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Capillary electrophoretic separation of mono- and dinucleotides of adenosine using cyclodextrin solutions with $MgCl_2$ additive

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Abstract

The capillary electrophoretic separation of adenine, adenosine and nucleotide isomers of adenosine has been studied using several kinds of buffer solutions with cyclodextrin and metal additives in the presence and absence of sodium dodecyl sulfate. Optimum resolution of 2′,5′- and 3′,5′-adenylyladenosine monophosphate was obtained using β-cyclodextrin solution containing 10^{-4} *M* $MgCl_2$. The mixture of adenine, adenosine and nine types of adenosine nucleotide was able to be separated simultaneously within 15 min under the optimum conditions. The method was applicable to the analysis of the simulation reaction for the prebiotic formation of adenosine nucleotides. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Nucleotides; Adenosine; Adenine; Adenylyladenosine monophosphate

1. Introduction

The observation of the catalytic role of RNA in processing RNA transcripts [1,2] suggests that RNA-like molecules had a central role in the first life on earth [3,4]. If this hypothesis is correct, then RNA-like molecules formed spontaneously under primitive earth conditions. There have been a number of reports of successful studies of the condensation of activated nucleotides to form RNA oligonucleotides [5–12]. These oligonucleotides formed from the prebiotic simulation reactions generally contain 2′,5′-linked, pyrophosphate-incorporated and cyclic RNA isomers as well as 3′,5′-linked RNA that preserves genetic information in modern life [6,8,9]. However, chemical analysis of the prebiotic simulation products is a tedious and time-consuming procedure. The oligonucleotides are normally separated and analyzed by anion-exchange high-performance liquid chromatography (HPAEC). 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 an incorporated pyrophosphate linkage. Thus, the fractions containing oligonucleotides are collected from HPAEC and then analyzed by reversed-phase HPLC (RP-HPLC) after enzymatic hydrolysis of the fractions with ribonuclease T_2 and alkaline phosphatase, which selectively cleave 3′,5′-linked RNA and phosphate terminals, respectively. It was necessary to spend a few months determining the structure and composition of the oligonucleotides by this method. Thus, it was important to develop reliable and rapid techniques for the separation and analysis of 2′,5′- and 3′,5′-linked or pyrophosphate-incorporated RNA, to extend the study on the origin of life.

HPLC and capillary electrophoresis (CE) have both been developed as the main techniques for the separation and quantitative analysis of RNA and DNA [13–18]. However, only HPLC has been used in the analysis of RNA and RNA-like molecules

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formed from prebiotic simulation products, since CE methods have not been substantially tested yet to determine the short oligonucleotides.

With CE, extremely high resolution and low size requirements of nucleotides are obtained compared to HPLC, therefore, capillary gel electrophoresis (GCE) has developed rapidly for the sequential analysis of DNA fragments of 100–12 000 base pairs in length using capillaries filled with polyacrylamide gel [19–21]. However, GCE has not been extensively applied to the analysis of short oligonucleotides because GCE generally requires highly concentrated and extraordinarily cross-linked polyacrylamide gel to separate short oligonucleotides. On the other hand, there have been a number of studies using micellar electrokinetic chromatography (MEKC) and capillary zone electrophoresis (CZE) for the analysis of bases, nucleosides and short oligonucleotides [22–29]. Examples include MEKC using high concentrations of urea and metal additives for oligonucleotides [23], MEKC using hexadecyltrimethylammonium bromide [27] and CZE using β-cyclodextrin and borate additives [28]. However, these MEKC and CZE methods have not been applied to the analysis of RNA mixtures that contain 2′,5′- and 3′,5′-linked and pyrophosphate isomers.

In this study, several kinds of buffer solution, with and without sodium dodecyl sulfate (SDS), have been tested as electrophoretic media in the development of a separation method for oligonucleotides that contain 2′,5′- and 3′,5′-linked or pyrophosphate-incorporated isomers within twenty sequences. Based on these investigations, the separation of 2′,5′- and 3′,5′-linked adenylyladenosine monophosphates has been successful using a buffer solution containing cyclodextrin and a small amount of $MgCl_2$ additive. In the course of this study the, electrophoretic behavior was investigated for the separation of adenine, adenosine and adenosine nucleotides.

2. Experimental

2.1. Chemicals

Nucleotides were purchased from Sigma (St. Louis, MO, USA) and Seikagaku (Tokyo, Japan) and cyclodextrins were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and Wako (Osaka, Japan). Na^+-montmorillonite was prepared from Volclay using a method that was described previously [10]. All other reagents used were of analytical grade from Wako and Kanto Chemicals (Tokyo, Japan). Abbreviations for the reagents are as follows; 5′-AMP, adenosine 5′-monophosphate; 3′-AMP, adenosine 3′-monophosphate; 2′-AMP, adenosine 2′-monophosphate; 3′,5′-cAMP, adenosine 3′,5′-cyclicmonophosphate; $A^{2'}pA$, 2′,5′-adenylyladenosine monophosphate; $A^{3'}pA$, 3′,5′-adenylyladenosine monophosphate; $A^{5'}ppA$, P_1,P_2-diadenosine 5′,5′-pyrophosphate; 5′-ADP, adenosine 5′-diphosphate; 5′-ATP, adenosine 5′-triphosphate; $U^{2'}pU$, 2′,5′-uridylyluridine monophosphate; $U^{3'}pU$, 3′,5′-uridylyluridine monophosphate; $G^{2'}pG$, 2′,5′-guanylylguanosine monophosphate; $G^{2'}pG$, 3′,5′-guanylylguanosine monophosphate; $C^{3'}pC$, 3′,5′-cytidylylcytidine monophosphate; TBABr, tetrabutylammonium bromide; TMACl, tetramethylammonium chloride; α-CD, α-cyclodextrin; β-CD, β-cyclodextrin; γ-CD, γ-cyclodextrin; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and Na^+-Vol, Na^+-montmorillonite volclay.

2.2. Apparatus and CE procedure

Capillary electrophoretic separations were performed using a CE-990 power supply, a CE-971UV detector and a 807-IT integrator (Jasco, Tokyo, Japan). Fused-silica capillaries (50 mm I.D., 375 mm O.D.) (Jasco), with an effective length of 45 cm and a total length of 61 cm, were used. All buffers were filtered using a membrane filter (pore size, 0.2 μm; Advantec, DISMIC-CP020). The CE procedures are as follows: The capillary was rinsed prior to each analysis with 0.2 *M* NaOH for 1 min, water for 1 min, and a running buffer for 3 min. Samples were introduced by a dynamic compression injection system using a pressure difference at 5–50 mbar for 0.05–0.1 min and were separated at 15 kV with negative polarity. All CE separations were carried out at 40°C and a wavelength of 260 nm was used for detection.

2.3. Preparation of prebiotic simulation samples

Prebiotic experiments were carried out using a

modification of a previous method [10,12]. A 1-ml volume of a solution containing 0.02 *M* adenosine, 0.02 *M* 5′-AMP and 0.2 *M* EDAC in 0.2 *M* NaCl, 0.075 *M* $MgCl_2$, 0.1 *M* HEPES, pH 8.0, was added to 50 mg of Na^+-Vol. The condensation of adenosine and 5′-AMP in the presence of Na^+-Vol was carried out for six days at 20°C. The supernatant was diluted 40-fold and analyzed by CE. A 5-μl aliquot of the supernatant was added to 200 μl of 0.2 *M* NaOH, to hydrolyze adenylyladenosine monophosphates. The hydrolysis reaction was carried out for 24 h at 37°C and the sample was directly introduced to the CE system without further treatment.

3. Results and discussion

3.1. MEKC separation using SDS micelles

The separation of eleven types of solute, namely, adenine, adenosine and adenosine nucleotides (selected structures of the nucleotides are shown in Fig. 1) were studied using several kinds of buffer solutions containing SDS and other additives. The migration times of the nucleotides are summarized in Table 1 and electropherograms are shown in Fig. 2 (systems 1–8). In systems 1–7, adenine and adenosine were resolved and other nucleotides were separated into groups of nucleotides with the same number of negative charges. However, $A^{2'}pA$ and $A^{3'}pA$ and three isomers of AMP were not resolved in these systems. The migration time in MEKC was somewhat longer than that in CZE without surfactant, while the order of migration for the nucleotides was not changed dramatically from that obtained using CZE. This fact implies that partitioning of the nucleotides to SDS micelles is not strong enough to result in better resolution of the nucleotides in MEKC than found using CZE.

The electrophoretic velocity of micelles is pre-

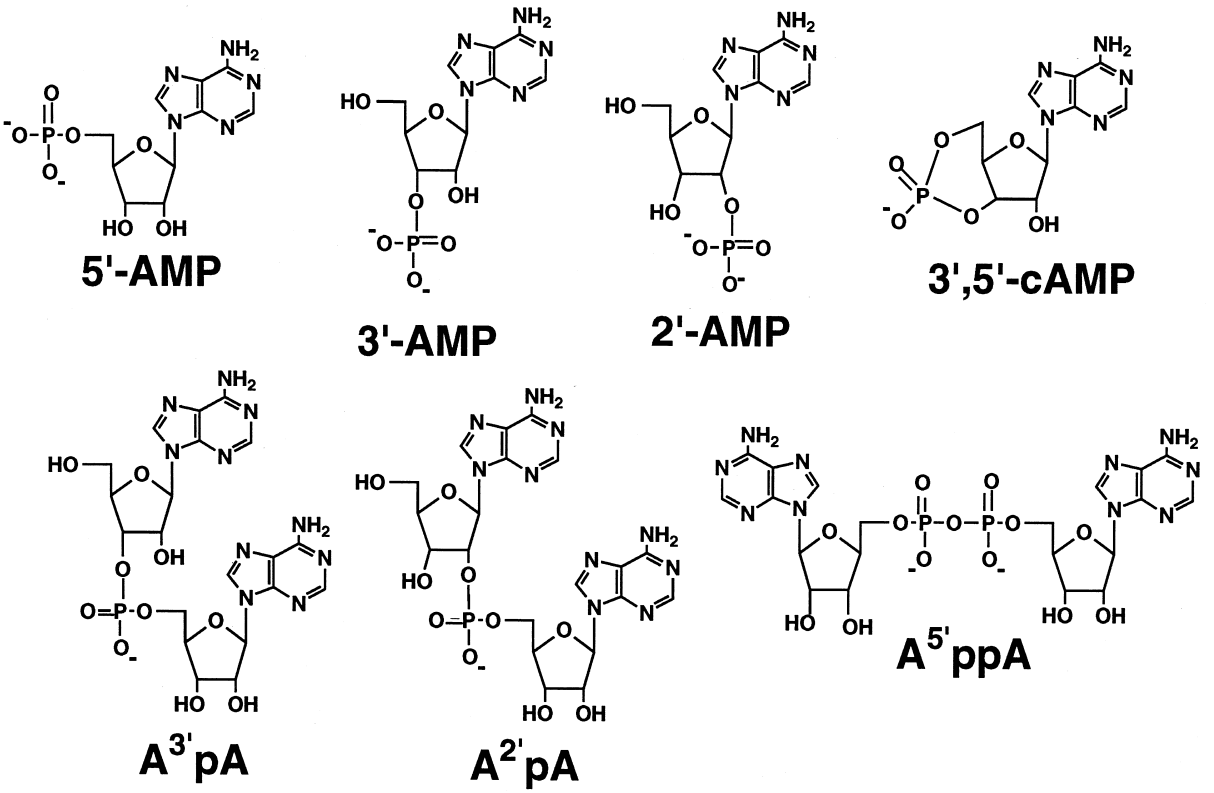

Fig. 1. Selected structures for the adenoside nucleotides used in this study.

Table 1
Migration time (min) for adenine, adenosine and nucleotide isomers in CZE and MEKC systems

	Migration time (min)						
	System 1	System 2	System 3	System 4	System 5	System 6	System 7
Solvent	4.43	4.80	7.95	5.04	4.61	5.57	8.82
Adenine	4.77	5.89	8.27	7.13	5.84	6.54	9.42
Adenosine	4.43	5.24	8.10	5.52	4.88	5.91	9.17
$A^{3'}pA$	5.84	6.42	10.49	7.07	6.08	7.67	12.01
$A^{2'}pA$	5.84	6.42	10.59	7.07	6.08	7.81	12.14
3′,5′-cAMP	6.54	7.29	12.23	8.54	6.88	8.14	14.20
$A^{5'}ppA$	7.52	8.51	13.77	9.78	7.91	10.60	16.18
5′-AMP	9.01	10.35	16.60	12.15	9.40	12.08	20.32
3′-AMP	9.44	10.90	18.06	12.72	9.83	12.08	22.43
2′-AMP	9.56	11.08	18.16	12.84	9.96	12.20	22.64
5′-ADP	12.47	14.76	19.38	17.59	12.57	10.03	26.72
5′-ATP	14.27	17.73	18.62	20.67	14.60	10.38	27.50

Operating conditions: System 1, 0.02 *M* Na_2HPO_4; pH=8.0; System 2, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, pH=8.0; System 3, 0.02 *M* Na_2HPO_4, 0.05 *M* $C_7F_{15}COOH$, pH=8.0; System 4, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.04 *M* TBACl, pH=8.0; System 5, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.02 *M* TBABr; System 6, 0.02 *M* borate, 0.05 *M* SDS, 0.005 *M* $MgCl_2$, pH=8.0; System 7: 0.005 *M* Na_2HPO_4, 0.05 *M* SDS, 0.005 *M* Tris, $3\cdot10^{-4}$ *M* $CuCl_2$, 7 *M* urea, pH=8.0 [23]; applied voltage, 15 kV; thermostated at 40°C; sample was introduced by a dynamic compression injection system at a pressure difference of 50 mbar for 0.1 min; sample concentration, $1\cdot10^{-4}$ *M*.

sumed to increase with decreasing molecular size. Thus, the migration time of a micelle should increase if surfactants with a smaller molecular mass were used. Therefore, the migration time window between elution of the solvent front and the micelles increases and the resolution of the nucleotides can be improved. The result in system 5, where the migration time of the nucleotides increased and the separation of $A^{2'}pA$ and $A^{3'}pA$ was improved, is consistent with this assumption.

The addition of a quaternary ammonium salt to the SDS solution has been known to improve the resolution of ionic substances [30], therefore, TMACl and TBABr were added to SDS solutions (systems 3 and 4 in Table 1). However, a dramatic improvement in the resolution between $A^{3'}pA$ and $A^{2'}pA$ or among three types of AMP was not observed in the presence of quaternary ammonium salt. The migration time for the nucleotides increased in the presence of TMACl. The reason is thought to be that the TMA^+ ion adsorbed onto the fused-silica surface and slowed down the electroosmotic flow.

It is known that metal ions form complexes with nucleotides through interaction with the phosphate and/or the bases, thus, the separation behavior of nucleotides in MEKC can be controlled by the addition of metal ions. In this study, $MgCl_2$ was added to the SDS solution, as Mg^{2+} ions interact with the phosphate group of the adenosine nucleotides rather than with the adenine base. The migration time of 5′-ADP and 5′-ATP decreased in the presence of $MgCl_2$. Borax was used to control the pH instead of phosphate, since Mg^{2+} possibly forms a $Mg_3(PO_4)_2$ precipitate (system 6 in Table 1). The separation of $A^{2'}pA$ and $A^{3'}pA$ was somewhat improved, but the migration-time window seems to become narrower.

On the other hand, the addition of a small amount of Cu(II), Zn(II) or Mg(II) in the presence of 7 *M* urea was used to successfully resolve oligonucleotides up to 18-mer in length [23]. This method was used to try to separate $A^{2'}pA$ and $A^{3'}pA$ or three types of AMP isomer, but the separations was not successful (system 7 in Table 1). Moreover, it was found that a copper hydroxide precipitate formed in the buffer solution at room temperature within one day, which may cause serious inconvenience.

3.2. CE separations with cyclodextrin additives

3.2.1. Effect of cyclodextrin on MEKC

Host–guest complexation with cyclodextrin additives has been used to resolve chiral isomers and nucleotide monomers [28]. The addition of cyclo-

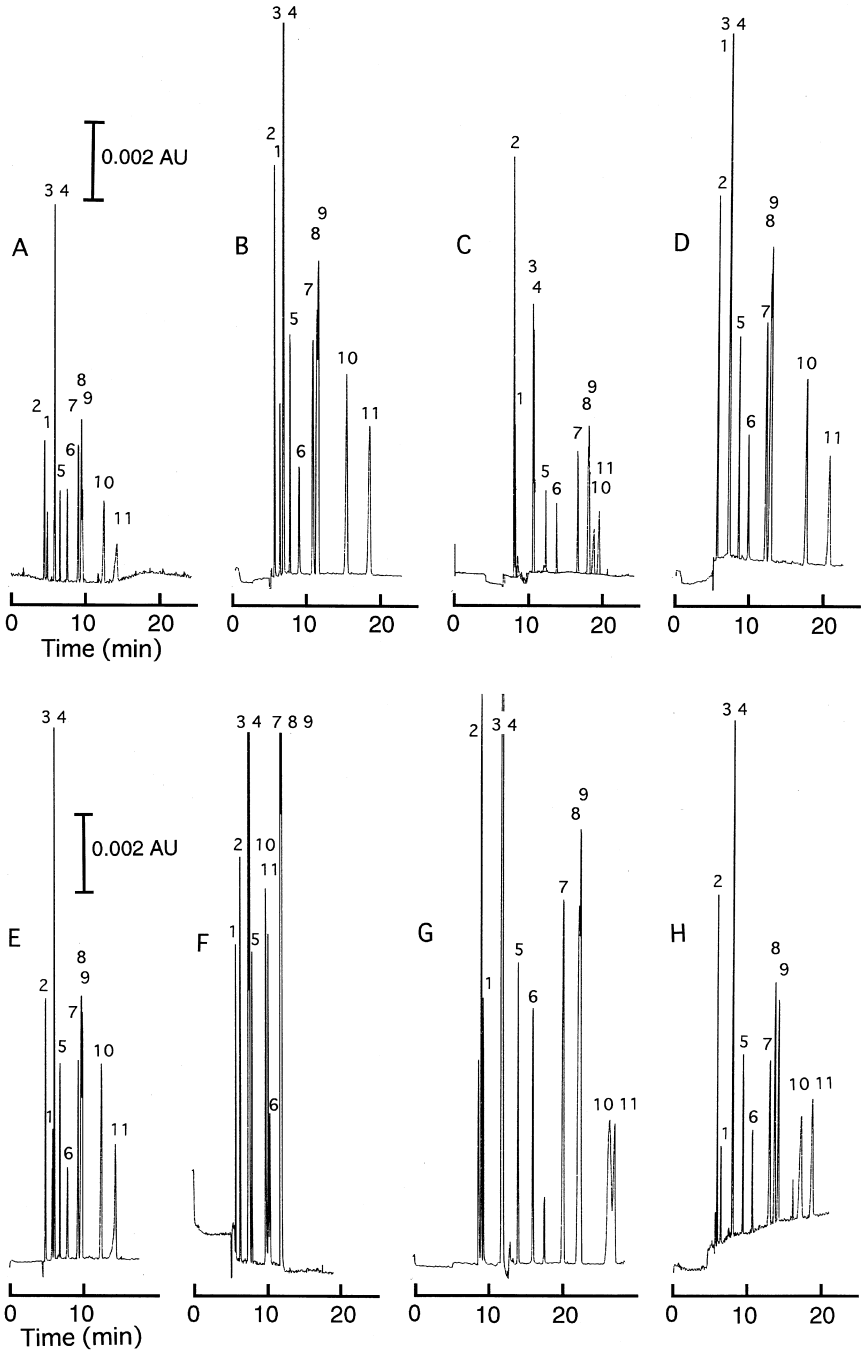

Fig. 2. Separation of adenosine and adenosine nucleotides using several types of electrophoretic buffer. Operating conditions: (A) system 1, 0.02 *M* Na_2HPO_4, pH=8.0; (B) system 2, 0.02 *M* Na_2HPO_4, 0.05 *M* $C_7F_{15}COOH$; (D) 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.04 *M* TMACl, pH=8.0; (E) system 4, 0.02 *M* Na_2HPO_4, 0.05 *M* $MgCl_2$, pH=8.0; (G) system 7, 0.005 *M* Na_2HPO_4, 0.05 *M* SDS, 0.005 *M* Tris, $3\cdot10^{-4}$ *M* $CuCl_2$, 7 *M* urea, pH=8.0 [23]; (H) system 8, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.015 *M* β-CD, pH=8.0; applied voltage, 15 kV; thermostated at 40°C; sample was introduced by a dynamic compression injection system at a pressure difference of 50 mbar for 0.1 min. Sample concentration, $1\cdot10^{-4}$ *M*. Peaks: 1=adenine, 2=adenosine, 3=$A^{3'}$pA, 4=$A^{2'}$pA, 5=3′,5′-cAMP, 6=$A^{5'}$ppA, 7=5′-AMP, 8=3′-AMP, 9=2′-AMP, 10=5′-ADP and 11=5′-ATP.

Table 2
Migration time (min) for adenine, adenosine and adenosine nucleotide isomers in MEKC containing cyclodextrin

	Migration time (min)			
	System 8	System 9	System 10	System 11
Solvent	4.9	4.62	5.18	5.55
Adenine	5.71	5.59	5.94	5.89
Adenosine	5.30	5.04	5.47	5.83
$A^{3'}$pA	6.61	6.11	6.53	7.48
$A^{2'}$pA	6.61	6.11	6.53	7.48
3′,5′-cAMP	7.55	7.01	7.73	8.72
$A^{5'}$ppA	8.81	8.21	8.78	9.73
5′-AMP	10.72	9.90	10.52	11.58
3′-AMP	11.33	10.50	11.14	12.52
2′-AMP	11.73	10.62	11.34	12.82
5′-ADP	14.55	14.20	14.05	15.09
5′-ATP	16.43	17.00	15.93	16.49

Operating conditions, System 8, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.025 *M* β-CD, pH=8.0; System 9, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.05 *M* β-CD, pH=8.0; System 10, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.025 *M* α-CD, pH=8.0; System 11, 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.05 *M* γ-CD, pH=8.0. All other conditions are the same as in Table 1.

dextrin enhances the differences between neutral molecules or those with similar charge-to-mass ratios, resulting in better resolution. The effects of the size and concentration of cyclodextrin on the migration time of the nucleotides are summarized in Table 2. The addition of β-CD resulted in the separation of the three AMP isomers, but was not successful at resolving $A^{2'}$pA and $A^{3'}$pA. This indicates that the difference in the partition of $A^{3'}$pA and $A^{2'}$pA to SDS is not enhanced sufficiently, even in the presence of cyclodextrin.

3.2.2. Effect of cyclodextrin on the CZE system

The migration times of the nucleotides were measured in the presence of α-, β- or γ-CD in the absence of SDS micelles (Table 3). The influence of

Table 3
Migration time (min) for adenine, adenosine and nucleotide isomers in CZE with cyclodextrin

	Migration time (min)				
	System 12	System 13	System 14	System 15	System 16
Solvent	5.08	5.21	4.68	4.63	4.68
Adenine	5.08	5.28	4.68	4.63	4.68
Adenosine	5.08	5.21	4.68	4.63	4.68
$A^{3'}$pq	6.75	6.78	5.89	6.24	6.23
$A^{2'}$pq	6.88	7.00	6.03	6.24	6.27
3′,5′-cAMP	7.39	7.39	6.39	6.96	6.89
$A^{5'}$ppA	8.99	8.98	7.51	7.97	7.92
5′-AMP	9.89	9.73	7.94	9.50	9.39
3′-AMP	9.54	9.30	7.63	9.96	9.62
2′-AMP	11.08	10.89	8.75	10.06	9.97
5′-ADP	13.75	13.39	10.28	12.84	12.71
5′-ATP	14.69	14.9	11.43	14.29	13.07

Operating conditions: System 12, 0.02 *M* Na_2HPO_4, 0.0125 *M* β-CD, pH=8.0; System 13, 0.02 *M* Na_2HPO_4, 0.015 *M* β-CD, pH=8.0; System 14, 0.02 *M* Na_2HPO_4, 0.02 *M* β-CD, pH=8.0; System 15, 0.02 *M* Na_2HPO_4, 0.0125 *M* α-CD, pH=8.0; System 16, 0.02 *M* Na_2HPO_4, 0.0125 *M* γ-CD. All other conditions are the same as in Table 1.

the addition of cyclodextrin decreased in the order of β-CD>γ-CD>α-CD, with the trend being similar to that found in the presence of SDS. This indicates that the complexation of adenosine nucleotides with β-CD is stronger that with γ-CD or α-CD. Furthermore, the migration times of the nucleotides decreased on addition of β-CD. The reason for this is assumed to be that complexation of the nucleotides with β-CD slows the electrophoretic flow towards the anode. The order of migration for three AMP isomers was 5′-AMP<3′-AMP<2′-AMP in the absence of β-CD (system 1 in Table 1) while the order was 3′-AMP<5′-AMP<2′-AMP in the presence of β-CD (Table 3). This indicates that the complexation of 3′-AMP with β-CD is stronger than that of 2′-AMP or 5′-AMP and the trend was similar to that shown in a previous study using borax buffer with β-CD additive [28]. The separation of $A^{2'}$pA and $A^{3'}$pA was improved considerably in the presence of β-CD, while they were found to coelute in the presence of α-CD or γ-CD. This suggests that $A^{3'}$pA and $A^{2'}$pA interact more strongly with β-CD than with α-CD or γ-CD. Furthermore, the migration of $A^{3'}$pA is faster than that of $A^{2'}$pA, therefore, it is assumed that the complexation of $A^{3'}$pA is stronger than that of $A^{2'}$pA.

The relationship between the migration times of the nucleotides and the concentration of β-CD (system 12, 13, 14 in Table 3) was investigated. The separation factor between $A^{2'}$pA and $A^{3'}$pA was maximal at 15 m*M* β-CD (Fig. 3). At 20 m*M* β-CD, the migration time of 3′,5′-cAMP decreased and 3′,5′-cAMP coeluted with $A^{2'}$pA. Furthermore, a precipitate formed in a 20 m*M* β-CD solution at room temperature. Based on these facts, the optimum concentration of β-CD was determined to be 15 m*M*.

3.2.3. Effect of $MgCl_2$

The resolution between $A^{2'}$pA and $A^{3'}$pA was still insufficient, even in the presence of 15 m*M* β-CD, therefore, the addition of metal salt was investigated. The resolution between $A^{2'}$pA and $A^{3'}$pA was improved in the presence of $MgCl_2$. The migration time for the nucleotides was determined at several concentrations of $MgCl_2$ (Fig. 5). The resolution between $A^{2'}$pA and $A^{3'}$pA was maximal at $1 \cdot 10^{-4}$ *M* $MgCl_2$ and the separation factor was higher than one (Fig. 4).

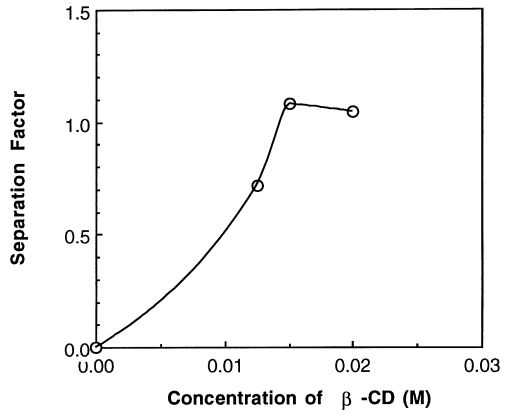

Fig. 3. Effect of the concentration of β-CD on the selectivity factor between $A^{3'}$pA and $A^{2'}$pA. Operating conditions: 0.02 *M* Na_2HPO_4, pH=7.5, without the addition of $MgCl_2$. All other conditions are the same as in Fig. 2.

It is known that Mg^{2+} ions form complexes with phosphate groups of nucleotide and this is thought to be one of the reasons why $MgCl_2$ had an influence on the migration times of the nucleotides. It is assumed that the formation of a complex between the nucleotides and the Mg^{2+} reduces the net negative charge and slows the electrophoretic velocity of the nucleotides towards the anode. Thus, complex formation between the nucleotides and Mg^{2+} is expected to decrease the migration time, as was found

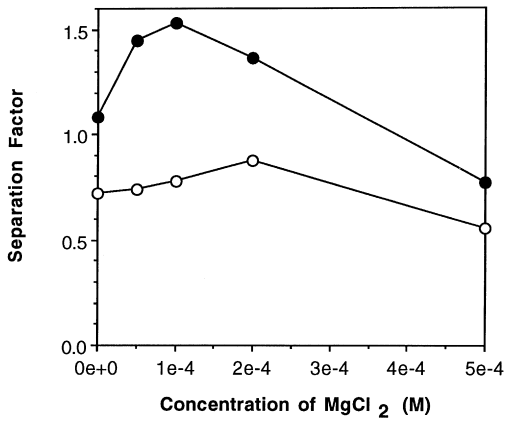

Fig. 4. Effect of the concentration of $MgCl_2$ on the selectivity factor between $A^{3'}$pA and $A^{2'}$pA. Operating conditions: 0.02 *M* Na_2HPO_4, pH=7.5 (a) 0.0125 *M* β-CD, (b) 0.015 *M* β-CD. All other conditions are the same as in Fig. 2.

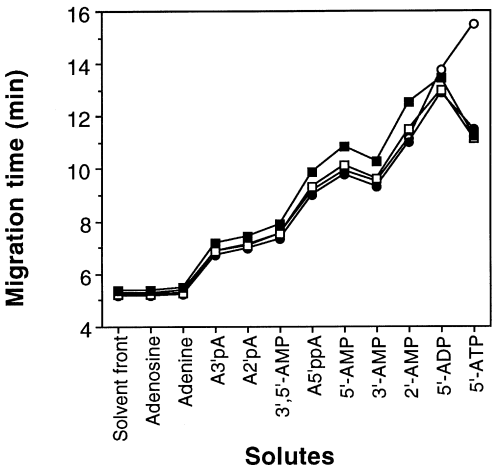

Fig. 5. Effect of $MgCl_2$ on the separation of adenosine and adenosine nucleotides using a solution containing β-cyclodextrin. Operating conditions: 0.02 *M* Na_2HPO_4, pH=7.5, with 0.015 *M* β-CD; (○) 0 *M* $MgCl_2$, (●) $5\cdot10^{-5}$ *M* $MgCl_2$, (□) $1\cdot10^{-4}$ *M* $MgCl_2$, (■) $2\cdot10^{-4}$ *M* $MgCl_2$. All other conditions are the same as in Fig. 2.

for 5′-ADP and 5′-ATP. However, other nucleotides do not interact with Mg^{2+} as strongly as 5′-ATP or 5′-ADP do, so that the decrease in the migration time of other nucleotides is not significant. The stability constants for 5′-AMP ($10^{1.97}$ *M*), 5′-ADP ($10^{3.17}$ *M*) and 5′-ATP ($10^{4.22}$) [31] are consistent with the degree of influence of $MgCl_2$ on the migration times of 5′-AMP, 5′-ADP and 5′-ATP.

The adsorption of Mg^{2+} ion to the fused-silica surface is another possible reason that the migration time changed. Adsorption of Mg^{2+} ions decreases the density of negative charges on the fused-silica surface, which slows electroosmotic flow towards the cathode. The migration time for the solvent peak (t_0) increased with increasing concentrations of $MgCl_2$. The values of t_0 were 5.20 min at 0 *M* $MgCl_2$, 5.39 at $2\cdot10^{-4}$ *M* and 5.92 at $5\cdot10^{-4}$ *M*. This supports the view that the adsorption of Mg^{2+} onto the fused-silica surface slows the electroosmotic flow.

3.2.4. Effect of pH

The influence of pH on the migration time was investigated at pH values ranging from seven to nine (Fig. 6). Although the resolution of $A^{2'}pA$ and $A^{3'}pA$ was better at pH 7 than at pH values of 7.5 to 9.0, the resolution of $A^{5'}ppA$, 3′-AMP and 5′-AMP was poorer at pH 7. The resolution between adenine and adenosine or that among $A^{5'}ppA$, 3′-AMP and 5′-AMP was worse at pH 8 than at pH 7–7.5. Therefore, the optimal pH for resolving $A^{2'}pA$ and $A^{3'}pA$ and other nucleotides was determined to be 7.5. The migration time increased with increasing pH and peak-broadening was observed. This is understood to be due to the decrease in electroosmotic flow at higher pH values.

The reproducibility of both the peak area and the migration time was approximately 0.5% R.S.D. (n= 6) under the optimum conditions.

3.2.5. Resolution of other kinds of dinucleoside monophosphate

The resolution of 2′,5′- and 3′,5′-linked dinucleoside monophosphates with other bases was investigated using the optimum conditions for the separation of the adenosine nucleotides (Table 4). However, 2′,5′- and 3′,5′-linked isomers of uridylyluridine and guanylylguanosine monophosphates were not resolved clearly, indicating that the interaction of these nucleotides with β-CD is weaker than that of adenosine nucleotides.

The migration time of 3′,5′-linked isomers is shorter than that of 2′,5′-linked isomers for adenosine, guanosine and uridine nucleotides. This implies that the 3′,5′-linked isomers interact with β-CD better than the 2′,5′-linked isomers, since strong host–guest complexation causes a decrease in electrophoretic velocity to the anode. To evaluate this assumption, it will be necessary to carry out quantitative analysis of the interaction between 2′,5′- and 3′,5′-linked nucleotides and β-CD.

3.3. Determination of prebiotic simulation samples

Finally, the optimized method was applied to the analysis of the prebiotic simulation products formed from the condensation of adenosine and 5′-AMP using water-soluble carbodiimide in the presence of a clay catalyst (Fig. 7). Both $A^{3'}pA$ and $A^{2'}pA$ are formed by the condensation of adenosine and 5′-AMP and $A^{5'}ppA$ is formed by the condensation of two molecules of 5′-AMP [12]. The electropherogram in Fig. 7A clearly shows that $A^{3'}pA$ (peak 3), $A^{2'}pA$ (peak 4) and $A^{5'}ppA$ (peak 6) are formed from the reaction and both adenosine (peak 2) and

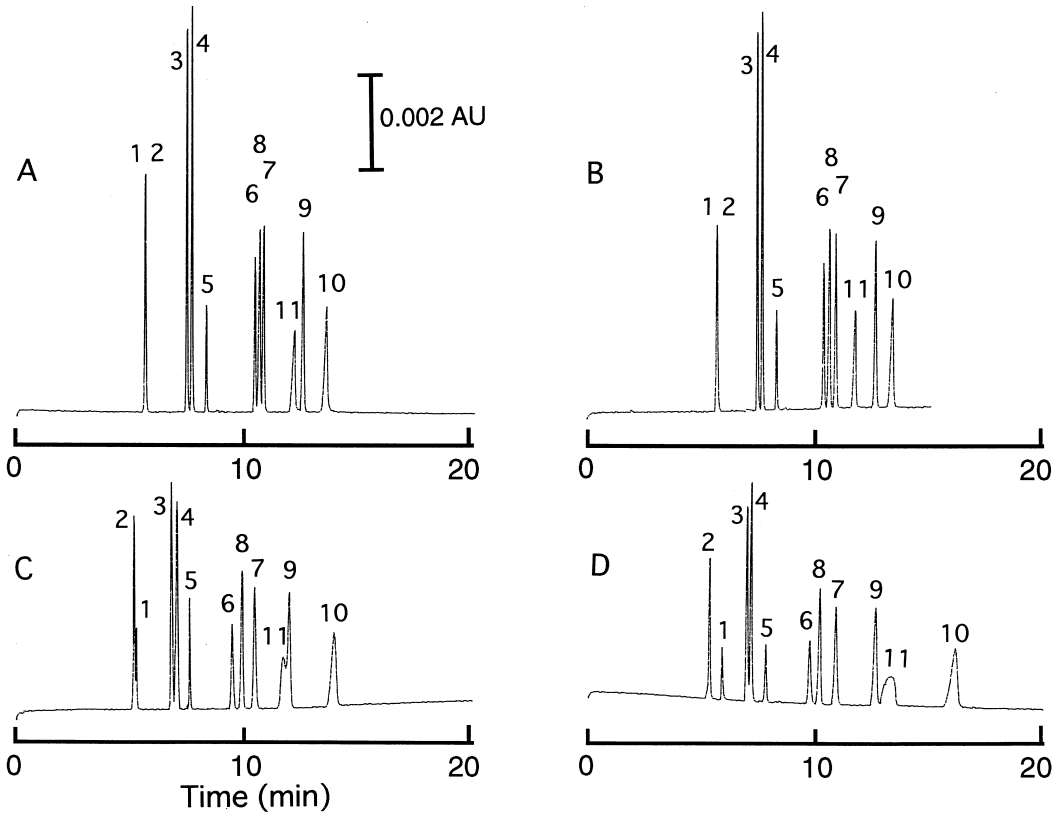

Fig. 6. Effect of pH on the separation of adenosine and adenosine nucleotides using a solution containing β-CD. Operating conditions: 0.02 *M* Na_2HPO_4, 0.05 *M* SDS, 0.015 *M* β-CD, $1 \cdot 10^{-4}$ *M* $MgCl_2$, at (A) pH=7.0, (B) pH=7.5, (C) pH=8.0 and (D) pH=9.0. All other conditions are the same as in Fig. 2.

5′-AMP (peak 7) are reactants. The sample was hydrolyzed in 0.2 *M* NaOH for 24 h at 37°C (Fig. 7B) and injected directly without further treatment. This treatment yields both 3′-AMP (peak 8) and 2′-AMP (peak 9), either from hydrolysis of $A^{3'}pA$ or $A^{2'}pA$, whereas $A^{5'}ppA$, adenosine and 5′-AMP are not hydrolyzed. The migration time was influenced less, even the sample dissolved in 0.2 *M* NaOH. The repeatability of the measurement of the migration times for all peaks was in the range of 1–2.5% R.S.D. (n=4–12). This indicates that high concentrations of NaOH do not interfere with the determination of adenosine nucleotides. This demonstrates that the method is simple and convenient for the analysis of adenosine nucleotides in prebiotic simulation samples.

Table 4
Migration time (min) for the dinucleoside monophosphate isomers in CZE with cyclodextrin

Nucleotides	Migration time (min)
$A^{2'}pA$	6.52
$A^{3'}pA$	6.67
$U^{2'}pU$	6.80
$U^{3'}pU$	6.88
$G^{2'}pG$	6.57
$G^{3'}pG$	6.65
$C^{3'}pC$	6.77

Operating conditions: 0.02 *M* Na_2HPO_4, 0.015 *M* β-CD, $1 \cdot 10^{-4}$ *M* $MgCl_2$, pH=7.5. All other conditions are the same as shown in Table 1.

4. Conclusions

The analytical method for 2′,5′- and 3′,5′-linked adenylyladenosine monophosphates was developed

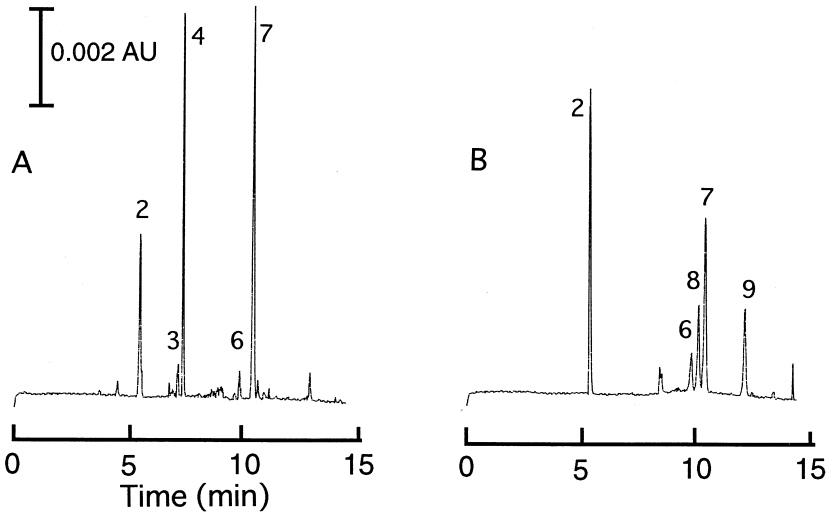

Fig. 7. Separation of model samples for the prebiotic experiments. Prebiotic experimental conditions: (A) 0.02 *M* adenine, 0.02 *M* 5′-AMP, 0.2 *M* EDAC, 0.2 *M* NaCl, 0.075 *M* $MgCl_2$, 0.1 *M* HEPES, pH=7.0. A 50-mg amount of Na^+-Vol was added to 1 ml of the reaction mixture; reaction time, six days at 20°C. (B) Sample A was treated with 0.2 *M* NaOH for 24 h at 37°C to hydrolyze $A^{2'}pA$ and $A^{3'}pA$ to form $A^{2'}p$, $A^{3'}p$ and A. CE conditions are the same as those given in Fig. 6 at pH 7.5.

using capillary electrophoresis and a solution containing β-CD and $MgCl_2$ additive. This method separates simultaneously adenosine and nine types of adenosine nucleotides within 15 min. Investigation of the separation behavior using several kinds of electrophoresis media shows that CZE containing β-cyclodextrin is more suitable for the separation of 2′,5′-linked and 3′,5′-linked or pyrophosphate-incorporated adenosine nucleotides than MEKC. The separation of longer oligonucleotides with 2′,5′- and 3′,5-linkages or pyrophosphate-incorporated isomers is being investigated at present.

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