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Gradient Hydroxyapatite Chromatography with Small Sample Loads. IV. Gaussian Expression of the Chromatogram and a Further Consideration on the Resolving Power of the Columns

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Abstract

In earlier papers a pair of equations that represent a theoretical chromatogram using an intermediate parameter was derived. In the present paper the two equations are reduced into a single Gaussian form; essentially the same chromatogram can be calculated from the new equation. For a practical purpose, this equation is much more useful. Introducing a further approximation, the change in the chromatographic resolution occurring with a change in the experimental condition can explicitly be understood. This approximation, with a slight modification, is useful for roughly estimating the optimal chromatographic condition with a very simple calculation. A limit of the application of the theory occurring in an extreme case is discussed.

INTRODUCTION

Earlier (*1*, *2*) a theory of hydroxyapatite (HA) chromatography with small sample loads was developed for linear gradient elution; the elution of molecules is carried out by competition with particular ions from the buffer for adsorbing sites on the HA crystal surfaces (*1*). With small sample loads the chromatographic behavior of any single component in the mixture is independent of the presence of the other components. The theory in Refs. *1* and *2* was confirmed experimentally (*3*). In the theory (*1*, *2*), however, a chromatogram is described in terms of a pair of equations by using an intermediate parameter [eqs. 36 (or 36′) and 34 in Ref. *1*, or eqs. 62 and 73 in Ref. *2*; reproduced as Eqs. (1) and (2) in this article]; it is not easy to understand intuitively the chromatographic behavior of molecules from these equations.

In Ref. *3* it was shown numerically that the shape of a theoretical chromatogram calculated from the two equations is almost Gaussian (in agreement with experiment). In the present paper an approximation is introduced to reduce the two equations into a single Gaussian expression. It can be confirmed that essentially the same chromatogram can be calculated from the new equation. Introducing a further approximation for the standard deviation of a chromatographic peak, a change in the chromatographic resolution occurring with change in the experimental condition can explicitly be understood. This approximation (with a slight modification) is useful for roughly estimating the optimal chromatographic condition with a very simple calculation.

THEORETICAL

Gaussian Approximation

A pair of equations representing a theoretical chromatogram for any component of the mixture that have been derived in earlier papers (*1*, *2*) (see "Introduction") can be written as

$$f_s(m) = \frac{1}{\sqrt{4\pi\theta_0 g s}} e^{-(m-m_\lambda)^2/4\theta_0 g s} B_\lambda(m_\lambda) \tag{1}$$

and

$$m = m_\lambda + r(m_\lambda) - s \tag{2}$$

where m_λ is an intermediate parameter. s and $r(m_\lambda)$ are defined as

$$s = gL \tag{3}$$

and

$$r(m_\lambda) = \int_{m_{in}}^{m_\lambda} \frac{B_\lambda(m_\lambda)}{1 - B_\lambda(m_\lambda)} dm_\lambda \tag{4}$$

respectively. Physical meanings of the symbols involved in Eqs. (1)–(4) are:

m = molarity of competing ions in the chromatogram; the chromatogram is represented as a function of m [when the experimental parameter s (see below) is given]. Since f_s (Eq. 1) is equal to the mean concentration of sample molecules within the last section at

the bottom of the column, m also represents the mean ion molarity within that column section (which increases linearly with increase in elution volume V); within a column section, the ion molarity generally is heterogeneous due to longitudinal diffusion in the total column.

m_λ = local molarity of competing ions within the last section at the bottom of the column.

m_{in} = initial molarity of competing ions at the beginning of the gradient introduced at the top of the column.

$B_\lambda(m_\lambda)$ = partition of sample molecules (under consideration) in solution or in the mobile phase, occurring in a local interstitial elementary volume δV_λ within the section at the bottom of the column; in δV_λ, the ion molarity is m_λ. B_λ moves between 0 and 1, increasing monotonically with an increase of m_λ (see Eq. 10).

L = length of the column.

g = positive constant representing the slope of the molarity gradient of competing ions in the column; this is expressed as the increase in mean ion molarity (within a column section) per unit column length, measured from the bottom to the top. Therefore, the experimental parameter s (Eq. 3) has a dimension of molarity; when s is given, the chromatogram can be represented as a function of m.

τ_θ = positive constant with a dimension of length that measures the longitudinal diffusion in the column.

In the idealized case of no longitudinal diffusion in the column, the chromatogram for any component of the mixture is a sharp peak with an infinitesimal width; the elution molarity, μ, for this peak (when s is given) can be represented as

$$r(\mu) = s \tag{5}$$

(see Eq. A23 in Appendix II of Ref. *1*). Let us now introduce an approximation such that, even with the actual case with longitudinal diffusion in the column, the molarity range over which appears a chromatographic peak (with finite width) is small around the mean value μ; within a chromatographic peak, the partition, $B_\lambda(m_\lambda)$, of molecules in solution is essentially constant, being equal to $B_\lambda(\mu)$. This means that the derivative of the function $r(m_\lambda)$ (Eq. 4) is essentially constant, and that it is equal to $[dr(m_\lambda)/dm_\lambda]_{m_\lambda=\mu}$; by using a Taylor expansion around μ, $r(m_\lambda)$ can be represented at

$$r(m_\lambda) = r(\mu) + \left[\frac{dr(m_\lambda)}{dm_\lambda}\right]_{m_\lambda=\mu} (m_\lambda - \mu) \tag{6}$$

On the basis of Eqs. (2) and (4)–(6), m_λ can now be represented in terms of m and μ as

$$m_\lambda = (1 - B_\lambda(\mu))m + B_\lambda(\mu)\mu \tag{7}$$

By substituting both Eq. (7) and the approximate expression, $B_\lambda(\mu)$, of $B_\lambda(m_\lambda)$ into Eq. (1), a Gaussian approximation of $f_s(m)$:

$$f_s(m) = \frac{B_\lambda[\mu(s)]}{\sqrt{4\pi\theta_0 gs}} e^{-\{B_\lambda|\mu(s)|\}^2/4\theta_0 gs|m-\mu(s)|^2} \tag{8}$$

can be obtained (where it is emphasized that μ is a function of s; cf. Eqs. 5, 12, and 13); the standard deviation, σ, can be represented as

$$\sigma = \frac{\sqrt{2\theta_0 gs}}{B_\lambda[\mu(s)]} \tag{9}$$

It should be noted that if both g and s (or L; see Eq. 3) are given, σ is simply proportional to $\sqrt{\theta_0}$ (Eq. 9). Therefore, the dependences of σ upon g and s (or L) is independent of the value of θ_0; the optimal chromatographic condition can be given independently of the value of θ_0 (see "General Discussion").

The function $B_\lambda(m_\lambda)$ or $B_\lambda(\mu)$ is given by Eq. (A1) in Appendix I of Ref. *1* or Eq. (75) in Ref. *2*, which can be written as

$$B_\lambda(\mu) = \frac{1}{1 + q(\varphi'\mu + 1)^{-x'}} \tag{10}$$

where

$$q = \beta\tau e^{x\varepsilon/kT} \tag{11}$$

Physical meanings of the parameters in Eqs. (10) and (11) are:

φ' = positive constant representing the property of competing ions.
β = positive constant representing the property of the column.
x' = average number (in equilibrium state) of adsorbing sites of HA on which the adsorption of competing ions is impossible due to the presence of an adsorbed molecule; x' represents, therefore, the effective dimensions of the sample molecule.
x = average number (in equilibrium state) of functional groups per molecule that react with sites of HA.
$-\varepsilon(\varepsilon > 0)$ = adsorption energy of a functional group of the molecule onto

one of the sites of HA. Therefore, $-x\varepsilon$ represents the energy per molecule on the HA surface.

$\tau =$ the number of effective geometrical configuration(s) of a molecule on the crystal surface (in equilibrium state). Therefore

$$Q \equiv -kT(\ln q - \ln \beta)$$
$$= -x\varepsilon - kT \ln \tau$$

represents the free energy per molecule on the HA surface (neglecting the solvent effect).

By using Eqs. (10) and (4), Eq. (5) can be rewritten as

$$s = \frac{1}{(x' + 1)\varphi' q}[(\varphi'\mu + 1)^{x'+1} - (\varphi' m_{in} + 1)^{x'+1}] \quad (12)$$

which can be solved with respect to μ, giving the function $\mu(s)$:

$$\mu(s) = \frac{1}{\varphi'}\{[(x' + 1)\varphi' qs + (\varphi' m_{in} + 1)^{x'+1}]^{1/(x'+1)} - 1\} \quad (13)$$

Further Approximations

In the usual case of the molecules that are initially retained at the top of the column, the relationship

$$(\varphi'\mu + 1)^{x'+1} \gg (\varphi' m_{in} + 1)^{x'+1} \quad (14)$$

should, in general, be fulfilled (cf. "Theoretical" Section in Ref. *2* and "General Discussion"). Therefore, if x' is large enough, then, with Eqs. (14) and (12), the second term in the denominator on the right-hand side of Eq. (10) can be reduced to

$$q(\varphi'\mu + 1)^{-x'} \approx q^{1/(x'+1)}[(x' + 1)\varphi' s]^{-x'/(x'+1)} \approx \frac{q^{1/x'}}{x'\varphi' s} \quad (15)$$

By applying Eq. (15) to Eq. (9), an approximate expression of σ:

$$\sigma \approx \sqrt{2\theta_0 gs}\left(1 + \frac{q^{1/x'}}{x'\varphi' s}\right)$$
$$= \sqrt{2\theta_0}\left(g\sqrt{L} + \frac{q^{1/x'}}{x'\varphi'}\frac{1}{\sqrt{L}}\right) \quad (16)$$

can be obtained. Equation (16) shows that, if the column length L is constant, then σ increases linearly with an increase in the slope g of the molarity gradient. If g is constant, σ decreases with an increase of L when L is small. σ increases, however, after the first decrease; the L value giving σ the minimum value (denoted by L^*) and the minimum σ value (denoted by σ^*) can be written as

$$L^* = \frac{q^{1/x'}}{x'\varphi' g} \tag{17}$$

and

$$\sigma^* = \sqrt{8\theta_0 L^*}\, g = \sqrt{\frac{8\theta_0 q^{1/x'} g}{x'\varphi'}} \tag{18}$$

respectively. (For further argument, see "General Discussion").

In many instances the molecular weights of the samples applied to HA chromatography are in the range of 10^4 (globular protein) to 10^8 (virus particle and intact DNA molecule) daltons; it can be deduced that the x' value varies in the range of 10 to more than 1000 (cf. "General Discussion"). However, the elution molarity, μ, does not vary so much, being usually of the order of 0.1 to 0.2 M. Now, if x' and q are both large, Eq. (15) can be represented roughly as

$$q^{1/x'} \approx \varphi'\mu + 1 \tag{19}$$

Therefore, Eqs. (17) and (18) can be written roughly as

$$L^* \approx \frac{\varphi'\mu + 1}{x'\varphi' g} \tag{20}$$

and

$$\sigma^* \approx \sqrt{\frac{8\theta_0 g(\varphi'\mu + 1)}{x'\varphi'}} \tag{21}$$

respectively. Equations (20) and (21) show that both L^* and σ^* decrease with an increase of x' if μ is (almost) constant. (For further argument, see "General Discussion").

DISCUSSION

General Discussion

In Ref. *3*, on the basis of Eqs. (1) and (2) (and Eq. 10), σ was calculated under different experimental conditions for some typical model molecules (Figs. 1–3 in Ref. *3*; cf. Figs. 2 and 3 herein); the resolution, R_s, of the column was also estimated from Eqs. (1) and (2) (and Eq. 10) (Figs. 4, 7, and 10 in Ref. *3*). It can be shown, however, that both σ and R_s that coincide almost completely with those obtained in Ref. *3* can be calculated by using Eqs. (9), (10), and (13) (see below). The shape of the chromatogram calculated from Eq. (8) (and Eqs. 10 and 13) is essentially identical with that calculated from Eqs. (1) and (2) (and Eq. 10); a typical example of such a calculation is shown in Fig. 1 for the lysozyme model molecule. (For details, see the legend of Fig 1.) For practical purposes, a simple equation, Eq. (8), is much more useful than a pair of equations, Eqs. (1) and (2).

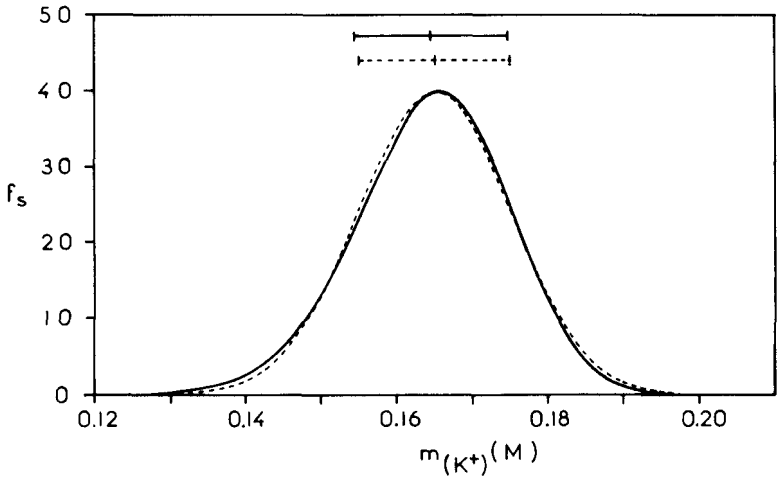

FIG. 1. Two theoretical chromatograms for lysozyme [when $L = 21$ (cm) and $g = 1.18 \times 10^{-3}$ (M/cm)] as functions of m, calculated from Eqs. (1), (2), and (10) (——), and Eqs. (8), (10), and (13) (- -); the former chromatogram is identical with the left-hand side pattern in Fig. 5(a) in Ref. *3*. Both g and m concern competing potassium ions from the buffer; m is written, therefore, as $m_{(K^+)}$ (abscissa of the figure). Both the centers of gravity of the chromatograms and twice the standard deviations are also shown. Lysozyme is characterized by $x' = 7$ and $\ln q = 6.7$ (eqs. 10 and 11; see Ref. *3*). The numerical values of the other parameters are: $\varphi' = 9.0$ (M^{-1}), $\theta_0 = 0.3$ (cm) and $m_{in} = 0.0015$ (M) (*3*).

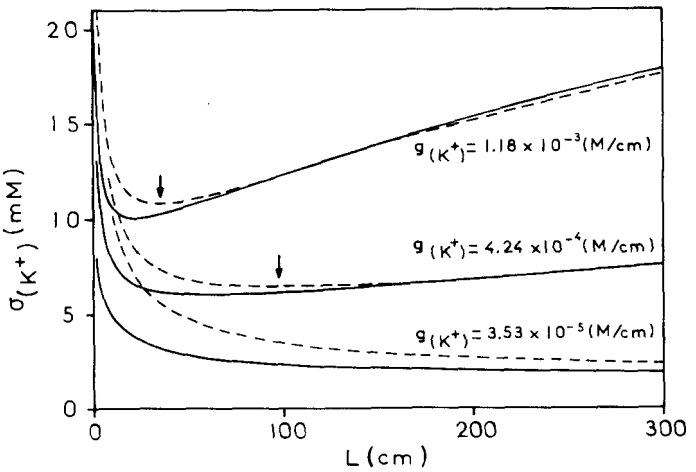

FIG. 2. Standard deviations σ for the lysozyme model as functions of L for three different g, calculated from Eqs. (9), (10), and (13) (or Eqs. 1, 2, and 10) (——), and from Eq. (16) (- -); the former curves are identical with the corresponding curves in Fig. 2 in paper (*3*). Arrows show the L^* values (Eq. 17). In order to show that both σ and g concern competing potassium ions from the buffer, these are written as $\sigma_{(K^+)}$ and $g_{(K^+)}$, respectively. The numerical values of the parameters necessary for the calculation are shown in the legend of Fig. 1.

Let us examine the validity of Eq. (16). Three continuous curves in Fig. 2 represent σ for the lysozyme model molecule (see above) as a function of L for three different g values, calculated from Eqs. (9), (10), and (13); these curves cannot be distinguished from the corresponding curves in Fig. 2 in Ref. *3*, calculated from Eqs. (1) and (2). Now, the three discontinuous curves in Fig. 2 are those obtained from Eq. (16); arrows indicate L^* values (Eq. 17). For the curve with $g = 3.53 \times 10^{-5}$ M/cm, however, L^* (= 1170 cm) giving $\sigma^* = 1.87$ (mM) (Eq. 18) is outside the figure. It can be seen in Fig. 2 that, at least in the neighborhoods of the column lengths L^*, the coincidences of the σ values calculated from the different methods are good; σ^* represents, with a good approximation, the true minimal σ value that can be obtained for a given g value.

It can be shown that the precision in the approximation of Eq. (16) increases, in general, with an increase in x' or the dimensions of the participating molecule. For instance, the σ curves for a molecule with $x' = 70$ (i.e., the molecule with x' ten times as large as that for lysozyme; cf. the legends of Figs. 1 and 3) that cannot be distinguished from those shown in Fig. 2 in Ref. *3* (calculated from Eqs. (1) and (2)) can be calculated even by using Eq. (16). The increase in the precision in Eq. (16) occurring with an increase of x' can also be understood from Fig. 3. Thus the continuous curve

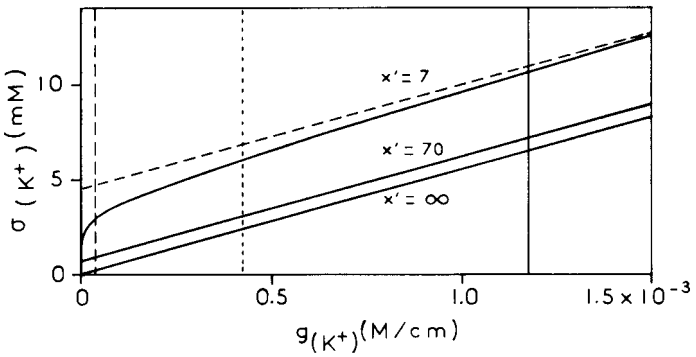

FIG. 3. Dependences of σ upon g when $L = 50$ (cm) for three model molecules with different x' values. The molecule with $x' = 7$ is the lysozyme model ($\ln q = 6.7$) used in both Figs. 1 and 2; the molecule with $x' = 70$ (and $\ln q = 100.3$) is identical with that considered in Ref. *3* (the section entitled "Analysis of Several Experiments," and the molecule with $x' = \infty$ can have any $\ln q$ value. The continuous curve with $x' = 7$ is calculated from Eqs. (9), (10), and (13) (or Eqs. 1, 2, and 10); the discontinuous curve with $x' = 7$ (which is a straight line) is calculated from Eq. (16). Concerning the molecules with $x' = 70$ and $x' = \infty$, (essentially) identical straight lines can be obtained from any method of calculation. The continuous curves with $x' = 7$ and $x' = \infty$ are both identical with the corresponding curves in Fig. 3 in Ref. *3*. Three vertical lines show the three g's in Fig. 2.

indicated by $x' = 7$ in Fig. 3 represents σ for the lysozyme model molecule (when $L = 50$ cm) as a function of g, calculated from Eqs. (9), (10), and (13); this cannot be distinguished from the corresponding curve in Fig. 3 in Ref. *3* calculated from Eqs. (1) and (2). The discontinuous straight line (for $x' = 7$) in Fig. 3 is calculated from Eq. (16). With the molecule with $x' = 70$ (considered above), however, the curve obtained from Eq. (16) cannot be distinguished from that obtained from Eqs. (9), (10), and (13) (or Eqs. 1 and 2), giving a straight line (see Fig. 3); with a further increase of x', this latter tends to a straight line indicated by $x' = \infty$ in Fig. 3.

Except for the mixture of molecules with both small and heterogeneous dimensions, the resolution, R_s, of the column is almost inversely proportional to the mean σ value in the mixture (see Ref. *3*); by replacing the molecular parameters x' and q (Eq. 11) by mean values in the mixture, the optimal column length and corresponding reciprocal of the chromatographic resolution (relatively speaking) can be represented approximately by Eqs. (17) and (18), respectively. Equation (18) shows that the optimal column resolution ($1/\sigma^*$) increases with a decrease of g; the optimal column length L^* increases at the same time with a decrease of g (Eq. 17). However, in the neighborhood of L^*, the variation in σ occurs only slightly with a change of L; the width in the region of the column length (around L^*) where σ is essentially constant

increases with a decrease of g (Fig. 2). From a practical point of view, a minimal σ or a maximal chromatographic resolution would generally be obtained even with a column with a length of about $L^*/4$ (see Fig. 2); this would be the best column length since the shorter the column, the easier is the experiment.

On the basis of Eq. (20), let us deduce best column lengths for some typical molecular mixtures; we here always assume that $g = 3.53 \times 10^{-5}$ M/cm (cf. Fig. 2). Now, for lysozyme [molecular weight = 1.43×10^4 daltons (*4*); lysozyme competes with potassium ions from the buffer for adsorption on the HA surfaces (cf. the legend of Fig. 1)], we have: $\varphi' = 9\ M^{-1}$, $x' = 7$ (legend of Fig. 1), and $\mu \approx 0.1\ M$ (Fig. A1 in Appendix II of Ref. *3*); from Eq. (20) we obtain $L^* \approx 1000$ cm. A best column length, $L^*/4$, for a mixture of lysozyme-like molecules would, therefore, be about 250 cm (cf. Fig. 2). Collagen [which is heterogeneous (*3*, 5); molecular weight = 3×10^5 daltons (*6*)] competes with phosphate ions from the buffer with $\varphi' = 6.7\ M^{-1}$ (*3*), and it is characterized by $x' = 40$ and $\mu \approx 0.1\ M$ (*3*). For collagen, we therefore obtain $L^* \approx 200$ cm and $L^*/4 \approx 50$ cm. DNA also competes with phosphate ions [$\varphi' = 6.7\ M^{-1}$ (*1*)]; it is usual that $\mu \approx 0.2\ M$ (*7, 8*). From the experiment of Wilson and Thomas (*8*), it can be deduced under some assumptions that, for DNA's with molecular weights of the order of 10^6 daltons, $x' \approx 1000$; for these molecules we obtain $L^* \approx 10$ cm and $L^*/4 \approx 2.5$ cm. With DNA it should be noted, however, that except when the sample load is extremely small, the mutual interactions among molecules occurring on the HA surfaces play a fundamental role in chromatography (5); the present theory cannot be applied to this case. It should also be noted that the effective molecular dimensions x' per unit molecular weight of collagen is much smaller than those for both lysozyme and DNA (see above); an explanation for this difference is given in Ref. 5.

Limit of the Theory When the *s* Value is Extremely Small

Equations (1) and (2) are given as a solution of the continuity equation for an abstract molecular flux occurring on the molarity gradient of competing ions that is obtained under a boundary condition given by Eq. (74) in Ref. 2; this equation can be written as

$$\lim_{\substack{s \to +0 \\ m_\lambda \to m_{in}}} f_s/B_\lambda = \delta(m - m_{in}) \tag{22}$$

However, Eq. (22) shows that Eqs. (1) and (2) are valid only for the case of molecules that are initially retained at the top of the column, forming a narrow band (*2*). Practically, only the case of retained molecules is important

since, unless this is the situation, it is unnecessary to apply the molarity gradient. Now, the retained molecules are characterized by the fact that when $m_\lambda = m_{in}$, B_λ is almost equal to zero with a large q value (see Eqs. 10 and 11) since when $B_\lambda \approx 0$, then $R_F \approx 0$. Mathematically, however, the initial B_λ value at the top of the column is finite even with retained molecules. Equations (1) and (2) show that when s (or L) approaches zero, then σ, in general, increases (Figs. 1 and 2 in Ref. *3* or Fig. 2 in this paper). In other words, with a decrease of s, the f_s value, or the mean concentration of molecules in the interstitial liquid within the last section at the bottom of the column, approaches the order of the initial concentration of molecules in the interstitial liquid at the top of the column (occurring due to the finite B_λ value; see above). On the basis of Eqs. (1) and (2), it can be shown that, in this situation, the left-hand part of the chromatogram is eluted out of the column even before the application of the gradient, because σ increases excessively. It can be assumed, however, that the case of such a small s value is beyond the limit of the application of the present theory [constituted on the basis of the introduction of a delta-function (Eq. 22)]. Actually, only experimental conditions where molecules can migrate on the column with practically finite R_F values can be applied. Under these experimental conditions, the B_λ value within the last section at the bottom of the column is not close to zero; the mean concentration, f_s, of molecules in the interstitial liquid within this column section, generally, is much higher than the initial concentration in the interstitial liquid at the top of the column. Therefore, the width in the chromatographic peak is small enough, and virtually no molecules can be eluted out of the column before the application of the gradient (see Fig. 1); for this situation to occur, the s value should be large enough. Of course, it is under these experimental conditions that Eq. (8) can be applied as an approximate expression of Eqs. (1) and (2).

Some arguments related to the introduction of the delta-function were made in both Refs. *1* and *3*; for the case of stepwise chromatography, see Appendix III of Ref. *1*.

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REFERENCES

1. T. Kawasaki, *Sep. Sci. Technol.*, *16*(4), 325–364 (1981). (Part I of this series.)
2. T. Kawasaki, *Ibid.*, *16*(7), 817–851 (1981). (Part III of this series).

3. T. Kawasaki, *Ibid.*, *16*(5), 439–473 (1981). (Part II of this series).
4. T. Imoto, L. N. Johnson, A. C. T. North, D. C. Phillips, and J. A. Pulpley, in *The Enzymes*, Vol. 7, (P. D. Boyer, ed.), Academic, New York, 1972, p. 665.
5. T. Kawasaki, "A Theory of Gradient Hydroxyapatite Chromatography: Approximate Calculations of Chromatograms Taking into Account Both Mutual Molecular Interactions and Longitudinal Diffusion," *Sep. Sci. Technol.*, In Press; "Microheterogeneous Collagen Model on the Basis of a Chromatographic Study on Hydroxyapatite Columns," *Sep. Sci. Technol.*, In Press.
6. W. Traub and K. A. Piez, *Adv. Protein Chem.*, *25*, 243 (1971).
7. G. Bernardi, *Methods Enzymol.*, *21*, 95 (1971).
8. D. A. Wilson and C. A. Thomas, Jr., *Biochim. Biophys. Acta*, *331*, 333 (1973).

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