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Dilution effect of carbonate ion on isotachophoretic zones observed with scanning UV photometric detection

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ABSTRACT

A problem in the isotachophoretic analysis of anions at high pH is the so-called 'dilution effect' originating from aerial carbon dioxide dissolved in the terminating electrolyte. Elongation of isotachophoretic zones due to this dilution effect was studied with a scanning UV photometric detector, varying the carbonate concentration in the terminating electrolyte from 0 to 3 m*M*. The zone of a component with a smaller effective mobility than that of carbonate ion was elongated in proportion to the carbonate concentration, and the present system was still isotachophoretic, although it had two criteria of migration velocity. A practical example of the dilution effect in protein analysis at high pH using a model mixture of globulin, transferin and albumin is presented.

INTRODUCTION

In the isotachophoretic separations of anions, a problem encountered at relatively high pH (> 7) is disturbance due to carbonate ions from the terminating electrolyte solution. The carbonate ions originate from aerial carbon dioxide dissolved in water, and cause the elongation of sample zones and a decrease in the step heights in isotachopherograms due to the dilution of the sample (dilution effect). The carbonate zone can be suppressed to some extent by adding barium hydroxide to the terminating electrolyte [1]. This conventional technique may sometimes cause unwanted complex formation and blocking of the separation tube due to the precipitation of barium carbonate. As discussed by Verheggen *et al.* [2], the use of an isotachophoretic analyser with a complete closed system is useful for preventing the dilution effect; doubly distilled water is used to fill the electrode compartments, which are separated from the separation compartment by a semi-permeable membrane placed at the boundary between the electrolyte compartment and the separation compartment. The system was especially useful when the operational electrolytes were prepared in a closed system under a nitrogen atmosphere [2].

Fidler *et al.* [3] discussed several situations that may occur when the operational electrolyte system contains impurities. The dilution effect is one of these situations and is the most frequently encountered effect in the analysis of anions at high pH. Although a method for coping with the dilution effect is available [2], the theoretical approach [3] based on the simulation of the transient state should be confirmed by experiment and the feature of this unusual migration should be studied in detail from a dynamic viewpoint.

In this work, the mechanism of the elution and the degree of the dilution effect in relation to the concentration of carbonate in the terminating electrolyte was investigated by the use of a scanning UV photometric zone detection system [4], which permitted the observation of the transient state of electrophoretic migration. The dynamic feature of the elution is briefly discussed and a practical sample of the dilution effect in protein analysis at high pH is presented.

EXPERIMENTAL

Apparatus

The scanning UV photometric detection system used was equipped with a scanning assembly of a UV lamp and a detector. The assembly was driven by a linear head with a stepping motor [4]. The separation tube, a fused-silica capillary (I.D. 0.53 mm, O.D. 0.66 mm), was scanned over a length of 32 cm. A single scanning cycle took 7.027 s and 5333 photometric signals (a UV position spectrum) were acquired. The practical resolution was 0.15 mm and the detection limit of picric acid was 20 pmol. The migration current was 50 μA throughout. No special care was taken to prevent carbon dioxide from the air. The electrolyte compartments were conventionally filled with each electrolyte.

Samples and electrolyte system

The model sample used to study the dilution effect was a mixture of 4,5-dihydroxy-3-(*p*-sulphophenylazo)-2,7-naphthalenedisulphonic acid (SPADNS) and picric acid from Tokyo Kasei Kogyo (Tokyo, Japan). The dilution effect in the actual analysis was demonstrated using a model mixture of globulin, transferin and albumin. Transferin (molecular weight (MW) 70 000–90 000, p*I* 5.8) and albumin (MW 66 000, p*I* 4.7) were obtained from Midori Juji (Tokyo, Japan) and globulin (MW) 90 000–150 000, p*I* 6.3–8.4) from Sigma (St. Louis, MO, USA). The other chemicals for the electrolyte system were obtained from Tokyo Kasei Kogyo in the purest form available.

The leading electrolyte was 10 m*M* hydrochloric acid buffered by adding 2-amino-2-methyl-1,3-propanediol (ammediol, pH of leading electrolyte $\mathrm{pH_L} = 8.6$). Hydroxypropylcellulose (HPC) was added to the leading electrolyte (0.2%). The terminator was 10 m*M* β-alanine. In order to clarify the dilution effect, sodium carbonate was added to the terminating electrolyte in the concentration range 1–4 m*M*. Water was distilled once after being processed by an anion exchanger.

In the analysis of globulin, transferin and albumin, the terminating electrolyte (10 m*M* β-alanine) was prepared in a conventional manner: the pH was adjusted to 10 by adding barium hydroxide and sodium carbonate was not added. The pH measurements were carried using a Horiba (Tokyo, Japan) Model F7ss expanded pH meter. Data processing was carried out with an NEC PC9801VX microcomputer.

RESULTS AND DISCUSSION

Fig. 1A illustrates the normal separation diagram of a two-component mixture of SPADNS (S) and picric acid (P). After the constituents had been separated, the zone length of the separated samples did not change, as shown in Fig. 1A, *i.e.*, all of the boundaries migrate at constant speed. Fig. 1B shows the migration model of SPADNS and picrate zones when carbonate ions were contained only in the terminating zone (CT) before starting electrophoretic migration. When SPADNS and picric acid are separated at $pH_L = 8.6$, the effective mobility of carbonate ions is higher than that of picrate and smaller than that of SPADNS. At this pH, most of carbonic acid ($pK_a = 6.352$ and 10.329, mobility $= 46.1 \times 10^{-5}$ and 71.8×10^{-5} cm^2 V^{-1} s^{-1}) is in the form of hydrogencarbonate ion, HCO_3^-. The abundance of carbonate ion, CO_3^{2-}, is negligibly small. The simulated effective mobility was 42.7×10^{-5} cm^2 V^{-1} s^{-1} at $pH_L = 8.6$. After the migration had started, the carbonate ions were eluted from the terminating zone (zone electrophoresis). It forms a pure carbonate zone (C) behind the SPADNS zone (S) after penetrating the picrate zone (CP).

As indicated by Fig. 1B, the SPADNS zone (S) will never be influenced by the carbonate ions from the terminating zone, but the picrate zone will be seriously affected: the zone length will be elongated and the conductivity of the zone will be decreased by the dilution due to carbonate ions. The extent of the dilution effect may depend on the concentration of carbonate ions in the terminating electrolyte, as the concentration in the picrate zone depends on it.

Zone elongation effect

Fig. 2 shows the observed transient isotachopherograms of an equimolar mixture of SPADNS and picric acid at $pH_L = 8.6$. In all experiments, the amount of sample was kept constant (total amount 20 nmol). On the other hand, the concentration of the added carbonate ions in the terminating electrolyte was varied in the range

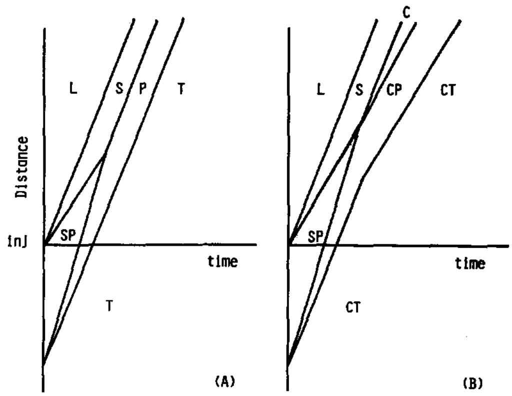

Fig. 1. (A) Normal separation process of two-component system of SPADNS and picrate ions. (B) Separation process with elution of carbonate from the terminating electrolyte. L = leading zone; S = SPADNS zone; P = picrate zone; T = terminating zone; SP = mixed zone of SPADNS and picrate ions; CP = mixed zone of carbonate and picrate ions; CT = terminating zone containing carbonate ion; inj = sample injection point.

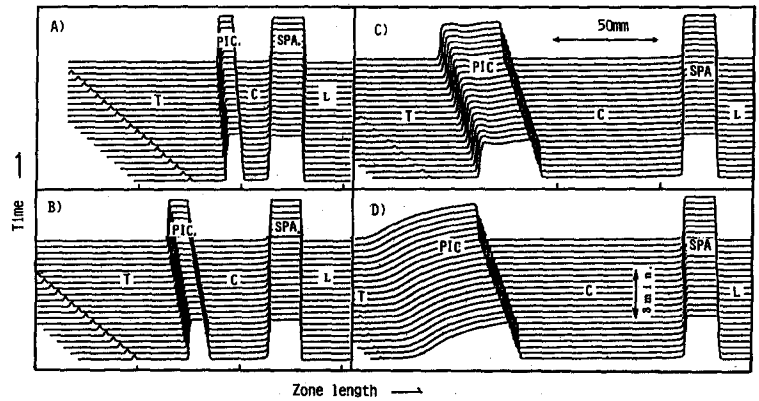

Fig. 2. Observed transient isotachopherograms of SPADNS (SPA) and picric acid (PIC) by the use of the scanning UV photometric zone detection system. L = leading zone; C = carbonate zone; T = terminating zone. The concentration of carbonate ion added to the terminating electrolyte was (A) 0, (B) 1, (C) 2 and (D) 3 m*M*. Leading electrolyte: 10 m*M* HCl–ammediol (pH_L = 8.6). Terminating electrolyte: 10 m*M* β-alanine. The migation current was 50 μA.

of 0–3 m*M*. In Fig. 2, the boundaries between the leading and SPADNS zones were rearranged at the same abscissa position to demonstrate the change in the individual zone lengths at the transient state. It is apparent from Fig. 2 that the length of the picrate and carbonate zones increased with increasing concentration of carbonate ion in the terminating electrolyte, although the increment was not linearly related to concentration.

Fig. 3 shows the concentration dependence of the ratio of zone length of SPADNS and picrate ions. Although the change in the zone length was negligible when the carbonate concentration was low, serious disturbances were observed at high concentrations. According to our simulation, the concentration of the pure

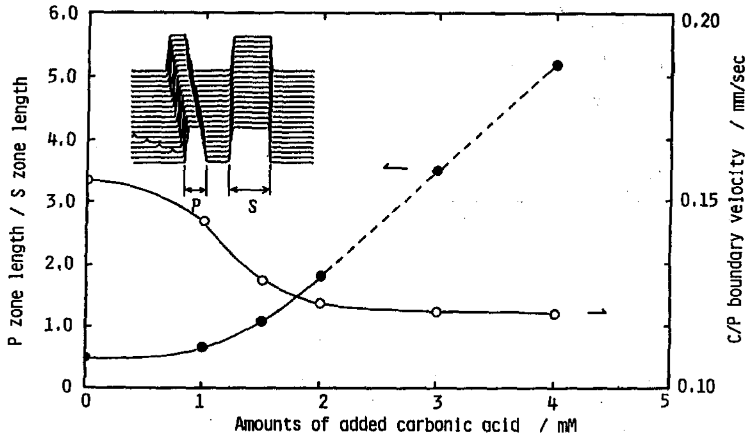

Fig. 3. Observed elongation of the zone length of picrate and the observed velocity of the boundary between carbonate and picrate zones. Operational conditions as in Fig. 2.

picrate zone was 6.8 m*M*. The concentration of picrate coexisting with carbonate ion was estimated from the value and the concentration ratio in Fig. 3 as 5.7, 3.1 and 1.8 m*M* for the terminating electrolyte containing 1, 1.5 and 2 m*M* carbonate ion, respectively.

Verheggen *et al.* [2] suggested that the calibration line was distorted owing to the dilution effect. It should be noted that the degree of the distortion depends on the carbonate concentration in the terminating zone.

Migration mode

Table I summarizes the evaluated boundary velocity. As estimated from the constant zone length in Fig. 2, the velocity of the boundary of the leading and SPADNS zone (L/S) and that of the SPADNS and carbonate zone (S/C) agreed with each other, suggesting that SPADNS migrated isotachophoretically and reached the steady state without any disturbance by the eluted carbonate ions. An interesting observation was that the boundary velocity of the carbonate and picrate zone (C/P) and that of the picrate and terminating zone (P/T) agreed with each other, within experimental error. This suggested that the zone length of the picrate zone was also constant during detection, in spite of the significant dilution effect. Hence the picrate zone also reached the steady state, although the boundary velocity was smaller than that of SPADNS. The migration in the present system is not isotachophoresis, in the sense that the criteria of migration velocity should be unique in isotachophoresis. However, it should be noted that the present system was still isotachophoretic in the sense that all sample zones reached the steady state, although it had two criteria of migration velocity, namely L/S and C/P boundaries.

As shown in Fig. 3, the velocity of the C/P boundary decreased and reached a constant value. Moreover, the boundary between the carbonate and the terminating zones was obscure at high concentrations of the carbonate ion. This might be caused by the similar potential gradient of the zones owing to the addition of carbonate. The simulated R_E values of the pure carbonate zone and the pure picrate zone were 1.73

TABLE I

BOUNDARY VELOCITIES OBSERVED WITH THE USE OF THE SCANNING UV DETECTION SYSTEM

L = Leading zone (Cl^-); S = SPADNS zone; C = carbonate zone; P = picrate zone; T = terminating zone [(β-alanine)$^+$].

Amount of carbonate (m*M*)[a]	Boundary velocity × 10^2 (cm s^{-1})			
	L/S	S/C	C/P	P/T
0	1.504	1.498	1.373	1.371
1	1.547	1.544	1.311	1.303
1.5	1.519	1.520	1.145	1.136
2	1.529	1.533	1.098	1.080
3	1.476	1.476	1.040	–
4	1.522	1.525	1.071	–

[a] Added to the terminating electrolyte.

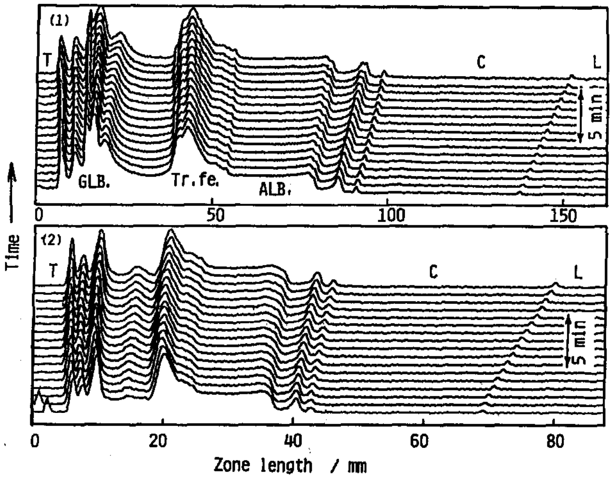

Fig. 4. Observed transient isotachopherograms of albumin (ALB), transferin (Tr.fe.) and globulin (GLB) by the use of the scanning UV photometric zone detection system. Total amount: (1) 553 μg and (2) 267 μg. The constituent ratio was 5:1:10. L = leading zone; C = carbonate zone; T = terminating zone. No carbonate ion was added to the terminating electrolyte (10 m*M* β-alanine; pH adjusted to 10 by adding barium hydroxide. The migration current was 50 μA.

and 2.61, respectively. The R_E value of the picrate zone with eluted carbonate ions may decrease to 1.73 with increase in the amount of eluted carbonate ion.

Migration of proteins with elution of carbonate

In the analysis of protein anions and amino acid anions, the pH of the leading electrolyte is usually chosen in the range *ca.* 8–10. Under such pH conditions, the carbonate zone migrates before the sample zones. Fig. 4 shows the observed transient isotachopherograms of a mixture of albumin, transferin and globulin (5:1:10, w/w/w). Apparently a zone of eluted carbonate was observed. Although the 553-μg mixture did not reach the steady state during the observation, it seemed that the 267-μg sample reached the steady state in spite of the dilution effect.

In a conventional isotachophoretic analyser, a potential gradient detector and a UV detector are used simultaneously. If the dilution effect exists, the zone lengths found in the recorder traces do not agree with each other even if the separation is complete. The reason is obvious from the above discussion. In protein analysis the patterns of the isotachopherograms were compared with each other. However, it must be noted that the difference, if it exists, may be caused by the dilution effect. Also, it should be noted that such an unfavourable effect is not restricted to carbonate ion, but can be caused by any mobile impurity in the terminating electrolyte, as generally discussed in ref. 3, if the abundance is significant.

If the leading electrolyte contains carbonate ion, a similar dilution effect will be observed for the sample zones sandwiched by the carbonate zone and the leading zone. However, the magnitude of the effect will be much smaller than that caused by the carbonate ions in the terminating electrolyte if the carbonate concentration in both electrolyte solutions is the same.

REFERENCES

1 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis*, Elsevier, Amsterdam, 1976.
2 Th. P. E. M. Verheggen, J. C. Reijenga and F. M. Everaerts, *J. Chromatogr.*, 260 (1983) 471.
3 Z. Fidler, V. Fidler and J. Vacik, *J. Chromatogr.*, 320 (1985) 175.
4 T. Hirokawa, Y. Yokota and Y. Kiso, *J. Chromatogr.*, 538 (1991) 403.