+0.0	7.93	24.8	1.46	14.8	16.2	9.58	3.98	1.81	0.96	0.17	+	+	-
+0.01	8.46	33.7	0.73	20.8	14.6	8.83	3.36	1.53	0.79	0.31	+	+	-
+0.1	7.98	26.2	1.15	22.3	15.8	10.0	4.23	2.06	0.90	0.34	+	+	-
+1	8.34	35.1	1.30	22.3	14.5	8.29	2.97	1.29	0.46	+	+	-	-
+2	10.5	51.9	1.12	21.5	7.24	3.24	0.99	0.32	+	-	-	-	-
+5	11.2	50.5	0.30	26.4	5.60	2.3	0.28	+	-	-	-	-	-
+10	11.1	64.6	1.06	16.0	3.89	0.97	0.15	-	-	-	-	-	-
+15	12.6	71.0	0.90	10.4	1.49	0.48	+	-	-	-	-	-	-
+20	13.1	74.9	0.78	8.28	0.69	+	-	-	-	-	-	-	-
+25	8.86	81.0	0.52	3.80	0.29	-	-	-	-	-	-	-	-
+30	11.5	83.1	0.47	2.83	+	-	-	-	-	-	-	-	-
+36	10.7	84.8	0.39	2.13	-	-	-	-	-	-	-	-	-

^a See footnotes to Table IV.

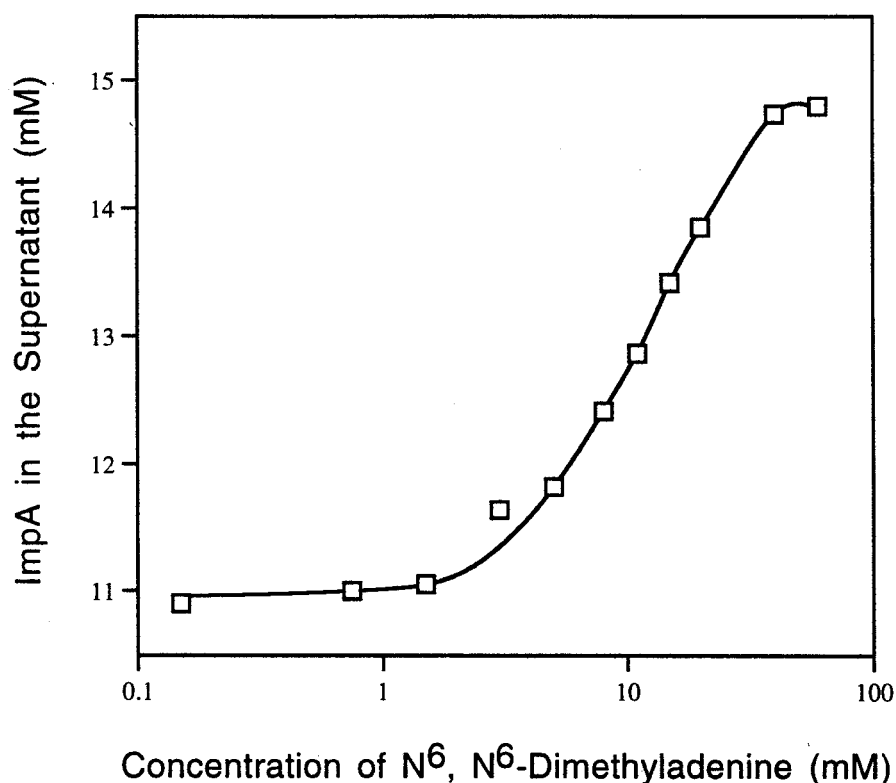


Figure 6. The binding of 15 mM ImpA in the presence of N⁶, N⁶-dimethyladenine measured at 4 °C.

of the supernatant from the binding measurement was determined by HPLC. The compounds were separated on a μ Bondapak C18 column (Waters, 3.9×300 mm) at a flow rate of 1 mL min^{-1} (isocratic) using $0.02 \text{ M KH}_2\text{PO}_4$ buffer (pH 6) as eluant. Varying the amounts of methanol in the eluant compensated for the difference in hydrophobicity of the substrates. For example, 2% methanol and 40% methanol were added to the buffer for the elution of CH₃ppA and N⁶, N⁶-dimethyladenine, respectively.

Binding times of 24–32 hr were used for the binding studies on ImpA and ImpU (Ding *et al.*, 1996; Ertem and Ferris, 1998). Here it was observed that binding of ImpU and ImpA appears to increase slowly after 60 min. This may be due to some oligomerization and hydrolysis of the activated monomers. For adsorption isotherm measurements, the binding of ImpU and ImpA to the clay was determined after incubating the solution at 4 °C for 15 min to minimize the reaction of ImpU and ImpA. An incubation time of 60 min was used for the binding of ImpU and ImpA in the presence of dA^{5'}ppdA and N⁶, N⁶-dimethyladenine. No oligomer formation was observed in anion exchange chromatography of the ImpU and ImpA solutions used in these binding studies.

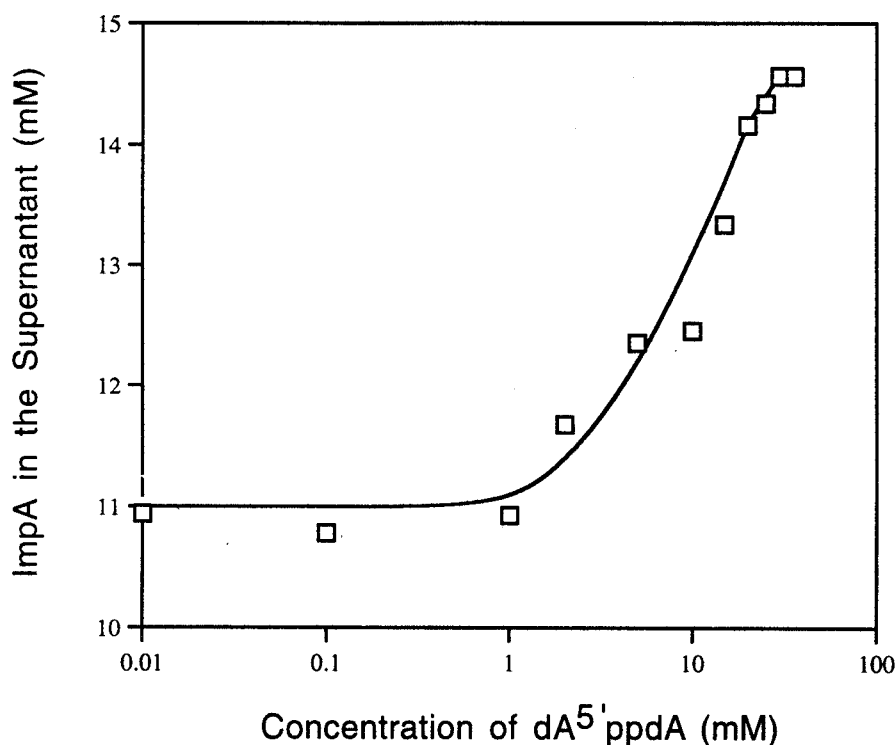


Figure 7. The binding of 15 mM ImpA in the presence of dA^{5'}ppdA measured at 4 °C.

2.3. SYNTHESIS OF dA^{5'}ppdA (Smith *et al.*, 1961)

This synthesis is described because no purification procedure was described in the previous preparation. A solution of 5'-dAMP (free acid, 1 mmol), morpholine-dicyclohexylcarbodiimide (Moffatt and Khorana, 1961) (1 mmol) in pyridine (120 mL) was warmed with vortexing until a clear solution was resulted. After addition of dicyclohexylcarbodiimide (10 mmol), the solution was refluxed (116 °C) for 20 min with stirring. HPLC analysis indicates that dA^{5'}ppdA was 92% of the total reaction chromophore. After cooling the solution, it was poured into 300 mL of cold water and a white precipitate was formed immediately. This solution was kept at 4 °C overnight, filtered, the filtrate was concentrated to about 30 mL using a rotary evaporator and the dA^{5'}ppdA was purified by passing through a preparative C-18 reverse phase column (column volume was about 300 mL). After washing the column with 300 mL 50% acetonitrile and 600 mL double distilled water, the sample was eluted with 600 mL double distilled water and then 500 mL 5% acetonitrile sequentially. The fractions containing dA^{5'}ppdA were passed through a Dowex 50X-X8 (Na⁺) cation exchange column to change dA^{5'}ppdA (H⁺) to dA^{5'}ppdA (Na⁺). The fractions containing dA^{5'}ppdA (Na⁺) were collected and

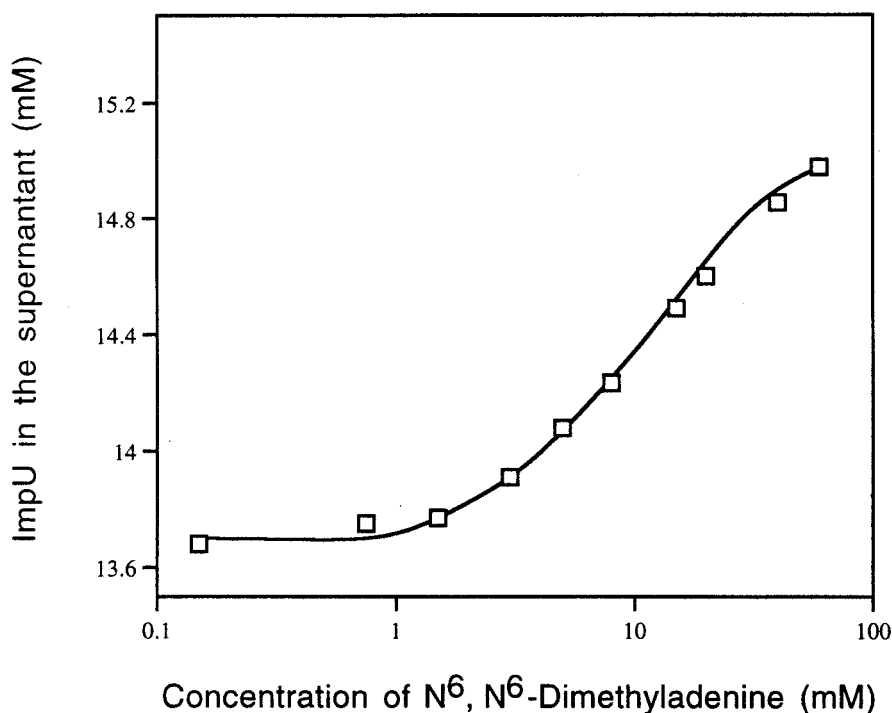


Figure 8. The binding of 15 mM ImpU in the presence of N⁶, N⁶-dimethyladenine measured at 4 °C.

lyophilized. Reverse phase chromatography showed that the purity of dA^{5'}ppdA was 98% while quantitative UV demonstrated that the product was 84% pure. ¹H NMR (D₂O): δ 2.480–2.516 (m, 1, H_{4'}), δ 2.584–2.637 (m, 1, H_{3'}), δ 4.160–4.232 (m, 3, H_{2'} or H_{5'}), δ 6.304 (t, *J* = 6.5 Hz, 1, H₁), δ 8.037 (s, 1, H₈), δ 8.168 (s, 1, H₂).

2.4. SYNTHESIS OF CH₃ppA (Darzynkiewicz *et al.*, 1985; Ding *et al.*, 1996)

This preparation is described because a new purification procedure is described because the previous one yielded an impure product (Ding *et al.*, 1996). Adenosine 5'-diphosphate (5'-ADP) (Na⁺, 1 mmol) was converted to its triethylammonium salt by passing through a Dowex 50W-X8 (TEA-H⁺ form). The combined fractions were evaporated to dryness using a rotary evaporator. Then 100 mL anhydrous methanol and dicyclohexylcarbodiimide (20 mmol) were added. The solution was stirred at room temperature for 14 hr. HPLC analysis indicates the depletion of 5'-ADP and 5'AMP impurity in the 5'-ADP. Ion exchange HPLC (HEMA) analysis demonstrated that CH₃pA accounts for 2.8% of the total UV absorbing materials. The mixture was passed through a Dowex 50W-X8 cation exchange column (H⁺) to remove triethylammonium ion and other cations. The solution was then passed through a Dowex 50X-X8 (Na⁺) will convert H⁺ form of CH₃ppA to Na⁺ form.

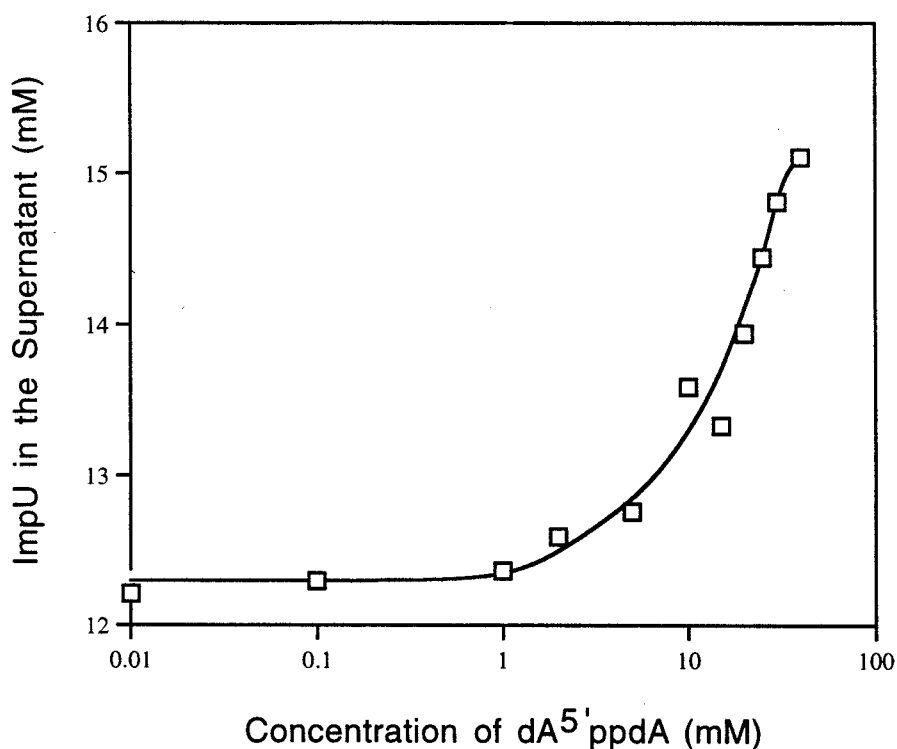


Figure 9. The binding of 15 mM ImpU in the presence of dA^{5'}ppdA measured at 4 °C.

The lyophilized product was found to be 94% pure by anion exchange and 97% pure by reverse phase chromatography. In reverse phase HPLC analysis, it was found that CH₃pA accounts for 0.8% of the final product. Quantitative UV analysis demonstrated that the product was 83% pure. The purity of CH₃ppA prepared previously was 15% as determined by UV adsorption (Ding *et al.*, 1996). ¹H NMR (D₂O): δ 3.626 (d, *J* = 11.5 Hz, 3, H_{CH₃}, the doublet is due to the coupling between the methyl group and the β-phosphorous), δ 4.195–4.230 (m, 2, H_{5'}), δ 4.438–4.420 (m, 1, H_{3'} or H_{4'}), δ 4.535 (t, *J* = 4 Hz, 1, H_{2'}), δ 6.133 (d, *J* = 6 Hz, 1, H_{1'}), δ 8.230 (s, 1, H₈), δ 8.491 (s, 1, H₂).

2.5. SYNTHESIS OF CH₃pA

The synthesis of CH₃pA was first reported by the Khorana group (Smith *et al.*, 1958) but here the procedure used for the preparation of CH₃ppA (Darzynkiewicz *et al.*, 1985) was used for the synthesis of CH₃pA. 5'-AMP (free acid, 1 mmol) was dissolved in DMF (10 mL) and the solution was evaporated to dryness at a reduced pressure to remove H₂O. The evaporation was repeated twice to remove water of crystallization. Then 5 mL triethylamine, 70 mL anhydrous methanol and dicyclohexylcarbodiimide (40 mmol) were added into the mixture and it was

TABLE VI
Inhibition of binding and oligomerization^{a, b}

	ImpA (mM)			ImpU (mM)		
	Initial	Half	Final	Initial	Half	Final
N ⁶ , N ^{6'} -dimethyladenine (mM)						
Binding	2	11	40	1.5	10	60
Reaction	3	5–8 (11–15) ^c	40	1.5	4–11 (15) ^c	40
dA ^{5'} ppdA (mM)						
Binding	1–1.5	9	36	0.5	12	36
Reaction	0.5	1.5 (1.5) ^c	25	0.5	1.5 (3–7) ^c	25

^a 'Binding' refers to the action of inhibitors on the binding of ImpA and ImpU shown in Figures 6–9. 'Initial' is the concentration of inhibitor at the first increase in concentration of activated monomer. 'Half' is the midpoint of the S-curve and 'Final' is the inhibitor concentration where there is no further increase in the concentration of the activated monomer.

^b 'Reaction' refers to the formation of oligomers from ImpA and ImpU in Tables VI–IX. 'Initial' is the concentration of inhibitor where the number of oligomers decreases by one. 'Half' is the concentration of inhibitor where the yield of 4–6 mers decreases by half and 'Final' is where no 4 mers are formed.

^c The values in parenthesis was obtained from considering the effect of inhibitors on 2- and 3-mer formation as described in the text.

stirred at room temperature for 60 hr. The solution was poured into cold water (300 mL), allowed to stand at room temperature overnight and the precipitate of dicyclohexylurea was filtered. The filtrate was concentrated to 30 mL. Reverse phase HPLC analysis indicated that CH₃pA was 96% of the total chromophore. The CH₃pA was purified on a preparative C-18 reverse phase column (column volume was about 300 mL). The column was cleaned with 300 mL 50% acetonitrile and 600 mL double distilled water, the loaded sample was eluted with 600 mL double distilled water, and 500 mL 3% acetonitrile consequently. The collected fractions containing CH₃pA were passed through Dowex Na⁺ cation exchange column (50X-X8)(Na⁺) to generate CH₃pA (Na⁺) and the eluate was lyophilized after concentrating the water solution on a rotary evaporator. HPLC analysis on reverse phase and anion exchange HPLC showed the purity to be about 97% while quantitative UV demonstrated that the product was 88% pure. ¹H NMR (D₂O): δ 3.540 (d, *J* = 11.0 Hz, 3, H_{CH₃}, the doublet is due to the coupling between the methyl group and the α-phosphorous), δ 4.090–4.170 (m, 2, H_{5'}), δ 4.378–4.400

(m, 1, H_{3'} or H_{4'}), δ 4.522 (t, $J = 4.5$ Hz, 1, H_{2'}), δ 6.158 (δ , $J = 5.5$ Hz, 1, H_{1'}), δ 8.331 (s, 1, H₈), δ 8.522 (s, 1, H₂).

2.6. MOLECULAR MODELING OF dA^{5'}ppdA

Modeling of the binding of both adenine rings of dA^{5'}ppdA to one platelet of montmorillonite was accomplished by Professor Curt Brenneman using the Tripos Force Field Pullman Charges. The two adenine rings were constrained to a coplanar orientation while minimizing the remainder of the structure. Modeling of the binding of dA^{5'}ppdA as a pillar between two clay platelets was accomplished using MMFF Force Field Kollman by constraining the adenine rings to parallel orientations.

3. Results and Discussion

3.1. BINDING TO MONTMORILLONITE

The first step in the investigation of the inhibition of RNA oligomer formation was the measurement of the relative affinity of the activated monomers and the inhibitors to the montmorillonite. The adsorption isotherms of ImpA, ImpU, dA^{5'}ppdA and N⁶, N⁶-dimethyladenine and CH₃pA were measured (Figures 2–4). Binding studies were also performed with P¹-methyl, P²-adenosine-5'-diphosphate (CH₃ppA) but it was discovered that its previously reported strong inhibition of ImpN reactions was due to an impurity of unknown structure in the preparation (Ding *et al.*, 1996). It was established that the inhibitor was not P-methyl-5'-adenosine phosphate (CH₃pA), dicyclohexylurea or bicarbonate but its structure was not determined. Purified CH₃ppA has a weak inhibitory effect on the reaction of ImpU. The adsorption isotherm for ImpA was reported previously where a 32 hr equilibration time was used and oligo(A)_s were formed (Kawamura and Ferris, 1994). A 15 min equilibration time was used in the present study to avoid the formation of oligomers and to monitor the binding and reaction using short time intervals. ImpA and ImpU exhibit a slow continued increase in binding with the time (Figure 5). The other compounds used in this study reach a binding equilibrium within 10 min after mixing with the montmorillonite.

The observed isotherms (Figures 2–4) are consistent with Langmuir type binding where the high binding at low concentrations of the compound levels off at the saturation concentrations (Giles *et al.*, 1960, 1974). This isotherm is postulated to be due to the formation of a monolayer of the adsorbate on the mineral surface and the absence of stacking or other interactions (Saenger, 1984) between the bound nucleotide and the nucleotides in solution. Such nucleotide-nucleotide interactions would be recognized by an S-shaped adsorption isotherm. Thus the Langmuir isotherms observed for ImpA and ImpU are not consistent with stacking interactions between the nucleotides bound to the surface of the montmorillonite

(Kawamura and Ferris, 1999). It is concluded that RNA oligomers are formed as the result of the proximate binding between the activated nucleotides on the montmorillonite surface. The stronger binding of ImpA over ImpU reflects the stronger Van der Waals bonding between the purine ring and the surface of the clay platelets. This strong binding may constrain the geometry of the transition state for the reaction between two ImpAs and can account for the higher regioselectivity for 3', 5'-phosphodiester bond formation than is observed for ImpU (Ferris and Ertem, 1993a; Ding and Kawamura, 1996).

The equilibrium binding constants (K_L) and the saturation binding constants (a_s) were determined from plots of the Langmuir absorption isotherm ($c/a = c/a_s + 1/K_L a_s$) where a = moles of nucleotide adsorbed, c = the molar equilibrium concentration of adsorbate, a_s = the moles of nucleotide adsorbed per mole of Na^+ at saturation (42.2×10^{-6} mol per 50 mg of montmorillonite (Kawamura and Ferris, 1994) and K_L = Langmuir adsorption coefficient (Langmuir, 1918; Lawless and Edelson, 1980; Ferris and Hagan, 1986). The values of K_L and a_s are consistent with the extent of the binding that was observed. The trends in the values of K_L , a measure of the affinity of the montmorillonite for the adsorbates, decrease in the order N^6 , N^6 -dimethyladenine, $\text{dA}^{5'}$ ppdA, ImpA and ImpU (Table I). The saturation binding, a_s , decreases in the same order as does K_L . The a_s for N^6 , N^6 -dimethyladenine is almost equal to that of the cation exchange capacity of montmorillonite. The values of K_L and a_s for $\text{dA}^{5'}$ ppdA are about 35% those of N^6 , N^6 -dimethyladenine. There exists the possibility that both adenine rings of $\text{dA}^{5'}$ ppdA bind to the surface of the montmorillonite so that the value of a_s per adenine is about 0.18, a value close to that of ImpA.

3.2. INHIBITION OF MONOMER BINDING AND OLIGOMER FORMATION

The inhibitory effects of N^6 , N^6 -dimethyladenine and $\text{dA}^{5'}$ ppdA on the binding and reaction of ImpA and ImpU were investigated. These inhibitors were selected because of their strong binding to montmorillonite and for the potential to compare the binding of compounds with one and two adenine rings. If two catalytic sites were in close proximity then the $\text{dA}^{5'}$ ppdA has the potential of binding at both of them. The effect of increasing concentrations of N^6 , N^6 -dimethyladenine and $\text{dA}^{5'}$ ppdA on the binding of ImpA and ImpU are shown in Figures 6–9 and on the formation of oligomers in Tables II–V. The competitive binding studies were performed by mixing increasing amounts of inhibitor with 15 mM activated nucleotide in the presence of 50 mg of Na^+ -montmorillonite. The amount of activated nucleotide not bound to montmorillonite was determined by HPLC analysis after a 60 min incubation time. The mixture was centrifuged and the amount of activated nucleotide was determined from its HPLC peak area. This was compared to the amount of activated nucleotide in a control with no added montmorillonite. The concentrations of inhibitor used to block the binding of ImpA and ImpU are given in Table VI.

The inhibition of oligomer formation was estimated from the decrease in the yields of 4–6 mers formed from ImpA and ImpU (Tables II–V). The 2- and 3-mers were not used in the evaluation since they form in the absence of montmorillonite (Lohrmann and Orgel, 1978). The concentrations of inhibitor required to inhibit the reactions of ImpA and ImpU are given in Table VI.

A reviewer requested that the extent of inhibition of oligomer formation be based on the yields of 2- and 3-mers since they are formed in larger amounts. Corrections for the non-catalyzed formation of these dimers were determined from control reactions performed in the absence of both clay and inhibitor. This analysis was performed and the amount of inhibitor required for the half inhibition of the ImpU reaction was significantly less with dA^5ppdA than it was for N^6 , N^6 -dimethyladenine for the 2- and 3-mer fractions of the ImpU reaction (Table VI). The same result was obtained for the amount of inhibitor needed for the half inhibition of 3-mer formation in the ImpA reaction (Table VI). Comparable amounts of each inhibitor (N^6 , N^6 -dimethyladenine was 20–40 mM and dA^5ppdA was 15–20 mM) were required for the half inhibition of 2-mer formation in the ImpA reaction. Since these latter findings differ significantly from those obtained from the analysis of the 4–6 mer formation, 2- and 3-mer formation in the ImpU reaction and 3-mer formation in the ImpA reaction they are not given in Table VI. It is not clear why the values for the half inhibition for 2-mer formation in the ImpA reaction are not consistent with those observed for the formation of the other oligomers.

A close correlation was observed between the concentration of each inhibitor used and its effect on binding both ImpA and ImpU (Table VI). The concentration of N^6 , N^6 -dimethyladenine and dA^5ppdA (~ 10 mM) which result in blocking the binding of half the amount of the activated monomer present, is the same for both ImpU and ImpA. The similarity in the amounts of inhibitor is consistent with the binding observed for 10 mM concentrations of each. Essentially all of the 10 mM N^6 , N^6 -dimethyladenine binds to montmorillonite while about 70% of the dA^5ppdA binds (Figure 2). Since both adenine rings of dA^5ppdA bind to the clay surface it has the potential block even more sites than the more strongly binding N^6 , N^6 -dimethyladenine.

The same amount of inhibitor was required to block the binding of the more strongly binding ImpA as was required to block the weaker binding ImpU. This can be explained by the proposal that these strongly binding inhibitors bind with equal strength to all the binding sites on the montmorillonite. So the same concentration of inhibitor blocks the binding of ImpA to this site as blocks the binding of ImpU. The latter only binds to some of these sites.

The N^6 , N^6 -dimethyladenine concentration which results in the decrease in the yields 4–6 mers by one-half is approximately the same required to inhibit the binding both ImpA and ImpU by one-half (4–11 mM) (Table VI). dA^5ppdA is a much more potent inhibitor of oligomer formation (1.5–2 mM) than N^6 , N^6 -dimethyladenine (4–11 mM). This finding is consistent with the proposal that each

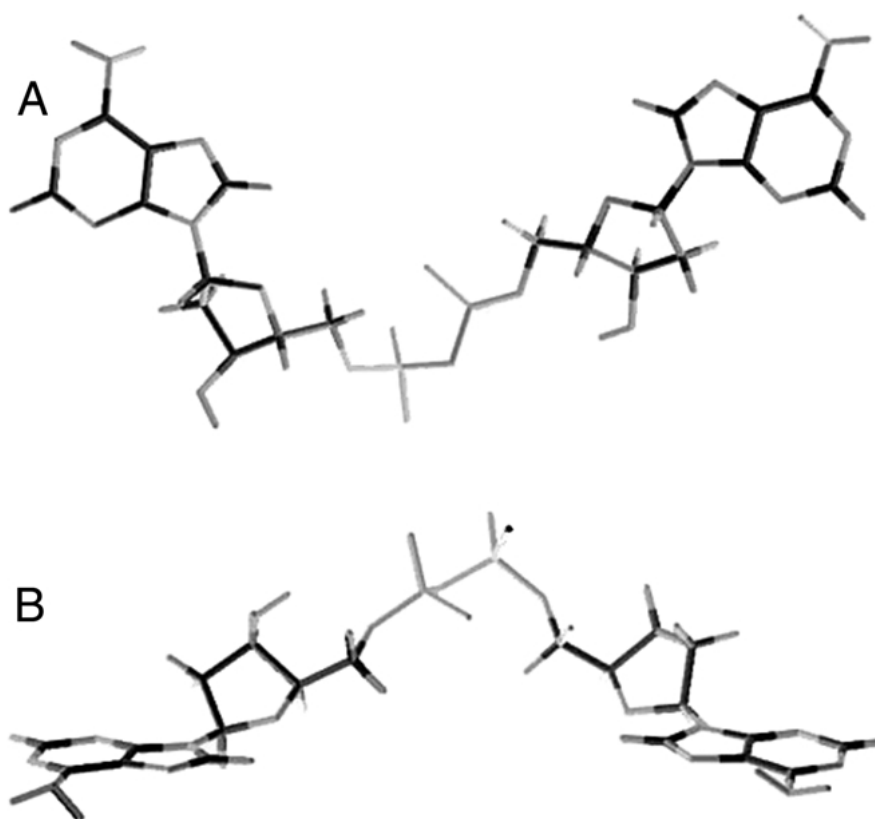


Figure 10. Structure generated by molecular modeling of $dA^{5'}$ ppdA where the adenine rings are constrained to be in the same plane to mimic the simultaneous binding of both adenines to montmorillonite. (A) View to show the conformation of $dA^{5'}$ ppdA. (B) View shows the same conformation as in (A) where the $dA^{5'}$ ppdA is assumed to be bound to a clay platelet.

of the adenine rings of $dA^{5'}$ ppdA are bound to adjacent catalytic sites on the montmorillonite so that $dA^{5'}$ ppdA prevents the juxtaposition of two monomers as required to link them together. This postulate was tested by molecular modeling the geometry of $dA^{5'}$ ppdA where the rings are constrained to be in the same plane, as if they were bound to a flat (clay) surface (Figure 10). The remainder of the molecule was minimized to its lowest energy conformation and it was found that the 4-position atoms of each adenine ring were 1.5 nm apart. This distance is sufficiently close to prevent the binding of two activated monomers from reacting with each other. Two N^6, N^6 -dimethyladenines will have a much lower probability of binding on adjacent sites so N^6, N^6 -dimethyladenine is a less effective inhibitor.

Molecular modeling suggests that $dA^{5'}$ ppdA could also serve as a pillar in the interlayer between two clay platelets. In this calculation the adenine rings are constrained to be parallel and then the structure was minimized (Figure 11). Here the adenine rings are 1.7 nm apart and the vertical distance between them is 0.4 nm. It

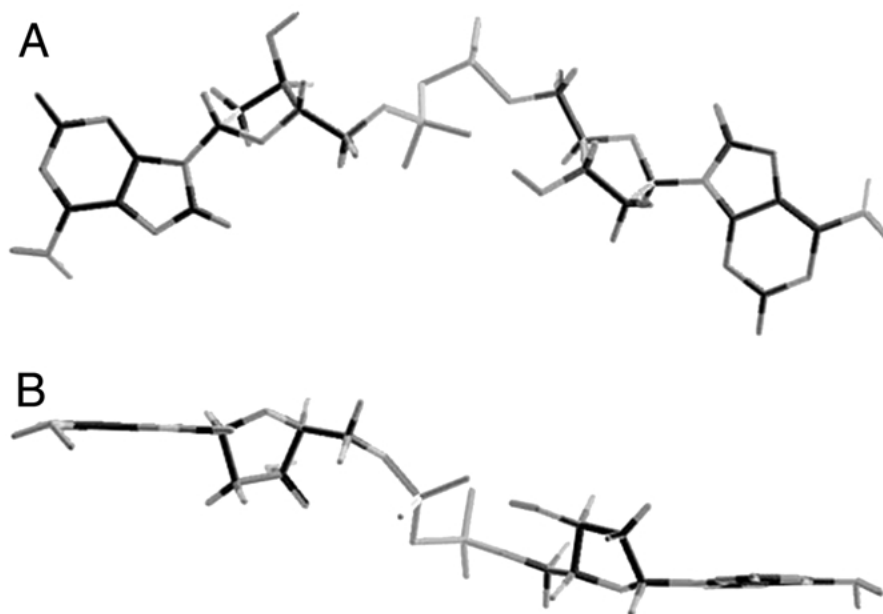


Figure 11. Structure generated by molecular modeling of dA^{5'}ppdA where the adenine rings are constrained to be in parallel planes to mimic the simultaneous binding of both adenines in the interlayer to two clay platelets. (A) View to show the conformation of dA^{5'}ppdA. (B) View shows the same conformation as in (A) where the dA^{5'}ppdA is assumed to be bound between two clay platelets.

seems less likely that this conformation can explain the strong inhibitory effect of dA^{5'}ppdA.

It was possible to determine the binding of ImpU to montmorillonite in this study by the difference in its HPLC peak areas in the presence and absence of clay. UV absorption measurements were not sufficiently sensitive to determine the binding previously (Kawamura and Ferris, 1999). It was observed that about 5.2% of the ImpU bound in the presence and absence of Mg²⁺, a value that corresponds to 7.8×10^{-7} mol. This corresponds to 2% of the cation exchange capacity (CEC) (42.2×10^{-6} equivalents) for 50 mg of montmorillonite (Kawamura and Ferris, 1994). If it is assumed that ImpU binds only at catalytic sites then there are 4.7×10^{15} catalytic sites on 50 mg of montmorillonite.

The number of catalytic sites can also be determined from the amount of dA^{5'}ppdA required for the one-half inhibition of the oligomerization reaction. This value, 1.5 mM (Table VI), can be estimated with greater precision than the value for complete inhibition of the reaction of ImpU. The one-half inhibition value is divided by 2 since both adenine rings can bind to the montmorillonite and is multiplied by 2 to estimate the value for total inhibition. This calculates to 1.5×10^{-7} equivalents of catalytic sites or 4% of the CEC of the montmorillonite, a result close to the 2% value calculated for ImpU binding. A similar determination based

on the mean one-half concentration of N^6 , N^6 -dimethyladenine (8 mM) required gives a value of 1.6×10^{-5} equivalents of catalytic sites that is 40% of the CEC. It is unlikely that this value is correct since it is much greater than the 5.2% value calculated for the binding of ImpU to montmorillonite. The much higher value using N^6 , N^6 -dimethyladenine is consistent with the proposal that purine nucleotides exhibit both productive (catalytic) and non-productive (not catalytic) binding to montmorillonite. It should be noted that the 2–4% CEC values are upper limits since it is not known if ImpU is also binding at non-catalytic sites.

4. Conclusions

It is concluded from absorption isotherm measurements that the activated RNA monomers bind only to the silicate surface of the clay interlayer and do not stack on top of each other. The oligomerization takes place between proximate monomers bound to adjacent positions on the clay surface and not between stacked nucleotides. The strong inhibitory effect of $dA^{5'}$ ppdA on the oligomerization reaction is due to the binding of both its adenine rings to the montmorillonite surface where they block proximate catalytic sites required for linking two monomers together. It was estimated from ImpU binding measurements and $dA^{5'}$ ppdA inhibition studies that there are a maximum of $5\text{--}10 \times 10^{15}$ catalytic sites on 50 mg of montmorillonite where oligomerization reactions proceed. The sites are about 1.5 nm apart on the silicate surface as determined from the molecular modeling of the conformation of $dA^{5'}$ ppdA if both adenine rings are binding simultaneously to the surface of a clay platelet. It is proposed that purine nucleotides bind more strongly and are oriented differently than the pyrimidine nucleotides on the clay surface. This may account for the differences in the regioselectivity for phosphodiester bond formation (Ferris and Ertem, 1993a; Ding *et al.*, 1996) and sequence selectivity (Ertem and Ferris, 2000) observed in the reaction products.

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