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# Effect of the Buffering Capacity of a Supporting Solution on Paper Electrophoretic Migrations of Adenine, Adenosine, and Adenosine Nucleotides

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Paper electrophoretic migrations of adenine, adenosine, and adenosine nucleotides as a function of pH-values of various supporting solutions have been studied. The buffering capacity of the supporting solution played an important role in the migration and is discussed along with the electroosmosis. The $R_f$-values of adenines in the supporting solutions are also presented.

In the previous paper,[1] we reported the electrophoretic behavior of adenine, adenosine, and adenosine nucleotides as a function of the pH and of the concentration of inorganic acids. In the case of a supporting solution of lower buffering capacity, the deprotonation of protonated adenine or the dissociation of the secondary hydrogen of the phosphate moiety in the nucleotides did not take place at the pH-value expected from other chemical analyses. In order to elucidate the cause of this behavior, we carried out more paper electrophoresis in supporting solutions having different buffering capacities in a range of pH=2—13. Electroosmotic flows and the $R_f$-values of samples in solutions to be used for the electrophoresis are also shown.

## Experimental

*Materials.* Adenine and adenosine were purchased from Wako (Osaka, Japan), and the sodium salts of the nucleotides from Sigma (St. Louis, Mo., U.S.A). Guaranteed grades of chemicals were used for preparation of supporting solutions without further purification.

*Paper Electrophoresis.* The procedures and apparatus[1] used were similar to those described previoulsy. Supporting solutions (ionic strength $\mu=0.1$) of NaOH in various concentrations were prepared by diluting 0.1 M NaOH solution with 0.1 M NaCl solution (1 M=1 mol dm$^{-3}$). The supporting solution of pH=13 was a 0.1 M NaOH solution containing no NaCl. Sørensen's buffer solutions[2] were prepared in the following way. A 0.1 M sodium citrate solution was prepared by dissolving 21 g (0.1 M) of $C_6H_8O_7\cdot H_2O$ and 8.0 g (0.2 M) of NaOH in one liter of distilled water. The citrate solution was mixed with 0.1 N HCl or 0.1 N NaOH solution to obtain citrate buffer solutions of different pH values. Glycine buffer solutions were prepared by mixing 0.1 M glycine +0.1 M NaCl solution with 0.1 M NaOH, and phosphate buffers by mixing 0.2 M $Na_2HPO_4$ and 0.2 M $NaH_2PO_4$. The buffering capacity ($\beta$) of the supporting solution was calculated by this equation:[3]

$$\beta = 2.303\{[C]\cdot[H^+]\cdot K_a/([H^+]+K_a)^2+[H^+]+10^{-14}/[H^+]\},$$

where $C$ is the concentration of the solute, $K_a$ the dissociation constant of the solute, and [H+] the concentration of hydrogen ions. The calculated buffering capacities of the solutions are listed in Table 1.

Adenosine and adenosine nucleotides were dissolved in distilled water and adenine was dissolved in 0.1 M sodium acetate. A 5 μl volume of sample solution ($5\times10^{-2}$ M) was spotted at the center of a filter paper (Toyo Roshi No. 51A, 1×40 cm) wetted with a supporting solution. The filter paper was dipped in hexane in the migration chamber and a voltage gradient (1000 V/30 cm) was applied it for 30 min at *ca.* 20 °C. Adenines after migration on the filter paper were detected using the absorption band at 253 nm.

TABLE 1. BUFFERING CAPACITIES($\beta$) OF SUPPORTING SOLUTIONS

| Component | pH | $\beta$ |
|---|---|---|
| HCl+0.1 M NaCl | 2.0 | $2.3\times10^{-2}$ |
| | 3.0 | $2.3\times10^{-3}$ |
| | 4.0 | $2.3\times10^{-4}$ |
| | 5.0 | $2.3\times10^{-5}$ |
| | 6.0 | $2.5\times10^{-6}$ |
| 0.1 M NaCl | 7.0 | $4.6\times10^{-7}$ |
| NaOH+0.1 M NaCl | 8.0 | $2.5\times10^{-6}$ |
| | 9.0 | $2.3\times10^{-5}$ |
| | 10.0 | $2.3\times10^{-4}$ |
| | 11.0 | $2.3\times10^{-3}$ |
| | 12.0 | $2.3\times10^{-2}$ |
| | 13.0 | $2.3\times10^{-1}$ |
| Citric acid buffer | 2.0 | $3.3\times10^{-2}$ |
| | 3.0 | $2.5\times10^{-2}$ |
| | 4.0 | $2.2\times10^{-2}$ |
| | 5.0 | $5.6\times10^{-2}$ |
| | 6.0 | $3.0\times10^{-2}$ |
| | 6.7 | $2.6\times10^{-2}$ |
| Phosphoric acid buffer | 7.0 | $2.8\times10^{-2}$ |
| | 8.0 | $6.6\times10^{-3}$ |
| Glycine+NaOH buffer | 8.9 | $2.5\times10^{-2}$ |
| | 10.1 | $2.9\times10^{-2}$ |
| | 11.0 | $7.8\times10^{-3}$ |
| | 12.0 | $2.4\times10^{-2}$ |
| | 12.9 | $1.8\times10^{-1}$ |

The electroosmotic flows were estimated from the movement of glucose ($5\times10^{-2}$ M), which migrated along with the sample solution. The results are shown in Fig. 1. The different migrations of glucose among spotting positions are due to a capillary action, which is towards the center of a filter paper and almost disappears in 1 h.[4] The contribution of the capillary action to the movement of glucose is minimum at the center and usually zero within experimental errors. That is why the samples are spotted at the center of a filter paper. Since electroosmotic flows[5] should vary with the charge and porosity of filter paper, different batches of the same type of filter paper may give different results. The different electroosmotic flow for Toyo Roshi No. 51A filter papers were within 0.5 cm at 1000 V per 30 cm at 20 °C for 30 min. Glucose on a filter paper was detected by spraying *o*-aminophenol solution.[6]

*Paper Chromatography.* No ideal method to evaluate the adsorption effects of migrants on a filter paper has been proposed.[7] The measurement of $R_f$-value may be one convenient method, since it is useful for the estimation of

the interaction between a filter paper and the migrants in a given solvent. Samples were developed in solvents used for the paper electrophoresis for 3.5—4 h at 15 °C

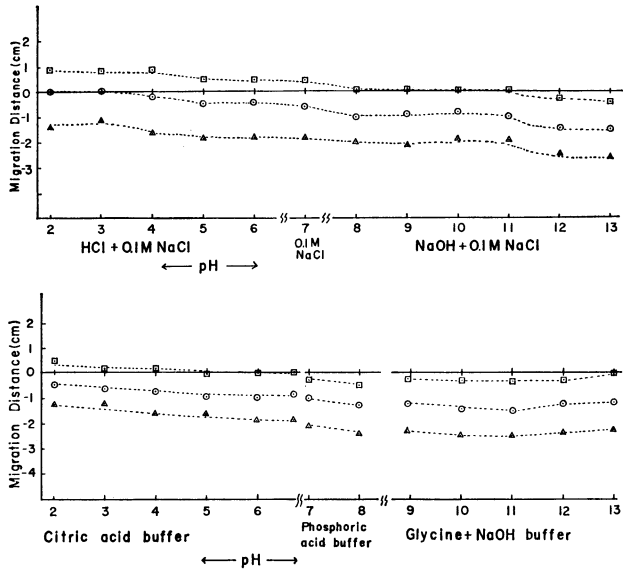


Fig. 1. The movement of glucose.
Conditions: voltage gradient, 1000 V per 30 cm; migration time, 30 min; migration temperature, *ca.* 20 °C; supporting solutions are referred to Experimental. Spotting positions: ⊡, 5 cm to the cathodic side from the center of the filter paper; ⊙, the center; ▲, 5 cm to the anodic side from the center. Positive movement is towards the anode, negative movement towards the cathode.

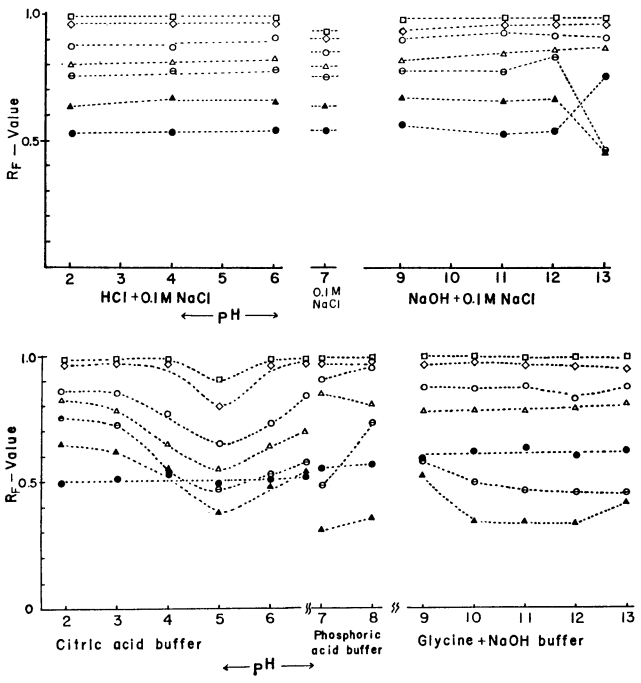


Fig. 2. $R_f$-values of paper chromatography in various solvents.
Conditions: developing time, 3.5—4.0 h; temperature, *ca.* 15 °C; ascending method. Developing solvents are referred to Experimental. ○: AMP, △: c-AMP, ◇: ADP, □: ATP, ▲: adenine, ⊖: adenosine, ●: picric acid.

on Toyo Roshi No. 51A (2×40 cm) filter paper by an ascending method. The results are shown in Fig. 2. Adenosine nucleotides showed larger $R_f$-values than adenosine and adenine; ATP moved nearly the same distances in all solvents as the solvent moved. The lower $R_f$-values of adenine and adenosine in phosphoric acid and glycine buffers suggest the presence of specific weak interactions in these solutions. The $R_f$-values of picric acid were almost constant in all solvents ($R_f$=0.5—0.6). The $R_f$-values decreased in the order: ATP>ADP>AMP>c-AMP>adenosine>adenine.

## Results and Discussion

Under ideal conditions, the relationship between the electrophoretic migration distance and the pH value of the supporting solution could be expressed by a sigmoid curve having an inflection point at the pH value equal to p$K_a$ of the migrant.[8] The p$K_a$-values of the adenine, adenosine, and adenosine nucleotides which are studied in this experiment are shown in Table 2.[9] The values are determined by the

Table 2. p$K_a$-Values
The ionic strength in each instance was 0.1 at 25 °C.

| Compound | p$K_a$(base) | p$K_a$ (secondary phosphate) | p$K_a$ (>$N_9$H) |
|---|---|---|---|
| Adenine | 4.2 | — | 9.7 |
| Adenosine | 3.6 | — | — |
| 5′-AMP | 3.8 | 6.2 | — |
| 5′-ADP | 3.9 | 6.4 | — |
| 5′-ATP | 4.1 | 6.5 | — |

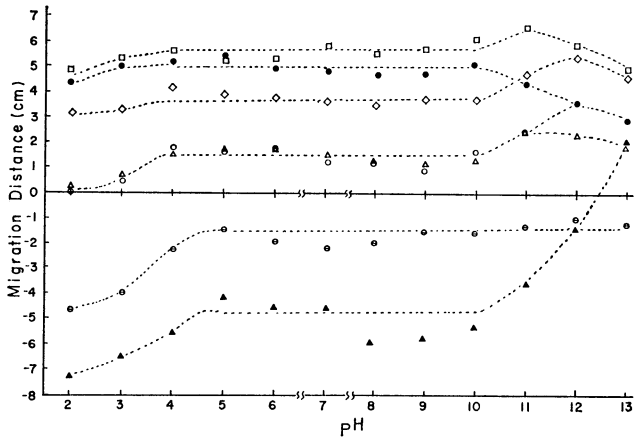


Fig. 3. Migration distances of adenine, adenosine and adenosine nucleotides in supporting solutions of low buffering capacity. Conditions: voltage gradient, 1000 V per 30 cm; migration time, 30 min, migration temperature, *ca.* 20 °C. Supporting solution, HCl+0.1 M NaCl (pH=2—6); 0.1 M NaCl (pH=7.0); NaOH+0.1 M NaCl (pH=8—13). Positive movement is towards the anode, negative movement towards the cathode. Symbols as in Fig. 2. Buffering capacity of each solution is referred to Table 1.

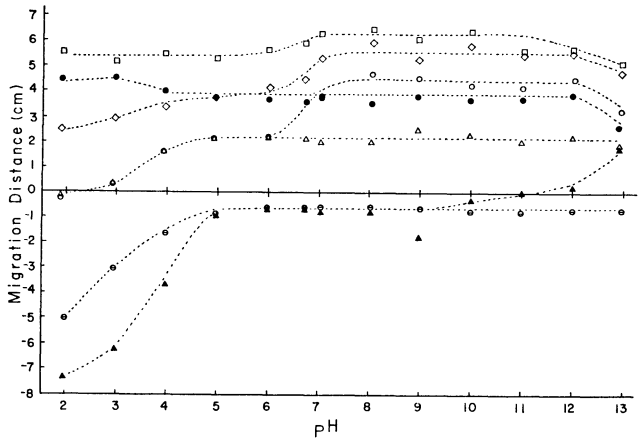


Fig. 4. Migration distances of adenine, adenosine, and adenosine nucleotides in supporting solutions of high buffering capacity. Electrophoresis conditions and symbols as in Fig. 3. Supporting solutions, citric acid buffer (pH=1.9—6.7); phosphoric acid buffer (pH=7—8); glycine+NaOH buffer (pH=8.9—12.9). Buffering capacity is referred to Table 1.

usual chemical methods, such as pH titration and spectrophotometry, rather than by electrophoresis.

The dependence of the observed migration distances of adenine, adenosine and adenosine nucleotides on the pH values of the supporting solutions are shown in Fig. 3. The migration distances in acidic region (HCl) in the previous experiment[1] and those in basic regions(NaOH) in this experiment are plotted against the pH values of the supporting solutions. Both adenine and adenosine moved to the cathode and showed an inflection point near pH=4. For pH values higher than pH=5, considerable migration was observed, showing that the deprotonation of protonated adenine or adenosine is incomplete. In the range of pH=11—13, the rapid decrease in the negative movement (the movement towards the cathode) of adenine and the increase in the positive movement with increasing pH values seem to be due to a dissociation of the imino hydrogen atom ($>N_9H$) of the purine ring, resulting in anions of adenine. Although the primary-hydrogen dissociation in the phosphate moiety of nucleotides took place near pH=1.0, AMP and c-AMP (adenosine cyclic 3′,5′-monophosphate) almost did not move in the range of pH=2—3 because adenine moiety of the nucleotide was protonated. ADP and ATP, which have two and three dissociable primary-hydrogen atoms respectively, moved to the anode. The migration distances of AMP, c-AMP, ADP and ATP increased in the range of pH=3—4. The nucleotides showed almost constant migration distances in the range of pH=5—10 and no inflection point near pH=7.

The graphs in Fig. 4 show the results obtained in the usual supporting solutions of rather high buffering capacity over a range of pH=2—13. Citric acid has three dissociable hydrogen atoms ($pK_a$=3.1, 4.8, and 6.4 at 25 °C[10]) and is a good supporting solution, having high buffering capacity, in the range of pH=2—7. Adenine and adenosine showed an inflection point near pH=3.5. The small negative migrations of adenine and adenosine (*ca.* −0.6 cm) at more than pH=5 are due to an electroosmotic flow (see Experimental). The electrophoretic behavior of adenine and adenosine in the strong basic region are similar to those in Fig. 3. The migration curves of the nucleotides in Fig. 4 show two inflection points, around pH=4 and 7.

Comparing the graphs in Fig. 3 with those in Fig. 4, we will find the differences of electrophoretic behavior to be larger in the range of pH=5—10. Assuming that an electroosmotic flow would coincide with the movement of glucose, we tried to correct the observed migrations for the flow. Although we subtracted the migration distance of glucose from that of a sample in a given solution (in the case of anions, we added the migration distances of glucose to that of a sample), graphs in Fig. 3 did not agree with those in Fig. 4, because the differences of the electroosmotic flow among graphs in Fig. 1 are within 0.5 cm. The considerable migrations of adenine and adenosine in Fig. 3 are unreasonable because the protonated adenine or adenosine should dissociate a proton in basic region and result in a neutral molecule, and because the contribution of the electroosmotic flow to the migration was within 1.0 cm, as seen in Fig. 1. The most probable explanation for the discrepancy between Figs. 3 and 4 would be made on the basis of the buffering capacity of the supporting solution. When the buffering capacity of a supporting solution is not large enough to neutralize the acid groups present as contaminants in a filter paper(probably -COOH), the pH value of the solution in the filter paper during electrophoresis will be shifted to be more acidic than the pH value initially prepared. The considerable negative migration distances of adenine and adenosine in pH=5—10 in Fig. 3, and the absence of dissociation of the secondary-hydrogen atom of the phosphate moiety (no inflection point of the curve near pH=7) are considered to reflect the effects of acid groups of the filter paper. The strength of the effect depends on the physicochemical nature of the filter paper as a supporting material. In the migration of a strong acid or base in a paper electrophoresis, we may not need to take so much a care about the buffering capacity. However, in the migration of a weak acid or base, its dissociation is often not observed at the expected pH value in a supporting solution of lower buffering capacity, as seen in Fig. 3; it is therefore necessary to consider the buffering capacity of the supporting solution. Further, to get good reproducibility and to lessen the chemical effects of the supporting material, a supporting solution of high buffering capacity is desirable. In our experimental conditions, a buffering capacity of more than $\beta$=0.023 is desirable to keep the pH value of the supporting solution constant during migration. These results remind us that the buffering capacity of the supporting solution should be paid attention to in paper electrophoresis in addition to its pH value.

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