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Gradient Hydroxyapatite Chromatography with Small Sample Loads. V. Effect of the Top of the Column

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Abstract

The earlier theory of gradient hydroxyapatite chromatography with small sample loads is further developed by taking into account the effect of the top of the column. The ambiguity in the theory occurring when the value of the parameter s is extremely small is eliminated. The resolving power of the column is discussed at the limit when the slope of the gradient tends to zero.

INTRODUCTION

In earlier papers (*1–4*) a theory of gradient chromatography on hydroxyapatite (HA) columns was developed for the case of small sample loads when a narrow band of molecules is formed initially at the top of the column. The process of chromatography is virtually a quasi-static process; a thermodynamic equilibrium is locally realized within any elementary volume, δV, in the column at any instant t (*1*). In the quasi-static process, longitudinal diffusions of both sample molecules and competing ions can be assumed to occur, caused essentially only by the local heterogeneity in the flow rate occurring in the column; the ions constitute a linear molarity gradient in the column (*1*). However, the molarity gradient itself is not disturbed by diffusion since the diffusion effect is canceled out among different parts of the gradient (*1, 3*).

The gradient chromatographic process is indescribable on the basis of a continuity equation for the actual molecular flux occurring in the column (*3*). In order to describe this process it is necessary to stand on a new point of view. From this point of view [called the second point of view on gradient

chromatography (*3*)] it is not the column but rather the molarity gradient of competing ions (actually migrating on the column) that *a priori* is fixed, and this latter is considered as a medium through which migrates a molecular flux (*3*). In contrast to the actual flux in the column, this flux is only endowed with an abstract meaning since molarity m of the ions chromatographically represents a "force" that drives sample molecules out of the crystal surfaces of HA [through a competition mechanism; see Ref. *1* (Appendix I) and Ref. *3*]. In other words, the latter flux migrates in an "intensive space" of the "force" (*3*). Further, the abstract flux is different from the actual flux in that the density C of the former corresponds to the concentration of molecules *in the interstitial liquid in the column* (i.e., the mobile phase) whereas the density Ω of the latter represents the molecular density *in the interstices, including the crystal surfaces, in the column* [i.e., both mobile and stationary phases (*3*)].

In Ref. *3* a continuity equation for the abstract flux has been derived from which a chromatogram can be calculated. This equation involves, as variables, both "position" m on the molarity gradient and parameter s which is proportional to time t provided the flow rate in the column is constant with respect to t (see Eq. 1). With gradient chromatography, the meaning of the "chromatogram" is the distribution in concentration C of molecules in solution that has just been eluted out of the column with length L, represented as a function of molarity m of competing ions in the solution eluted at the same time out of the column. m increases linearly with an increase in elution volume V (with linear gradient chromatography), and V is proportional to time t provided the flow rate is constant with respect to t. This means that m *increases linearly with* t. It can now be understood that two steps are involved in the process of the calculation of the chromatogram starting from the abstract continuity equation. Thus, in the first step, a solution, $C(s, m)$, of the abstract continuity equation is obtained under a suitable initial condition. In this step, based on the second point of view (see above), the solution $C(s, m)$ only has an abstract meaning. In the second step a transfer is made from the second point of view to another, called the first point of view. From this point of view it is the column itself, and not the molarity gradient of the ions, that *a priori* is fixed (*3*). By this transfer of the point of view the meaning of time (relatively speaking) that was given to s (see above) is translated into a meaning of the product of length L of the column and slope g of the molarity gradient (eq. 2). Since g is constant with linear gradient chromatography, it can now be considered that s represents the length of the column (relatively speaking); it is m that increases with time t. Thus the meaning of a chromatogram is given to $C(s, m)$ (for details, see Ref. *3*).

The point of argument in the present paper is concerned with the initial boundary condition to the abstract continuity equation. In the earlier theory

(*1–4*), an assumption was introduced that the initial boundary condition can be given (a) replacing the actual column by a hypothetical column with infinite length that extends upward beyond the top of the column (cf. Remark 1 below), and (b) representing the initial narrow band of molecules occurring at the top of the column in terms of a delta function that has a value only at a longitudinal position, $L = 0$, on the infinite column. Under this assumption, however, an unreasonable conclusion is attained that, when the value of the parameter s (see above and Eq. 2) is extremely small, the left-hand part of the chromatogram should, in general, be eluted out of the column even before the application of the gradient. This occurs independently of whether or not rinsing the column is made between the sample load and the application of the gradient by using the initial solvent [(*4*); cf. Remark 2 below]. In Ref. *4* a limit in the theory occurring in this extreme experimental condition was discussed. Further, the experimental chromatograms, in general, are slightly asymmetrical, with a slower decrease in height on the right-hand side of the pattern than on the other side. On the other hand, with theoretical chromatograms obtained under the assumption of the delta function occurring initially on the infinite column (see above), it is on the left-hand side of the pattern that the height decreases more slowly, although the difference in rates of decrease in height between the two sides of the chromatographic peak is extremely small, and the peak is almost symmetrical (*2, 4*). In Ref. *2* it was suggested that this slight difference between the theoretical and experimental results also arise from the introduction of the delta-function.

In an earlier paper (5) a theory of stepwise elution chromatography was developed in which account was taken of the existence of the top of the column. It can, in general, be assumed (5) that thermal Brownian diffusion of molecules in the interstitial liquid in the column occurs only in association with a diffusion that is provoked by a type [called the second type (5)] of flow heterogeneity. The effect of the column top is conceivable only in terms of the effect of Brownian diffusion plus diffusion due to the second type of flow heterogeneity (called, hereafter, B-dif. plus STFH-dif.) occurring near the top of the column (5). In both the present and the subsequent paper (*6*) this consideration of stepwise chromatography is applied with modifications to gradient chromatography.

In the present paper only the extreme case when the B-dif. plus STFH-dif. effect tends to zero is treated. Under this situation, longitudinal diffusion in the column is limited to a diffusion occurring, and caused by another type (called as the first type) of flow heterogeneity (5), which is identical with the flow heterogeneity considered in the earlier theory of gradient chromatography [(*1–4*); cf. Remark 3 below]. Thus, instead of the infinitesimal molecular band occurring at positon $L = 0$ on the infinite column which is represented by using a delta-function (*1–4*), an infinitesimal band occurring

at the *top* of the column is considered. This latter is conceivable only in terms of an extreme case when B-dif. plus STFH-dif. tends to zero at the column top. A similar consideration for stepwise chromatography was made in Ref. 5, Theoretical Section E. A conclusion is derived that the shape of the chromatogram calculated from the present theory is identical with that obtained earlier (*1–3*). However, the ambiguity in the earlier theory (*1–4*) occurring in extremely small s values (see above), is eliminated. Chromatographic resolution R_s, occurring at the limit when the slope g of the molarity gradient tends to zero, is also discussed (Theoretical Section C).

In the subsequent theory (*6*), account is taken of the finite effect of B-dif. plus STFH-dif. Theoretical chromatograms with slightly asymmetrical shapes, similar to those obtained experimentally (see above), can be calculated (*6*). For this purpose, however, it is first necessary to reduce the fundamental continuity equation (Eq. 1) to a simpler form (*6*).

Remark (1). In Refs. *1–4* the statement that the hypothetical column extends upward beyond the top is made only implicitly, however. The hypothetical column also extends downward beyond the bottom of the actual column. With the quasi-static chromatographic process (see aboe), it can, in general, be assumed (*1*) that density C of sample molecules and molarity m of competing ions in solution that has just been eluted out of the bottom of the column with length L are equal to the density and the molarity in the interstitial liquid that has just passed the longitudinal positon L in the infinite column, respectively. This assumption is necessary in order for $C(s, m)$ that has been calculated from the abstract continuity equation to have meaning for the chromatogram (*1, 3*). The assumption is applicable to some wider cases (*5, 6*).

Remark (2). Since $s = gL$ (Eq. 2), s can be small when either g or L is small. However, from the structure of the fundamental continuity equation (Eq. 1), it can be understood that the unreasonable theoretical result mainly arises from the small L value. Thus, in Eq. (1), the diffusion parameter θ_0 is involved within the term $g\theta_0/B(s, m)$, which constitutes as a whole the apparent diffusion coefficient. When g is small, $g\theta_0/B(s, m)$ is also small and diffusion decreases. This prevents the leak of molecules out of the beginning of the gradient.

Remark (3). In the theory in Refs. *1–4* the effect of the second type of flow heterogeneity *a priori* is neglected. This procedure is necessary in order for the initial boundary condition of the fundamental continuity equation (Eq. 1) to be represnted in terms of a delta function (i.e., for the initial molecular band on the column, in fact, to have an infinitesimal width). Some comment on this problem is made in Remark 2 in Ref. 5, Theoretical Section A. The assumption of a quasi-static chromatographic process, in which longitudinal thermal Brownian diffusion is negligible in comparison with diffusion due to

the total flow heterogeneity (see above), arises from the experimental fact that virtually no deformation of the chromatogram or the change in elution position occurs when the flow rate is changed (*1*). Even though a finite effect of B-dif. plus STFH-dif. is actually occurring, the assumption of the quasi-static process is valid provided that, for the B-dif. plus STFH-dif. effect, it is the second type of flow heterogeniety that plays a major role (cf. Discussion Section in Ref. *6*).

THEORETICAL

A. Some Consideration on the Earlier Theory (*1–4*): A Specification of the Initial Boundary Condition to the Fundamental Continuity Equation

The fundamental continuity equation for the abstract molecular flux obtained earlier (Eq. 17 in Ref. *3*; see Introduction Section) can be written as

$$\mathrm{div}_m\left[\frac{\overrightarrow{1-B(s,\,m)}}{B(s,\,m)}C-\frac{g\theta_0}{B(s,\,m)}\mathrm{grad}_m\frac{C}{B(s,\,m)}\right]+\frac{\partial C}{\partial s}=0 \quad (1)$$

where m = mean molarity of competing ions in solution within a vertical section of the column. By connection m occurring within respective column sections, the molarity gradient can be defined; this is linear with linear gradient chromatography. However, the abstract flux itself is a concept that belongs in the second point of view on gradient chromatography (Introduction Section). From this point of view, m simply represents the current coordinate along which the abstract flux proceeds.

g = positive constant representing the slope of the linear gradient of m. This is expressed as an increase in m per unit length of the column, measured from the bottom to the top.

s = parameter with a dimension of molarity. From the first point of view on gradient chromatography (Introduction Section), this can be defined as

$$s = gL \quad (2)$$

where L represents the length of the column. From the second point of view (i.e., in the abstract flux itself), however, s is a variable that increases with time t; s is proportional to t provided the flow rate is constant with respect to t. Therefore, provided g is given, fixing time t in the abstract flux (second point of view) means fixing length L of the column (first point of view).

C = mean concentration of sample molecules in solution in a vertical section of the column. From the first point of view, C represents the molecular density in solution that has just passed a given logitudinal position L on the infinite column. This can be assumed to be identical with the molecular density in solution that has just been eluted out of the column with length L (see Remark (1) in the Introduction Section). This means that, when both L and g are given, C represents a chromatogram as a function of m. From the second point of view, however, C simply represents the molecular density in the abstract flux occurring at "position" m at "time" s.

θ_0 = positive constant with a dimension of length that measures longitudinal diffusion provoked by the first type of flow heterogeneity in the column.

$B(s, m)$ = ratio of the amount of molecules existing in the interstitial liquid to the total amount in a vertical column section, i.e., partition of molecules in solution.

The function $B(s, m)$ is represented as

$$B(s, m) = B_\lambda[m_\lambda(s, m)] \tag{3}$$

where the function $B_\lambda(m_\lambda)$ is defined by Eq. (A-1) in Ref. *1*, Appendix I, as

$$B_\lambda(m_\lambda) = \frac{1}{1 + q(\varphi' m_\lambda + 1)^{-x'}} \tag{4}$$

in which

$$q = \beta\tau e^{x\varepsilon/kT} \tag{5}$$

The function $m_\lambda(s, m)$ is implicitly defined as

$$m = m_\lambda + r(m_\lambda) - s \tag{6}$$

where

$$r(m_\lambda) = \int_{m_{in}}^{m_\lambda} \frac{B_\lambda(m_\lambda)}{1 - B_\lambda(m_\lambda)} dm_\lambda \tag{7}$$

The physical meanings of the symbols involved in Eqs. (3)–(7) are summarized below:

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

$m_\lambda(s, m)$ = local molarity of competing ions in solution occurring in an infinitesimal part of the vertical column section. Based on the

second point of view, the value of m_λ is determined when both "time" s and "position" m on the molarity gradient are given, "position" m corresponding to the mean molarity of the ions in the column section.

$B_\lambda(m_\lambda)$ = partition of molecules in solution occurring locally in the infinitesimal part of the column section where the molarity of competing ions is m_λ. Equation (3) shows that the mean partition B is equal to the local partition B_λ. This apparently occurs on the basis of the hypothesis that the effect of B-dif. plus STFH-dif. should be canceled within the molecular band migrating on the column. Arguing in the other direction, this is the reason why the B-dif. plus STFH-dif. effect, in fact, is finally negligible. [See the argument in Ref. *3*, Theoretical Section, where thermodynamic diffusion (identical with Brownian diffusion) is considered instead of B-dif. plus STFH-dif. This argument is valid, however; cf. Remark 3 in the Introduction Section and Remark 2 in Ref. 5, Section A]. Equation (4) shows that B_λ increases monotonically with an increase of m_λ, tending to unity when m_λ tends to infinity.

φ = positive constant representing the property of competing ions.

β = positive constant representing the property of the column.

x' = average number (in the 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', therefore, represents the effective dimensions of the sample molecule.

x = average number (in the 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. $-x\varepsilon$ therefore represents the energy per molecule on the HA surface.

τ = number of effective geometrical configuration(s) of a molecule on the HA surface (in the equilibrium state). Therefore

$$Q \equiv -kT(\ln q - \ln \beta) = -x\varepsilon - kT \ln \tau \qquad (8)$$

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

In Ref. *3* the initial boundary condition to Eq. (1) was represented by using a delta-function as

$$\lim_{\substack{s \to +0 \\ m_\lambda \to m_{in}}} \Omega = \delta(m - m_{in}) \qquad (9)$$

(see Eq. 74 in Ref. *3*) where

$$\Omega = C/B \tag{10}$$

represents the total molecular density in the interstices, including the crystal surfaces, of a vertical column section. Equation (9) symbolically represents the situation occurring at time 0 when both relationships $s = 0$ and $m_\lambda = m_{\text{in}}$ are fulilled at position $L = 0$ of the infinite column on which the ion molarity m is extending in an infinite range of $[-\infty, \infty]$ (cf. Remark below). However, if Ω is considered as a function of s and r (Eq. 7), Eq. (9) should be rewritten as

$$\Omega(s = +0, r) = \delta(r) \tag{11}$$

since Eq. (6) shows that, both when $s = 0$ and $m_\lambda = m = m_{\text{in}}$, then $r = 0$. Under the boundary condition given by Eq. (9) or (11), Eq. (1) has a solution

$$C = \frac{1}{\sqrt{4\pi g\theta_0 s}} e^{-\frac{[r|m_\lambda(s,\, m)]-s|^2}{4g\theta_0 s}} B_\lambda[m_\lambda(s,\, m)] \tag{12}$$

(cf. Eq. 62 in Ref. *3*).

Now, by using Eqs. (6) and (7), Eq. (12) can be rewritten as

$$C\left[\frac{\partial m}{\partial m_\lambda}\right]_s dm_\lambda = \frac{1}{\sqrt{4\pi g\theta_0 s}} e^{-\frac{(r-s)^2}{4g\theta_0 s}} dr \tag{13}$$

(cf. Eq. 79 in Ref. *3*). As long as s is fixed, Eq. (13) can further be rewritten as

$$C\, dm = \frac{1}{\sqrt{4\pi g\theta_0 s}} e^{-\frac{(r-s)^2}{4g\theta_0 s}} dr \tag{14}$$

On the other hand, by using Eqs. (3), (10), and (12),

$$\Omega \frac{1}{\sqrt{4\pi g\theta_0 s}} e^{-\frac{(r-s)^2}{4g\theta_0 s}} \tag{15}$$

is derived. It can be verified that Eq. (15), in fact, fulfulls Eq. (11). By using Eq. (15), Eq. (14) can be rewritten as

$$C = \Omega\left[\frac{\partial r}{\partial m}\right]_s \tag{16}$$

This means that the boundary condition (Eq. 9 or 11) to Eq. (1) can finally be specified as

$$C(s = +0, m) = \delta(m - m_{in}) \tag{17}$$

Remark. Therefore, $m_\lambda$is also extended in the range of $[-\infty, \infty]$. Equation (4) only defines the actual B_λ occurring when $m_\lambda \geq m_{in}$. The hypothetical B_λ occurring when $m_\lambda < m_{in}$ can be defined, for instance by Eq. (76) in Ref. *3*, as

$$B_\lambda(m_\lambda) = \frac{1}{1 + qe^{-x'\varphi' m_\lambda}} \tag{4a}$$

r is now extended in the infinite range of $[-\infty, \infty]$ that corresponds to the range $[-\infty, \infty]$ for m_λ and m (see Eqs. 7 and 11–16).

B. Effect of the Top of the Column*

From its mechanism, it can be assumed that the first type of flow heterogeneity cannot occur near the top of the column (see Ref. 5, Theoretical Section D). It can also be asumed (5) that, within the small width

$$\Delta L = 4\hat{\theta}_0 \tag{18}$$

at the column top (where the effect of the first type of flow heterogeneity is negligible), molecules migrate virtually at random, receiving the B-dif. plus STFH-dif. effect. $\hat{\theta}_0$, with a dimension of length, is a positive constant measuring this effect (5). As with the derivation of Eq. (17) or (17′) in Ref. 5 for stepwise chromatography, let us consider the case when the width in the initial molecular band occurring at the column top is smaller than, or equal to, the critical width ΔL, and further when the partition B (Eq. 3) of molecules in solution occurring in any column section within ΔL is very small. This latter is a necessary condition for molecules to be initially retained on the column (cf. Remark in Ref. 5, Numerical Calculations of Idealized Chromatography in the Absence of the First Type of Flow Heterogeneity and Discussion Section). Under this situation a much larger volume of the solvent than the total interstitial volumes

$$\Delta L' = \alpha \Delta L \tag{19}$$

*The consideration made in this section partially originates in the considerations made in both Ref. *7* and Appendix II in Ref. *8*.

within ΔL (where α represents the pore volume per unit length of the column) would pass ΔL while (almost) all molecules are eluted out of ΔL (5). This would mean that the amount of molecules that are eluted out of ΔL while a volume $\Delta L'$ of the solvent passes is virtually equal to the mean amount of molecules that stay in the mobile phase in ΔL during this time interval (5). This hypothesis can be represented in terms of a differential equation

$$-\frac{d\chi}{d(V/\Delta L')} = C = \frac{B(m)}{1 - B(m)}\chi \tag{20}$$

where V is the elution volume. C is defined here as the total amount of molecules existing in the interstitial liquid in ΔL. However, we represent C in such a unit that it is numerically equal to the molecular concentration in solution occurring at longitudinal position $L = \Delta L$ on the column (i.e., the position the distance ΔL apart from the top). At positions $L > \Delta L$, C is always defined as the molecular concentration in the interstitial liquid in the column. This definition is applied to C in Eq. (1). χ represents the total amount of molecules existing on the crystal surfaces in ΔL. As with C, however, χ also represents the molecular density on the crystal surfaces at position $L = \Delta L$. Finally, $B(m)$ represents the partition of molecules in solution occurring in ΔL. This is equal to $B(s, m)$ (Eq. 3) occurring in the infinitesimal column section at position $L = \Delta L$. From the second equality in Eq. (20) and Eq. (10), a general relationship among the three quantities Ω, C, and χ,

$$\Omega = C + \chi \tag{21}$$

can be obtained. (It is possible to add χ, the meaning of the molecular density on the crystal surfaces at column positions $L > \Delta L$. It is also possible to add Ω, the extensive meaning of the total amount of molecules in ΔL.)

In comparison with Eq. (17) in Ref. 5 for stepwise chromatography in which it is Ω that changes with $V/\Delta L'$, in Eq. (20) above it is χ that changes. (although actually $\chi \approx \Omega$; see above). This is due to the fact that, with gradient chromatography, it is χ, and not Ω, that decreases directly with an increase in m which represents the "force" that drives molecules out of the crystal surfaces (Introduction Section; see Ref. *3* Theoretical Section); V increases with m (Introduction Section). In fact, since

$$dm/dV = g/\alpha \tag{22}$$

(which gives a definition itself of g; see the explanation of Eq. 1), Eq. (20) can be rewritten by using Eqs. (18) and (19) as

$$-\frac{d\chi}{dm} = \frac{1}{4g\hat{\theta}_0}\,\frac{B(m)}{1 - B(m)}\chi \tag{23}$$

It is Eq. (23) rather than Eq. (20) that has a fundamental physical meaning (cf. Ref. *3*, Theoretical Section).

Equation (23) can easily be integrated under a normalized conservation conditon

$$\int_{m_{in}}^{\infty} C\, dm = 1 \tag{24}$$

to give

$$\chi = \frac{1}{4g\hat{\theta}_0} \exp\left[-\frac{1}{4g\hat{\theta}_0} \int_{m_{in}}^{m} \frac{B(m)}{1 - B(m)}\, dm \right] \tag{25}$$

which can be rewritten as

$$C = \frac{1}{4g\hat{\theta}_0} \frac{B(m)}{1 - B(m)} \exp\left[-\frac{1}{4g\hat{\theta}_0} \int_{m_{in}}^{m} \frac{B(m)}{1 - B(m)}\, dm \right] \tag{25'}$$

On the other hand, from Eqs. (3) and (7) and the equality between $B(m)$ and $B(s, m)$ occurring when $L = 4\hat{\theta}_0$ (see above) or when $s = 4g\hat{\theta}_0$ (Eq. 2), a relationship

$$r = \int_{m_{in}}^{m} \frac{B(m)}{1 - B(m)}\, dm \tag{26}$$

is obtained. In Eq. (26) the expression $r(m)$ for r should be avoided. In fact, if we give $B(m)$ the meaning of $B(s, m)$ occurring when $s = 4g\hat{\theta}_0$ (see above), then r should be written as $r[m_\lambda(s = 4g\hat{\theta}_0, m)]$ rather than $r(m)$. This is because r originally is defined by Eq (7), and m_λ is a function of s and m (Eq 6). r in Eq. (26) is different from $r(m_\lambda = m)$. Now, by using Eq. (26), Eq. (25') can further be rewritten as

$$\left.\begin{aligned} C &= \frac{1}{4g\hat{\theta}_0} \left[\frac{dr}{dm} \right]_{s=4g\hat{\theta}} e^{-\frac{r}{4g\hat{\theta}_0}} \qquad (\text{for } m \geq m_{in}) \\ \text{and} \\ C &= 0 \qquad\qquad\qquad\qquad\quad (\text{for } m < m_{in}) \end{aligned}\right\} \tag{27}$$

where the second equality has been added only for convenience sake. Here, let us introduce the hypothesis that the B-dif. plus STFH-dif. effect tends to zero (Introduction Section). This means that $\hat{\theta}_0 \rightarrow +0$. Under this situation, Eq. (27) is identical with Eq. (17). Under the boundary condition given by

Eq. (17), Eq. (1) has a solution of Eq. (12). According to the earlier theory (*1–4*), Eq. (12) should represent a theoretical chromatogram with Eq. (6) (see Introduction Section). Equation (12) (with Eq. 6) shows, however, that when s is extremely small, the left-hand part of the chromatogram should be eluted out of the column even before the application of the molarity gradient. This is an unreasonable conclusion (see Introduction Section).

It should be emphasized, however, that it is the first equality in Eq. (27) that involves the physical meaning. At the limit of $\hat{\theta} \to +0$, this represents the infinitesimal molecular band occurring initially at the *top* of the column. It is only by adding the second equality that Eq. (27) coincides with the delta function (Eq. 17). An interpretation should now be introduced that, in Eq. (12), it is the part $m \geq m_{in}$ of C that has the physical meaning; the other part, $m < m_{in}$, that formally occurs in Eq. (12) actually should occur at the beginning, $m = m_{in}$, of the molarity gradient forming a sharp peak. This peak gradually disappears in early stages of the development process. The corresponding argument for stepwise chromatography was made in Ref. 5 Theoretical Section E. In contrast to stepwise chromatography in which the band at the column top keeps the infinitesimal width as long as it remains (see Ref. 5 Theoretical Section E), with gradient chromatography it can be assumed that the width in the sharp peak at the beginning of the molarity gradient (which initially was infinitesimal at the top of the column) increases slightly with the development process. This occurs in association with diffusion at the beginning of the gradient (*3*). However, the peak under consideration actually survives only in the early stages of the development process when the difussion at the beginning of the gradient has just begun. Further, in these stages the total width in the chromatogram (in which is involved, as part, the sharp peak) increases rapidly (cf. Fig. 1). As a result, the width in the sharp peak at the beginning of the gradient can be considered to be virtually infinitesimal.

C. Chromatographic Resolution, R_s, at the Limit of $g \to +0$

In Ref. *4* it was shown that, provided the molarity range over which a chromatogram appears is small around the mean elution molarity μ, Eqs. (12) and (6) reduce to a single equation with a Gaussian form:

$$C = \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{[m-\mu(s)]^2}{2\sigma^2}} \tag{28}$$

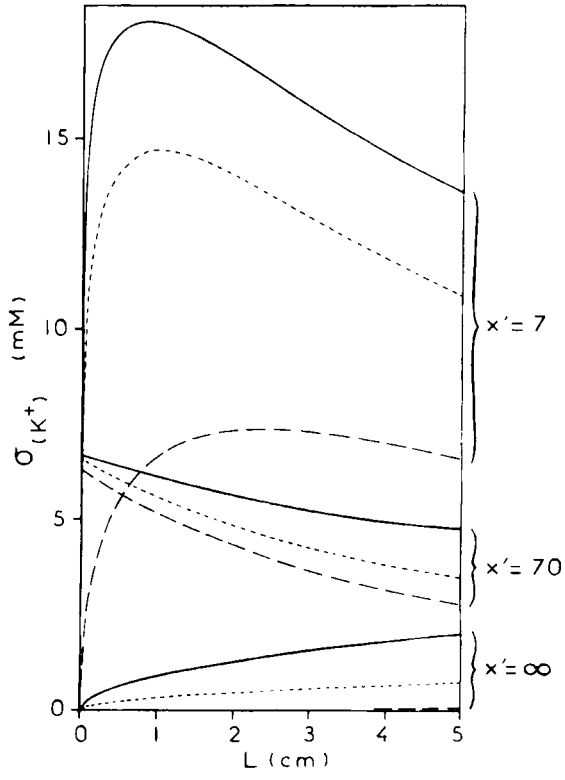

FIG. 1. Recalculations of (some of) the ambiguous parts of both Figs. 1 and 2 in an earlier paper (*2*) occurring when the length L of the column is small. The dependence of the standard deviation, $\sigma_{(K^+)}$, of the theoretical chromatographic peak upon L is shown; the standard deviation is represented in terms of the range of molarities of competing potassium ions over which appears the peak, and $\sigma_{(K^+)}$ is concerned with the part of the total chromatogram from which the infinitesimal peak occurring at the beginning ($m_{(K^+)} = m_{in(K^+)}$) of the potassium gradient is eliminated. $\sigma_{(K^+)}$ is plotted for three different slopes, $g_{(K^+)}$, of the potassium molarity gradient [1.18×10^{-3} (——), 4.24×10^{-4} (- -) and 3.53×10^{-5} (– –) M/cm] for three different molecules with $x' = 7$ and $\ln q = 6.7$ (lysozyme model), with $x' = 70$ and $\ln q = 100.3$, and with $x' = \infty$ and $\ln q = \infty$. For the diffusion parameter θ_0, the best value, 0.3 cm, is used. For any curve, $\sigma_{(K^+)}$ tends to zero when L tends to zero. For the three curves for $x' = 70$, however, the decrease in $\sigma_{(K^+)}$ with a decrease of L occurs in such small values of L that this aspect cannot explicitly be drawn in the figure. The three curves for $x' = \infty$ are parabolas.

where

$$\mu(s) = \frac{1}{\varphi'} \{[(x' + 1)\varphi' qs + (\varphi' m_{in} + 1)^{x'+1}]^{\frac{1}{x'+1}} - 1\} \tag{29}$$

and

$$\sigma = \frac{\sqrt{2g\theta_0 s}}{B_\lambda[\mu(s)]} = \sqrt{2g\theta_0 s}\ \{1 + q[\varphi'\mu(s) + 1]^{-x'}\} \tag{30}$$

(see Eq. 4). Since the width (concerning m) in the chromatogram tends to zero when s tends to zero (Section B), Eq. (28) should hold precisely for extremely small s values. For small s values, Eq. (29) reduces to

$$\mu(s) \approx \frac{qs}{(\varphi' m_{in} + 1)^{x'}} + m_{in} \tag{31}$$

which shows that, when $s \to +0$, then $\mu \to m_{in} + 0$.

s tends to zero when either g or L tends to zero (Eq. 2; cf. Remark 2 in the Introduction Section). We here consider the chromatographic resolution R_s occurring when g tends to zero and L is constant (for R_s, see Ref. 2). Under this situation, the diffusion occurring at the beginning of the molarity gradient (see Section B) is negligible since, when $g \to +0$, the volume of the solvent over which a chromatogram appears is infinity. We also limit ourselves within the case of a mixture of components "1" and "2" with the same effective molecular dimensions x' (for x', see the explanation of Eqs. 3–7). The resolution R_s at the limit of $g \to +0$ can be defined as

$$\lim_{g \to +0} R_s = \lim_{g \to +0} \frac{|\mu_{(2)} - \mu_{(1)}|}{2(\sigma_{(1)} + \sigma_{(2)})} \tag{32}$$

where the two subscripts refer to the two components in the mixture (cf. Eq. 1 in Ref. 2). In the distribution C in Eq. (28), it is only the part $m \geq m_{in}$ that has physical meaning (see Section B). Equation (28) shows, however, that when s tends to zero, then C tends to a delta function occurring at $m = \mu = m_{in}$. This means that, at the limit of $s \to +0$, the total distribution C in Eq. (28) coincides with its significant part $m \geq m_{in}$; $\lim_{g \to +0} R_s$ would, in fact, be definable in terms of Eq. (32). Now by using Eqs. (30) and (31) and taking into account Eq. (2), Eq. (32) can be rewritten as

$$\lim_{g \to +0} R_s = \frac{|q_{(2)} - q_{(1)}|}{\sqrt{32\theta_0}\left[\frac{q_{(1)} + q_{(2)}}{2} + (\varphi' m_{in} + 1)^{x'}\right]} \sqrt{L} \tag{33}$$

or as

$$\lim_{g\to+0} R_s = \frac{\left|1 - \frac{q_{(1)}}{q_{(2)}}\right|}{\sqrt{32\theta_0}\left[\frac{1 + \frac{q_{(1)}}{q_{(2)}}}{2} + \frac{(\varphi' m_{in} + 1)^{x'}}{q_{(2)}}\right]}\sqrt{L} \qquad (33')$$

Let us consider an extreme case when $x'\to\infty$ and $-Q_{(2)}/x' =$ constant (> 0; for $Q_{(2)}$, see Eq. 8). This means that the dimensions of any molecules in the mixture are infinity, but that the free energy per unit molecular dimensions on the HA surface is finite (i.e., not equal to zero), at least concerning Component "2." A proof is given below that, under this situation, a relationship $q_{(2)} \gg (\varphi' m_{in} + 1)^{x'}$ is fulfilled. As a result, Eq. (33') reduces to

$$\lim_{g\to+0} R_s = \frac{\left|1 - \frac{q_{(1)}}{q_{(2)}}\right|}{\sqrt{8\theta_0}\left[1 + \frac{q_{(1)}}{q_{(2)}}\right]}\sqrt{L} \qquad (34)$$

Proof: Since $-Q_{(2)} = \infty$ (see above), Component "2" is perfectly retained at the top of the column at the beginning of chromatography where the relationship $m = m_{in}$ is fulfilled. This verifies the fact that the mean elution molarity $\mu_{(2)}$ of Component "2" is higher than m_{in}. In other words, if $s \neq 0$, then $\mu_{(2)} > m_{in}$, it follows from this that $(\varphi'\mu_{(2)} + 1)^{x'} \gg (\varphi' m_{in} + 1)^{x'}$. Equation (29) reduces to $(\varphi'\mu_{(2)} + 1)^{x'} \approx q_{(2)} s\varphi' x' \approx q_{(2)}$, where the second approximate equality arises from the fact that $q = \beta e^{-Q/kT}$ (Eq. 8) and that $-Q = 0(x')$ (see above). This means that the factor $s\varphi' x'$ that is involved in the intermediate term in the approximate equation (where $x' = \infty$) is virtually equal to unity when $s \neq 0$. Hence the relationship $q_{(2)} \gg (\varphi' m_{in} + 1)^{x'}$ is obtained.

Practically, Eq. (34) gives a very good approximation for Eq. (33) or (33') (cf. Fig. 2).

Remark. In Ref. *2*, Analysis of Several Experiments Section, it was mentioned that, when $x' = \infty$, the optimal length L^* of the column tends to zero, independent of the value of g. This statement appears to be inconsistent with the conclusion that can be derived from Eq. (34) that, when both $x' = \infty$ and $g = +0$, R_s should increase with an increase of L (see Eq. 34). This inconsistency arises from the fact that, in the argument in Ref. *2*, the limit of $x'\to\infty$ *a priori* was considered whereas in Eq. (34) the limit of $x'\to\infty$ is considered after the limit of $g\to+0$ has been obtained. Actually, however, the limit of $x'\to\infty$ and $g\to+0$ is unrealizable; the formal mathematical argument under the unrealizable situation has no practical meaning.

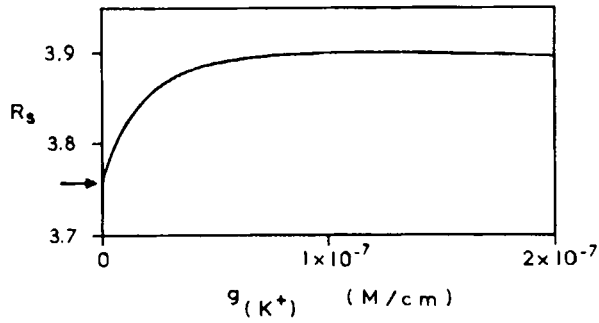

FIG. 2. Representation, by greatly extending the abscissa scale, of the part of the curve for $L = 300$ (cm) in Fig. 4 in an earlier paper (*2*) occurring when the slope, $g_{(K^+)}$, of the potassium molarity gradient is extremely small. Thus the chromatographic resolution, R_s, is plotted as a function of $g_{(K^+)}$ for a column of length 300 cm for the mixture of molecules with $x' = 7$ and $\ln = 6.7$ (lysozyme model) and with $x' = 7$ and $\ln q = 7.4$. It can be seen that R_s increases very slowly with a decrease of $g_{(K^+)}$ when $g_{(K^+)} \gtrsim 10^{-7}$ (*M*/cm); when $g_{(K^+)} \lesssim 10^{-7}$, R_s decreases rapidly with a decrease of $g_{(K^+)}$, tending to a finite value when $g_{(K^+)}$ tends to zero. The arrow shows the limiting R_s values calculated from both Eqs. (33) (or 33') and (34), which cannot be distinguished from each other in the figure.

SOME NUMERICAL CALCULATIONS AND DISCUSSION

Due to the new interpretation given in Theoretical Section B to Eqs. (12) and (6), ambiguities can be eliminated from the results of earlier numerical calculations in Ref. *2* obtained on the basis of these equations. These occur for small s or L values (see Introduction Section and Theoretial Section B). Typical examples of such ambiguities can be seen in both Figs. 1 and 2 in Ref. *2* in which the theoretical dependence of the standard deviation $\sigma_{(K^+)}$ of the chromatographic peak upon the length L of the column is shown. The standard deviation is represented in terms of the range of molarities over which the peak appears, and $\sigma_{(K^+)}$ is plotted for three different slopes, $g_{(K^+)}$, of the potssium molarity gradient for several different model molecules. For very small L values, $\sigma_{(K^+)}$ is incalculable, however (see Figs. 1 and 2 in Ref. *2*).

Figure 1 illustrates results of recalculations of (some of) the ambiguous parts of both Figs. 1 and 2 in Ref. *2* carried out on the basis of the new interpretation for Eqs. (12) and (6) (see above); $\sigma_{(K^+)}$ in Fig. 1 is concerned with the part of the total chromatogram from which the infinitesimal peak occurring at the beginning ($m_{(K^+)} = m_{in(K^+)}$) of the potassium gradient is eliminated (see Theoretical Section B). Thus Fig. 1 shows the dependence of $\sigma_{(K^+)}$ upon L for three slopes, $g_{(K^+)}$, of the potassium gradient for molecules

with different effective dimensions x'. For the diffusion parameter θ_0, the best value, 0.3 cm (see Ref. *2*), is used. (For details, see the legend of Fig. 1.) It can be seen in Fig. 1 that, when x' is finite, $\sigma_{(K^+)}$, in general, increases with a decrease of L unless L is extremely small. $\sigma_{(K^+)}$ finally decreases, however, with a decrease of L, tending to zero when L tends to zero. When x' is large (but not infinite), the final decrease in $\sigma_{(K^+)}$ with a decrease of L occurs to such smal values of L that it cannot explicitly be drawn in Fig. 1 (see the curves for $x' = 70$). From the combination of Fig. 1 and Figs. 1 and 2 in Ref. *2*, a general conclusion can now be reached that, with a decrease of L, $\sigma_{(K^+)}$ decreases, increases, and again decreases, tending finally to zero when L tends to zero. When $x' = \infty$, however, $\sigma_{(K^+)}$ decreases monotonically with a decrease of L, resulting in a parabola for each $g_{(K^+)}$ (see the curves for $x' = \infty$ in both Fig. 1 in this paper and Fig. 2 in Ref. *2*).

Concerning the other figures in Ref. *2*, virtually identical patterns can be obtained even on the basis of the present theory. However, the limiting values $\lim_{g_{(K^+)} \to +0} R_s$, of the chromatographic resolution R_s in Figs. *4*, *7*, and 10 in Ref. *2* can easily be calculated from Eq. (33), (33'), or (34) in Theoretical Section C. For instance, for the mixture with $x' = 7$ in Fig. 4 in Ref. *2*, we obtain, from Eq. (33) or (33'), $\lim_{g(K^+) \to +0} R_s = 0.686$, 1.534, and 3.757 when $L = 10$, 50, dand 300 cm, respectively. These values are essentially equal to the corresponding values 0.687, 1.535, and 3.761 calculated from the approximate equation Eq. (34). In Fig. 4 in Ref. *2* it can be seen, however, that the corresponding values are slightly larger. This is due to the fact that R_s, is general, decreases slightly but very rapidly just before $g_{(K^+)}$ tends to zero. This aspect is drawn in Fig. 2 for the case when $L = 300$ cm by extending extremely the abscissa scale in Fig. 4 in Ref. *2*. In Fig. 2 the arrow shows the limiting R_s values obtained from both Eqs. (33) (or 33') and (34), which cannot be distinguished from each other. From a practical point of view, the slight decrease in R_s occurring in extremely small $g_{(K^+)}$ values is of no importance. The simplest equation, Eq. (34), is useful for the approximate estimations of R_s values that would correspond to minimum practically attainable $g_{(K^+)}$ values (see Fig. 4 in Ref. *2*).

Similar arguments can be made for both Figs. 7 and 10 in Ref. *2*.

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REFERENCES

1. T. Kawasaki, *Separ. Sci. Techol., 16*, 325 (1981).
2. T. Kawasaki, *Ibid., 16*, 439 (1981).
3. T. Kawasaki, *Ibid., 16*, 817 (1981).
4. T. Kawasaki, *Ibid., 16*, 885 (1981).
5. T. Kawasaki, "A Theory of Stepwise Chromatography," *Ibid.*, In Press.
6. T. Kawasaki, *Ibid., 17*, 337 (1982).
7. T. Kawasaki, *J. Chromatogr., 120*, 271 (1976).
8. T. Kawasaki, *Ibid., 161*, 15 (1978).

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