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# Molecular Recognition of the Macrocyclic Polyamine for the Hydrolysis of Nucleotides in the Electrophoretic Condition

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*Key words:* bonded phase, capillary electrophoretic, hydrolysis, macrocyclic polyamine, nucleotides

## Abstract

A 28-membered macrocyclic polyamine, 4,8,12,18,22,26-hexaaza-1,15-dioxacyclooctaeicosane ([28]ane-$N_6O_2$) with two dipropylenetriamine moieties bound together by diethylether unit was able to form polyprotonated, highly charged species in a wide pH region. It was covalently bonded on the inner surface of the fused silica capillary. The capillary showed reversed electroosmotic flow (EOF), allowing anions to be separated in the co-EOF mode. With the phosphate buffer (30 mM) and an applied voltage of –15 kV, the influence of buffer pH and temperature on the interaction between nucleotides and the bonded phase were investigated. The results indicated that a coordination reaction occurs between the analytes and the bonded phase, and this is followed by cleavage of the terminal phosphate. As a result we found three peaks for each nucleoside triphosphate and two peaks for the diphosphates after samples were injected at 35 °C, while only two peaks for the triphosphates and no splitting for the diphosphates at 20 °C were indicated. In other words, a significant effect of temperature on the hydrolysis was observed, and the bonded phase showed a preference for the binding of nucleoside triphosphate over nucleoside diphosphate.

## Introduction

Anion coordination chemistry is a rapidly emerging field stimulated by the continuous interest displayed in anions by researchers from the environmental, industrial and health-related domains [1, 2]. Phosphates are ubiquitous anions in biological structure, function and regulation; thus their interaction with their corresponding receptors is of special interest [3, 4].

Macrocyclic compounds have extraordinary properties because of the stereochemistry of the donor atoms and the size and shape of the central cavity [5, 6]. The ligands in their protonated forms have been shown to bind neutral molecules and anions including phosphates in aqueous solution and in the solid state [7, 8]. The macrocyclic polyamine, 1,4,7,13,16,19-hexaaza-10,22-dioxatetraeicosan ([24]ane-$N_6O_2$), which catalyzes the hydrolysis of ATP to ADP and phosphate has been reported by Bethell *et al.* [9]. Zhu *et al.* [10] investigated the catalytic carboxyester hydrolysis by new macrocyclic polyamine zinc complexes with a phenolic-pendant as novel nucleophile. Phosphate anion binding by macrocyclic dinucleating ligands and their metal complexes has been reported by Martell *et al.* [11]. With the aid of the species distribution curves in competitive systems, the use of macrocycles for the separation of ATP, ADP and AMP was suggested.

Hydrolysis of carboxyesters catalyzed with a resin containing histidine groups has been studied by Hung *et al.* [12], and that of phosphate esters with polymer-supported catalysts containing cyclodextrin pendant group has also been investigated in our lab [13]. Recently a 28-membered macrocyclic polyamine, 4,8,12,18,22,26-hexaaza-1,15-dioxacyclooctaeicosane ([28]ane-$N_6O_2$) bonded phase was prepared and evaluated for its use in the electrophoretic separation of organic and inorganic anions and metal ion speciation [14–18]. A highly selective property was attributed to anion complexation, anion exchange and reversal of the electroosmotic flow (EOF) provided by the wall-bonded functional groups. Supercomplexation formation resulting from the second-sphere interaction between metallocyanide and the polyamine was also indicated [14]. As an extension of these investigations, more applications of the bonded phases were expected.

Here we report a systematic evaluation of the recognition phenomena of the macrocyclic polyamine-bonded phase toward the nucleotides that are of importance in studying various aspects of cellular metabolism.

## Experimental

### Apparatus

A high-voltage power supply with a 30 kV capacity (Model 890-CE, Jasco, Tokyo, Japan) and a variable-wavelength UV/Vis detector (Jasco 870-CE) were employed for capillary electrophoresis. Electropherograms were recorded and

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processed with a PC SISC-Lab data acquisition system (Scientific Information Service Co. Taiwan). The separations were carried out on a fused-silica capillary column with an external coating of polyimide (Resteck, Bellefonte, PA, USA) and chemically modified with a 28-membered macrocyclic ligand containing oxygen and nitrogen as donor atoms. The modified capillaries were of 75 $\mu$m ID and the total length of the capillary was 70 cm, with a distance of 50 cm between the injection end and the detection window.

*Reagents*

Most chemicals were of analytical reagent grade from Merck (Darmstadt, Germany). Purified water (18 MΩ-cm) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare all solutions. Adenosine diphosphate (ADP), cytidine diphosphate (CDP), guanosine diphosphate (GDP) and uridine diphosphate (UDP); adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP); phosphoric acid and sodium phosphate were purchased from Sigma (St. Louis, MO, USA).

$\gamma$-Glycidoxypropyltrimethoxysilane was obtained from Aldrich (Milwaukee, WI, USA). Benzyl alcohol and dimethyl formamide were obtained from Merck. All liquid reagents and solvents used in moisture-sensitive reactions were distilled and collected over type 4Å molecular sieves.

Stock solutions (5 mg ml$^{-1}$) of the nucleotide were prepared in pure water and diluted appropriately prior to use. All solvents and solutions for CEC analysis were filtered through a 0.45 $\mu$m cellulose ester membrane (Advantec MFS, Inc.).

*Preparation of capillary column [14]*

Prior to any column modifications, fused silica capillaries (75 $\mu$m ID, 70 cm long) were first flushed with 1 M NaOH (30 min), then pure water (15 min), 1 M HCl (30 min) and pure water (15 min) sequentially. The capillaries were purged with nitrogen for 20 min, then dried at 110 °C overnight. For coating, the capillary was filled with a 10% (w/v) solution of $\gamma$-glycidoxypropyltrimethoxysilane in toluene under the pressure of 30 psi. The filling rate was about 0.1 ml min$^{-1}$. The capillary was kept for 3 h at 110 °C for silylization. After purging with toluene to remove the unreacted reagent for several minutes, the capillaries were dried in a vacuum oven. The capillary was then filled with a 1% (w/v) solution of the macrocyclic compound, [28]ane-$N_6O_2 \cdot$ 6HCl in N,N-dimethylforamide. After standing for 10 h at 120 °C for functionalization, the dried capillaries were purged with ethanol and pure water for several minutes before equilibration with the buffer solution. They were then ready for use.

*Capillary electrophoretic condition*

Samples were introduced electrokinetically at the cathodic end of the capillary column. The appropriate background electrolyte was phosphate buffer. Benzyl alcohol was used as the neutral marker. The analytes were detected by monitoring their absorbance at 254 nm.

## Results and discussion

The column preparation and the characterization were reported in a previous paper [14]. Simple polyammonium macrocycles have been found to be useful biomimics with respect to catalysis of phosphoryl transfer reactions. These molecules are capable of forming high-affinity complexes with a variety of anionic substrates, including nucleotides, through both electrostatic and hydrogen bonding interactions [19]. Therefore, to get a deeper understanding of the host–guest interaction of the prepared column, nucleotides were chosen as the guests in this study.

In the case of nucleotides, complexation is followed by dephosphorylation, i.e., cleavage of the terminal phosphate for di- and triphosphates. There are a number of potential macrocyclic catalysts for these reactions, large-rate accelerations for dephosphorylation are noted only for a limited number of rings and appear to be related to a size between 21 and 24 atoms. For both larger and smaller ring rates are markedly slower [20]. Here a 28-membered macrocyclic polyamine, 28 ane-$N_6O_2$ was chosen for this study. Whether the hydrolysis reaction for nucleoside triphosphate or diphosphate would be catalyzed by the macrocyclic polyamine-bonded phase or not was investigated by the capillary electrophoretic method.

*Interaction of nucleoside triphosphates with the macrocyclic polyamine-bonded phase*

To illustrate this effect, first of all we injected four kinds of nucleoside triphosphate, ATP, CTP, GTP and UTP respectively, with phosphate buffer (30 mM, pH 3.0) as a background electrolyte under the applied voltage of –15 kV. Here we must mention that samples should be prepared freshly in order to avoid hydrolysis prior to the injection. However, preliminary tests showed that three peaks were obtained for each analyte at pH 6. Rather similar migration times but different signal responses were exhibited. The phenomena are shown in Figure 1. Some impurity (less 1%) of diphosphate or monophosphate might coexist with triphosphate from commercial sources. Moreover, according to our experience with the bare fused silica there was no evidence for such dramatic additional peaks appearing when triphosphate was injected into the electrophoresis system similar as that shown in Figure 1 except the applied voltage of +15 kV.

Therefore the influence of phosphate buffer pH on the hydrolysis reaction was further investigated systematically. The migration behaviors of the hydrolysis product as a function of pH for each nucleoside triphosphate are shown in Figure 2. On increasing the pH at constant ionic strength (30 mM phosphate buffer), a longer migration time was demonstrated. This might be a greater effective charge of the analyte (Table 1) resulting in more interaction for the analyte and the bonded phase. Moreover at pH values higher than 7, a smaller EOF would be the predominant factor for

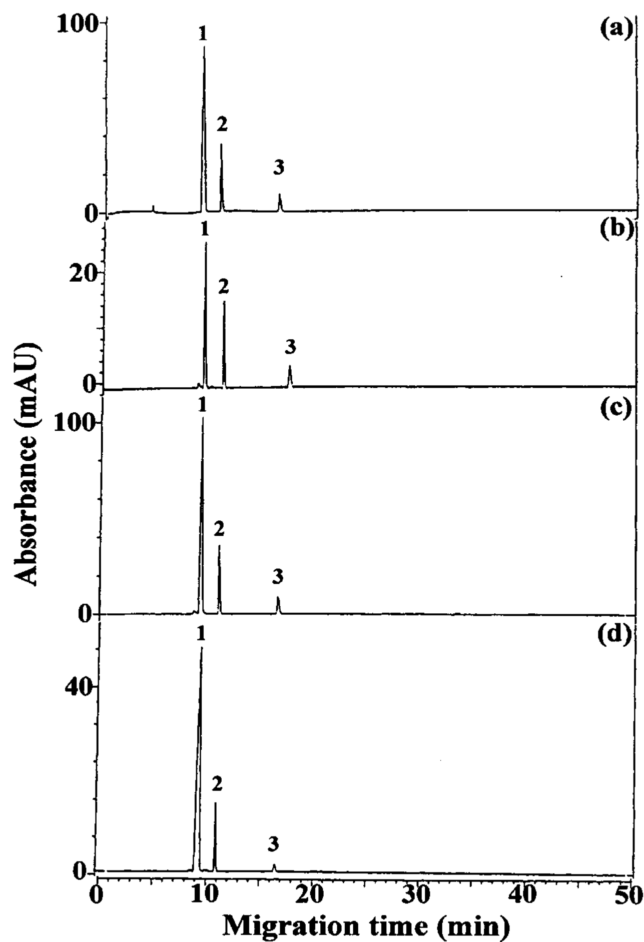


*Figure 1.* Hydrolysis reaction occurring for the nucleoside triphosphate in the column of wall-coated macrocyclic polyamine. Column: [28]ane-$N_6O_2$ bonded phase fused silica capillary; 70 cm (50 cm) × 75 $\mu$m ID. Sample injection: electrokinetic (–5 kV, 15 sec). Sample concentration: 0.05 mg $ml^{-1}$. Background electrolyte: phosphate buffer (30 mM, pH 6.0). Applied voltage: –15 kV. Detection at 254 run. Sample: (a) ATP (b) CTP (c) GTP (d) UTP. Peak identification: 1. nucleoside triphosphate 2. nucleoside diphosphate 3. nucleoside monophosphate.

the slower migration. Here it seems much too complicated for explaining the distinct inflection point at pH 6 for each nucleotide. However, it was suggested that the inflection point might be correlated with the protonation constant of the bonded macrocylic polyamine [14]. The migration times for each analyte at various pH values are summarized as Table 2.

The observed hydrolysis rate constants for ATP to ADP in a homogeneous system catalyzed by a 24-membered macrocyclic polyamine containing an acridine unit at pH 7 and 84 °C was 0.0126 $min^{-1}$, while that of ADP to AMP was 0.0015 $min^{-1}$ [21]. In this work, a 28-membered macrocyclic polyamine bonded phase in the electrophoretic condition (30 mM phosphate buffer and –15 kV) and different temperature were employed. Although it is a heterogeneous system, as expected, the time difference of the migration between ADP and AMP was significantly longer than that of between ATP and ADP (Table 2). Of course some contribution from the difference of electrophoretic mobility among them might be considered. But this is not much. Since the nucleoside itself is not involved in the binding process, all triphosphates

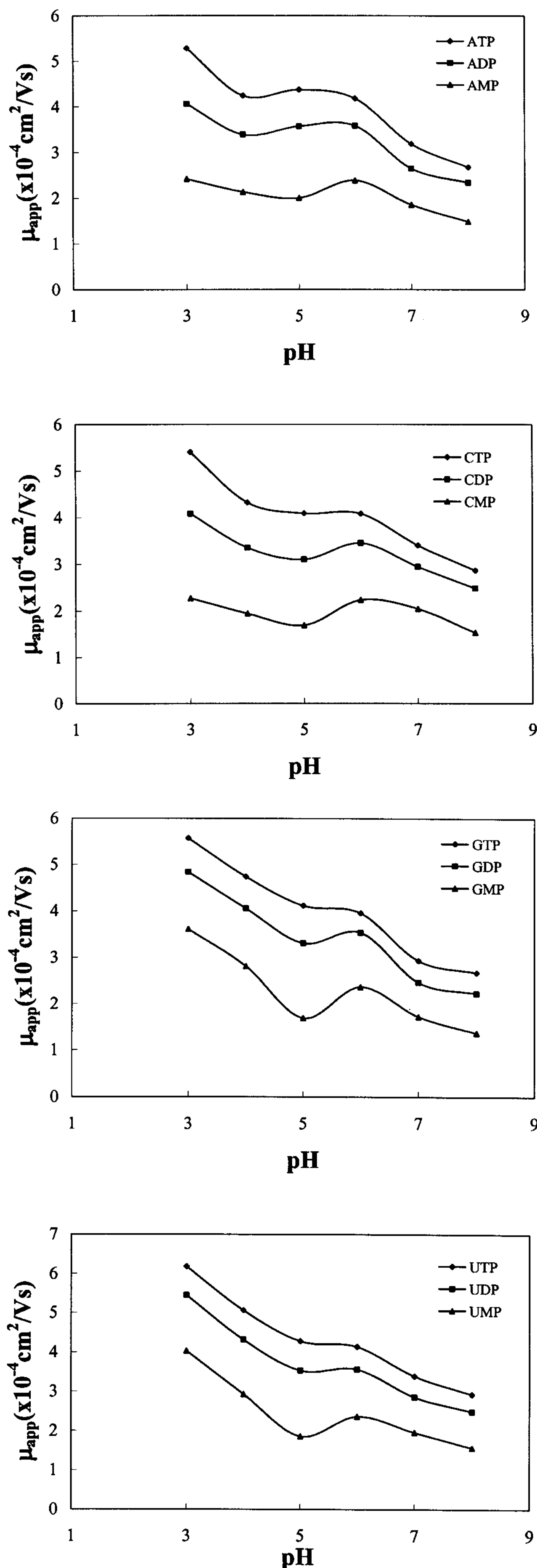


*Figure 2.* Observation of reaction of nucleoside triphosphate as a function of pH. Conditions as in Figure 1.

*Table 1.* Chemical and physical properties of the nucleotides

| Nucleotide | $pKa_1$ | $pKa_2$ | $pKa_3$ | Effective charge | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | pH 2 | pH 3 | pH 4 | pH 5 | pH 6 | pH 7 | pH 8 |
| AMP | 3.9 | <2 | 6.4 | –0.022 | –0.154 | –0.645 | –0.948 | –0.995 | –1.999 | –2 |
| CMP | 4.5 | <2 | 6.6 | –0.004 | –0.031 | –0.240 | –0.760 | –0.969 | –1.997 | –2 |
| GMP | 2.4; 9.4 | <2 | 6.7 | –0.334 | –0.799 | –0.975 | –0.997 | –1 | –2.004 | –2.038 |
| UMP | 9.5 | <2 | 6.6 | –1 | –1 | –1 | –1 | –1 | –2.003 | –2.031 |
| ADP | 4.2 | <2 | 7.0 | –1.014 | –1.101 | –1.428 | –1.918 | –1.991 | –2.999 | –3 |
| CDP | 4.6 | <2 | 7.2 | –1.005 | –1.035 | –1.266 | –1.784 | –1.973 | –2.997 | –3 |
| GDP | 2.9; 9.6 | <2 | 7.2 | –1.147 | –1.560 | –1.976 | –1.992 | –1.996 | –3.003 | –3.024 |
| UDP | 9.4 | <2 | 7.2 | –2 | –2 | –2 | –2 | –2 | –3.003 | –3.038 |
| ATP | 4.1 | <2 | 7.0 | –2.012 | –2.091 | –2.5 | –2.909 | –2.99 | –3.999 | –4 |
| CTP | 4.8 | <2 | 7.6 | –2.003 | –2.024 | –2.201 | –2.715 | –2.962 | –3.996 | –4 |
| GTP | 3.3; 9.6 | <2 | 7.7 | –2.056 | –2.334 | –2.834 | –2.98 | –2.998 | –4.005 | –4.048 |
| UTP | 9.6 | <2 | 7.6 | –3 | –3 | –3 | –3 | –3 | –4.003 | –4.024 |

Dissociation constant for base moiety: $pKa_1$, for primary phosphate group: $pKa_2$, for secondary phosphate group: $pKa_3$. Data are from *Handbook of Biochemistry and Molecular Biology*, 3rd edn., Vol. 1, CRC Press, Boca Raton, Florida.

*Table 2.* Summary for the migration time of the hydrolysis products for each nucleoside triphosphate*

| Analyte | Migration time ($t_m$, min) | | | | | |
|---|---|---|---|---|---|---|
| | pH 3 | pH 4 | pH 5 | pH6 | pH7 | pH8 |
| ATP | 7.36 | 9.18 | 8.90 | 9.30 | 12.23 | 14.56 |
| | 9.58 | 11.47 | 10.89 | 10.87 | 14.75 | 16.67 |
| | 16.14 | 18.24 | 19.47 | 16.31 | 21.04 | 26.33 |
| | $\Delta t_1 = 2.22$ | $\Delta t_1 = 2.29$ | $\Delta t_1 = 1.99$ | $\Delta t_1 = 1.57$ | $\Delta t_1 = 2.52$ | $\Delta t_1 = 2.11$ |
| | $\Delta t_2 = 6.56$ | $\Delta t_2 = 6.77$ | $\Delta t_2 = 8.58$ | $\Delta t_2 = 5.44$ | $\Delta t_2 = 6.29$ | $\Delta t_2 = 9.66$ |
| CTP | 7.20 | 9.00 | 9.51 | 9.53 | 11.44 | 13.60 |
| | 9.53 | 11.62 | 12.55 | 11.26 | 13.22 | 15.65 |
| | 17.10 | 20.00 | 23.02 | 17.41 | 19.03 | 25.38 |
| | $\Delta t_1 = 2.33$ | $\Delta t_1 = 2.62$ | $\Delta t_1 = 3.04$ | $\Delta t_1 = 1.73$ | $\Delta t_1 = 1.78$ | $\Delta t_1 = 2.05$ |
| | $\Delta t_2 = 7.57$ | $\Delta t_2 = 8.38$ | $\Delta t_2 = 10.47$ | $\Delta t_2 = 6.15$ | $\Delta t_2 = 5.81$ | $\Delta t_2 = 9.73$ |
| GTP | 6.98 | 8.20 | 9.44 | 9.83 | 13.26 | 14.56 |
| | 8.03 | 9.57 | 11.76 | 10.98 | 15.79 | 17.54 |
| | 10.78 | 13.83 | 22.98 | 16.51 | 22.66 | 28.33 |
| | $\Delta t_1 = 1.05$ | $\Delta t_1 = 1.37$ | $\Delta t_1 = 2.32$ | $\Delta t_1 = 1.60$ | $\Delta t_1 = 2.53$ | $\Delta t_1 = 2.98$ |
| | $\Delta t_2 = 2.75$ | $\Delta t_2 = 4.26$ | $\Delta t_2 = 11.22$ | $\Delta t_2 = 5.53$ | $\Delta t_2 = 6.87$ | $\Delta t_2 = 10.97$ |
| UTP | 6.29 | 7.68 | 9.08 | 9.39 | 11.47 | 13.27 |
| | 7.13 | 9.02 | 11.03 | 10.91 | 13.58 | 15.61 |
| | 9.65 | 13.27 | 20.86 | 16.50 | 19.85 | 24.72 |
| | $\Delta t_1 = 2.22$ | $\Delta t_1 = 2.29$ | $\Delta t_1 = 1.99$ | $\Delta t_1 = 1.57$ | $\Delta t_1 = 2.52$ | $\Delta t_1 = 2.11$ |
| | $\Delta t_2 = 6.56$ | $\Delta t_2 = 6.77$ | $\Delta t_2 = 8.58$ | $\Delta t_2 = 5.44$ | $\Delta t_2 = 6.29$ | $\Delta t_2 = 9.66$ |

*Capillary: [28]ane-$N_6O_2$ bonded phase, 70 cm (50 cm to detector) $\times 75 \mu$m ID; sample injection: electrokinetic (–5 kV; 10 sec); sample concentration: 0.05 mg $ml^{-1}$; background electrolyte: phosphate buffer 30 mM; applied potential: –15 kV; detection at 254 nm. $\Delta t_1$: the migration time difference between nucleoside diphosphate and triphosphate. $\Delta t_2$: the time difference between nucleoside monophosphate and diphosphate.

would be expected to show similar association constants. The phenomena were indeed demonstrated for CTP, GTP and UTP.

We also investigated the kinetic preference at various pH values. By plotting the peak area ratio against pH for each analyte (figures not shown), the results indicated that the extent of the dephosphorylation reaction does not seem to be significantly different from pH 3 to 8 for most analytes, both at a temperature of 35 and 20 °C. The peak area ratio was around 70% for ATP, 20% for ADP and 10% for AMP; 67% for CTP, 20% for CDP and 13% for CMP, 88% for GTP, 11% for GDP and 1% for GMP; 87% for UTP, 12% for UDP and 1% for UMP at 35 °C, while it was around 96% for ATP and 4% for ADP; 86% for CTP and 14% for CDP; 94% for GTP and 6% for GDP; 97% for UTP and 3% for UDP at 20 °C. The pH range chosen due to the extreme high or low pH would lead to the acid or alkaline hydrolysis of the analytes. In this case, the peak area ratio indicated the fraction of each hydrolysis product. The synthetic macrocyclic polyamine has a propylene unit between the neighboring nitrogen atoms

*Table 3.* Summary for the migration time of the hydrolysis products for each nucleoside diphosphate*

| Analyte | Migration time ($t_m$, min) | | | | | | |
|---|---|---|---|---|---|---|---|
| | pH 2.1 | pH 3 | pH 4 | pH 5 | pH 6 | pH 7 | pH 8 |
| ADP | 12.42 | 13.68 | 14.61 | 13.73 | 15.35 | 19.17 | 17.08 |
| | 24.20 | 31.12 | 29.78 | 25.57 | 27.79 | 32.63 | 28.70 |
| | Δt = 11.78 | Δt = 17.44 | Δt = 15.17 | Δt = 11.84 | Δt = 12.44 | Δt = 13.46 | Δt = 11.62 |
| CDP | 11.85 | 13.95 | 15.83 | 14.40 | 15.24 | 17.25 | 15.35 |
| | | 35.75 | 38.48 | 26.66 | 27.85 | 30.18 | 24.94 |
| | | Δt = 21.8 | Δt = 22.65 | Δt = 12.26 | Δt = 12.61 | Δt = 12.93 | Δt = 9.59 |
| GDP | 11.47 | 11.48 | 12.76 | 14.40 | 16.57 | 21.21 | 18.14 |
| | 18.66 | 17.11 | 20.12 | 27.29 | 30.80 | 35.54 | 28.99 |
| | Δt = 7.19 | Δt = 5.63 | Δt = 7.34 | Δt = 12.89 | Δt = 14.23 | Δt = 14.33 | Δt = 10.85 |
| UDP | 8.29 | 9.20 | 13.05 | 13.34 | 15.28 | 17.49 | 15.83 |
| | 10.45 | 12.96 | 20.94 | 24.10 | 27.30 | 29.53 | 24.98 |
| | Δt = 2.16 | Δt = 3.76 | Δt = 7.89 | Δt = 10.76 | Δt = 12.02 | Δt = 12.04 | Δt = 9.15 |

*Capillary: [28]ane-$N_6O_2$ bonded phase, 70 cm (50 cm to detector) $\times 75\mu$m ID; sample injection: electrokinetic (–5 kV, 10 sec); sample concentration: 0.05 mg ml$^{-1}$; background electrolyte: phosphate buffer 30 mM; applied potential: –15 kV; detection at 254 nm. Δt means the migration time difference between nucleoside monophosphate and diphosphate.

like spermidine and spermine that could bind tightly to the nucleotides to participate in numerous biological reactions [8]. Therefore, even at neutral pH a rather great percentage of the bonded phase is still protonated. Meanwhile the effective charge of the analyte is greater as the pH increases. Consequently, even at the higher pH conditions, there could be coordinative binding of the analyte toward the bonded phase.

The analyte and the bonded phase interaction was also confirmed by comparison with Cahour's work [22]. They separated the mixture of ATP, ADP and AMP with the capillary electrophoretic system [47 cm (40 cm to detector) $\times 50\mu$m ID] of the citrate-citric acid buffer that was conditioned for 2 min with aqueous hexadimethrine bromide solution before each injection and an applied voltage of –20 kV [22]. A condition similar to ours is the EOF reversal. However, neither macrocyclic nor a host–guest effect was involved in their work. At pH 5, the EOF for Cahours' work was $8.51 \times 10^{-5}$ cm$^2$ V$^{-1}$s$^{-1}$ which is greater than ours, $5.95 \times 10^{-5}$ cm$^2$ V$^{-1}$s$^{-1}$. The calculated $\mu_{ep}(\times 10^{-4}$ cm$^2$ s$^{-1}\cdot$V$^{-1})$ for the hydrolysis product, ATP (8.90 min), ADP (10.89 min) and AMP (19.47 min) in our work are 3.77, 2.98 and 1.40, respectively. While those for the injected mixture of ATP (3.73 min), ADP (4.40 min) and AMP (6.26 min) in Cahour's work are 3.35, 2.71 and 1.65, respectively [22]. The electrophoretic mobilities ($\mu_{ep}$) were 12.54% greater for ATP, 9.97% greater for ADP and 5.15% less for AMP compared with Cahour's work. The greater viscosity BGE that was used in their work might be the reason for the phenomena mentioned. Meanwhile we can conclude that the bonded macrocyclic polyamine facilitated the hydrolysis and that the rate for the ATP hydrolysis to ADP is greater than that for ADP to AMP.

*Interaction of nucleoside diphosphates with the macrocyclic polyamine-bonded phase*

Under the condition of a phosphate buffer (30 mM) and an applied voltage of –15 kV, two peaks were observed for each nucleoside diphosphate. The migration times of each analyte at various acidities are summarized in Table 3.

*Comparison of the interaction of the bonded group toward nucleoside triphosphate with that toward nucleoside diphosphate*

A comparison of the time differences between the neighboring peaks in the electropherogram as a function of pH for nucleoside diphosphates with those of triphosphates is illustrated in Figure 3. By carefully considering the shape of the curves, we may conclude that there are two kinds of catalytic hydrolysis reaction. At neutral and acidic conditions, the catalytic reaction for the hydrolysis of nucleotide seems to occur primarily based on anion coordination, while base hydrolysis seems the predominant factor at alkaline conditions. Meanwhile the hydrolysis behaviors of adenosine and cytidine phosphates show more similarity than those of guanosine and uridine phosphates. Base hydrolysis occurring could also be concluded by seeing the similar time difference shown in Tables 2 and 3 for the hydrolysis of nucleoside diphosphate to monophosphate at pH 8, irrespective of whether the starting material is nucleoside triphosphate or nucleoside diphosphate.

*Temperature effect on the interaction of nucleotides with the bonded phase*

In this work, three peaks were found for each nucleoside triphosphate injected at 35 °C (Figure 1), while only two peaks were found at 20 °C. In a similar manner, only two peaks were found for each nucleoside diphosphate at the former condition, and no peak splitting was found for the nucleoside diphosphate at latter condition. The results showed that the

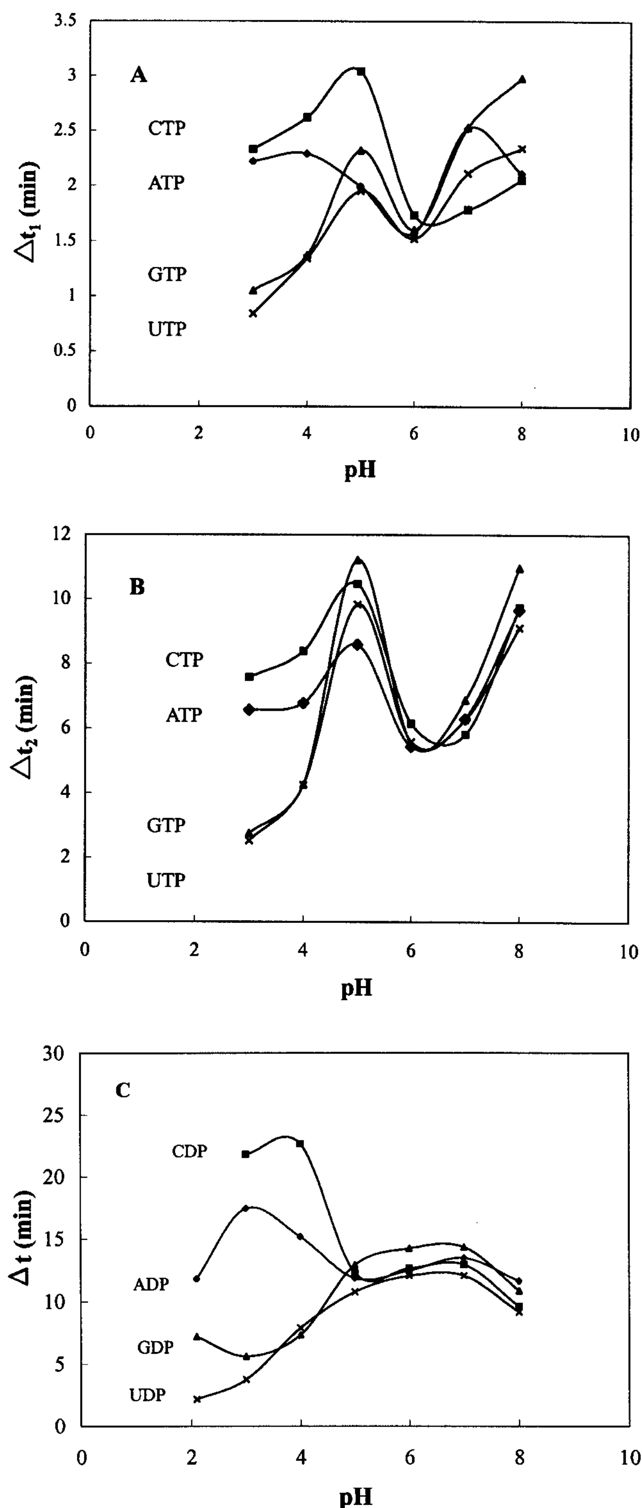


*Figure 3.* The migration time difference for each product of nucleotide in the stepwise hydrolysis as a function of pH. Conditions as in Figure 2. $\Delta t_1$: the time difference between the migration time of nucleoside diphosphate and that of triphosphate. $\Delta t_2$: the time difference between the migration time of nucleoside monophosphate and that of diphosphate. $\Delta t$ means the time difference between the migration time of nucleoside monophosphate and that of diphosphate.

dephosphorylation reaction was easier in the higher temperature. Therefore a greater percentage of triphosphate was converted to the diphosphate and monophosphate, and the remaining fraction of triphosphate at 35 °C was less than that at 20 °C. A similar behavior in temperature effect was also shown for the nucleoside diphosphate (data not shown).

A significant temperature effect on the nucleotide hydrolysis in the electrophoretic condition was seen (Figure 4). Of course the solutes' mobilities decrease at a lower temperature, probably because of a change in their diffusion coefficients [23]. Meanwhile the mobilities decrease as the pH increases due to the lower EOF. All parameters would result in a greater $\Delta t_1$ in the lower temperature. However, the change is not so great.

## Conclusion

There are three factors determining the migration of the nucleotides in this work. They are electrophoretic mobility of the solute, electroosmotic flow and the interaction between the solute and the bonded group on the capillary wall. Sample–wall interactions can produce some retention of solutes in the column, and this affects the separation resolution in CE. Macrocyclic polyamines have long been demonstrated to be good host molecules for polyanions and form stable 1 : 1 complexes even at neutral pH in aqueous solution.

For separation the mixture of nucleoside triphosphate, diphosphate and monophosphate, the EOF modifier [22] or polymeric coating [24] were needed. Initially, we hoped that the bonded phase could be used for the separation of the mixture mentioned, with only the simple buffer system. However, three peaks existed for each nucleoside triphosphate and two peaks indicated the nucleoside diphosphate after the sample was injected at a higher temperature, while there are only two peaks for the former and one peak for the latter at the lower temperature. The phenomena indicated that there were stepwise dephosphorylation reactions catalyzed by the macrocyclic polyamine bonded phase. Here one may wonder if hydrolysis occurs on-column during the separation. Then peak tailing and fronting should be observable as the triphosphates hydrolyze to the diphosphates and monophosphates as they migrate down the capillary. But in this work the phosphate buffer was only used as the background electrolyte. This was because the inorganic phosphate could displace the strong binding between the nucleotide and the bonded phase. Therefore a shorter migration time could be anticipated than with any other non-complexing buffer.

The results of this work indicate that the bonded phase in the electrophoretic conditions could be used as a special method for the hydrolysis reaction. Of course further investigation should be made for this purpose and a packed column containing this phase will be anticipated to be more promising for this use. If it is available, only a small amount of the reagent in combination with the applied voltage could be employed for the hydrolysis. Meanwhile it could be regenerated and used once again. Although the bonded phase could not be employed for the determination of nucleoside

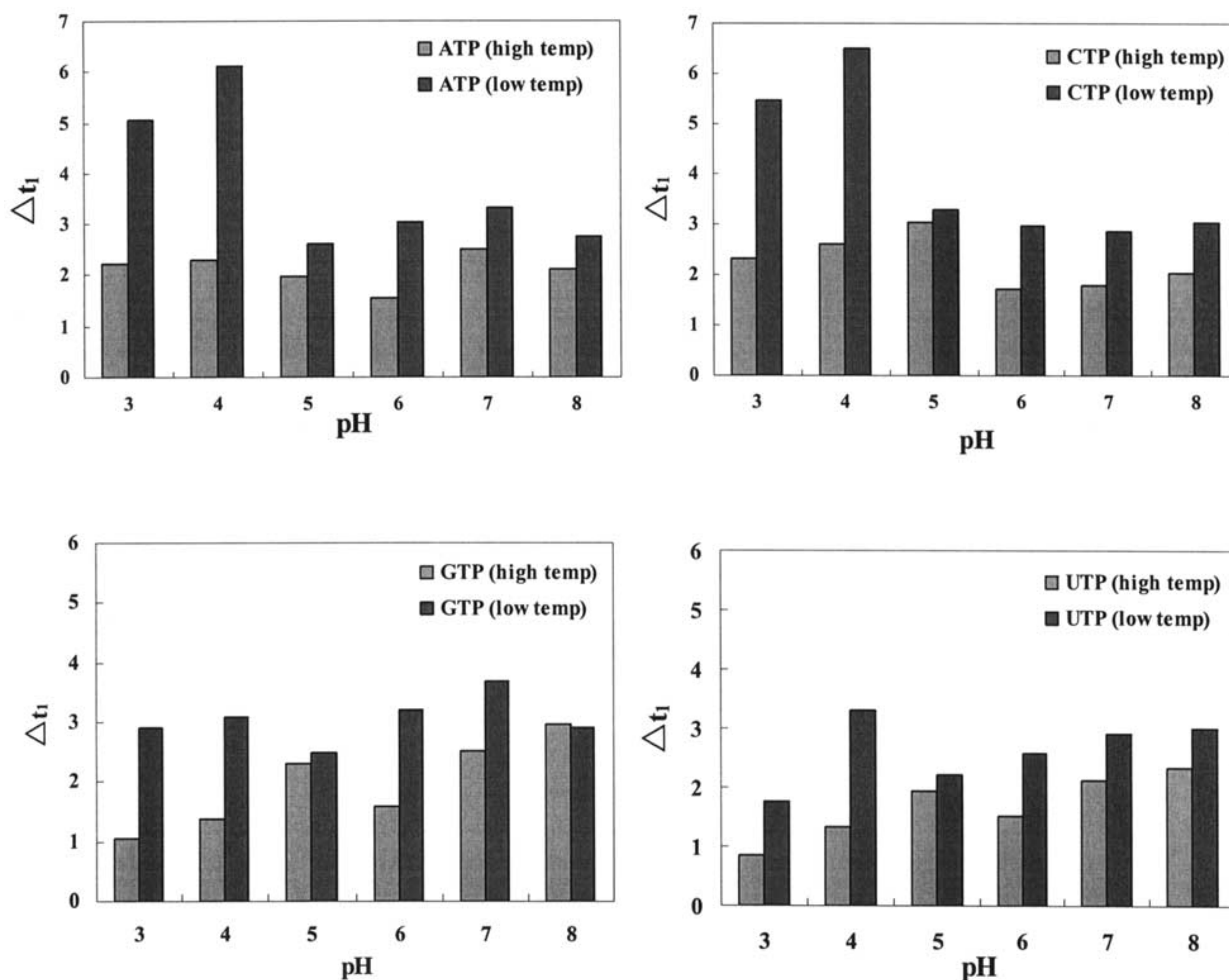


*Figure 4.* Comparison of $\Delta t_1$ at high temperature with that at low temperature. Conditions as in Figure 2. High temperature (35 °C), low temperature (20 °C).

triphosphate or diphosphate, satisfactory results have been obtained for the separation of nucleoside monophosphate isomers [17, 18].

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