Reprinted from

Journal of Chromatography, 168 (1979) 241–245
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,328

Note

Study of the ionic species in aqueous solutions of adenine, adenosine and adenosine nucleotides by paper electrophoresis

Y. KITAOKA

Research Reactor Institute, Kyoto University, Kumatori-cho, Sennan-gun, Osaka (Japan)

(Received July 17th, 1978)

Adenosine nucleotides are among the most important compounds in biochemical reactions, and many reports have been published on their chemical nature[1,2]. However, some controversial statements have been made about the proton-accepting site of the adenine moiety. Some workers[3–6] regard the hydrogen atom as being bound to the C_6–NH_3^+, and others[7–11] to the N_1H^+ position, of the adenine ring. Thus, protonated adenine, adenosine or adenosine nucleotides are interesting chemical species in solution.

As electrophoresis offers a useful means of studying ionic species in solution, we have investigated the electrophoretic behaviour of ions of adenine, adenosine, and adenosine 5′-mono-, 5′-di-, 5′-tri- and cyclic 3′,5′-monophosphates (AMP, ADP, ATP and cAMP, respectively) in aqueous solution under various conditions. We have found evidence for the existence of "zwitterions" of nucleotides, sometimes even in near neutral medium.

REAGENTS AND METHODS

Adenine and adenosine were purchased from Wako (Osaka, Japan), and the sodium salts of the nucleotides from Sigma (St. Louis, Mo., U.S.A.).

The techniques and apparatus used were similar to those described previously[12]. Acetate buffer solutions were prepared by mixing 1 *M* acetic acid (AcOH) and 1 *M* sodium acetate (AcONa), and phosphate buffers by mixing 0.2 *M* Na_2HPO_4 and 0.2 *M* NaH_2PO_4; the compositions of the solutions are shown in Table I. Supporting solutions of acids (HCl, AcOH and H_3PO_4) in various concentrations were prepared by diluting 0.1 *M* solutions of the acids with 0.1 *M* NaCl; supporting solutions of AcONa were similarly prepared.

Adenine was dissolved in 0.1 *M* AcOH, and the other samples in distilled water. A 5-μl portion of sample solution (5×10^{-3} *M*) was spotted at the centre of a filter paper (Toyo roshi No. 51A; 1 × 40 cm) wetted with a supporting solution. The paper was then dipped in hexane in the electrophoresis chamber, and a constant stabilized voltage was applied, the temperature of the chamber being kept constant. Adenine compounds after migration were detected by means of their absorption at 253 nm, and H_3PO_4 was located by the formation of molybdo-complexes.

TABLE I

COMPOSITIONS OF BUFFER SOLUTIONS

The ionic strength in each instance was 0.1.

Molarity in				*pH*
AcOH	*AcONa*	Na_2HPO_4	NaH_2PO_4	
0.35	0.1	—	—	4.1
0.05	0.1	—	—	5.0
—	—	0.0024	0.092	5.3
—	—	0.01	0.07	6.0
—	—	0.025	0.025	7.0
—	—	0.033	0.002	8.0

RESULTS AND DISCUSSION

The migration distances of the adenine species are shown in Fig. 1, from which it can be seen that the migration distances are strongly affected by the pH value and components of the supporting solution; similar results have been reported by Morales-Vallarta and Castañeda[13]. The nucleotides moved towards the anode, and adenine and adenosine to the cathode, as expected, the migration distances increasing in the order AMP $\approx$ cAMP $<$ ADP $<$ ATP (this order was maintained in all supporting solutions). The relationship between charge state and pH of supporting solution could be expressed by a sigmoid curve having an inflection point at the pH value equal to pK_a[14]. The primary-hydrogen dissociation in the phosphate moiety takes place near pH 1.0. If we neglect the positive charge on the adenine moiety, then, over the pH range 3–5.5, ATP, ADP, AMP and cAMP should have negative charges of *ca.* 3, 2, 1 and 1 units, respectively. The increase in migration distances of AMP,

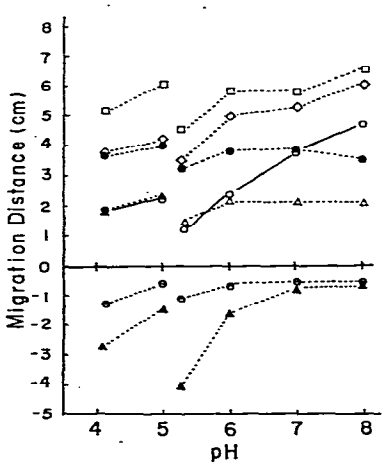

Fig. 1. Migration distances of adenine, adenosine and adenosine nucleotides. Conditions: voltage gradient, 1000 V per 30 cm; migration time, 30 min; supporting solution, AcOH–AcONa; Na_2HPO_4–NaH_2PO_4; migration temperature, *ca.* 20°. Positive movement is towards the anode, negative movement towards the cathode. ⊙, H_3PO_4; ○, AMP; △, cAMP; ◇, ADP; □, ATP; ▲, adenine; ⊖, adenosine; ●, picric acid.

ADP and ATP in the pH range 6–8 is due to dissociation of the secondary hydrogen of the phosphate moiety (cAMP has no secondary hydrogen, so that its migration did not change as much as did that of the other nucleotides[15]). If the protonated adenine or adenine moiety dissociates in media of pH near 4 (as has been reported[1,2]), an increase in the migration distances of the nucleotides (and a decrease in those of adenine and adenosine) should be observed in this pH region. There was an increase in the migration distances of the nucleotides, and a decrease in those of adenine and

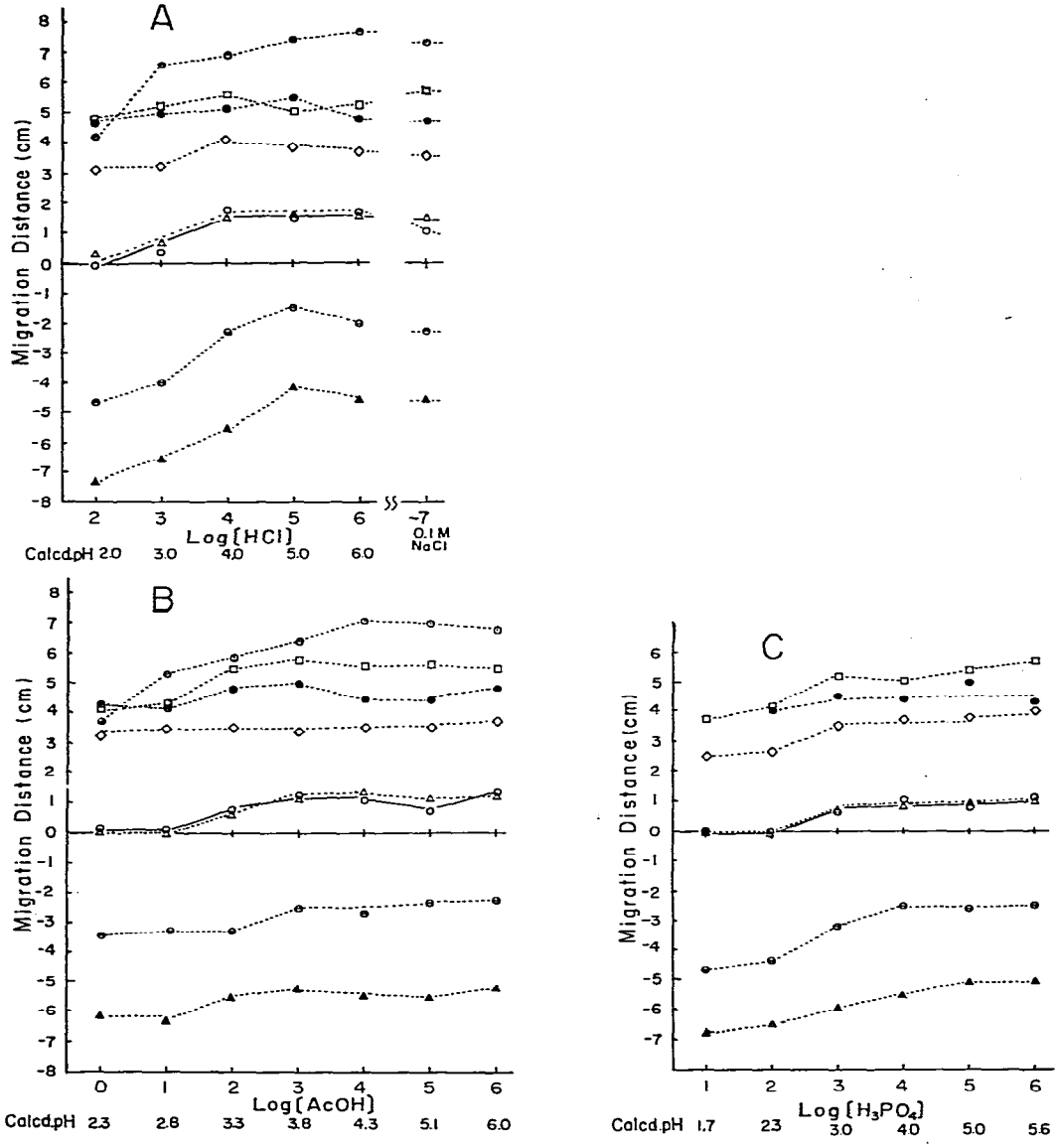

Fig. 2. Relationship between migration distance and the negative logarithm of the acid concentration. Electrophoresis conditions and symbols as in Fig. 1. Supporting solution: A, HCl–NaCl; B, AcOH–NaCl; C, H_3PO_4–NaCl.

adenosine, but the changes in the distances differed with the components of the supporting solutions. In the acetate buffer solution, the change was observed at pH 4–5 and in the phosphate buffer at pH 5–6. Such electrophoretic behaviour differs from that of normal primary amines, and we therefore studied the effect of the concentration of acid on the migration distance in solutions of which the ionic strength was adjusted to 0.1 with 0.1 *M* NaCl. The results are plotted in Fig. 2, in which the calculated pH values of the supporting solutions, together with the concentration of acids, are shown. At higher concentrations of acid ($\geqslant 10^{-2}$ *M*), AMP and cAMP mostly did not migrate, but they began to move with decreasing concentration of acid, and the migration distances of ADP and ATP became larger and attained a constant value, respectively. On the other hand, the migration distances of adenine and adenosine decreased with decreasing concentration of acid until a constant value was reached. However, the distances migrated by adenine and adenosine were never zero, indicating that part of the adenine and adenosine remained protonated, even in near neutral medium. Comparison of Fig. 2 with Fig. 1 shows that the migration distances of adenine and adenosine (Fig. 2) never decreased to *ca.* 0.7 cm (as in Fig. 1) and that all the migration distances of nucleotides (Fig. 2) are smaller than those shown in Fig. 1 for the same pH. These facts show that the ratio of protonated adenine or adenine moieties in the nucleosides or nucleotides to non-protonated species is larger for the graphs in Fig. 2 than for those in Fig. 1.

The effects of a basic salt are shown in Fig. 3. At higher concentrations of the salt, the ratio of protonated to non-protonated adenine or adenine moiety is lower, increases with decreasing concentration of the salt, and reaches a constant value. This tendency is similar to that observed in acid media and suggests that deprotonation is not complete unless the buffering capacity of the supporting solution exceeds a limiting value.

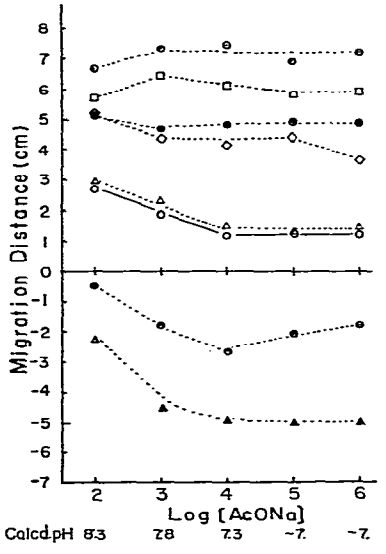

Fig. 3. Relationship between migration distance and the negative logarithm of the concentration of basic salt. Electrophoresis conditions and symbols as in Fig. 1. Supporting solution, AcONa–NaCl.

In order to remove most of the protons from the adenine or adenine moiety, a proton acceptor (such as AcO^- or $H_2PO_4^-$) must be present in a concentration higher than a limiting value, and, in addition, the supporting solution must have an adequate pH value. This means that complete deprotonation of adenine, adenosine or an adenosine nucleotide will sometimes not occur, even if the pH of the solution is greater than the so-called pK_a of adenine or the adenine moiety (pK_a *ca.* 4). If this is true, then part of the adenosine nucleotides will exist as "zwitterions" in the usual physiological solutions or in a cell solutions. Thus, the role of the "zwitterions" of nucleotides as well as that of anions should be considered in studies on biochemical reactions.

REFERENCES

1 R. Phillips, *Chem. Rev.*, 66 (1966) 501.
2 R. M. Izatt, J. J. Christensen and J. H. Rytting, *Chem. Rev.*, 71 (1971) 439.
3 G. E. Cheney, H. Freiser and Q. Fernando, *J. Amer. Chem. Soc.*, 81 (1959) 2611.
4 S. Lewin and N. W. Tann, *J. Chem. Soc.*, (1962) 1466.
5 R. F. Beers and R. F. Steiner, *Nature (London)*, 179 (1957) 1076.
6 S. Lewin, *J. Chem. Soc.*, (1964) 792.
7 B. Pullman, *J. Chem. Soc.*, (1959) 1621.
8 W. Cochran, *Acta Crystallogr.*, 4 (1951) 8.
9 G. Zubay, *Biochim. Biophys. Acta*, 28 (1958) 644.
10 J. J. Christensen and R. M. Izatt, *J. Phys. Chem.*, 66 (1962) 1030.
11 C. D. Jardetzky and O. Jardetzky, *J. Amer. Chem. Soc.*, 82 (1960) 222.
12 Y. Kiso, M. Kobayashi, Y. Kitaoka, K. Kawamoto and J. Takada, *J. Chromatogr.*, 33 (1968) 561.
13 M. Morales-Vallarta and M. Castañeda, *J. Chromatogr.*, 68 (1972) 298.
14 Y. Kiso, M. Kobayashi and Y. Kitaoka, in M. Halmann (Editor), *Analytical Chemistry of Phosphorus Compounds*, Wiley, New York, 1972, p. 105.
15 Y. Kitaoka, *J. Chromatogr.*, 111 (1975) 206.