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ISOTACHOPHORETIC DETERMINATION OF MOBILITY AND pK_a BY MEANS OF COMPUTER SIMULATION

IV. EVALUATION OF m_0 AND pK_a OF TWENTY-SIX AMINO ACIDS AND ASSESSMENT OF THE SEPARABILITY

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SUMMARY

Isotachophoretic qualitative indices, R_E, of twenty-six amino acids were measured at several pH_L values in the range of 8.6–9.6. The absolute mobility, m_0, and pK_a values were evaluated by the use of a least-squares method utilizing a simulation of the isotachophoretic steady state. The pK_a values were in good agreement with values cited in the literature. The R_E values simulated using the evaluated constants were in good agreement with R_E values converted from step heights observed previously. By comparing the previously observed separation behaviour of amino acids with their simulated effective mobilities, it is concluded that when the effective mobility of samples differs by *ca.* $1 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} at the steady state, *ca.* 10-nmol samples can be separated by the use of a 80 cm × 0.5 mm I.D. tube. The simulated effective mobilities of twenty-two amino acids were tabulated to assess the separability under some typical electrolyte conditions.

INTRODUCTION

As reported previously[1,2], the isotachophoretically steady state can be treated theoretically and isotachopherograms can be simulated, when the absolute mobilities, m_0, and the thermodynamic acid dissociation constants, pK_a, of the samples and electrolyte constituents are available. This technique can be used for estimation of the optimum separation condition[3,4]. A microcomputer program, SIPS (simulation of isotachophoretic separation), based on a data base including the m_0 and pK_a values of *ca.* 500 ionic species, has been developed[5] and can be used for the practical purpose stated above.

However, in our data base many important samples such as amino acids are not included due to the lack of the physico-chemical constants, especially m_0. This

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is due to the fact that the conventional conductivity method cannot be applied simply to the measurement of m_0 for amphoteric electrolytes such as neutral amino acids. Therefore, among the natural amino acids, the m_0 values of only two acidic amino acids, Asp^- and Glu^-, have been reported. In contrast, the pK_a values of amino acids have been extensively studied, although the thermodynamic values obtained are not always available. In isotachophoresis the ionic strengths of the leading, sample and terminating zones are always different. Therefore, in simulations, the thermodynamic pK_a values must be corrected for the ionic strength by using the Debye–Hückel equation.

The m_0 and pK_a values can be evaluated as reported[6,7] by use of the least-squares method to fit the observed potential gradient ratios of the sample zones separated isotachophoretically. To increase the utility of the SIPS program, in the present study, the m_0 and pK_a values of twenty-six amino acid were evaluated and then added to our data base. Further, the effective mobilities of twenty-two amino acids under several typical electrolyte conditions were simulated using the valuated values to clarify the limitation of separability, taking into account the previous systematic experimental studies by Kopwillem and Lundin[8] and Everaerts *et al.*[1].

EXPERIMENTAL

The amino acids treated were DL-Ala, β-Ala, α-amino-*n*-butyric acid (DL-α-Amin), L-Arg, L-Asn, L-Cys, L-Glu, L-Gln, Gly, L-His, L-Hyp, 3,5-I_2-L-Tyr, DL-Ile, L-Leu, L-Lys, DL-Met, L-Orn, L-Phe, L-Pro, DL-Ser, Tau, DL-Thr, DL-Trp, L-Tyr and DL-Val (guaranteed grade, Tokyo Kasei Co.). Sample solutions (3–10 m*M*) were prepared by dissolving these amino acids in distilled water or diluted sodium hydroxide solution (Cys). CysH was not considered since it is converted in to Cys in the alkaline solution.

Most of the treated amino acids are neutral amino acids and the pK_a values of their cationic forms are in the range of 1.5–3.6 and those of their anionic forms are 9.5–10.5. The cationic amino acids are not very mobile at pH = *ca.* 3.5, the lower limit of isotachophoretically "safe" pH in cationic analysis*, except for some basic amino acids, *e.g.*, Arg, Lys and Orn, and a neutral amino acid β-Ala with relatively large pK_a (3.6). Therefore qualitative indices, R_E, of the anions of neutral and acidic amino acids were measured in the pH_L (pH of the leading electrolyte) range of 8.6–9.6. The R_E is the ratio of the potential gradient, E (V cm^{-1}), of a sample zone, E_S, to that of the leading zone, E_L, which corresponds to the ratio of the effective mobility of the leading ion $\bar{m}_L$, to that of the sample ion, $\bar{m}_S$, *i.e.*, $R_E = E_S/E_L = \bar{m}_L/\bar{m}_S$.

For the R_E measurements of neutral and acidic amino acids the electrolyte systems used were as follows (Nos. 1–6 in Table II): the leading electrolytes were 10 m*M* hydrochloric acid solutions and the pH_L was adjusted to 8.64, 9.00 and 9.40 by adding 2-amino-2-methyl-1,3-propanediol (amediol) and to 9.03, 9.3 and 9.62 by

* According to our simulation, when a leading electrolyte of 10 m*M* potassium hydroxide buffered by formic acid (pH of leading electrolyte, pH_L = 3.5) is used, model cations of $m_0 > ca.\ 45 \cdot 10^{-5}$ ($pK_a > 6$) can migrate isotachophoretically. For the others, H^+ migrates instead. A neutral amino acid β-Ala, for which the pK_1 is the largest of those presently treated, the isotachophoretically steady state is not achieved when formic acid is used as the buffer. When glutamic acid is used as the buffer, the limiting pH for the analysis of β-Ala is *ca.* 4.

adding ethanolamine, respectively. The low pH limit was chosen in order that the effective mobilities would not be too small. If this were not the case, the temperature increment in the zones could not be neglected. The terminator was 10–30 m*M* β-Ala and the pH was adjusted to *ca.* 10 by adding barium hydroxide to suppress the disturbance caused by HCO_3^-. For precise measurement of R_E, the asymmetric potential of the potential gradient detection (PGD) used must be corrected[6]. Gly was used as an internal standard for this purpose, since its precise thermodynamic pK_a has been reported (9.7796 at 25°C)[9]. The m_0 value of Gly was first evaluated by isotachophoresis using similar electrolyte conditions as with the other samples. The internal standards were propionate and caproate ions. The evaluated m_0 value was $37.4 \cdot 10^{-5}$cm^2 V^{-1} s^{-1} (pK_a value fixed at 9.7796 in the least-squares method). The R_E values of Gly simulated using these constants were used for the correction of asymmetric potential under the electrolyte conditions. When Gly was unsuitable for this purpose because of mixed zone formation, Thr, Asn and β-Ala were used indirectly. Since Pro was not very mobile under these conditions, and the R_E values were large, β-Ala was used as the standard and Pro as the terminator.

For the analysis of basic amino acids, Arg, Lys and Orn, the leading electrolytes used were 10 m*M* potassium hydroxide solutions (Nos. 7–10 in Table II). The pH_L was adjusted in the range of 6.4–9.4 by adding 2-(N-morpholino)ethanesulphonic acid (pH_L = 6.43) and Phe (8.84, 9.03, 9.37). The terminator was tris(hydroxymethyl)aminomethane (Tris). The internal standards used were His and Tris and the R_E values are listed in Table II. All of the leading electrolytes contained 0.02% hydroxypropylcellulose to suppress electrode reactions and electroendosmosis.

The isotachopherograms were obtained using a Shimadzu isotachophoretic analyzer, IP-1B, equipped with PGD. The temperature was thermostatted at 25°C. The separating tube used was *ca.* 40 cm × 0.5 mm I.D. The driving current applied was 50 μA and a single experiment took *ca.* 35 min. The pH measurements were carried out using an Horiba expanded pH meter, Model F7ss.

Table I shows the m_0 and pK_a values of the electrolyte constituents used in the calculations. These values were taken mainly from the literature[10–15], but most of the

TABLE I

PHYSICO-CHEMICAL CONSTANTS USED IN THE SIMULATIONS (25°C)

m_0 = Absolute mobility (cm^2 V^{-1} s^{-1}) · 10^5; pK_a = thermodynamic acid dissociation constant; Tris = tris(hydroxymethyl)aminomethane; amediol = 2-amino-2-methyl-1,3-propanediol; MES = 2-(N-morpholino)ethanesulphonic acid; BDB = 5-bromo-2,4-dihydroxybenzoic acid.

Cation	m_0	pK_a	*Anion*	m_0	pK_a
K^+	75.72	–	Cl^-	79.08	–
Histidine	29.5*	6.042	Butyric acid	33.8	4.820
Imidazole	52.0*	7.15	MES	28.0*	6.15
Tris	29.5*	8.076	BDB	27.6*	3.0**
Amediol	32.0*	8.78		50.7*	7.60*
Ethanolamine	44.3*	9.498			

* Obtained isotachophoretically; other constants were taken from refs. 9–13.

** Assumed value.

mobilities used were determined by our isotachophoretic method. The m_0 value of amediol was changed from the previously used value of $29.5 \cdot 10^{-5}$ to $32.0 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}, taking into account the result of the conductivity measurement for the leading electrolyte. The observed conductivity of the leading electrolyte (10.02 m*M* hydrochloric acid solution, pH_L = 8.64, amediol buffer) was 0.998 mS cm^{-1} cm and the simulated value was 0.997 mS cm^{-1}. The conductivity meter used was a TOA Electronics Model CM-30ET.

Table II summarizes the leading electrolyte conditions together with the calculated concentrations, effective mobilities of the leading electrolyte constituents and the R_E values of the internal standard. Fig. 1 shows two typical isotachopherograms obtained by the use of electrolyte systems 1 and 2 in Table II.

For the data processing and the simulation, SIPS programs on SORD M223 MkIII and NEC PC9801E microcomputers and the SIPS-LSQ program on a NEC minicomputer MS120 were used. For plotting the figures, a Watanabe X-Y plotter WX4671 and a Roland DXY-980 were used.

RESULTS AND DISCUSSION

Table III summarizes the observed R_E values for all amino acids treated under the electrolyte conditions 1–10 in Table II. The R_E values, measured from the electropherograms for several completely separable combinations of the amino acids under each electrolyte condition (see Fig. 1), were the averages of at least three experimental determinations. The experimental errors were less than *ca.* ± 0.05 R_E units.

TABLE II

EXPERIMENTAL CONDITIONS FOR THE EVALUATION OF ABSOLUTE MOBILITIES AND pK_a VALUES OF AMINO ACIDS, CALCULATED CONCENTRATIONS AND EFFECTIVE MOBILITIES OF LEADING ZONE CONSTITUENTS

pH_L = pH of leading electrolyte; C_L^* = total concentration (m*M*) of leading ion; $\bar{m}_L$ = effective mobility (cm^2 V^{-1} s^{-1}) of leading ion · 10^5; $C_{B,L}^*$ = total concentration (m*M*) of buffer ion; $\bar{m}_{B,L}$ = effective mobility (cm^2 V^{-1} s^{-1}) of buffer ion · 10^5; Std(R_E) = internal standard used for correction of asymmetric potential and the corresponding R_E value. Leading ions: chloride (systems 1–6); potassium (systems 7–10).

System	*Buffer*	pH_L	C_L^*	$\bar{m}_L$	$C_{B,L}^*$	$\bar{m}_{B,L}$	*Std(R_E)*
Anionic analysis							
1	Am	8.64	10.02	74.69	16.57	17.28	Gly (6.12)
2	Am	9.00	10.02	74.69	25.04	11.45	Gly (5.02)
3	Am	9.40	10.02	74.69	47.79	6.004	Gly (3.89)
4	EA	9.03	10.02	74.69	13.11	31.08	Gly (4.01)
5	EA	9.30	10.02	74.69	15.78	25.85	Gly (3.66)
6	EA	9.62	10.02	74.68	22.07	18.51	Gly (3.20)
Cationic analysis							
7	MES	6.43	9.85	71.43	14.51	16.75	His (4.28)
8	Phe	8.84	10.21	71.36	30.82	7.988	Tris (6.23)
9	Phe	9.03	10.21	71.36	23.51	10.47	Tris (7.08)
10	Phe	9.37	10.21	71.36	16.26	15.12	Tris (8.64)

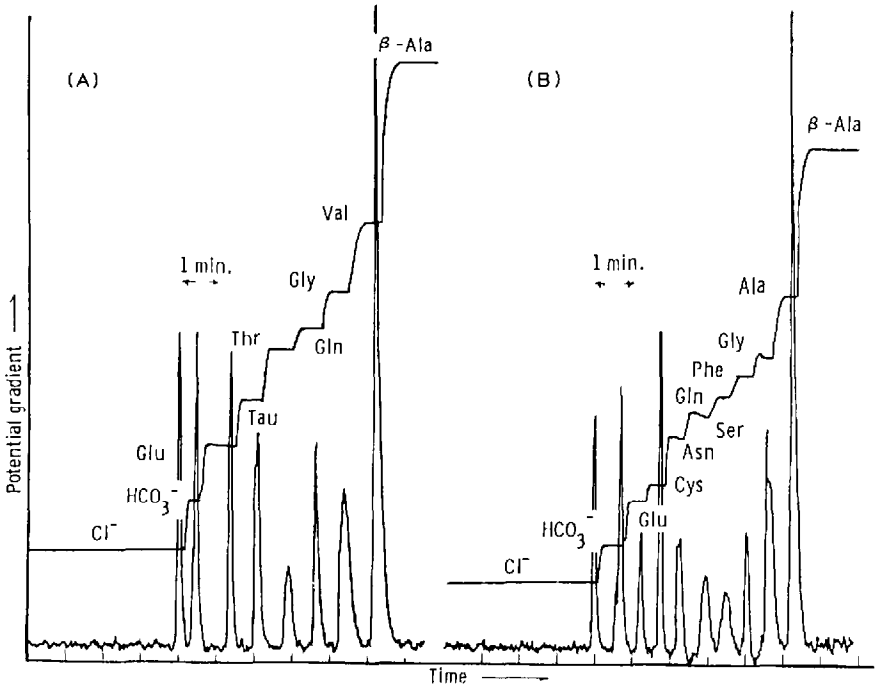

Fig. 1. The observed isotachopherograms for Glu, Tau, Thr, Gln, Gly and Val at pH_L = 9.00 buffered by amediol (A), and for Glu, Cys, Asn, Ser, Gln, Phe, Gly and Ala at pH_L = 6.84 (B). The leading ion was 10 m*M* chloride and the terminator was 30 m*M* β-Ala (pH = *ca.* 10 by adding barium hydroxide). The sample amounts were *ca.* 5–10 nmol and migration current was 50 μA.

The black circles in Figs. 2 and 3 show the pH_L dependence of the observed R_E values of the anionic amino acids in the pH_L range of 8.6–9.6 (buffers: amediol and ethanolamine). Using these R_E values, the m_0 and pK_a values were determined by the least-squares method. The curves in Figs. 2 and 3 were plotted using such values. The discontinuities in the curves are due to the different buffers. Even if the pH_L value is the same, the pH of the separated sample zones, and consequently the effective mobilities and R_E values, will depend on the mobility and pK_a of the buffers used. Table IV shows the observed and the best-fitted R_E values, the effective mobilities and the concentrations of the zone constituents, Ala, Glu, Gln, Leu, Thr, Val and Lys. The observed and the simulated R_E values were in good agreement, the mean error being in the range of 0.46(Glu)–1.43%(Tyr). The evaluated m_0 and pK_a were listed in Table V together with the pK_a values obtained by previous methods. In the least-squares method, several pK_a values were fixed at the literature values as shown in Table V, taking into account the pH range used in the R_E measurement. If the R_E values for in the completely dissociated state could be measured, m_0 and pK_a could be evaluated independently. This is not the case, since the pK_a values of the anionic forms of the samples are large and isotachophoretic equilibria could not be achieved at the completely dissociated state. Concerning 3,5-I_2-Tyr, the reported pK_a values of pK_2 = 6.48 and pK_3 = 7.12 were used as the initial values in the least-squares method; however, our best-fitted pK_3 = 9.69 was significantly different

TABLE III

OBSERVED R_E VALUES OF TWENTY-FIVE AMINO ACIDS

Electrolyte systems numbered as in Table II. R_E = Ratio of potential gradients, E_S/E_L.

Sample	*Electrolyte system and pH_L*					
	1 *8.64*	*2* *9.00*	*3* *9.40*	*4* *9.03*	*5* *9.30*	*6* *9.62*
Asp	2.59	2.41	2.20	2.37	2.24	2.03
Glu	2.84	2.61	2.31	2.52	2.35	2.11
Cys	3.21	2.72	2.28	2.50	2.33	2.11
3,5-I_2-Tyr	3.49	–	2.79	2.99	2.75	2.46
Tau	3.94	3.30	2.76	2.97	2.77	2.52
Asn	4.27	3.63	3.10	3.34	3.14	2.88
Ser	4.77	3.99	3.27	3.46	3.26	2.90
Thr	4.81	4.06	3.38	3.63	3.38	3.04
Gln	5.21	4.42	3.66	3.90	3.65	3.28
Met	5.58	4.65	3.76	4.00	3.77	3.36
Phe	5.70	4.78	3.91	4.28	3.95	3.52
His	5.64	4.78	3.87	4.14	3.88	3.46
Tyr	5.92	4.71	3.88	4.20	3.69	3.17
Gly*	6.12	5.02	3.89	4.02	3.66	3.20
Val	7.47	6.07	4.67	5.01	4.51	3.92
Ala	7.49	5.94	4.69	4.80	4.41	3.82
Trp	7.59	6.11	4.91	5.23	4.73	4.12
Hyp	7.74	6.19	4.78	4.93	4.54	3.94
α-Amin	7.67	6.16	4.80	4.88	4.56	3.93
Ile	8.22	6.64	5.11	5.43	4.94	4.25
Leu	8.00	6.57	5.12	5.36	4.90	4.27
β-Ala	10.64	8.45	6.38	6.18	5.65	4.86
	7 *6.43*	*8* *8.84*	*9* *9.03*	*10* *9.37*		
Arg	2.98	4.01	4.27	5.02		
Lys	3.00	3.77	3.94	4.56		
Orn	2.81	4.20	4.55	5.49		

* Internal standard; simulated value.

from the reported value. For 3,5-I_2-Tyr, Tyr and Cys, the pH_L conditions were unsuitable for the independent evaluation of two pK_a values and two m_0 values; we therefore assumed $m_2 = 2m_1$ in order to decrease unknown constants in the least-squares method. Except for the pK_a values obtained by means of this assumption, the evaluated pK_a agreed well with the values reported previously. Fig. 4 shows the pH dependence of the effective mobility of several amino acids, Asp, Glu, Cys, Tau, Gly, Ala, β-Ala, Leu, His, Orn, Arg and Lys. The curves were plotted using the evaluated absolute mobility and thermodynamic constants and are not for the isotachophoretic steady state. Fig. 5 shows the simulated isotachopherograms for Glu, Cys, Asn, Ser, Gln, Phe, Gly and Ala at $pH_L = 8.64$ and for Glu, Tau, Thr, Gln, Gly and Val at $pH_L = 9.00$ (amediol buffer). The terminator is β-Ala. The simulated and the observed isotachopherogram (Fig. 1) are in good agreement.

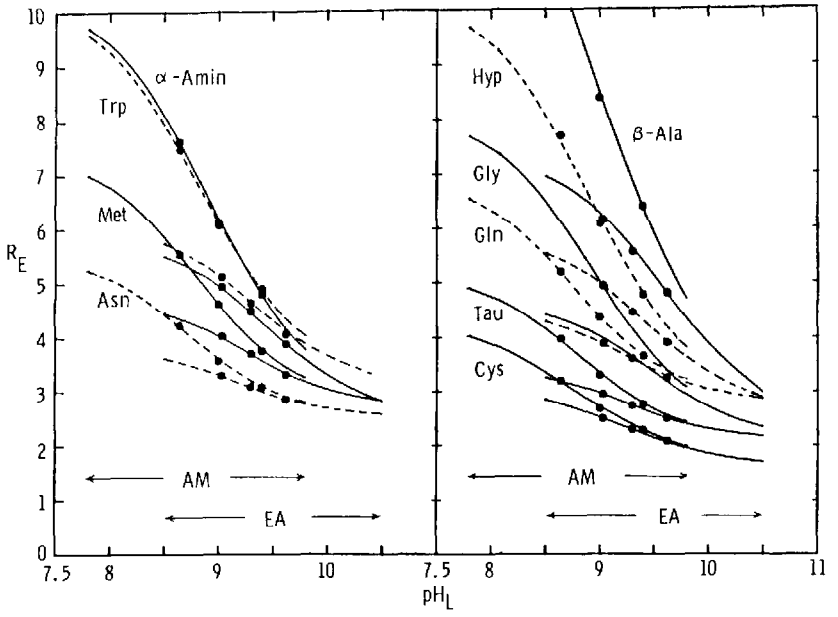

Fig. 2. The observed R_E values of Asn, Met, Trp, Amin, Cys, Tau, Gln, Hyp and β-Ala. The leading ion was 10 mM chloride. The curves were plotted using the best-fitted mobility and pK_a. The pH_L dependence of the R_E values of the internal standard, Gly, is also shown. AM and EA denote the buffers used, amediol and ethanolamine.

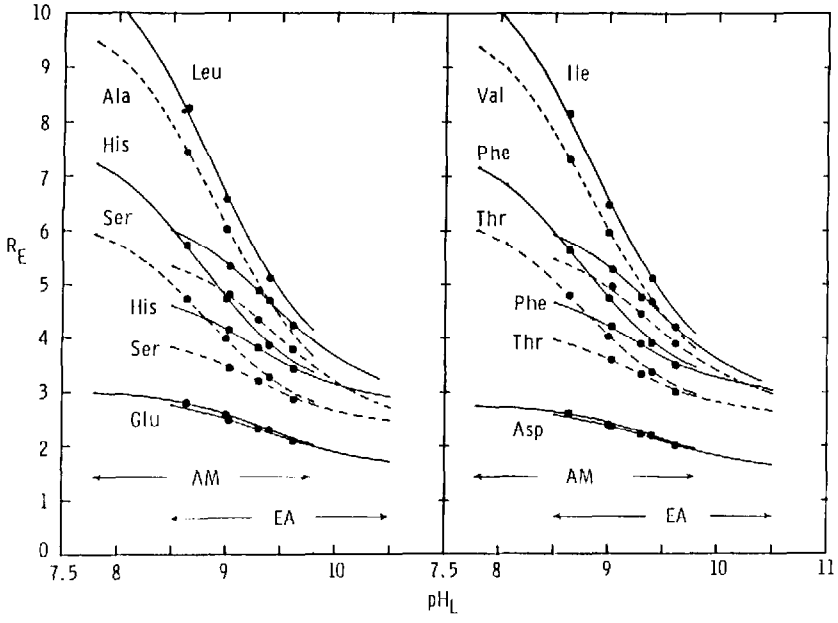

Fig. 3. The observed R_E values of Glu, Ser, His, Ala, Leu, Asp, Thr, Phe, Val and Ile. The leading ion was 10 mM chloride. Other details as in Fig. 2.

To confirm the evaluated m_0 and pK_a values, the step heights of eighteen amino acids observed by Everaerts *et al.*[1] using a conductometric detector were converted into R_E and these values were compared with the simulated R_E values. The amino

TABLE IV

OBSERVED AND SIMULATED R_E VALUES OF SEVEN AMINO ACIDS, EFFECTIVE MOBILITIES AND CONCENTRATIONS OF ZONE CONSTITUENTS (25°C)

System = electrolyte system (see Table II); dev./% = percentage deviation; $\bar{m}_S$ = effective mobility ($cm^2\ V^{-1}\ s^{-1}$) of sample ion · 10^5; pH_S = pH of sample zone; C_S^t = total concentration (mM) of sample; $C_{B,S}^t$ = total concentration (mM) of buffer ion; $\bar{m}_{B,S}$ = effective mobility ($cm^2\ V^{-1}\ s^{-1}$) of buffer ion · 10^5; I = ionic strength · 10^3.

System No.	R_E		*dev./%*	$\bar{m}_S$	pH_S	C_S^t	$C_{B,S}^t$	$\bar{m}_{B,S}$	I
	Obs.	*Calc.*							
Ala									
1	7.49	7.44	0.69	10.04	9.525	6.573	13.78	4.824	2.195
2	5.94	6.07	−2.17	12.31	9.666	6.533	22.28	3.643	2.695
3	4.69	4.67	0.44	15.99	9.883	6.440	45.10	2.316	3.497
4	4.80	4.79	0.12	15.58	9.858	6.006	9.995	13.36	3.170
5	4.41	4.36	1.14	17.13	9.950	5.960	12.70	11.51	3.478
6	3.82	3.79	0.68	19.68	10.11	5.852	19.10	8.704	3.970
	Mean error		0.87						
Glu									
1	2.84	2.83	0.32	26.39	8.862	5.793	13.39	13.82	6.919
2	2.61	2.62	−0.51	28.47	9.205	5.433	22.26	8.453	7.538
3	2.31	2.32	−0.57	32.15	9.577	4.972	45.53	4.310	8.617
4	2.52	2.51	0.30	29.73	9.338	4.932	10.05	25.18	7.359
5	2.35	2.33	0.77	32.03	9.557	4.707	13.06	20.07	8.042
6	2.11	2.12	−0.27	35.30	9.824	4.451	19.73	13.98	8.997
	Mean error		0.46						
Gln									
1	5.21	5.22	−0.12	14.32	9.259	6.408	13.20	7.814	3.452
2	4.42	4.40	0.42	16.97	9.442	6.375	21.69	5.619	4.102
3	3.66	3.67	−0.36	20.33	9.720	6.293	44.51	3.253	4.907
4	3.90	3.91	−0.15	19.12	9.606	5.819	9.301	19.04	4.237
5	3.65	3.62	0.81	20.63	9.743	5.774	12.01	15.84	4.561
6	3.28	3.31	−0.91	22.56	9.966	5.665	18.42	11.17	4.943
	Mean error		0.46						
Leu									
1	8.00	8.03	−0.43	9.296	9.486	5.886	13.09	5.198	2.247
2	6.57	6.56	0.11	11.38	9.640	5.834	21.59	3.836	2.752
3	5.12	5.12	−0.08	14.58	9.876	5.715	44.45	2.355	3.504
4	5.36	5.34	0.36	13.98	9.829	5.258	9.284	13.98	3.079
5	4.90	4.86	0.88	15.38	9.935	5.195	12.02	11.79	3.370
6	4.27	4.26	0.18	17.52	10.12	5.051	18.49	8.586	3.785
	Mean error		0.34						

TABLE IV *(continued)*

System No.	R_E		dev./%	$\bar{m}_S$	pH_S	C_S^*	$C_{B,S}^*$	$\bar{m}_{B,S}$	I
	Obs.	*Calc.*							
Thr									
1	4.81	4.81	0.08	15.54	9.241	6.682	13.45	8.043	3.625
2	4.06	4.07	−0.12	18.37	9.424	6.654	21.93	5.814	4.300
3	3.38	3.40	−0.56	21.97	9.700	6.582	44.74	3.385	5.141
4	3.63	3.61	0.43	20.66	9.586	6.116	9.550	19.51	4.462
5	3.38	3.35	0.74	22.26	9.721	6.077	12.25	16.32	4.801
6	3.04	3.07	−1.00	24.32	9.943	5.981	18.64	11.62	5.211
	Mean error		0.49						
Val									
1	7.47	7.43	0.55	10.06	9.470	6.179	13.31	5.362	2.361
2	6.07	6.08	−0.18	12.28	9.622	6.134	21.81	3.979	2.887
3	4.67	4.75	−1.79	15.71	9.855	6.030	44.65	2.458	3.681
4	5.01	4.96	1.08	15.07	9.808	5.573	9.489	14.41	3.249
5	4.51	4.51	−0.07	16.55	9.913	5.519	12.21	12.23	3.556
6	3.92	3.96	−1.14	18.84	10.09	5.392	18.64	8.995	4.005
	Mean error		0.80						
Lys									
7	3.00	3.01	−0.49	23.69	6.250	6.415	11.09	14.60	6.408
8	3.77	3.72	1.36	19.19	8.548	6.701	30.14	4.363	5.382
9	3.94	3.98	−1.11	17.91	8.684	6.690	21.85	5.606	5.004
10	4.56	4.56	0.08	15.66	8.879	6.660	13.65	7.799	4.346
	Mean error		0.76						

acids were L-Ala, β-Ala, L-Asp, L-Cys, L-Glu, Gly, L-His, L-Hyp, 3,5-I_2-L-Tyr, L-Ile, L-Leu, DL-Met, L-Phe, L-Ser, L-Thr, L-Trp, DL-Tyr and L-Val. 5-Bromo-2,4-dihydroxybenzoate anion (BDB) was used as the leading ion to prevent the disturbance caused by HCO_3^-*. The buffers were ethanolamine and Lys, and the pH_L was in the range of 9–9.5. Table VI summarizes the seven leading electrolyte conditions used together with the calculated concentrations and effective mobilities of the electrolyte constituents. Since the given step heights (mm) were those from the leading zone to sample zones in recorder traces (Tables 13.2 and 13.3 in ref. 1), to convert these step heights into R_E values, the step heights of the leading zones, h_L, were estimated by

$$h_L = h_{std}/(R_{E,std} - 1) \quad (1)$$

* The BDB ions are divalent under the conditions used in ref. 1 and the effective mobility is almost the same as with HCO_3^-. Therefore the zone of HCO_3^- cannot be distinguished from that of BDB by a conductometric detector.

TABLE V

ABSOLUTE MOBILITIES AND THERMODYNAMIC DISSOCIATION CONSTANTS OF TWENTY-SIX AMINO ACIDS (25°C)

Amino acid	*Present method*		*Other methods* pK_a
	m_0	pK_a	
Ala	−32.2	9.857	9.866[9,11], 9.87[10,12,13]
β-Ala	−30.8	10.241	10.240[9,11]
α-Amin	−30.5	9.827	9.830[9,11], 9.833[10]
Arg	26.9	8.919	9.143[10], 8.991[11], 9.05[12], 8.994[13]
Asp	−30.1	3.900*	3.900[9−11], 3.63[12], 3.86[13]
	−55.4	10.002*	10.002[9 11], 9.47[12], 9.82[13]
Asn	−31.6	9.030	8.870[10,11], 8.85[12,13] (20°C)
Cys	−27.0	8.405	7.854[10], 8.00[11]
	−53.9	9.845	9.854[10], 9.850[12]
Glu	−27.0	4.324*	4.288[9], 4.324[10,11], 4.25[12]
	−54.3	9.960	9.387[9], 9.475[10], 9.96[11], 9.67[12]
Gln	−28.8	9.224	9.131[10,11]
Gly	−37.4	9.7796*	9.780[9], 9.7796[10,12,13], 9.778[11]
His	29.6*	6.04*	6.04[10,12,13], 6.00[11]
	−28.3	9.330	9.33[10], 9.17[11,13], 9.12[12]
Hyp	−30.1	9.816	9.662[9−11], 9.58[12] (20°C)
3,5-I_2-Tyr	−21.0	6.5*	6.48[9−11]
	−42.0	9.469	7.12
Ileu	−26.7	9.765	9.758[9,13], 9.761[10], 9.752[11]
Leu	−26.4	9.728	9.744[9,11,13], 9.748[10], 9.77[12]
Lys	26.4	9.127	8.951[10,12], 9.18[11,12]
	−26.4*	10.79*	10.53[10,13], 10.79[11], 10.72[12] (0.01 *M*, 20°C)
Met	−29.3	9.344	9.210[10,11,12], 9.27[13]
Orn	28.4	8.712	8.65[10,12], 8.690[11,13]
	−28.4*	10.755*	10.76[10], 10.755[11], 10.67[12] (0.02 *M*)
Phe	−26.9	9.262	9.119[10], 9.13[11,12]
Pro	−25.4	10.640*	10.640[9,10,13], 10.643[11], 10.60[12]
Ser	−33.6	9.302	9.208[9 11,13]
Tau	−37.9	9.182	9.061[9 −11,13]
Thr	−30.9	9.200	9.100[9,10,13], 9.099[11]
Trp	−25.4	9.594	9.377[10], 9.39[11], 9.55[12] (0.01 *M*, 20°C), 9.43[12] (1 *M*, 20°C), 9.44[13]
Tyr	−20.0	9.099	9.108[10], 9.11[11−13], 9.19[12]
	−40.0	10.189	10.07[10,12], 10.13[11,13], 10.47[12]
Val	−28.4	9.710	9.719[9,13], 9.722[10], 9.716[11], 9.62[12]

* Value fixed in the least-squares method.

where h_{std} is the observed step height of a standard sample and $R_{E,std}$ the R_E value of the standard. Using the estimated h_L, the R_E values of the sample zones, $R_{E,S}$, can be evaluated

$$R_{E,S} = (h_S + h_L)/h_L \quad (2)$$

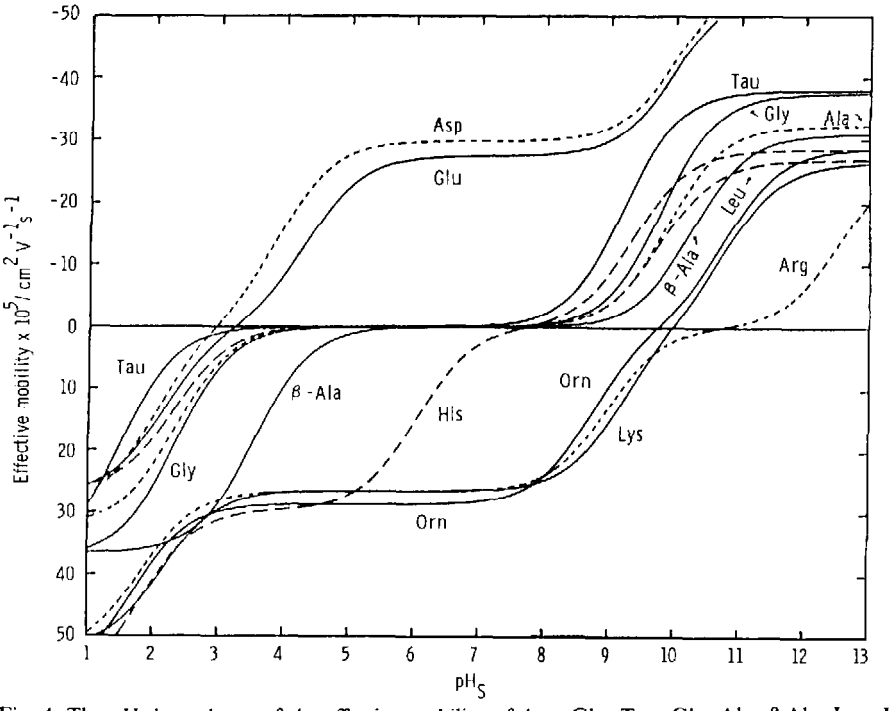

Fig. 4. The pH dependence of the effective mobility of Asp, Glu, Tau, Gly, Ala, β-Ala, Leu, His, Orn, Arg and Lys, not for the isotachophoretically steady state. The ionic strength is zero. pH_S = pH of sample zone.

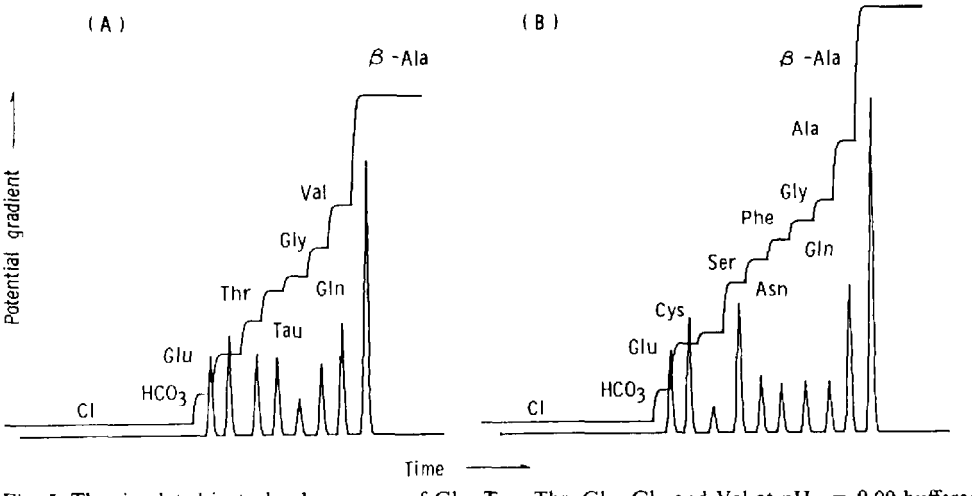

Fig. 5. The simulated isotachopherograms of Glu, Tau, Thr, Gln, Gly and Val at pH_L = 9.00 buffered by amediol (A), and Glu, Cys, Asn, Ser, Gln, Phe, Gly and Ala at pH_L = 8.64 (B). The leading ion was 10 m*M* chloride. The sample amounts were 10 nmol of each acid. Migration current: 50 μA.

TABLE VI

EXPERIMENTAL CONDITIONS USED FOR THE MEASUREMENT OF STEP HEIGHTS FOR EIGHTEEN AMINO ACIDS BY EVERAERTS *et al.*[1], CALCULATED CONCENTRATIONS AND EFFECTIVE MOBILITIES OF LEADING ZONE CONSTITUENTS

For the abbreviations used, see Table II. The leading ion is 5-bromo-2,4-dihydroxybenzoate.

System	*Buffer*	pH_L	C_L	$\bar{m}_L$	$C_{B,L}$	$\bar{m}_{B,L}$	I
1	Lys	9.07	4.00	42.05	14.18	12.74	12.0
2	Lys	9.22	4.00	42.12	17.13	10.58	12.1
3	Lys	9.42	4.00	42.09	23.84	7.613	12.6
4	EA	9.00	4.00	42.21	10.14	31.47	11.8
5	EA	9.20	4.00	42.40	11.52	27.86	11.9
6	EA	9.36	4.00	42.51	13.16	24.47	11.9
7	EA	9.55	4.00	42.61	16.07	20.12	12.0

where h_S is the step height of the sample from the leading to the sample zone. The simulated values of $R_{E,std}$ for the standard Gly ware shown in Table VI. Substituting $R_{E,std}$ and the reported h_{std} (135, 124.5 and 117 mm for systems 1–3 in Table VI) into eqn. 1 gave the estimated h_L values of 80.8, 83.0 and 92.1 mm respectively. Table VII

TABLE VII

CORRECTED AND SIMULATED R_E VALUES OF EIGHTEEN AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS 1–3 IN TABLE VI

The original step heights were measured by Everaerts *et al.*[1]. Corr. = Corrected R_E values; Sim. = simulated R_E values using the evaluated m_0 and pK_a.

Amino acid	pH_L = 9.07			pH_L = 9.22			pH_L = 9.42		
	Corr.	*Calc.*	*dev./%*	*Corr.*	*Calc.*	*dev./%*	*Corr.*	*Calc.*	*dev./%*
Asp	1.40	1.39	0.7	1.35	1.35	0	1.30	1.28	1.5
Cys	1.50	1.53	−2.0	1.43	1.44	−0.7	1.34	1.33	0.7
Glu	1.46	1.49	−2.1	1.40	1.44	−2.9	1.36	1.35	0.7
I_2-Tyr	1.75	1.77	−1.1	1.70	1.70	0	1.62	1.60	1.2
Ser	2.15	2.19	−1.9	1.99	2.06	−3.5	1.85	1.90	−2.7
Thr	2.18	2.24	−2.8	2.05	2.12	−3.4	1.96	1.97	−0.5
Tyr	2.51	2.62	−4.4	2.34	2.43	−3.8	2.13	2.18	−2.3
Met	2.47	2.53	−2.4	2.33	2.38	−2.1	2.15	2.19	−1.9
Gly	2.67	–	std	2.50	–	std	2.27	–	std
His	2.51	2.58	−2.8	2.55	2.43	4.7	2.18	2.23	−2.3
Phe	2.61	2.62	−0.4	2.46	2.47	−0.4	2.26	2.28	−0.9
Ala	3.18	3.17	0.3	2.99	2.96	1.0	2.68	2.67	0.4
Val	3.09	3.19	−3.2	2.93	2.97	−0.4	2.66	2.68	−0.8
Trp	3.12	3.26	−4.5	2.95	3.04	−3.1	2.69	2.74	−1.9
Hyp	3.10	3.25	−4.8	2.95	3.03	−2.7	2.66	2.73	−2.6
Ile	3.33	3.44	−3.3	3.17	3.20	−0.9	2.86	2.87	−0.3
Leu	3.35	3.40	−1.5	3.15	3.16	−0.3	2.85	2.84	0.4
β-Ala	3.97	–	–	3.81	–	–	3.23	–	–
Mean error	(%)		2.4			1.9			1.3

shows the converted R_E values from the observed step heights for the electrolyte systems using Lys as buffer (electrolyte systems 1–3) together with the simulated R_E. The mean deviations, except for β-Ala, between the observed and the simulated R_E values were 2.4, 1.9 and 1.3% respectively. However, the simulation of the steady state of β-Ala as the terminator failed: the pH of the β-Ala zone increased over the isoelectric point of Lys buffer corresponding to conversion of Lys cations into anions. This may suggest that β-Ala was no longer the actual terminator and that hydroxide ions may fulfil this rôle[1].

Table VIII summarizes the converted and the simulated R_E values for the electrolyte systems 4–7 (ethanolamine buffer). The mean deviations were 3.5, 3.2, 2.1 and 3.9% respectively. Except for system 6, the deviations were about twice as large as those found in the the electrolyte systems buffered by Lys. Apparently, from Table VIII, these relatively large mean errors are caused by the large deviations between the observed and the simulated R_E of Trp, Hyp, Ile, Leu and β-Ala. Since ethanolamine was also used in our R_E measurement, such deviations for the electrolyte systems 4, 5 and 7 were not expected.

We confirmed these experimental facts for twenty-two amino acids using a leading electrolyte of 4 m*M* BDB buffered by ethanolamine at pH_L = 9.06. The mean error between the observed and the simulated values was 2.2% for all samples, and 4.1% for Amin, Hyp, Trp, Ile, Leu and β-Ala. Since the migration current was

TABLE VIII

CORRECTED AND SIMULATED R_E VALUES OF EIGHTEEN AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS 4–7 IN TABLE VI

The original step heights were measured by Everaerts *et al.*[1]. For the abbreviations used, see Table VII.

Amino acid	pH_L = 9.00			pH_L = 9.20			pH_L = 9.36			pH_L = 9.55		
	Corr.	*Sim.*	*dev/%*	*Corr.*	*Sim.*	*dev/%*	*Corr.*	*Sim.*	*dev/%*	*Corr.*	*Sim.*	*dev/%*
Asp	1.40	1.37	2.1	1.34	1.32	1.5	1.32	1.28	3.0	1.34	1.22	9.0
Cys	1.50	1.49	0.7	1.41	1.40	0.7	1.38	1.33	3.6	1.30	1.25	3.8
Glu	1.48	1.47	0.7	1.42	1.40	1.4	1.36	1.35	0.7	1.31	1.27	3.1
I_2-Tyr	1.74	1.75	−0.6	1.66	1.66	0.0	1.55	1.59	−2.6	1.45	1.51	−4.1
Ser	2.10	2.04	2.9	1.92	1.95	−1.6	1.89	1.86	1.6	1.75	1.76	−0.6
Thr	2.10	2.12	−1.0	2.03	2.02	0.5	1.95	1.94	0.5	1.83	1.84	−0.5
Tyr	2.32	2.46	−6.0	2.19	2.30	−5.0	2.13	2.16	−1.4	1.92	1.99	−3.6
Met	2.30	2.37	−3.0	2.21	2.25	−1.8	2.19	2.15	1.8	2.03	2.03	0.0
Gly	2.35	–	std	2.24	–	std	2.12	–	std	1.97	–	std
His	2.41	2.43	−0.8	2.29	2.31	−0.9	2.25	2.21	1.8	2.08	2.08	0.0
Phe	2.44	2.48	−1.6	2.33	2.36	−1.3	2.28	2.26	0.9	2.11	2.14	−1.4
Ala	2.76	2.82	−2.2	2.61	2.68	−2.7	2.55	2.53	0.8	2.31	2.35	−1.7
Val	2.79	2.92	−4.7	2.66	2.76	−3.8	2.60	2.61	−0.4	2.36	2.43	−3.0
Trp	2.86	3.04	−6.3	2.68	2.88	−7.5	2.66	2.73	−2.6	2.41	2.55	−5.8
Hyp	2.80	2.93	−4.6	2.69	2.77	−3.0	2.60	2.63	−1.2	2.35	2.43	−3.4
Ile	2.98	3.17	−6.4	2.80	3.00	−7.1	2.72	2.84	−4.4	2.47	2.63	−6.5
Leu	3.00	3.14	−4.7	2.80	2.97	−6.1	2.74	2.81	−2.6	2.49	2.61	−4.8
β-Ala	3.33	3.68	−10.5	3.19	3.48	−9.1	3.12	3.29	−5.4	2.67	3.03	−13.5
Mean error (%)			3.5			3.2			2.1			3.9

TABLE IX

SIMULATED EFFECTIVE MOBILITIES OF TWENTY-TWO AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS OF pH_L = 8.6, 9.0, 9.4 AND 9.7 BUFFERED BY AMEDIOL

The leading ion is 10 m*M* chloride. The values cited are mobility · 10^5 ($cm^2V^{-1}s^{-1}$). The diagonal values are the effective mobilities and the others are the differences.

	Asp	*Glu*	*Cys*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Met*	*His*	*Phe*	*Tyr*	*Gly*	*Val*	*Ala*	*Trp*	*Amin*	*Hyp*	*Leu*	*Ile*	*β-Ala*
pH_L = 8.6																						
1 Asp	28.8	2.6	5.8	7.5	10.1	11.5	13.3	13.5	14.7	15.6	15.9	15.9	16.4	16.8	18.9	19.0	19.1	19.2	19.2	19.7	19.8	21.9
2 Glu		26.2	3.2	4.9	7.5	9.0	10.8	11.0	12.2	13.0	13.3	13.3	13.8	14.3	16.4	16.4	16.5	16.6	16.7	17.1	17.3	19.3
3 Cys			23.0	1.7	4.3	5.7	7.6	7.8	8.9	9.8	10.1	10.1	10.6	11.0	13.1	13.2	13.3	13.4	13.4	13.9	14.1	16.1
4 I_2-Tyr				21.3	2.6	4.1	5.9	6.1	7.3	8.1	8.4	8.4	8.9	9.3	11.5	11.5	11.6	11.7	11.8	12.2	12.4	14.4
5 Tau					18.7	1.5	3.3	3.5	4.7	5.5	5.8	5.8	6.3	6.8	8.9	8.9	9.0	9.1	9.2	9.6	9.8	11.8
6 Asn						17.3	1.8	2.0	3.2	4.0	4.3	4.4	4.8	5.3	7.4	7.4	7.5	7.7	7.7	8.1	8.3	10.3
7 Ser							15.5	0.2	1.4	2.2	2.5	2.6	3.0	3.5	5.6	5.6	5.7	5.9	5.9	6.3	6.5	8.5
8 Thr								15.3	1.2	2.0	2.3	2.4	2.8	3.3	5.4	5.4	5.5	5.7	5.7	6.1	6.3	8.3
9 Gln									14.1	0.9	1.2	1.2	1.6	2.1	4.2	4.2	4.3	4.5	4.5	4.9	5.1	7.1
10 Met										13.2	0.3	0.3	0.8	1.2	3.4	3.4	3.5	3.6	3.7	4.1	4.3	6.3
11 His											12.9	0.0	0.5	0.9	3.1	3.1	3.2	3.3	3.4	3.8	4.0	6.0
12 Phe												12.9	0.5	0.9	3.0	3.1	3.2	3.3	3.3	3.8	3.9	6.0
13 Tyr													12.4	0.5	2.6	2.6	2.7	2.8	2.9	3.3	3.5	5.5
14 Gly														12.0	2.1	2.1	2.2	2.4	2.4	2.9	3.0	5.0
15 Val															9.9	0.0	0.1	0.3	0.3	0.7	0.9	2.9
16 Ala																9.8	0.1	0.2	0.3	0.7	0.9	2.9
17 Trp																	9.7	0.1	0.2	0.6	0.8	2.8
18 Amin																		9.6	0.0	0.5	0.6	2.7
19 Hyp																			9.6	0.4	0.6	2.6
20 Leu																				9.1	0.2	2.2
21 Ile																					9.0	2.0
22 β-Ala																						6.9

	Asp	*Glu*	*Cys*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Met*	*His*	*Phe*	*Tyr*	*Gly*	*Ala*	*Val*	*Trp*	*Amin*	*Hyp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.0																						
1 Asp	30.6	2.2	3.3	6.8	8.2	10.2	11.9	12.3	13.7	14.5	14.9	15.0	15.1	15.8	18.3	18.3	18.6	18.6	18.7	19.2	19.4	21.9
2 Glu		28.5	1.2	4.6	6.0	8.0	9.8	10.1	11.5	12.4	12.8	12.9	12.9	13.6	16.2	16.2	16.4	16.5	16.5	17.1	17.3	19.7
3 Cys			27.3	3.5	4.9	6.8	8.6	8.9	10.3	11.2	11.6	11.7	11.7	12.4	15.0	15.0	15.2	15.3	15.3	15.9	16.1	18.5
4 I_2-Tyr				23.9	1.4	3.4	5.1	5.5	6.9	7.8	8.1	8.2	8.3	9.0	11.6	11.6	11.8	11.8	11.9	12.5	12.6	15.1

5 Tau				22.4	2.0	3.7	4.1	5.5	6.4	6.7	6.8	6.9	7.6	10.1	10.1	10.4	10.4	10.5	11.0	11.2	13.7
6 Asn					20.5	1.7	2.1	3.5	4.4	4.7	4.9	4.9	5.6	8.2	8.2	8.4	8.5	8.5	9.1	9.3	11.7
7 Ser						18.7	0.4	1.7	2.6	3.0	3.1	3.1	3.8	6.4	6.4	6.6	6.7	6.7	7.3	7.5	9.9
8 Thr							18.4	1.4	2.3	2.6	2.8	2.8	3.5	6.1	6.1	6.3	6.4	6.4	7.0	7.2	9.6
9 Gln								17.0	0.9	1.3	1.4	1.4	2.1	4.7	4.7	4.9	5.0	5.0	5.6	5.8	8.2
10 Met									16.1	0.4	0.5	0.5	1.2	3.8	3.8	4.0	4.1	4.1	4.7	4.9	7.3
11 His										15.7	0.1	0.1	0.9	3.4	3.4	3.6	3.7	3.8	4.3	4.5	7.0
12 Phe											15.6	0.0	0.7	3.3	3.3	3.5	3.6	3.6	4.2	4.4	6.8
13 Tyr												15.6	0.7	3.3	3.3	3.5	3.6	3.6	4.2	4.4	6.8
14 Gly													14.9	2.6	2.6	2.8	2.9	2.9	3.5	3.7	6.1
15 Ala														12.3	0.0	0.2	0.3	0.3	0.9	1.1	3.5
16 Val															12.3	0.2	0.3	0.3	0.9	1.1	3.5
17 Trp																12.1	0.1	0.1	0.7	0.9	3.3
18 Amin																	12.0	0.0	0.6	0.8	3.2
19 Hyp																		12.0	0.6	0.8	3.2
20 Leu																			11.4	0.2	2.6
21 Ile																				11.2	2.4
22 β-Ala																					8.8

	Asp	*Cys*	*Glu*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Tyr*	*Met*	*Gly*	*His*	*Phe*	*Ala*	*Val*	*Amin*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.4																						
1 Asp	33.8	1.4	1.7	6.7	6.9	10.0	11.1	11.9	13.5	14.1	14.2	14.6	14.7	15.1	17.8	18.1	18.3	18.3	18.6	19.2	19.4	22.1
2 Cys		32.4	0.3	5.3	5.5	8.6	9.7	10.5	12.1	12.7	12.8	13.3	13.3	13.7	16.5	16.7	16.9	16.9	17.2	17.8	18.0	20.7
3 Glu			32.2	5.0	5.3	8.3	9.4	10.2	11.8	12.5	12.5	13.0	13.0	13.4	16.2	16.4	16.6	16.7	16.9	17.6	17.8	20.4
4 I_2-Tyr				27.1	0.2	3.3	4.4	5.2	6.8	7.4	7.5	7.9	8.0	8.3	11.1	11.4	11.6	11.6	11.9	12.5	12.7	15.4
5 Tau					26.9	3.0	4.1	4.9	6.5	7.2	7.2	7.7	7.8	8.1	10.9	11.2	11.3	11.4	11.7	12.3	12.5	15.1
6 Asn						23.9	1.1	1.9	3.5	4.1	4.2	4.7	4.7	5.1	7.9	8.1	8.3	8.4	8.6	9.3	9.4	12.1
7 Ser							22.8	0.8	2.4	3.1	3.1	3.6	3.6	4.0	6.8	7.0	7.2	7.3	7.5	8.2	8.3	11.0
8 Thr								22.0	1.6	2.2	2.3	2.8	2.8	3.2	6.0	6.2	6.4	6.5	6.7	7.4	7.5	10.2
9 Gln									20.3	0.6	0.7	1.2	1.2	1.6	4.4	4.6	4.8	4.9	5.1	5.7	5.9	8.6
10 Tyr										19.7	0.1	0.5	0.6	0.9	3.7	4.0	4.2	4.2	4.5	5.1	5.3	8.0
11 Met											19.6	0.5	0.5	0.9	3.7	3.9	4.1	4.2	4.4	5.0	5.2	7.9
12 Gly												19.2	0.0	0.4	3.2	3.5	3.6	3.7	4.0	4.6	4.8	7.4
13 His													19.1	0.4	3.2	3.4	3.6	3.6	3.9	4.5	4.7	7.4
14 Phe														18.8	2.8	3.0	3.2	3.3	3.6	4.2	4.4	7.0
15 Ala															16.0	0.3	0.4	0.5	0.8	1.4	1.6	4.2
16 Val																15.7	0.2	0.2	0.5	1.1	1.3	4.0
17 Amin																	15.6	0.1	0.3	1.0	1.1	3.8
18 Hyp																		15.5	0.3	0.9	1.1	3.7

(*Continued on p. 74*)

TABLE IX (*continued*)

	Asp	*Cys*	*Glu*	*I_2-Tyr*	*Tau*	*Asn*	*Ser*	*Thr*	*Gln*	*Tyr*	*Met*	*Gly*	*His*	*Phe*	*Ala*	*Val*	*Amin*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
19 Trp																			15.2	0.6	0.8	3.5
20 Leu																				14.6	0.2	2.9
21 Ile																					14.4	2.7
22 β-Ala																						11.7

	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Tyr*	*Gly*	*Gln*	*Met*	*His*	*Phe*	*Ala*	*Amin*	*Val*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.7																						
1 Asp	36.9	0.6	1.3	7.1	7.4	11.1	11.3	12.6	13.9	13.9	14.4	14.8	15.4	16.1	17.6	18.3	18.3	18.4	19.3	19.7	19.8	22.2
2 Cys		36.3	0.7	6.5	6.7	10.4	10.7	12.0	13.2	13.3	13.8	14.2	14.8	15.4	17.0	17.6	17.7	17.7	18.6	19.0	19.2	21.6
3 Glu			35.6	5.8	6.0	9.7	10.0	11.3	12.5	12.6	13.1	13.5	14.1	14.7	16.3	16.9	17.0	17.0	17.9	18.3	18.5	20.9
4 Tau				29.8	0.3	4.0	4.3	5.5	6.8	6.8	7.3	7.7	8.3	9.0	10.6	11.2	11.3	11.3	12.2	12.6	12.7	15.1
5 I_2-Tyr					29.6	3.7	4.0	5.3	6.5	6.6	7.0	7.5	8.1	8.7	10.3	10.9	11.0	11.0	11.9	12.3	12.4	14.9
6 Asn						25.9	0.3	1.6	2.8	2.9	3.3	3.7	4.4	5.0	6.6	7.2	7.3	7.3	8.2	8.6	8.7	11.1
7 Ser							25.6	1.3	2.5	2.6	3.1	3.5	4.1	4.7	6.3	6.9	7.0	7.0	7.9	8.3	8.5	10.9
8 Thr								24.3	1.2	1.3	1.8	2.2	2.8	3.4	5.0	5.6	5.7	5.7	6.6	7.0	7.2	9.6
9 Tyr									23.1	0.1	0.5	0.9	1.6	2.2	3.8	4.4	4.5	4.5	5.4	5.8	5.9	8.3
10 Gly										23.0	0.5	0.9	1.5	2.1	3.7	4.3	4.4	4.4	5.3	5.7	5.9	8.3
11 Gln											22.5	0.4	1.0	1.7	3.2	3.9	3.9	4.0	4.9	5.3	5.4	7.8
12 Met												22.1	0.6	1.2	2.8	3.4	3.5	3.6	4.4	4.9	5.0	7.4
13 His													21.5	0.6	2.2	2.8	2.9	2.9	3.8	4.2	4.4	6.8
14 Phe														20.9	1.6	2.2	2.3	2.3	3.2	3.6	3.7	6.1
15 Ala															19.3	0.6	0.7	0.7	1.6	2.0	2.1	4.6
16 Amin																18.7	0.1	0.1	1.0	1.4	1.5	4.0
17 Val																	18.6	0.0	0.9	1.3	1.5	3.9
18 Hyp																		18.6	0.9	1.3	1.4	3.8
19 Trp																			17.7	0.4	0.5	2.9
20 Leu																				17.3	0.1	2.5
21 Ile																					17.1	2.4
22 β-Ala																						14.7

TABLE X

SIMULATED EFFECTIVE MOBILITIES OF TWENTY-TWO AMINO ACIDS UNDER THE ELECTROLYTE CONDITIONS OF pH_L = 9.0, 9.2, 9.4 AND 9.6 BUFFERED BY ETHANOLAMINE

Other details as in Table IX.

	Asp	*Glu*	*Cys*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gln*	*Gly*	*Met*	*His*	*Tyr*	*Phe*	*Ala*	*Val*	*Amin*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.0																						
1 Asp	31.4	1.9	2.0	6.3	6.5	9.1	10.1	10.9	12.4	12.9	13.1	13.5	13.6	13.9	16.0	16.5	16.5	16.5	17.1	17.5	17.7	19.6
2 Glu		29.5	0.1	4.4	4.6	7.2	8.2	9.0	10.5	11.0	11.2	11.6	11.7	12.0	14.1	14.6	14.6	14.6	15.2	15.6	15.8	17.7
3 Cys			29.4	4.3	4.4	7.1	8.1	8.9	10.4	10.9	11.0	11.5	11.5	11.9	14.0	14.4	14.4	14.5	15.0	15.5	15.6	17.5
4 Tau				25.1	0.2	2.8	3.8	4.6	6.1	6.6	6.8	7.3	7.3	7.6	9.7	10.2	10.2	10.3	10.8	11.2	11.4	13.3
5 I_2-Tyr					24.9	2.7	3.7	4.5	6.0	6.5	6.6	7.1	7.1	7.4	9.5	10.0	10.0	10.1	10.6	11.1	11.2	13.1
6 Asn						22.3	1.0	1.8	3.3	3.8	3.9	4.4	4.4	4.8	6.9	7.3	7.3	7.4	8.0	8.4	8.5	10.5
7 Ser							21.3	0.8	2.3	2.8	2.9	3.4	3.4	3.8	5.9	6.3	6.3	6.4	6.9	7.4	7.5	9.4
8 Thr								20.5	1.5	2.0	2.1	2.6	2.6	3.0	5.1	5.5	5.5	5.6	6.1	6.6	6.7	8.7
9 Gln									19.0	0.5	0.6	1.1	1.1	1.5	3.6	4.0	4.0	4.1	4.6	5.1	5.2	7.1
10 Gly										18.5	0.1	0.6	0.6	1.0	3.1	3.5	3.5	3.6	4.1	4.6	4.7	6.6
11 Met											18.4	0.5	0.5	0.8	2.9	3.4	3.4	3.5	4.0	4.5	4.6	6.5
12 His												17.9	0.0	0.3	2.4	2.9	2.9	3.0	3.5	4.0	4.1	6.0
13 Tyr													17.9	0.3	2.4	2.9	2.9	3.0	3.5	4.0	4.1	6.0
14 Phe														17.5	2.1	2.6	2.6	2.7	3.2	3.6	3.8	5.7
15 Ala															15.4	0.5	0.5	0.6	1.1	1.5	1.7	3.6
16 Val																15.0	0.0	0.1	0.6	1.1	1.2	3.1
17 Amin																	15.0	0.1	0.6	1.1	1.2	3.1
18 Hyp																		14.9	0.5	1.0	1.1	3.0
19 Trp																			14.3	0.5	0.6	2.5
20 Leu																				13.9	0.1	2.0
21 Ile																					13.8	1.9
22 β-Ala																						11.8

(*Continued on p. 76*)

TABLE X (*continued*)

	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gln*	*Gly*	*Met*	*Tyr*	*His*	*Phe*	*Ala*	*Amin*	*Val*	*Hyp*	*Thp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.2																						
1 Asp	32.8	1.5	1.7	6.4	6.5	9.4	10.3	11.2	12.8	13.2	13.4	13.5	13.9	14.3	16.4	16.9	16.9	17.0	17.5	18.0	18.1	20.2
2 Cys		31.3	0.2	4.9	5.0	7.9	8.8	9.7	11.3	11.6	11.9	12.0	12.4	12.8	14.8	15.3	15.4	15.4	16.0	16.5	16.6	18.6
3 Glu			31.1	4.7	4.8	7.7	8.6	9.5	11.1	11.5	11.7	11.8	12.2	12.6	14.7	15.2	15.2	15.3	15.8	16.3	16.4	18.5
4 Tau				26.5	0.1	3.1	4.0	4.8	6.4	6.8	7.0	7.2	7.5	7.9	10.0	10.5	10.5	10.6	11.2	11.6	11.8	13.8
5 I_2-Tyr					26.3	2.9	3.8	4.7	6.3	6.7	6.9	7.0	7.4	7.8	9.9	10.4	10.4	10.5	11.0	11.5	11.6	13.7
6 Asn						23.4	0.9	1.8	3.4	3.7	4.0	4.1	4.5	4.9	6.9	7.4	7.4	7.5	8.1	8.6	8.7	10.7
7 Ser							22.5	0.9	2.5	2.8	3.1	3.2	3.6	4.0	6.0	6.5	6.5	6.6	7.2	7.7	7.8	9.8
8 Thr								21.6	1.6	1.9	2.2	2.3	2.7	3.1	5.1	5.6	5.7	5.7	6.3	6.8	6.9	8.9
9 Gln									20.0	0.4	0.6	0.7	1.1	1.5	3.6	4.1	4.1	4.2	4.7	5.2	5.3	7.4
10 Gly										19.7	0.2	0.4	0.7	1.1	3.2	3.7	3.7	3.8	4.4	4.8	5.0	7.0
11 Met											19.4	0.1	0.5	0.9	3.0	3.5	3.5	3.6	4.1	4.6	4.7	6.8
12 Tyr												19.3	0.4	0.8	2.8	3.3	3.3	3.4	4.0	4.5	4.6	6.6
13 His													18.9	0.4	2.5	3.0	3.0	3.0	3.6	4.1	4.2	6.3
14 Phe														18.5	2.1	2.6	2.6	2.7	3.2	3.7	3.8	5.9
15 Ala															16.5	0.5	0.5	0.6	1.2	1.6	1.8	3.8
16 Amin																16.0	0.0	0.1	0.7	1.1	1.3	3.3
17 Val																	16.0	0.1	0.7	1.1	1.3	3.3
18 Hyp																		15.9	0.6	1.1	1.2	3.2
19 Trp																			15.3	0.5	0.6	2.6
20 Leu																				14.8	0.1	2.2
21 Ile																					14.7	2.0
22 β-Ala																						12.7

	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gln*	*Gly*	*Tyr*	*Met*	*His*	*Phe*	*Ala*	*Amin*	*Val*	*Hyp*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.4																						
1 Asp	34.6	1.0	1.5	6.6	6.7	10.0	10.6	11.7	13.3	13.3	13.5	13.8	14.4	14.9	16.7	17.3	17.3	17.4	18.1	18.5	18.7	20.7
2 Cys		33.5	0.5	5.5	5.6	8.9	9.6	10.6	12.3	12.3	12.5	12.8	13.4	13.8	15.7	16.2	16.3	16.3	17.1	17.5	17.6	19.7
3 Glu			33.0	5.0	5.2	8.4	9.1	10.1	11.8	11.8	12.0	12.3	12.9	13.4	15.2	15.7	15.8	15.8	16.6	17.0	17.1	19.2
4 Tau				28.0	0.1	3.4	4.1	5.1	6.7	6.8	7.0	7.3	7.8	8.3	10.2	10.7	10.8	10.8	11.5	12.0	12.1	14.2
5 I_2-Tyr					27.9	3.3	3.9	5.0	6.6	6.6	6.8	7.2	7.7	8.2	10.0	10.6	10.6	10.7	11.4	11.8	12.0	14.1
6 Asn						24.6	0.7	1.7	3.3	3.4	3.6	3.9	4.4	4.9	6.8	7.3	7.4	7.4	8.1	8.6	8.7	10.8
7 Ser							23.9	1.0	2.7	2.7	2.9	3.2	3.8	4.3	6.1	6.6	6.7	6.7	7.5	7.9	8.0	10.1
8 Thr								22.9	1.6	1.7	1.9	2.2	2.7	3.2	5.1	5.6	5.7	5.7	6.4	6.9	7.0	9.1
9 Gln									21.3	0.0	0.2	0.5	1.1	1.6	3.4	4.0	4.0	4.1	4.8	5.2	5.4	7.4
10 Gly										21.2	0.2	0.5	1.1	1.6	3.4	3.9	4.0	4.0	4.8	5.2	5.3	7.4

11 Tyr										21.0	0.3	0.9	1.3	3.2	3.7	3.8	3.8	4.6	5.0	5.1	7.2
12 Met											20.7	0.6	1.0	2.9	3.4	3.5	3.5	4.3	4.7	4.8	6.9
13 His												20.2	0.5	2.3	2.9	2.9	3.0	3.7	4.1	4.3	6.3
14 Phe													19.7	1.8	2.4	2.4	2.5	3.2	3.6	3.8	5.9
15 Ala														17.8	0.5	0.6	0.6	1.4	1.8	1.9	4.0
16 Amin															17.3	0.1	0.1	0.8	1.3	1.4	3.5
17 Val																17.2	0.0	0.8	1.2	1.3	3.4
18 Hyp																	17.2	0.7	1.2	1.3	3.4
19 Trp																		16.5	0.4	0.6	2.6
20 Leu																			16.0	0.1	2.2
21 Ile																				15.9	2.1
22 β-Ala																					13.8
	Asp	*Cys*	*Glu*	*Tau*	*I_2-Tyr*	*Asn*	*Ser*	*Thr*	*Gly*	*Tyr*	*Gln*	*Met*	*His*	*Phe*	*Ala*	*Amin*	*Hyp*	*Val*	*Trp*	*Leu*	*Ile*	*β-Ala*
pH_L = 9.6																					
1 Asp	36.5	0.7	1.4	6.9	7.1	10.8	11.0	12.3	13.4	13.5	14.0	14.4	15.1	15.7	17.0	17.6	17.7	17.8	18.7	19.1	19.2	21.2
2 Cys		35.8	0.7	6.2	6.4	10.1	10.4	11.6	12.7	12.9	13.4	13.8	14.4	15.0	16.3	17.0	17.1	17.1	18.1	18.4	18.5	20.5
3 Glu			35.1	5.5	5.8	9.4	9.7	11.0	12.0	12.2	12.7	13.1	13.7	14.3	15.6	16.3	16.4	16.4	17.4	17.7	17.8	19.9
4 Tau				29.6	0.2	3.9	4.2	5.4	6.5	6.6	7.1	7.5	8.2	8.8	10.1	10.7	10.8	10.9	11.8	12.2	12.3	14.3
5 I_2-Tyr					29.4	3.7	3.9	5.2	6.3	6.4	6.9	7.3	8.0	8.6	9.9	10.5	10.6	10.7	11.6	12.0	12.1	14.1
6 Asn						25.7	0.3	1.5	2.6	2.8	3.3	3.7	4.3	4.9	6.2	6.9	7.0	7.0	8.0	8.3	8.4	10.4
7 Ser							25.4	1.3	2.3	2.5	3.0	3.4	4.0	4.6	6.0	6.6	6.7	6.7	7.7	8.0	8.1	10.2
8 Thr								24.2	1.1	1.2	1.7	2.1	2.7	3.4	4.7	5.3	5.4	5.5	6.4	6.8	6.9	8.9
9 Gly									23.1	0.2	0.7	1.1	1.7	2.3	3.6	4.3	4.4	4.4	5.4	5.7	5.8	7.8
10 Tyr										22.9	0.5	0.9	1.5	2.1	3.5	4.1	4.2	4.2	5.2	5.5	5.6	7.7
11 Gln											22.5	0.4	1.0	1.6	3.0	3.6	3.7	3.8	4.7	5.1	5.2	7.2
12 Met												22.1	0.6	1.2	2.6	3.2	3.3	3.4	4.3	4.6	4.8	6.8
13 His													21.4	0.6	1.9	2.6	2.7	2.7	3.7	4.0	4.1	6.2
14 Phe														20.8	1.3	2.0	2.1	2.1	3.1	3.4	3.5	5.5
15 Ala															19.5	0.6	0.7	0.8	1.7	2.1	2.2	4.2
16 Amin																18.9	0.1	0.2	1.1	1.5	1.6	3.6
17 Hyp																	18.8	0.1	1.0	1.3	1.5	3.5
18 Val																		18.7	1.0	1.3	1.4	3.4
19 Trp																			17.8	0.3	0.4	2.5
20 Leu																				17.4	0.1	2.1
21 Ile																					17.3	2.0
22 β-Ala																						15.3

25 or 50 μA, the behaviour observed under such conditions is not be due to a temperature effect. This was apparent from the fact that the observed R_E value for the other standard, propionate ion, was 1.25 and the simulated value is the same when Gly was used as the internal standard. Since such a situation was not found in the BDB–Lys system, the nature of the BDB ethanolamine system is not properly reflected by the simulation.

Separability assessment

Using the evaluated constants, the separability of the amino acids can be assessed by the use of the SIPS program and the results can be compared with those from experiments. The order of appearance and the separability of samples are determined by the magnitude of the mobilities and the differences between them in the transient mixed zone. They are a complicated function of the m_0 and pK_a of the samples, the selected buffer and pH_L, the pH of the injected mixture, etc. Therefore, strictly speaking, a discussion of the order of appearance and the separability should take account of these factors besides steady state information. However, for such complicated sample systems as treated in this paper, the analysis of the mixed zones is a difficult problem. At present, even for a three-component system, no practical elucidation of the separation process has been reported, although two-component systems (monovalent ions) have been relatively well analyzed[14,15]. Although the SIPS program has a routine which is applicable to general multivalent ions, at present its utility is limited to two-component systems (the details will be published in due course). As a first approximation, the difference between the effective mobilities of samples at the steady state can be a good measure of their separation, since the pH of the mixed zone lies in the middle of the pH values of the adjacent separated zones and the effective mobilities in the mixed zone are not very different from those in the steady state.

Table IX summarizes the differences between the simulated effective mobilities of the twenty-two amino acids at the steady state when amediol was used as the pH buffer. The four pH conditions (pH_L = 8.6, 9.0, 9.4 and 9.7) were the same as those used by Kopwillem and Lundlin[8]. In the simulation, the leading ion concentration was 10 m*M*. Table X also summarizes the differences between the simulated effective mobilities in another conveniently used leading electrolyte, 10 m*M* hydrochloric acid ethanolamine (pH_L = 9.0, 9.2, 9.4 and 9.6). Apparently, from these tables, the differences for adjacent samples are very small and sometimes zero, suggesting that the separation of all of them is not practical as long as a pH effect on the effective mobility is utilized. In comparison with Table IX and X, amediol may be superior in separability.

Kopwillem and Lundin[8] studied the pH dependence of the separation of seventeen amino acids, using thermometric and UV detectors. The samples (each 2.5–10 nmol) were Ala, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr and Val. They found that fourteen amino acids can be separated at pH_L = 8.6 buffered by amediol. The leading ion used was chloride (5–10 m*M*). A capillary tube (81 cm × 0.5 mm I.D.) was used and a single experiment took *ca.* 70 min (driving current = 50 μA).

At pH_L = 8.6 (amediol buffer) the observed order of appearance of the samples in their experiment, the simulated R_E values and the effective mobilities, $\bar{m}$, of the

amino acids were Asp (R_E = 2.60, $\bar{m}$ = 28.8 · 10^{-5}cm^2 V^{-1} s^{-1}), Glu (2.85, 26.2), Cys (3.25, 23.0), Asn (4.32, 17.3), [Ser (4.83, 15.5), Thr (4.89, 15.3)], Gln (5.31, 14.1), Met (5.65, 13.2), Tyr (6.01, 12.4), [His (5.78, 12.9), Phe (5.79, 12.9)], Gly (6.23, 12.0), Trp (7.67, 9.74), Val (7.57, 9.87), Ala (7.59, 9.84), [Leu (8.18, 9.12), Ile (8.33, 8.96)] and β-Ala (10.76, 6.95). The pairs of samples in square brackets could not be separated when the injected sample amounts were 2.5 and 5 nmol of each. It was also reported that the pairs [Ser, Thr], [Tyr, His], [His, Phe], [Ala, Leu] and [Ile, Leu] could not be separated when the sample amounts were 7.5 and 10 nmol of each. For these pairs the differences between the simulated effective mobilities of the individual components are 0.2 · 10^{-5}, 0.5 · 10^{-5}, 0.0 · 10^{-5}, 0.72 · 10^{-5} and 0.16 · 10^{-5}cm^2 V^{-1} s^{-1} respectively. The reported order of appearance is in approximate agreement with both the increasing order of R_E values and the decreasing order of effective mobility, except for Tyr, His, Phe, Trp and Val. The behaviour of the last compounds is contradictory to the estimation not only from the simulation but also to independently observed R_E values with experimental errors of *ca.* 0.05* (5.92, DL-Tyr; 5.70, L-Phe; 5.64, L-His; 7.59, DL-Trp and 7.47, DL-Val). However, UV observation supported the first appearance of Tyr when the separation of Tyr, Phe and His, for example, was attempted at pH_L = 8.64 (amediol buffer). These facts can not be explained as the result of errors in the observed R_E values or the evaluated constants. Most probably, they were caused by the enforced phenomena**.

A tentative simulation of the separation process for the two-component system gave evidence which supported this estimation. When a 10 m*M* hydrochloric acid solution buffered by amediol (pH_L = 8.6) was the leading electrolyte and the pH of the injected 1:1 mixture was 9 (amediol buffer), for example, the simulated pH of the transient mixed zone was 9.255 and the effective mobilities of His and Tyr in the zone were 12.39 · 10^{-5} cm^2 V^{-1} s^{-1}. The mobility of Tyr was larger than that of His, contrary to the steady state, suggesting that enforced migration is occurring. The time needed for the resolution, t_{res}, of 10-nmol samples was 2060 s, when the migration current was 50 μA. The simulated t_{res} for the other inseparable pairs (10 nmol of each) were 2814 (Ser, Thr), 3344 (His, he), 7047 (Ala, Leu) and 2124 s (Ile, Leu). When the other samples coexist, it was confirmed experimentally that the observed t_{res} is larger than the simulated value for a two-component system. The t_{res} values for the amino acids in the electropherogram of Fig. 1 were simulated. The pH_L was 8.64, the pH of the mixture was 9 (amediol buffer respectively) and the migration current was 50 μA. The estimated values were 530 (Glu, Cys), 206 (Cys, Asn), 348 (Asn, Ser), 1055 (Ser, Gln), 745 (Gln, Phe), 228 (Phe, Gly) and 280 s (Gly, Ala).

At pH_L = 9.4 (amediol buffer), the order of appearance reported was Asp (R_E = 2.18, $\bar{m}$ = 34.7), [Glu (2.30, 33.0), Cys (2.28, 33.3)], Asn (3.10, 24.5), Ser (3.25,

* The R_E values could be measured repeatedly within an error of *ca.* ±0.05 R_E units for the completely separable sample combinations when the internal standard was selected properly. However, we found that the R_E value of some samples, *e.g.*, Tyr, varied over a greater range according to the selected combination of the samples, in spite of no mixed zone formation (usually this means that an isotachophoretic steady state is being achieved). The reason for this small but significant fluctuation is not yet known.

** In isotachophoresis the order of appearance of samples usually agrees with the decreasing effective mobilities. When this is not valid in relation to the pH of a sample zone and the preceding zone, it is called an enforced isotachophoretic system (see also ref. 1).

23.3), Thr (3.36, 22.6), Gln (3.62, 20.9), Tyr (3.71, 20.4), Met (3.75, 20.2), His (3.85, 19.7), Phe (3.91, 19.4), Gly (3.84, 19.8), [Trp (4.78, 15.9), Val (4.64, 16.4)], Ala (4.56, 16.6), [Ile (5.03, 15.1), Leu (4.97, 15.3)] and β-Ala (5.97, 12.7). The samples in square brackets could not be separated when the injected amounts were 2.5 nmol of each. When the sample amounts were 10 nmol, the pairs [Glu, Cys], [Tyr, Met], [His, Phe], [Trp, Val], [Val, Ala] and [Leu, Ile] could not be separated. The differences between the simulated effective mobilities are $0.3 \cdot 10^{-5}$, $0.2 \cdot 10^{-5}$, $0.3 \cdot 10^{-5}$, $0.5 \cdot 10^{-5}$, $0.2 \cdot 10^{-5}$ and $0.2 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ respectively. A discrepancy between the reported order of appearance and the order of R_E values was found for several samples. Similarly to the preceding case ($pH_L = 8.6$), some of these could be attibuted to enforced phenomena.

Comparing the observed separations of the amino acids in the electrolyte systems of $pH_L = 8.6$, 9.0 and 9.4[8] and the differences in the simulated mobilities, it was found that when the difference in the simulated effective mobilities of the samples were less than *ca.* $1 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ they could not be separated. The sample amounts were 10 nmol or less and the separating tube used was *ca.* 80 cm × 0.5 mm I.D.[8].

At $pH_L = 9.7$, several exceptions to the above mentioned rule were found for the pairs Thr, Tyr, (mobility difference = $1.2 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$), Gly, His (1.5), Gly, Phe (2.1) and Ala, Trp (1.5). All of these pairs have not been separated. By simulation of the separation process, when 5 m*M* hydrochloric acid solution buffered by amediol ($pH_L = 9.7$) was the leading electrolyte and the pH of the injected 1:1 mixture was 9, the t_{res} (s) were estimated as 2518 (Thr, Tyr), 3213 (Gly, His), 1916 (Gly, Phe) and 1618 (Ala, Trp).

When the length of the separation tube, l, is less than 80 cm, the threshold value of the difference in effective mobility can be simply estimated as $(80/l) \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$.

As previously concluded experimentally[1], eight to ten amino acids can be separated simultaneously in a single experiment, which is in good agreement with the estimation from the simulated mobility differences. It should be noted that, in some cases, samples having the same effective mobility at the steady state could be separated, and samples with different effective mobilities at the steady state could not be separated[14,15]. In the separation of amino acids, a similar situation can be found in Table IX. However, the separation of amino acids for which the effective mobilities and R_E values are almost the same is not practical. Even when separated, the dynamic range of the separable amount may be small and the separation process may be time-consuming.

So long as only the amino acids are treated, the separability of isotachophoresis using the pH effect on the effective mobility is not competitive with ion-exchange chromatography. To improve this situation, use of Schiff base formation with propanal may be effective for several amino acids[1]. For the separation of mixtures of amino acids and other anionic samples, however, isotachophoresis can be a powerful technique. Especially by utilizing the SIPS program[5], the separability can be assessed and the optimum separation conditions can be estimated conveniently. An example will be found in the succeeding paper on several oligo-peptides[16].

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