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Estimation of the Molecular Weight of Ions by Isotachophoresis.[1] II. Influence of Electric Charge on the Precision of the Correlative Equations[2]

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Correlative equations relating the molecular weight of ions of some organic acids and amino acids to the relative step height (or R_E) in isotachopherograms were derived. The equation for organic acids was $R_E=0.580+0.016M/|Z|$, and that for amino acids was $h_R=0.500+0.021\ M/|Z|$, where the relative step height (h_R) is the ratio of the step height of the sample ion to that of chloride ion, R_E is the ratio of the potential gradient of the sample zone to that of the leading zone, and M and Z are the molecular weight and the electric charge of ions, respectively.

A good inverse proportionality of electric charge (Z) to R_E or h_R was confirmed, even though dissociation was not complete. The slope value (b) of the correlative equation for each ion may represent the ratio of the resistance of the ion to the molecular weight, R_E/M or h_R/M, and may depend on the structure of the ion.

The h_R–pH curves based on the equation for amino acids and the experimental results showed that the order of h_R value was the same as that of molecular weight for Gly, Gly$_2$, and Gly$_3$ in the case of pH$_L$ 10.2.

Keywords——isotachophoresis; ion separation isotachophoresis; electric charge isotachophoresis; carboxylic acid isotachophoresis; organic acid isotachophoresis; amino acid isotachophoresis; glycine oligomer isotachophoresis; molecular weight correlative equation; step-height isotachophoresis; potential gradient isotachophoresis

In the previous study,[1] we derived a correlative equation relating the molecular weight of some carboxylic acids to the relative step height in an isotachopherogram, *i.e.*, $h_R=a+b\ M^n/|Z|$, where h_R, M, n, Z, a and b are the relative step height with chloride ion as a standard, molecular weight, exponent, the electric charge of ions, and two constants, respectively. According to this equation, the molecular weight of ions may be estimated from the relative step height in an isotachopherogram, when the electric charge is known.

On the other hand, simulated indices of many anionic substances were published by Hirokawa *et al.*[3] The R_E value for the qualitative index given by them is the ratio of the potential gradient of the sample zone (E_S) to that of the leading zone (E_L): $R_E=E_S/E_L=(h_S+\Delta h)/(h_L+\Delta h)$. The R_E value was corrected for the relative step height (h_R) by us: $h_R=h_S/h_L$.[1]

This paper describes our studies on the influence of electric charge (Z) on the precision of the correlative equation, using the data for some organic acids given by Hirokawa *et al.*,[3] and on the application of the correlative equation for some amino acids to peptides, using our experimental data.[2]

Calculation

The significance of the correlation coefficient (r) was tested by means of the t-test in each case after transformation of r to z.

Derivation of the Correlative Equation between the Molecular Weight and R_E for Organic Acids in the Case of pH_L 10.0

The values of R_E, molecular weight and electric charge in the case of pH_L 10.0 given by Hirokawa *et al.*[3] were used, where pH_L is the pH of the leading electrolyte. The organic acids were selected ($N=101$) and classified[4] as follows: aliphatic saturated monocarboxylic acids ($N=14$), aliphatic saturated dicarboxylic acids ($N=20$), aliphatic unsaturated monocarboxylic acid ($N=6$), aliphatic unsaturated dicarboxylic acids ($N=6$), hydroxy acids ($N=16$), and aromatic carboxylic acids and phenols ($N=39$).

Relationship between the Exponent of Molecular Weight and the Correlation Coefficient

The correlation coefficient (r) between the n-th power of molecular weight (M^n) and R_E for aliphatic saturated monocarboxylic acids was calculated for various values of the exponent (n) of molecular weight. The values of R_E, molecular weight and electric charge in the case of pH_L 10.0 given by Hirokawa *et al.*[3] were used ($N=10$).

Derivation of the Correlative Equation between the Molecular Weight per Electric Charge and R_E for Some Organic Acids

Phenol in the Range of pH_L 6.5—10.0 ($N=15$) and Capric Acid in the Range of pH_L 3.0—10.0 ($N=31$)——The values of R_E, molecular weight and electric charge given by Hirokawa *et al.*[3] were used.

Aliphatic Saturated Monocarboxylic Acids in the Cases of pH_L 3.0 and 10.0 (each $N=2$)——The values of R_E, molecular weight and electric charge in the cases of pH_L 3.0 and 10.0 given by Hirokawa *et al.*[3] were used.

Derivation of the Correlative Equation between the Molecular Weight and the h_R for Some Amino Acids

Derivation of the Correlative Equation betwen the Mobility and the Molecular Weight for Organic Acids——The values of mobility, molecular weight and electric charge for organic acids given by Hirokawa *et al.*[3] were used ($N=101$, described above).

Determination of the Mobility for Some Monovalent Amino Acids and the Electric Charge in the Cases of pH_L 8.2, 8.9 and 10.2——The electric charge (Z) was calculated from the equation of Kiso and Falk[5]: $Z=-1/2\sum_{i=1}^{n}[1+\tan h\ \{2.303/2\ (\mathrm{pH}-\mathrm{p}K_i)\}]$. The zone pH of the amino acid (A^-) was calculated from the equation of Martin and Everaerts[6]: $[H^+]_{A^-}=K_{A^-}[\{1+4\alpha(\alpha+1)K_B/K_A\}^{1/2}-1]/2(\alpha+1)$, $\alpha=[H^+]_{Cl^-}(1+m_{BH^+}/m_{Cl^-})/K_B(1+m_{BH^+}/m_{A^-})$, where K_B and m_{BH^+} are the dissociation constant and mobility of the buffer ion. The values of m_{BH^+} and K_B of the buffer and m_{Cl^-} were taken from those given by Hirokawa *et al.*[3]: the m_0 and $\mathrm{p}K_a$ values are 29.5 and 8.076 for Tris used in the case of pH_L 8.2, 29.5 and 8.78 for Amediol used in the case of pH_L 8.9, and 44.3 and 9.498 for ethanolamine used in the case of pH_L 10.2, respectively, and the value of m_{Cl^-} is 79.08 ($cm^2V^{-1}s^{-1}\times 10^5$). The mobility ($m_{A^-}$) was calculated according to Eq. 7. The values of $\mathrm{p}K$ and molecular weight for some amino acids were taken from the literature.[7]

Derivation of the Correlative Equation between the Molecular Weight and the h_R for Some Amino Acids——The values of electric charge (Z) and h_R shown in Table II were used.

Relationship between pH and the h_R in the Isotachopherogram

The Influence of Buffer on the h_R–pH Curve——As described above, the equation of Martin and Everaerts[6] shows that there is an influence of buffer on the h_R–pH curve, that is, the electric charge (Z) is affected by the zone pH, which is influenced by the mobility (m_{BH^+}) and the dissociation constant (K_B) of the buffer. Equation 10 was used for the h_R–pH curves. The electric charge (Z) was calculated from the equation of Kiso and Falk.[5] The values of m_0

(36.7) and pK_a (3.552) of β-alanine given by Hirokawa *et al.*[3] and those of the other buffers described above were used.

h_R–pH Curves for Gly, Gly_2 and Gly_3——The values of mobility (m_{A^-}) were calculated according to Eq. 7, and the values of dissociation constant (K_A) were taken from those given by Miyazaki and Katoh[8]: the pK_2values are 9.60 for Gly, 8.17 for Gly_2, and 7.91 for Gly_3.

Experimental

The Values of the h_R for Some Monovalent Amino Acids in the Cases of pH_L 8.2, 8.9 and 10.2——Samples and Reagents: Ala, Asn, Gln, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, Try, Val, Gly_2 and Gly_3 and the following reagents were purchased from Nakarai Chemicals, Ltd. Polyvinyl alcohol ($n = 1750 \pm 50$) was purchased from Tokyo Kasei Kogyo Co., Ltd.

Electrolyte Systems for Isotachophoresis and Instruments: The leading ion was 0.01 M chloride. The pH of the leading electrolyte pH_L was adjusted to 7.5, 8.2, 8.9, 9.5 or 10.2 by using Tris,[3,9] Tris, Amediol,[3,9] Amediol or ethanolamine,[3] respectively, and 0.1% Polyvinyl alcohol was added as a surfactant in order to suppress electroendosmosis and to increase the sharpness of the zone boundaries.[10] The terminating ions were 0.01 M β-alanine and 0.01 M L-lysine. The pH of the terminating electrolyte (pH10.0) was adjusted by using $Ba(OH)_2$; Ba^{2+} precipitates with CO_3^{2-} and should remove the influence of CO_3^{2-}. The migration currents were 75 μA in the case of pH_L 7.5 and 100 μA in the cases of other pH_L. An IP-1B isotachophoretic analyzer with the separating tube of 0.5 mm i.d. and 15 cm length, and a PGD-1 potential gradient detector (Shimadzu Seisakusho Ltd., Kyoto, Japan)[11] were used.

Results and Discussion

Derivation of the Correlative Equation between the Molecular Weight and R_E for Organic Acids in the Case of pH_L 10.0

In the case of pH_L 10.0, that is, of the maximum electric charge in the range of pH_L 3.0—10.0, the following correlative equations between the molecular weight and R_E for organic acids were derived ($N = 101$).

$$R_E = 0.580 + 0.016\ M/|Z| \qquad (r = 0.935,\ p < 0.0001) \qquad (1)$$

$$R_E = 0.346 + 0.092\ M^{2/3}/|Z| \qquad (r = 0.965,\ p < 0.0001) \qquad (2)$$

$$R_E = 0.294 + 0.213\ M^{1/2}/|Z| \qquad (r = 0.961,\ p < 0.0001) \qquad (3)$$

When the electric charge is known, the molecular weight may be estimated from the R_E of an unknown ion according to these equations more exactly than by using the previous equations.[1]

Relationship between the Exponent of Molecular Weight and the Correlation Coefficient

Figure 1 shows the relationship between the exponent (n) of molecular weight and the absolute values of correlation coefficient ($|r|$) for aliphatic saturated monocarboxylic acids.

The exponent in the proportionality of molecular weight to the mobility was reported by Jokl[12] and Offord[13] as $-1/2$, $-1/3$ or $-2/3$. The differences among four exponents in the proportionality of molecular weight to R_E (1, 2/3, 1/2 and 1/3) cannot be regarded as significant. Therefore, we used $n = 1$ as the exponent of the molecular weight for our studies on the influence of electric charge on the precision of the correlative equations. The equation in the case of $n = 1$ was:

$$R_E = 0.946 + 0.015M/|Z| \qquad (r = 0.972,\ p < 0.0016) \qquad (4)$$

Derivation of the Correlative Equation between the Molecular Weight per Electric Charge and R_E for Some Organic Acids

Phenol in the Range of pH_L 6.5—10.0 ($N = 15$) and Capric Acid in the Range of pH_L 3.0—10.0 ($N = 31$)——The following correlative equations between the molecular weight per

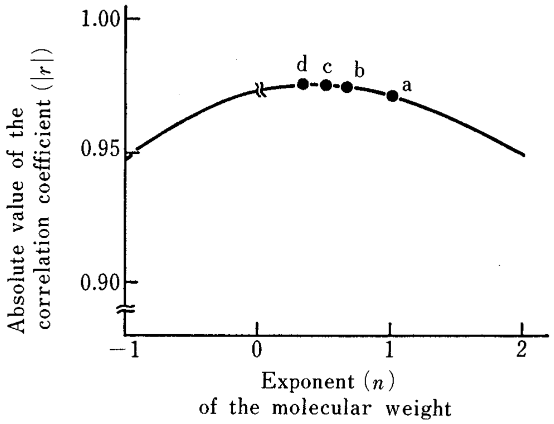

Fig. 1. Relationship between the Exponent (n) of Molecular Weight and the Absolute Value of Correlation Coefficient ($|r|$) for Aliphatic Saturated Monocarboxylic Acids ($N=10$)

a) $n=1$, $R_E=0.946+0.015M/|Z|$ ($r_1=0.972$, $p<0.0016$). b) $n=2/3$, $R_E=0.212+0.106M^{2/3}/|Z|$ ($r_2=0.975$, $p<0.0015$). c) $n=1/2$, $R_E=-0.522+0.303M^{1/2}/|Z|$ ($r_3=0.976$, $p<0.0014$). d) $n=1/3$, $R_E=-1.990+0.973M^{1/3}/|Z|$ ($r_4=0.976$, $p<0.0014$).

R_E is the ratio of the potential gradient of the sample zone to that of the leading zone, and M and Z are the molecular weight and the electric charge of ions, respectively.

electric charge and R_E for phenol (Eq. 5, $N=15$) and for capric acid (Eq. 6, $N=31$) were derived.

$$R_E=0.268+0.023M/|Z| \qquad (r=0.999,\ p<0.0001) \tag{5}$$

$$R_E=0.298+0.020M/|Z| \qquad (r=0.999,\ p<0.0001) \tag{6}$$

The pK of phenol is 9.998,[3] so the electric charge in the range pH_L 6.5—10.0 is small, and R_E was correlated in the range of molecular weight per electric charge ($M/|Z|$) from about 100 to 2000. In the case of $Z=1$, capric acid deviated from the trend for other aliphatic saturated monocarboxylic acids, but Eq. 6 shows a good correlation.

Aliphatic Saturated Monocarboxylic Acids in the Cases of pH_L 3.0 and 10.0 (each $N=2$)——Since a good inverse proportionality of electric charge (Z) to R_E was confirmed, the equations between $M/|Z|$ and R_E for aliphatic saturated monocarboxylic acids were derived, using the values in the cases of only pH_L 3.0 and 10.0. The values of the intercept (a) and the slope (b) of the equations are listed in Table I, and these constants, especially b, may be regarded as a qualitative index, because, when $a=0$ is assumed, the slope is represented by $b'=|Z|\cdot R_E/M$, and in the case of $Z=1$, $b'=R_E/M$. This value may show the ratio of the resistance of the ion to the molecular weight, and may depend on the structure of the ion. As Ohm's law, I=V/R, shows, the potential gradient (E) is proportional to the resistance (R), and we are now studying the relationship between the resistance and the structure of an ion. The values of b,b' for capric acid and the slope of Eq. 6 were 0.020, 0.022 and 0.020, and b, b' for phenol and the slope of Eq. 5 were 0.024, 0.025 and 0.023, respectively. The values of b' for aliphatic saturated monocarboxylic acids are listed in Table I. The correlation coefficient of b and b' was $r=0.996$ ($N=10$, $p<0.0006$).

Derivation of the Correlative Equation between the Molecular Weight and the h_R for Some Amino Acids

The Values of the h_R for Some Monovalent Amino Acids in the Cases of pH_L 8.2, 8.9 and 10.2——We used the h_R as a qualitative index instead of R_E, because for the measurements of R_E, the baseline drift in the isotachopherogram should be suppressed.[14]

The experimental values of h_R for some monovalent amino acids in the cases of pH_L 8.2, 8.9 and 10.2 are listed in Table II; these values were obtained independently, because of the unstable step height of the neighboring zone.

Derivation of the Correlative Equation between the Mobility and the Molecular Weight for Organic Acids——The following correlative equation between the mobility and the molecular weight for organic acids was derived ($N=101$).

$$m_0=2.357+318.0\ |Z|/M^{1/2} \qquad (r=0.970,\ p<0.0001) \tag{7}$$

TABLE I. Some Values for Aliphatic Saturated Monocarboxylic Acids

	Acid	$a^{a)}$	$b^{a)}$	$b'^{\,b)}$
1	Formic	0.04	0.030	0.031
2	Acetic	0.09	0.030	0.032
3	Propionic	0.11	0.028	0.029
4	Butyric	0.12	0.026	0.027
5	Valeric	0.13	0.024	0.025
6	Caproic	0.13	0.022	0.023
7	Enanthylic	0.15	0.021	0.022
8	Caprylic	0.16	0.020	0.021
9	Pelargonic	0.18	0.018	0.019
10	Capric	0.23	0.020	0.022

a) a and b are constants of the correlative equations for each acid: $R_E = a + bM/|Z|$. *b)* b' is slope, when $a=0$ is assumed and in the case of $Z=1$: $b' = R_E/M$.

TABLE II. Some Values for Amino Acids

		$m^{a)}$	$\mathrm{pH_L}$ 10.2		$\mathrm{pH_L}$ 8.9		$\mathrm{pH_L}$ 8.2		$b^{d)}$
			$\|Z\|^{b)}$	$h_R{}^{c)}$	$\|Z\|$	h_R	$\|Z\|$	h_R	
1	Ala	36.0	0.85	3.12	0.41	7.56	0.22	12.7	0.031
2	Asn	30.0	0.98	2.85	0.74	4.08	0.49	5.65	0.020
3	Gln	28.7	0.95	3.08	0.62	5.02	0.38	7.57	0.019
4	Gly	39.1	0.86	2.56	0.43	5.87	0.23	10.0	0.031
5	His	27.9	0.97	3.28	0.68	5.56	0.43	8.75	0.027
6	Ile	30.1	0.88	2.78	0.44	7.52	0.24	12.9	0.025
7	Leu	30.1	0.88	2.87	0.45	7.69	0.25	12.9	0.026
8	Met	28.4	0.94	3.12	0.60	5.23	0.35	8.52	0.020
9	Phe	27.1	0.96	3.40	0.63	5.54	0.38	8.56	0.020
10	Ser	33.4	0.95	2.64	0.61	4.67	0.36	7.10	0.024
11	Thr	31.5	0.95	2.84	0.62	4.59	0.38	7.22	0.023
12	Trp	24.6	0.93	2.98	0.54	6.83	0.31	11.6	0.019
13	Val	31.7	0.87	3.28	0.44	7.08	0.24	11.9	0.024

a) Mobility (m: $\mathrm{cm^2\,V^{-1}\,s^{-1}} \times 10^5$) was calculated according to Eq. 7. *b)* Electric charge (Z) was calculated from the equations of Kiso and Falk[5] and of Martin and Everaerts.[6] *c)* The values of relative step height (h_R) are experimental results. *d)* b is the slope of the correlative equations for each amino acid: $h_R = a + bM/|Z|$.

Determination of the Mobility for Some Monovalent Amino Acids and the Electric Charge in the Cases of $\mathrm{pH_L}$ 8.2, 8.9 and 10.2——The values of mobility for some monovalent amino acids and electric charge in the cases of $\mathrm{pH_L}$ 8.2, 8.9 and 10.2 are listed in Table II.

Derivation of the Correlative Equation between the Molecular Weight and the h_R for Some Amino Acids——The following correlative equations between the molecular weight and the h_R for some amino acids were derived ($N=39$).

$$h_R = 0.500 + 0.021\ M/|Z| \qquad (r=0.918,\ p<0.0001) \tag{8}$$

$$h_R = -0.098 + 0.012\ M^{2/3}/|Z| \qquad (r=0.970,\ p<0.0001) \tag{9}$$

$$h_R = -0.233 + 0.275\ M^{1/2}/|Z| \qquad (r=0.983,\ p<0.0001) \tag{10}$$

The values of the slope (b) of equations for each amino acid are listed in Table II.

Relationship between pH and the h_R in the Isotachopherogram

The Influence of Buffer on the h_R–pH Curve——Figure 2 shows h_R–pH curves for Gly,

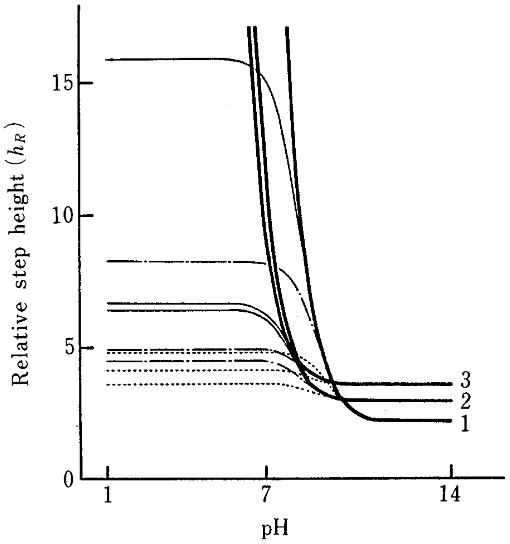

Fig. 2. Influence of Buffer on the Relative Step Height (h_R)–pH Curve

——, β-alanine; ——, Tris; - - - -, Amediol; ------, ethanolamine; 1, Gly; 2, Gly_2; 3, Gly_3.

The equation $h_R = -0.233 + 0.275M^{1/2}/|Z|$ (Eq. 10) was used for calculation. The electric charge (Z) was calculated from the equation of Kiso and Falk.[5] The zone pH values for Gly, Gly_2 and Gly_3 were calculated from the equation of Martin and Everaerts.[6]

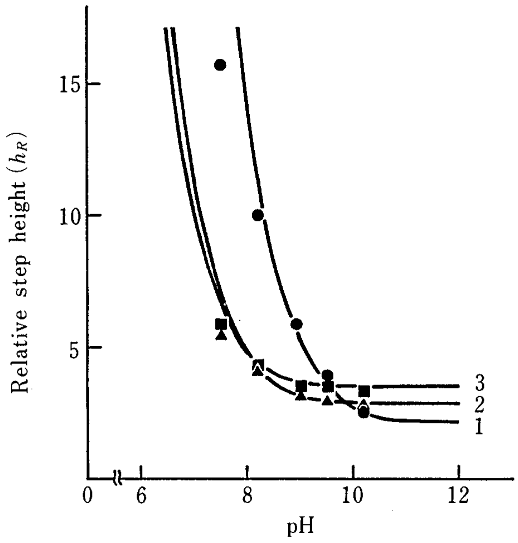

Fig. 3. Relative Step Height (h_R)–pH Curves for Gly, Gly_2 and Gly_3

Calculated results: 1, Gly; 2, Gly_2; 3, Gly_3.
Experimental results: ●, Gly; ▲, Gly_2; ■, Gly_3.
Calculated results were obtained from Eq. 10 with β-alanine as the buffer (Fig. 2).

Gly_2 and Gly_3 calculated using several buffers. The h_R–pH curves with β-alanine as the buffer contained those with ethanolamine, Amediol and Tris, and the pK_a value for β-alanine is smaller that the values for the other buffers.

h_R–pH Curves for Gly, Gly_2 and Gly_3——Figure 3 shows the h_R–pH curves for Gly, Gly_2 and Gly_3 based on Eq. 10, together with the experimental results; these values were obtained independently. In the case of pH_L 10.2, the order of h_R value was the same as that of molecular weight. The optimum pH of electrolytes for the separation of these ions may be near pH 9.0, based on Fig. 3 and the data of Miyazaki *et al.*[8]

These results show that the optimum pH of electrolytes for the estimation of molecular weight or for the separation of ions may be determined from the h_R–pH curves obtained on the basis of this equation.

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