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Gradient Hydroxyapatite Chromatography with Small Sample Loads. I. Fundamental Theory

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Abstract

On the basis of experimental data, it can be deduced that, on a hydroxyapatite column, the effect of thermodynamic longitudinal diffusion of molecules is "hidden" within diffusion occurring due to the heterogeneity in the flow rate of the solution. This can be assumed to occur caused by the heterogeneity in interspaces among hydroxyapatite crystals packed in the column. The chromatographic process is virtually a quasi-static process. By taking into account the longitudinal diffusion in the column, a theory of hydroxyapatite chromatography was developed for small sample loads for the linear gradient elution. The chromatographic mechanisms are fundamentally different between gradient and stepwise chromatographies. No theories that have been developed over many years for stepwise chromatography are applicable to gradient chromatography. Relations of the present theory to both classical "equilibrium" and recent "rate" theories are discussed.

INTRODUCTION

The chromatographic behavior on hydroxyapatite (HA) columns of any single component in a mixture is independent of the other components with small sample loads. The width of the chromatographic peak of a single component is independent of the sample load; it is the height of the peak that is proportional to the load. These facts have been verified experimentally (*1*). A theory was developed (*2–6*) for the case of small sample loads in a linear molarity gradient of competing ions (for competing ions, see below). In this theory, however, the effect of the longitudinal molecular diffusion in the column was not taken into consideration. (In Ref. *4*, this effect is partially considered, however. For some comment

on Ref. *4*, see Ref. *7*, Appendix II.) The purpose of the present paper and a subsequent paper (*8*) is to extend the earlier theory (*2–6*) to a theory in which account is taken of this effect. In a subsequent paper (*9*) the present theory (and the theory in Ref. *8*) will be confirmed experimentally.

Usually the sample initially adsorbed at the top of the column forming a narrow band is eluted from the column by increasing the ion concentration of the buffered solvent (in many instances sodium or potassium phosphate buffer, pH $\approx$ 6.8) stepwise or gradually; a linear molarity gradient is often applied. In gradient elution, when the ion concentration reaches some value, the band begins to broaden and to migrate. It is common practice that the chromatogram appears over a considerably large volume of the solvent. For instance, in a series of experiments with lysozyme where the initial band at the column top has a width much less than 1 cm, the chromatogram appears over 5–70 mL of the solvent when the column diameter is 1 cm (see Fig. 6 in Ref. *1*). This volume should extend over a range of about 10–100 cm provided it exists in the interior of the long column, since the interstice of the packed crystals occupies about 80% of the total volume (*10*). However, this does not mean that the band of the migrating molecules extends over this range in the interior of the column. The band should extend, in general, within a smaller range because the molecules only partially exist in solution or the mobile phase; they partially exist on the crystal surfaces of HA or in the stationary phase.

The flow rate applied is usually in the range of about 0.1–1 mL/min when the column diameter is 1 cm. It is important to note that, in spite of a variation in the flow rate, virtually no deformation of the chromatogram or the change in elution molarity is observed with gradient elution. Nevertheless, with gradient elution with small sample loads, it is observed that the width of the chromatogram increases with an increase in column length when it is high enough (see Fig. 6 in Ref. *1* or Fig. 1 in Ref. *9*). This demonstrates that there is a longitudinal diffusion of molecules in the column. [Broadening of the initial band at the column top at the beginning of the development process (see above) also demonstrates the existence of longitudinal molecular diffusion in the column.] In fact, unless there is longitudinal diffusion, the migration rate of molecules (or the R_F value) in the rear part of the band (where the ion concentration of the buffer is high) should always be higher than that in the front part (where the ion concentration is low), and the width of the band should decrease with the migration of the band on the column; the R_F should increase, in general, with an increase in ion concentration.

It can, in general, be stated that the longitudinal molecular diffusion on the column is contributed to by two types of diffusion: (a) diffusion due to the heterogeneity in the flow rate of the solution within a vertical section of

the column. Without investigating the hydrodynamic mechanism (since this is unnecessary for our purpose), it can simply be assumed that the heterogeneity in the flow rate does occur caused by the heterogeneity in interspaces among HA crystals packed in the column. (b) Thermodynamic diffusion, which is defined in this paper as any diffusion occurring provided the flow rate of the solution is homogeneous within any vertical section of the column. The generality of this statement can be justified by the additive property of flux. Now, if the column is divided into a number of parallel hypothetical columns with diameters of the order of magnitude of the interdistances among HA crystals being packed (the hypothetical column will be called column λ; see the section entitled "Outline of the Theory"), then in each microcolumn the effect of the first type of diffusion would be negligible. ("Parallel microcolumns" does not necessarily mean that the boundaries among them are microscopically smooth, and that they are always completely parallel with the axial direction of the total column. It is possible that the boundaries zigzag microscopically, fulfilling the condition that the flow is essentially homogeneous within each microcolumn.) Since the coexistence of the mobile and stationary phases in a column (which is a fundamental condition necessary for the occurrence of chromatography) should be realized by thermodynamic diffusion, and since it is physically impossible to separate the longitudinal diffusion from the total thermodynamic diffusion (cf. Appendix II), the existence of thermodynamic longitudinal diffusion in a microcolumn is also a necessary condition for chromatography. It is reasonable to assume that, caused by the heterogeneity in interspaces among HA crystals, the flow rate of molecules fluctuates at random not only among different longitudinal positions on the same microcolumns but also among parts of different microcolumns existing within the same vertical section of the total column; this brings about the first type of longitudinal diffusion. ["Diffusion due to the flow heterogeneity" is a concept that is intimately related to the concept of "eddy diffusion." However, the definition of this latter (slightly) differs depending upon the authors. "Diffusion due to the flow heterogeneity" is different at least from "eddy diffusion" defined, for instance, in Ref. *11* (cf. Theoretical Section, "Several Important Parameters").]

Now, the fact that virtually no deformation of the chromatogram nor change in elution molarity occurs when the flow rate is changed but that an increase in the width of the molecular band does occur when the band proceeds on the column (see above) can be explained only by assuming that the effect of thermodynamic molecular diffusion is "hidden" within diffusion due to the heterogeneity in the flow (assumption *a*). Thus the rate of increase in the width of the band due to the former diffusion should be negligible in comparison with the rate due to the latter diffusion;

the ratio of the rate of increase in the band width occurring caused by the heterogeneity in the flow rate to the mean flow rate should be constant (at least in the range of the variation in the mean flow rate examined experimentally; see above).

In order to explain the independence of both the shape of the chromatogram and the elution molarity from the mean flow rate of the solvent (see above), it is also necessary to introduce two further assumptions. Thus, even concerning the longitudinal diffusion of competing ions, the effect of thermodynamic diffusion should be negligible in comparison with the effect of diffusion due to heterogeneity in the flow rate (assumption *b*). This means that the longitudinal diffusions of both sample molecules and competing ions occur essentially in parallel on the column caused by heterogeneity in the flow rate. The chromatographic process is virtually a quasi-static process (assumption *c*). Thus, let us define an elementary volume δV representing the interstitial part, including the crystal surfaces of HA, of a vertical section of a microcolumn (see above). We assume that the width of the section of the microcolumn is of the same order of magnitude as its diameter. It would be reasonable to assume that, in this volume (where the effect of heterogeneity in the flow rate is negligible; see above), the total numbers of both sample molecules and competing ions (see below) are, in general, large enough to be an object of thermodynamics. δV should nevertheless be much smaller than the volume of the solvent that passes through δV during the time interval in which the total molecular bond passes. The system δV, therefore, should have a property such that both the dimensions of the system and the (apparent) total numbers of molecules and ions involved in it are maintained almost constant within a time much shorter than that necessary for the total band of molecules to pass through δV but long enough for an equilibrium state to be virtually achieved. Thus, in this time interval, δV should virtually be a canonical system in which the total Helmholtz energy is at a minimal value. The ratio $(R_F)_{\delta V}$ of the migration rate of sample molecules to the mean migration rate of the solvent occurring at any part of the molecular band should be equal to the partition $B_{\delta V}$ of molecules in solution or the mobile phase in the elementary volume δV existing at that part. This is a first principle of chromatography (*11*). The value of $B_{\delta V}$ should be determinable from the equilibrium theory.

The model which is chosen for the adsorption and desorption phenomena in a system δV is that adsorbing sites are arranged in some manner on the surfaces of HA. Sample molecules with functional or adsorption groups and particular ions from the buffer compete for these crystal sites. For reasoning behind this model, the reader is recommended to see, for instance, the Introduction in Ref. *6* and Appendix I in Ref. *12*. Actually,

it can be deduced that two types of sites, called C and P sites, exist on different surfaces of a crystal (*2*, *3*, *5*, *6*, and Appendix I in Ref. *12*). Both the distribution and the stereochemical structures of these sites on the crystal surfaces were explored on the basis of crystallographic data with the aid of chromatographic data (*5*, *6*). It can be deduced that nucleic acids and nucleoside phosphates are adsorbed onto C sites by using phosphate groups. Acidic proteins and acidic polypeptides are adsorbed mainly onto C sites by using carboxyl groups. All these molecules compete with phosphate ions from the buffer that are also adsorbed onto C sites. Basic proteins and basic polypeptides are mainly adsorbed onto P sites by using basic groups, and compete with cations (sodium or potassium ions) from the buffer that also are adsorbed onto P sites (*5*, *6*, and Appendix I in Ref. *12*; for details, see *10*, *13*, *14*). In many instances the adsorption of molecules occurs virtually onto only one of the two crystal sites (*2*, *3*, *14*, and Appendix I in Ref. *12*): this is the case treated in this paper. A theory in which the possibility of the adsorption onto both C and P sites is taken into consideration was developed in Ref. *3*.

On the basis of the competition model (see above), the partition $B_{\delta V}$ of sample molecules in solution (or the mobile phase) in a system δV can be represented as a function of molarity, $m_{\delta V}$, of competing ions in solution in that system. With small sample loads the density of molecules in the system δV is small in the development process of chromatography. $B_{\delta V}$ is independent of the molecular density. The concentration of sample molecules *in solution* in system δV is low throughout all the chromatographic process. With "retained" molecules, however, the molecular density *on the crystal surfaces of HA* in δV generally is high in the initial band at the top of the column. With a high molecular density on the crystal surfaces, the value of $B_{\delta V}$ depends upon the concentrations of both the molecules under consideration and the molecules of other components of the mixture. As a practical matter, however, the chromatography is carried out independently of the initial value of $B_{\delta V}$ at the column top since, at any rate, this value of $B_{\delta V}$ is virtually zero. This is the reason why molecules are retained on the column. In Appendix I the function $B_{\delta V}(m_{\delta V})$ is given for the case when only a single crystal site is used for chromatography.

THEORETICAL

Outline of the Theory

The general continuity equation for a flow of molecules (for a given component of the sample mixture) on a column can be written, by taking

into account both diffusion due to the heterogeneity in the flow rate and thermodynamic diffusion (see the "Introduction"), as

$$\mathrm{div}_L(\mathbf{v}\Omega - DB\,\mathrm{grad}_L\,\Omega - D_{\mathrm{therm}}\,\mathrm{grad}_L\,C) + \frac{\partial\Omega}{\partial t} = 0 \qquad (1)$$

With small sample loads, this equation should hold independently of the presence of the other components of the mixture. The physical meanings of the symbols involved in Eq. (1) are:

t = time.

L = longitudinal position on the column the distance L apart from the top.

Ω = total molecular density in the interstices, including the crystal surfaces, of a vertical column section at position L occurring at time t.

$\mathbf{v}$ = migration velocity of molecules at position L at time t occurring provided there is no longitudinal diffusion. $\mathbf{v}$, therefore, represents a mean velocity, and $\mathbf{v}\cdot\Omega$ represents the corresponding mean flux of molecules migrating in the interstices, including the crystal surfaces, of the column section. The flux $\mathbf{v}\cdot\Omega$ corresponds directly to the external driving force of chromatography; viz., gravity, pressure produced by the peristaltic pump, etc.

C = mean molecular density (or the concentraion) in the interstitial liquid (i.e., the mobile phase) in the column section at position L at time t.

B = partition of molecules in the interstitial liquid in the column section, or the ratio of the amount of molecules in solution to the total amount in that column section. B, in general, represents the mean value of $B_{\delta V}$ (see the "Introduction") or B_λ (see below) in the column section, and it can also be defined as

$$B = C/\Omega \qquad (2)$$

D and D_{therm} = diffusion coefficients (with dimensions of length^2/time concerning the longitudinal direction of the column) for diffusion due to the heterogeneity in the flow and thermodynamic diffusion, the corresponding fluxes being $-DB\,\mathrm{grad}_L\,\Omega$ and $-D_{\mathrm{therm}}\,\mathrm{grad}_L\,C$, respectively (see below). D is independent of the type of molecules whereas D_{therm} depends upon it. It is apparent that, concomitantly with the migration of the total band of molecules on the column, the centers of molecular bands occurring in the interstices, including

the crystal surfaces, within the corresponding microcolumns (see the "Introduction") are diffused from one another due to the heterogeneity in the flow. It can therefore be assumed that the flux concerning this diffusion is proportional to $-\text{grad}_L\,\Omega$. This flux should also be proportional to B because it must be proportional to the mean migration rate (due to diffusion) of molecules in the interstices, including the crystal surfaces, of the column section, and this latter should be proportional to B. Hence the flux due to heterogeneity in the flow should finally be proportional to $-B\,\text{grad}_L\,\Omega$. It can simply be assumed that the flux due to thermodynamic diffusion is directly proportional to the concentration gradient, $-\text{grad}_L\,C$, of molecules made in the interstitial liquid.

Since actually the effect of thermodynamic diffusion is negligible in comparison with that of diffusion due to heterogeneity in the flow (see the "Introduction"), the relationship

$$DB\,\text{grad}_L\,\Omega \gg D_{\text{therm}}\,\text{grad}_L\,C \tag{3}$$

should be fulfilled, and Eq. (1) reduces to

$$\text{div}_L(\mathbf{v}\Omega - DB\,\text{grad}_L\,\Omega) + \frac{\partial\Omega}{\partial t} = 0 \tag{4}$$

B is independent of the total amount of molecules in the column section (i.e., the quantity Ω) if the amount of molecules is small because, in this situation, the linear section of the adsorption isotherm is realized. With stepwise chromatography the migration of molecules on the column can be described by using Eq. (1) or (4) (see Appendix III). With gradient chromatography, however, the value of B within a given section of the column changes with time t due to a change (with time t) in molarity, m, of competing ions in the interstitial liquid in the same column section; m increases and B also increases gradually. Therefore, if the total amount of molecules in the column section is small, the increase in B with an increase in m should be carried out independently of the amount of molecules (see above). This means that with gradient chromatography it, as principle, is impossible to describe causally the migration of molecules on the column by using Eq. (1) or (4) (even though the conservation of the amount of molecules in a column section can be represented by this equation). This is because Eq. (1) or (4) gives a causal relationship between B (which is involved in $\mathbf{v}$; cf. Eq. A7) and Ω whereas, with gradient

chromatography, B is determined only by *m independently of* Ω (see above; cf. Ref. *8*).

It should be considered, however, that even with gradient chromatography, the migration of molecules on the column is describable by using a certain continuity equation because, even in this instance, the conservation of the amount of molecules should be predictable if the initial condition of chromatography is given. This leads to a consideration that, besides the actual flux of molecules occurring in the column, a certain flux should be conceivable; this flux, as a constituent of the new continuity equation, should play a fundamental role in gradient chromatography. The existence of the fundamental abstract flux in gradient chromatography can also be suggested from the consideration made in Appendix II. In Ref. *8*, the abstract flux is explored and the fundamental continuity equation for gradient chromatography (concerning this flux) is proposed. The equations representing the theoretical chromatograms are derived as a solution of the abstract continuity equation (see Appendix III).

In the present paper the same equations representing the gradient chromatogram are derived by using another method. Thus the column is divided into a number of parallel microcolumns λ ($\lambda = 1, 2, \ldots$) having diameters of the order of magnitude of the interdistances among HA crystals being packed (see the "Introduction"). We characterize the microcolumns in such a way that the volume of the solution that flows into any column λ is the same within unit time interval. We calculate (1) *the contribution of the band of molecules eluted out of column* λ *to the total chromatogram as a function of molarity* m_λ *of competing ions that also are eluted out of the column* λ *by assuming that there is no thermodynamic longitudinal diffusion in column* λ (for this assumption, see below), (2) *the distribution of the flow among different microcolumns*, and (3) *the relationship between the local molarity* m_λ *and the mean molarity* m *in the last section at the bottom of the column*. The fact that the effect of thermodynamic longitudinal diffusion is negligible in the actual column (see the "Introduction") means that the effects of thermodynamic diffusion in the interiors of microcolumns are canceled out among different microcolumns. The assumption of no thermodynamic longitudinal diffusion in a column λ introduced in Step (1) should be valid for the final result of the calculation. Now the final chromatogram obtained under a given experimental condition that can be represented by a parameter s (Eq. 25) can be written as a function of m as

$$f_s(m) = \int_0^\infty f_\lambda(m_\lambda, s_\lambda) \frac{dm_\lambda}{dm} \kappa(s_\lambda)\, ds_\lambda \tag{5}$$

where s_λ is the parameter characterizing the column λ. Column λ can be characterized in terms of the total interstitial volumes involved in it. s_λ is defined as proportional to these volumes (Eq. 22). $\kappa(s_\lambda)\, ds_\lambda$ is the probability of the occurrence of column λ characterized by a value of s_λ comprized between s_λ and $s_\lambda + ds_\lambda$. $\kappa(s_\lambda)$ represents the distribution of the flow among different microcolumns (cf. Step 2) because the flow heterogeneity in the total column is caused by the heterogeneity in interspaces among HA crystals packed in the column ("Introduction"). $f_\lambda(m_\lambda, s_\lambda)$ is the contribution of the molecular band eluted out of column λ (cf. Step 1). $f_\lambda(m_\lambda, s_\lambda)\, dm_\lambda$ represents the probability that molecules are eluted between molarity m_λ and $m_\lambda + dm_\lambda$ from a column λ which is characterized by the parameter s_λ. [$f_\lambda(m_\lambda, s_\lambda)$ is a delta-function (see Eq. 17). Therefore, the meaning of $f_\lambda(m_\lambda, s_\lambda)\, dm_\lambda$ is $\int_{m\lambda}^{m\lambda+dm_\lambda} f_\lambda(m_\lambda, s_\lambda)\, dm_\lambda$. Hereafter, the former symbolical expression will be used for any delta-function.] $f_\lambda(m_\lambda, s_\lambda)\, (dm_\lambda/dm)\, dm$, therefore, represents the probability that molecules are eluted between molarity m and $m + dm$ from the actual column. m_λ can be considered as a function of m [cf. Step 3; see the section entitled "Step 3: Calculation of $m_\lambda(m)$"]. It can now be understood that $f_\lambda(m_\lambda, s_\lambda)\, (dm_\lambda/dm)\, dm\, \kappa(s_\lambda)\, ds_\lambda$ represents the probability that the contributions of the bands of molecules that are eluted from microcolumns with characteristic values between s_λ and $s_\lambda + ds_\lambda$ of the parameter s_λ to the total chromatogram appear between molarity m and $m + dm$ of the ions. This means that $f_s(m)\, dm$ represents the proportion of the part of the actual chromatogram that appears between molarity m and $m + dm$, and that $f_s(m)$ represents the chromatogram itself.

Step 1: Calculation of $f_\lambda(m_\lambda, s_\lambda)$

In Appendix II, two differential equations are derived that represent the idealized chromatographic processes with stepwise and gradient chromatographies occurring in the absence of any type of longitudinal diffusion in the column; these are the DeVault equation and a Wilson-type equation, respectively. For these derivations, it is assumed that the pore volume α per unit length of the column is macroscopically constant. Through a procedure similar to the derivation of Eq. (A13), but with some attention to the fact that the pore volume $\alpha_\lambda\, \delta\lambda$ (or the apparent pore volume $\alpha_\lambda{}^*\, \delta\lambda$; see the section entitled "Several Important Parameters") per unit length of the microcolumn λ depends microscopically upon the longitudinal position, a Wilson-type equation representing the idealized elution process occurring on column λ provided there is no thermodynamic longitudinal diffusion:

$$\frac{\partial\left(\frac{B_\lambda}{1-B_\lambda}\chi_\lambda\right)}{\partial s_\lambda}+\frac{\partial\chi_\lambda}{\partial m_\lambda}=0 \tag{6}$$

can be derived where

$$\chi_\lambda=(1-B_\lambda)\Omega_\lambda \tag{7}$$

(cf. Eq. A4) represents the molecular density on the crystal surfaces in a section of the column λ. In contrast with s in Eq. (A13) that is defined by Eq. (A12), in Eq. (6) s_λ, which is a function of L, is defined as

$$s_\lambda(L)=\int_0^L g_\lambda(L)\,dL \tag{8}$$

where

$$g_\lambda(L)=-dm_\lambda/dL \tag{9}$$

represents the increase in local molarity of competing ions per unit length of the column λ measured from the bottom to the top. g_λ fluctuates at random around a mean value g with an increase of L due to the fluctuation in (apparent) pore volume per unit length of the column λ (see the section entitled "Several Important Parameters").

Partition B_λ (Eq. 6) can be represented as a function of m_λ simply by replacing $m_{\delta V}$ in Eq. (A1) with m_λ as

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

It can be assumed that B_λ is virtually independent of a microscopic variation in pore volume per unit length of the column λ because it is by local equilibrium between the adsorbed phase and the solution in a microscopic region that the value of B_λ is determined. This equilibrium is achieved due to thermodynamic motion of molecules. Therefore, if B_λ changed due to a microscopic positional dependence in the pore volume, this change should be canceled out by thermodynamic diffusion itself.

Corresponding to Eq. (A20), let us introduce a function

$$\begin{aligned} r(m_\lambda)&=\int_{m_{\text{in}}}^{m_\lambda}\frac{\mathrm{B}_\lambda}{1-B_\lambda}\,dm_\lambda \\ &=\frac{1}{q}\int_{m_{\text{in}}}^{m}(\varphi' m_\lambda+1)^{x'}\,dm_\lambda \\ &=\frac{1}{q\varphi'(x'+1)}[(\varphi' m_\lambda+1)^{x'+1}-(\varphi' m_{\text{in}}+1)^{x'+1}] \end{aligned} \tag{11}$$

or its derivatives

$$\frac{dr(m_\lambda)}{dm_\lambda} = \frac{B_\lambda}{1 - B_\lambda} = \frac{1}{q}(\varphi' m_\lambda + 1)^{x'} \tag{12}$$

where m_{in} represents the initial molarity of competing ions at the beginning of the gradient. This should be the same for any column λ. As B_λ is virtually independent of the pore volume per unit length of the column λ (see above), by substituting the first equality in Eq. (11) into Eq. (6),

$$\frac{\partial \chi_\lambda}{\partial s_\lambda} + \frac{\partial \chi_\lambda}{\partial r(m_\lambda)} = 0 \tag{13}$$

is obtained, which can be compared with Eq. (A13″).

With small sample loads when the band of molecules with an infinitesimal width is formed intially at the top of the column, the initial condition for Eq. (13) can be written as

$$[\chi_\lambda]_{r=0} = \delta(s_\lambda) \tag{14}$$

because, when $m_\lambda = m_{in}$, then $r = 0$ (Eq. 11). Under the condition of Eq. (14), Eq. (13) has a solution

$$\chi_\lambda = \delta(s_\lambda - r(m_\lambda)) \tag{15}$$

Due to the property of the delta-function, Eq. (15) only shows symbolically that the band of molecules with a very small width is formed at a longitudinal position on the column λ at which a relationship

$$s_\lambda = r(m_\lambda) \tag{16}$$

is fulfilled (cf. Eq. A23). Equation (15) does not give any information concerning the partition of molecules between the interstitial liquid and the adsorbed phase. This is consistent with the fact that the Wilson-type equation, Eq. (13), does not represent a continuity equation for the actual flow of molecules in the column; viz., it does not represent the conservation of the amount of molecules in the interstices, including the crystal surfaces, in the column. Equation (13) is independent of molecules in the interstitial liquid (see Appendix II).

However, let us examine the following hypothesis: we consider the flow of molecules at a given position L on a given column λ. This position is characterized by a parameter s_λ (Eq. 8). Then it should be observed that the value of the parameter r increases with a lapse of time because m_λ increases with a lapse of time (Eq. 11); all molecules appear at a certain value of r. The probability that the molecules appear between the value r and $r + dr$ of the parameter r should be given by $\chi_\lambda\, dr$, where χ_λ is defined by Eq. (15). This means that the probability that the molecules appear between molarity m_λ and $m_\lambda + dm_\lambda$ of competing ions should be equal to $\chi_\lambda\, [dr(m_\lambda)/dm_\lambda]\, dm_\lambda$. The molecules that appear between molarity m_λ and

$m_\lambda + dm_\lambda$ can now be *interpreted* to be those existing in the interstitial liquid in the column since m_λ represents the molarity in the interstitial liquid. This probability can also represent the probability that the molecules are eluted between molarity m_λ and $m_\lambda + dm_\lambda$ out of a column λ with length L, or the column λ characterized by the parameter s_λ (see above). The contribution f_λ of the band of molecules eluted out of column λ to the total chromatogram can now be represented as a function of both m_λ and s_λ as

$$f_\lambda(m_\lambda, s_\lambda) = \chi_\lambda \cdot \frac{dr(m_\lambda)}{dm_\lambda} = \delta(s_\lambda - r(m_\lambda)) \frac{dr(m_\lambda)}{dm_\lambda} \tag{17}$$

Several Important Parameters

We define $\delta\lambda$ as the ratio of the volume of the solution that flows into a column λ to the volume that flows into the actual whole column. Since the volume of the solution that flows into any column λ is the same within a unit time interval (see the section entitled "Outline of the Theory") the value of $\delta\lambda$ is independent of the value of λ, fulfilling the relationship

$$\int \delta\lambda = 1 \tag{18}$$

A local volume in the actual column that corresponds to the value of $\delta\lambda^{3/2}$ represents a canonical system δV (see the "Introduction").

Let us introduce a quantity $\alpha_\lambda(L)\,\delta\lambda$ that defines the pore volume per unit length of a column λ or the total area of the interstitial part on a vertical section of the column λ existing at a longitudinal position L. $\alpha_\lambda(L)\,\delta\lambda$ corresponds to α with the actual column (see Eq. A7) but is a function of L. It can be assumed that $\alpha_\lambda(L)$ fluctuates microscopically at random with an increase of L around the mean value α. Provided that there are no exchanges of liquids among different microcolumns and that the liquid is incompressible, the flow rate on a column λ should be inversely proportional to $\alpha_\lambda(L)$. The flow rate also should fluctuate virtually at random with an increase of L on a column λ. Actually, the exchange of liquids occurs among microcolumns. In this instance, it is possible to consider an apparent pore volume per unit length of the column λ, $\alpha_\lambda^*(L)\,\delta\lambda$, instead of $\alpha_\lambda(L)\,\delta\lambda$, and to attribute the variation in the flow rate on a column λ to the variation in the value of $\alpha_\lambda^*(L)$. It is reasonable to assume that $\alpha_\lambda^*(L)$ also fluctuates at random with an increase of L around the same value α as in the case of no exchanges of liquids (see above). However, the amplitude in fluctuation in $\alpha_\lambda^*(L)$ would be smaller than that for $\alpha_\lambda(L)$ because, in the former case, the fluc-

tuation would be partially canceled out by exchanges of liquids among microcolumns.

It is to random fluctuation in the flow rate within each column λ that the longitudinal molecular diffusion in the actual whole column is due (see above). If the mean flow rate in the actual column decreases, exchanges of liquids among microcolumns would increase within a time interval necessary for a unit increase in elution volume to occur. This might lead to the conclusion that the amplitude in fluctuation in $\alpha_\lambda^*(L)$ decreases and that the longitudinal molecular diffusion in the whole column also decreases with a decrease in the flow rate. This consideration is not true, however, since it should be to *thermodynamic* diffusion that the exchanges of liquids among microcolumns are mainly, or at least partially, due. The shape of the experimental chromatogram is virtually independent of the flow rate if it varies within the range applied in common practice (see the "Introduction"). This means that, within this range of the flow rate, the total longitudinal diffusion of molecules in the column receives hardly any influence from a change in thermodynamic diffusion of the liquids among microcolumns. This change occurs due to a change in the flow rate within a time interval necessary for a unit increase in elution volume to occur. If the mean flow rate in the column is reduced until considerable exchanges of the liquids begin among the microcolumns, then the width of the total chromatogram must also begin to extend due to thermodynamic diffusion. Thus the condition of a quasi-static process (see the "Introduction") is broken. This condition is also broken if the mean flow rate in the column is too high because, in this instance, the state of any position in the column should be far from the equilibrium state.

Let us again introduce a quantity defined as

$$L'_\lambda(L) = \int_0^L \alpha_\lambda^*(L)\, dL \tag{19}$$

From this definition it is evident that $L'_\lambda(L)\,\delta\lambda$ represents the apparent total interstitial volumes involved between the top ($L = 0$) and a longitudinal position L on column λ. The mean value L', concerning λ, of $L'_\lambda(L)$ at position L can be represented as

$$L'(L) = \alpha L \tag{20}$$

because both $\alpha_\lambda(L)$ and $\alpha_\lambda^*(L)$ fluctuate at random with an increase of L around the same mean value α (see above). L' also represents the total interstitial volumes involved between the top and position L of the actual column.

It is now possible to define the probability $\vartheta(L'_\lambda)\, dL'_\lambda$ that the apparent

total interstitial volumes involved between the top and position L on column λ takes a value between $L'_\lambda\,\delta\lambda$ and $L'_\lambda\,\delta\lambda + dL'_\lambda\,\delta\lambda$. It can be considered that the function $\vartheta(L'_\lambda)$ is directly related to the distribution of flow in a vertical section, existing at position L, of the actual column. The probability $\vartheta(L'_\lambda)\,dL'_\lambda$ can be written as

$$\vartheta(L'_\lambda)\,dL'_\lambda = \frac{1}{\sqrt{4\pi\theta L'}}\,e^{-(L'_\lambda - L')^2/4\theta L'}\,dL'_\lambda \tag{21}$$

where θ is a positive constant with dimensions of volume. Equation (21) can be derived as follows. First, the probability density $\vartheta(L'_\lambda)$ should be represented by a Gaussian distribution around a mean value L'. This is because $\alpha_\lambda{}^*(L)$ should fluctuate at random not only with an increase of L when λ is constant (see above) but also with an increase of λ when L is constant; i.e., at position L on the column, around the same mean value α. Therefore the integral of $\alpha_\lambda{}^*(L)\,dL$ between the column top and position L, i.e., $L'_\lambda(L)$ (Eq. 19), should also fluctuate at random with an increase of λ when L is constant. The mean value of $L'_\lambda(L)$ should be equal to the value of $L'(L)$ (see above). Further, the maximum value of λ can be assumed to be virtually infinity (see above). This means that $\vartheta(L'_\lambda)$ is a Gaussian distribution around a mean value L'. Second, the standard deviation of the Gaussian distribution should be proportional to the square-root of the distance L from the column top, or the square-root of L' (see Eq. 20). This is because the increase in width of a distribution of statistical elements, which is zero initially or when $L' = 0$, is attributed to a random fluctuation of these elements occurring with an increase of L'.

The parameter s_λ (Eq. 8) can now be represented as

$$s_\lambda(L) = \frac{g'}{\delta\lambda}\,L'_\lambda(L)\,\delta\lambda = g'L'_\lambda(L) \tag{22}$$

where

$$\frac{g'}{\delta\lambda} = -\frac{dm_\lambda}{d(L'_\lambda\,\delta\lambda)} \tag{23}$$

represents the slope of the molarity gradient of competing ions on column λ, expressed as an increase in molarity per unit interstitial volume in column λ measured from the bottom to the top. As the volume of the solution that flows into any column λ is the same within a unit time interval (see above), g' is a constant independent of both λ and L. This means that g' can also represent the gradient on the actual column (expressed as an increase per unit interstitial volume measured from the bottom to the top), or the increase in molarity per unit elution volume. Thus g' can also be defined as

$$g' = -\frac{dm}{dL'} = \frac{dm}{dV} \tag{24}$$

where it should be recalled that m represents the mean or macroscopic molarity of competing ions in a section of the actual column.

It can be shown experimentally that the slope of the molarity gradient on a column is essentially equal to the slope that should occur provided there is no adsorption of the ions on the crystal surfaces of HA (see Ref. *7*, Theoretical section *F*). This means that, even though the delay of the gradient occurs immediately after the gradient has been introduced because of the adsorption of the ions, any part of the gradient migrates (macroscopically) with the same rate after the initial delay on the column. This rate should be equal to the rate realized, provided there is no adsorption of the ions on the crystal surfaces. Thus molarity m of the ions in the interstitial liquid or the mobile phase on the column should be high enough, at least except at the beginning of the gradient, for almost all ions in a column section to be in the mobile phase (see Ref. *7*, Theoretical section *F*). Therefore m or m_λ should be virtually independent of the adsorption and desorption phenomena of sample molecules in the column. This is confirmed experimentally (*1*).

The final chromatogram should be expressed, instead of in terms of m_λ and s_λ, in terms of macroscopic molarity m in the last section of the column and a macroscopic parameter s which is introduced by Eq. (A12), or by

$$s = g'L' = gL \tag{25}$$

where

$$g = -\frac{dm}{dL} = \alpha\frac{dm}{dV} = \alpha g' \tag{26}$$

represents a mean slope (concerning λ) of the gradient of competing ions on the actual column, expressed as the increase in molarity per unit length of the column measured from the bottom to the top. This is macroscopically constant with a linear gradient. By comparing Eq. (25) with Eq. (8), it can be understood that s represents a mean value of $s_\lambda(L)$.

Step 2: Calculation of $\kappa(s_\lambda)$

By using Eqs. (21) and (22), $\kappa(s_\lambda)\,ds_\lambda$ can be calculated as

$$\kappa(s_\lambda)\,ds_\lambda = \vartheta(L'_\lambda)\frac{dL'_\lambda}{ds_\lambda}\,ds_\lambda = \frac{1}{\sqrt{4\pi\theta g's}}e^{-(s_\lambda - s)^2/4\theta g's}\,ds_\lambda \tag{27}$$

If both Eqs. (17) and (27) are substituted into Eq. (5),

$$f_s(m) = \frac{1}{\sqrt{4\pi\theta g's}} e^{-[r(m_\lambda)-s]^2/4\theta g's} \frac{dr(m_\lambda)}{dm_\lambda} \frac{dm_\lambda}{dm} \tag{28}$$

is obtained.

Step 3: Calculation of $m_\lambda(m)$

Intergrating Eq. (23),

$$m_0 - m_\lambda = g'L'_\lambda \tag{29}$$

is obtained where m_0 is the integration constant representing the molarity of competing ions at the top ($L'_\lambda = 0$) of column λ. This should be independent of the value of λ. Equation (29) can be rewritten, by using Eq. (22), as

$$m_0 - m_\lambda = s_\lambda(L) \tag{30}$$

Similarly, we obtain from Eq. (24)

$$m_0 - m = g'L' \tag{31}$$

which can be rewritten, by using Eq. (25), as

$$m_0 - m = s(L) \tag{32}$$

Now, by eliminating m_0 between Eqs. (30) and (32),

$$m = m_\lambda + s_\lambda(L) - s(L) \tag{33}$$

is obtained. Further, $s_\lambda(L)$ in Eq. (33) can be replaced with $r(m_\lambda)$ because $s_\lambda(L)$ simply represents the value of s_λ at any longitudinal position L on column λ, whereas $r(m_\lambda)$ represents the value of s_λ occurring at a position L on column λ *at which the band of molecules with an infinitesimal width exists*. This can be understood from the physical meaning of Eq. (16) or (15). Hence we have

$$m = m_\lambda + r(m_\lambda) - s(L) \tag{34}$$

from which we also have

$$\left[\frac{dm_\lambda}{dm}\right]_s = \frac{1}{1 + \dfrac{dr(m_\lambda)}{dm_\lambda}} \tag{35}$$

Equation (34) gives an implicit expression of the function $m_\lambda(m)$ occurring in the last section at the bottom of the column with length L. This column is characterized by the value of s when g' or g is given (Eq. 25). Therefore, the experimental condition can be represented by the parameter s.

Substituting Eq. (35) into Eq. (28), we obtain

$$f_s(m) = \frac{1}{\sqrt{4\pi\theta g' s}} e^{-[r(m_\lambda)-s]^2/4\theta g' s} \frac{\dfrac{dr(m_\lambda)}{dm_\lambda}}{1 + \dfrac{dr(m_\lambda)}{dm_\lambda}} \tag{36}$$

which can be rewritten by using, instead of g' and θ, g and

$$\theta_0 = \theta/\alpha \tag{37}$$

as

$$f_s(m) = \frac{1}{\sqrt{4\pi\theta_0 g s}} e^{-[r(m_\lambda)-s]^2/4\theta_0 g s} \frac{\dfrac{dr(m_\lambda)}{dm_\lambda}}{1 + \dfrac{dr(m_\lambda)}{dm_\lambda}} \tag{36'}$$

The reduced diffusion parameter θ_0 (Eq. 37) has a dimension of length. In both Eqs. (36) and (36') the factor

$$\frac{\dfrac{dr(m_\lambda)}{dm_\lambda}}{1 + \dfrac{dr(m_\lambda)}{dm_\lambda}} = B_\lambda \tag{38}$$

measures the partition of molecules in the interstitial liquid in column λ (see Eqs. 10 and 12).

Equation (36) or (36') represents, with Eq. (34), the chromatogram f_s as a function of m by using m_λ as an intermediate parameter. When the column has a macroscopic length and the slope g' or g of competing ions has a finite value (not too close to zero), then $f_s(m)$ is normalized such that

$$\int_{m_{in}}^{\infty} f_s(m)\, dm = 1 \tag{39}$$

which is common practice (*8*).

Finally, the shape of the chromatogram $f_s(m)$ is governed mainly by the factor

$$\frac{1}{\sqrt{4\pi\theta g' s}} e^{-[r(m_\lambda)-s]^2/4\theta g' s}$$

or

$$\frac{1}{\sqrt{4\pi\theta_0 g s}} e^{-[r(m_\lambda)-s]^2/4\theta_0 g s}$$

in Eq. (36) or (36'). This represents a Gaussian distribution concerning the parameter r with the maximum value at $r = s$. On the other hand, Eq. (34) shows that when $r(m_\lambda) = s$, then $m_\lambda = m$. This means that the

value of m which fulfills the relationship

$$r(m) = s \tag{40}$$

or

$$m = \frac{1}{\varphi'}\{[(x' + 1)\varphi' qs + (\varphi' m_{in} + 1)^{x'+1}]^{1/(x'+1)} - 1\} \tag{40'}$$

(see Eq. 11) represents approximately both the molarity of the ions at which the maximum height of the chromatographic peak occurs and the molarity at which the center of gravity of the peak is eluted. It should be noted that Eq. (40') is identical to Eq. (A25). This represents the elution molarity of the sharp peak occurring provided there is no longitudinal diffusion of molecules in the column. In a subsequent paper (*9*) it will be shown numerically that the theoretical chromatogram has a shape almost identical to a Gaussian shape.

The Case When $x' = \infty$

It can be understood from the physical meanings of the parameters x' and x (see Appendix I) that to change the value of x', while keeping the value of the parameter

$$\xi = x/x' \tag{41}$$

constant, corresponds to considering homologous molecules with different dimensions. We consider here an extreme case when the molecule has an infinite value of x' and a finite value of ξ, or when the molecule has infinite dimensions. It is easy to show that, for molecules with infinite dimensions, $B_{\delta V}$ (Eq. A1) or B_λ (Eq. 10) increases stepwise from 0 to 1 with an increase of $m_{\delta V}$ or m_λ at a critical value:

$$m^\circ = \frac{1}{\varphi'}(e^{\xi\varepsilon/kT} - 1) \tag{42}*$$

This means that, provided there is no longitudinal diffusion of sample molecules nor competing ions in the column, the chromatogram should always be a sharp peak appearing at molarity m° of the ions. This is independent of both the length of the column and the slope of the molarity gradient of the ions. Therefore the contribution, denoted by $f_\lambda(m_\lambda)$, of the band of molecules eluted from a column λ to the total chromatogram can be represented, instead of by Eq. (17), as

$$f_\lambda(m_\lambda) = \delta(m_\lambda - m^\circ) \tag{43}$$

*If the factor $(kT/\varepsilon)((\ln \tau)/x')$ does not converge to zero when x' tends to infinity, ξ in Eq. (42) should be replaced with the apparent value: $\xi_{app} \equiv \xi + (kT/\varepsilon)((\ln \tau)/x')$.

which is independent of s_λ. On the other hand, m_λ is always related to m through Eq. (33) or by the relationship

$$s_\lambda - s = m - m_\lambda \tag{33'}$$

Therefore, the quantity $\upsilon(m_\lambda)\, dm_\lambda$ which is obtained by substituting Eq. (33') into the term $s_\lambda - s$ in Eq. (27) and by replacing ds_λ with dm_λ*, i.e.,

$$\upsilon(m_\lambda)\, dm_\lambda = \frac{1}{\sqrt{4\pi\theta g' s(L)}} e^{-(m-m_\lambda)^2/4\theta g' s(L)}\, dm_\lambda \tag{44}$$

represents the probability that, when the mean molarity of the ions in the last section at the bottom of the column with length L is m, the local molarity in that section is between m_λ and $m_\lambda + dm_\lambda$. Hence the chromatogram $f_s(m)$ can be represented as

$$\begin{aligned} f_s(m) &= \int_{m_{1n}}^{\infty} f_\lambda(m_\lambda)\upsilon(m_\lambda)\, dm_\lambda \\ &= \frac{1}{\sqrt{4\pi\theta g' s}} e^{-(m-m^\circ)^2/4\theta g' s} \\ &= \frac{1}{\sqrt{4\pi\theta_0 g s}} e^{-(m-m^\circ)^2/4\theta_0 g s} \end{aligned} \tag{45}$$

which is a Gaussian distribution with both the maximum height and the center of gravity always at

$$m = m^\circ \tag{46}$$

(for m°, see Eq. 42). The standard deviation σ of the distribution $f_s(m)$ (Eq. 45) can be expressed in terms of the range of molarities of competing ions, as

$$\sigma = \sqrt{2\theta g' s} = \sqrt{2\theta_0 g s} \tag{47}$$

Equation (47) can be rewritten, by using Eqs. (20), (25), (26), and (37), as

$$\sigma = \sqrt{2\theta L' g'} = \sqrt{2\theta_0 L g} \tag{47'}$$

The standard deviation of the chromatogram also can be represented in units of both elution volume V and reduced elution volume

$$V_0 = V/\alpha \tag{48}$$

by dividing Eq. (47') by g' and g, giving

$$\sigma_V = \sqrt{2\theta L'} \tag{49}$$

*To be precise, the quantity obtained by substituting Eq. (33') into the term $s_\lambda - s$ in Eq. (27), by replacing $m - m_\lambda$ with $m_\lambda - m$, by replacing ds_λ with dm_λ, and by again replacing $m_\lambda - m$ with $m - m_\lambda$.

and

$$\sigma_{V_0} = \sqrt{2\theta_0 L} \tag{50}$$

respectively. Both σ_V and σ_{V_0} are independent of the slope of the molarity gradient, and increase with an increase of L' and L, respectively. This is a natural conclusion since the chromatogram occurring provided there is no longitudinal diffusion in the column is a sharp peak independent of both the slope of the molarity gradient and the length of the column (see above).

It is easy to show that Eq. (40′) reduces to Eq. (46) when x' approaches infinity and when ξ is constant. In a subsequent paper (*9*) it will be shown numerically that Eqs. (36) and (34) or Eqs. (36′) and (34) reduce to a single equation, Eq. (45), at the same time (Figs. 2 and 3 in Ref. *9*). This is shown theoretically in Ref. *8*.

DISCUSSION

Classical theories of adsorption chromatographies were developed over 40 years ago by Wilson (*15*), DeVault (*16*), and Weiss (*17*) for stepwise chromatography on the basis of simple assumptions of (a) instantaneous equilibrium of adsorbed phase and solution and (b) no longitudinal diffusion in the column (cf. the last paragraph in Appendix II). [The terminology "stepwise" includes, of course, the case when the development of the solute on the column is carried out by using the same solvent as that of the sample solution. In HA chromatography, however, the solvent used for the development usually is different from that of the sample solution (see the "Introduction").] The relation of these theories to the theory developed earlier (*2–6*) for gradient chromatography, also based on both assumptions (a) and (b), was discussed in Ref. *7*. This is reviewed (with some modifications) in Appendix II, limiting the case within small sample loads. Thus there is a fundamental difference in mechanisms between stepwise and gradient chromatographies (Appendix II; see also Appendix III). A more explicit explanation for this difference is given in Ref. *8*.

Stepwise and gradient chromatographies are also different in the following two respects. First, the equation representing the stepwise chromatogram (with small sample loads) involves the parameter B (see Eq. A35 or A35′). B represents the partition of molecules in the mobile phase, taking a constant value throughout the chromatographic process. In other words, the chromatographic process is independent of the mechanism itself due to which the parameter B takes the given value. As a result, even with gel chromatography, it is possible to assume a partition

B and to consider the elution process of the sample on the basis of the theory of adsorption chromatography with stepwise elution. This is a consideration first developed by Ackers (*18*). With gradient chromatography, however, it is the structure of B, and not the value of this parameter, that is directly concerned with chromatography. The structure of B is determined by the competition mechanism (see the "Introduction"), and this structure can, in general, be represented as the mean structure of B_λ (Eq. 10) concerning λ.

Second, with heavy sample loads when mutual interactions among sample molecules play an important role, chromatography depends upon the structure of B even in the case of stepwise chromatography. Here also, however, the manner of dependence is fundamentally different from that with gradient chromatography. In general, it is the shape of the adsorption isotherm of sample molecules on the stationary phase that determines the structure of B. This shape is determined by both geometrical and energetical interactions among molecules existing in the stationary phase, i.e., on the crystal surfaces with HA chromatography. The interactions occurring in the mobile phase or solution are negligible since the molecular concentration in solution is small. With HA chromatography the geometrical interactions among molecules on the crystal surface can be expressed in terms of the probability p that when a new molecule is added at random onto the crystal surface, a certain proportion of which is already occupied by molecules, it is not superimposed on the already adsorbed molecules (*19*, *20*). [With single component chromatography, let χ be the proportion of the crystal surface occupied by molecules that have already been adsorbed (see above). In this instance, provided the effect of energetical interactions among molecules is negligible, the Langmuir adsorption isotherm is obtained when $p = 1 - \chi$.] It can be shown, however, that the structure of B receives hardly any influence from the actual value of p in the case of gradient chromatography (*19*, *20*). Therefore, if the effect of the energetical interaction is negligible, the chromatography is carried out almost independently of both the sample load and the shape of the adsorption isotherm. With stepwise chromatography, however, the shape of the chromatogram depends directly upon the shape of the adsorption isotherm even in the absence of energetical molecular interaction.

Classical theories of adsorption chromatographies (see above) are also called "equilibrium" theories in contrast with "rate" theories developed more recently for the case of stepwise chromatography (*11*, *21–31*). In these theories both (a) the adsorption and desorption phenomena and (b) the longitudinal diffusion of molecules in the column are treated on kinetic bases. It is shown that slow adsorption and desorption rates of

molecules (in comparison with the flow rate) also bring about longitudinal diffusion. Therefore the total thermodynamic diffusion (defined in the "Introduction") would, in general, be caused both by thermal Brownian motion of molecules in solution and adsorption and desorption phenomena. If the flow rate is low enough, however, the latter effect should be negligible. Thermodynamic diffusion (being identical, in this instance, with thermal Brownian diffusion) is represented by the last term within the divergence term in Eq. (1). [Equation (1) is valid even by taking into acount the diffusion associated with slow adsorption and desorption rates; in this instance the third term within the divergent term in Eq. (1) is concerned with thermal Brownian diffusion, and the diffusion term due to slow adsorption and desorption rates does not explicitly appear in Eq. (1). It should be assumed (*11*, *21–31*), however, that C and χ (being related to Ω through Eq. A5) change with each other with time t. As a result, Eq. (1) can be rewritten into simultaneous equations for C and χ (cf. *22*, *25–29*). In the section entitled "Outline of the Theory," however, the explanation of Eq. (1) was made by neglecting *a priori* the effect of slow adsorption and desorption rates, since this hypothesis is sufficient for our purpose.] In the present theory it is further assumed that the effect of thermodynamic diffusion is "hidden" within diffusion due to heterogeneity in the flow rate in the column or, more generally, that the chromatography is a quasi-static process since it is carried out virtually independently of the flow rate (see the "Introduction"). In this situation, kinetic treatment of HA chromatography would gain no advantages over the quasi-static treatment.

Sørensen (*29*) reaches the conclusion that the migration velocity of the center of mass of a molecular band on the column is unaffected by any deviation from quasi-equilibrium conditions (at least with "linear" chromatography, which would be the case with small sample loads). A similar conclusion is also reached by Giddings on the basis of a random-walk consideration for the chromatographic process (*11*). This conclusion would be applied to any microcolumn λ, which would mean that, provided the effect of thermodynamic diffusion (i.e., both thermal Brownian diffusion and diffusion due to slow adsorption and desorption rates) is "hidden" within diffusion occurring due to the flow heterogeneity in the total column, the chromatography is carried out independently of the adsorption and desorption rates of molecules. The chromatographic process is virtually a quasi-static process in which the chromatogram is independent of the flow rate.

A differential equation similar to Eq. (1) or Eq. (A39) was given nearly 30 years ago by Lapidus and Amundson (*22*) for the purpose of describing a stepwise chromatographic process by taking into account the longi-

tudinal diffusion in the column. Mathematical solutions for this equation were obtained under wider initial conditions than that applied in Appendix III (Eq. A40). On solving the differential equations in both Ref. *22* and Appendix III, it is assumed for mathematical simplicity that the length of the column is infinity. In Ref. *22*, however, the physical interpretation of the longitudinal molecular diffusion in the column was made on a kinetic basis (even in the "equilibrium" treatment in Ref. *22*). As a result, the conclusion was reached that the mathematical solutions for the continuity equation are valid for describing the behaviors of molecules on a hypothetical column with infinite length, but that these cannot give the shapes of any actual chromatograms of the molecules that are eluted out of the column with finite length (*22*). The situation is different with the quasi-static treatment in the present paper in both stepwise (Appendix III) and gradient chromatographies. In this treatment the shape of the actual chromatogram can be represented by using the mathematical solution obtained by assuming that the column has an infinite length, since the flow of molecules that proceeds backward on the column is negligible (see Appendix III).

However, on calculating the theoretical chromatogram, Sørensen (*29*, *30*) introduced the following assumptions in a somewhat implicit way. Thus he assumed (a) that the longitudinal thermal Brownian diffusion in a column (see above) is negligible in comparison with both diffusion due to the flow heterogeneity and diffusion occurring associated with adsorption and desorption phenomena (cf. the last line on p. 200 in Ref. *29*), and (b) that the last diffusion occurs within the column independently of the total column length. Under these assumptions the chromatogram for any finite column length might be calculated by using a boundary condition for an infinite column (*29*, *30*). It has explicitly been shown (*29*, *30*) that the equation that is equivalent to Eq. (A34) can be derived for the special case when the elution velocity is low (but not too low, in order for thermal Brownian diffusion to be negligible).

As Sørensen points out (*29*, *30*), the equation representing the molecular distribution as a function of both column position and time (or elution volume) that he has derived (under some approximations) is much simpler and more tractable than those derived by other authors (*22*, *27*, *28*) on the basis of the same differential equation (Eq. 1, but see page 346). It should be noted, however, that in Bak's (*26*) or Sørensen's (*29*, *30*) equation, the molecular distribution is represented in terms of Ω (or c_{tot} according to the Sørensen's notation) in contrast with the equations in Refs. *22*, *27*, and *28* where the distribution is represented in terms of C. At least under suitable approximations simpler equations might, in general, be obtained in the case of Ω than with C. This is because, with stepwise

chromatography, it is the flux with density Ω, and not the flux of density C, that is chromatographically fundamental, at least when adsorption and desorption rates are high in comparison with the mean flow rate (cf. Appendix II and Ref. *8*).

However, the following fundamental assumption involved in Refs. *29* and *30* is unreasonable (although it does not influence the above argument). Thus it is assumed that the chromatogram for a column of given length L (or x in Refs. *29* and *30*) should be represented in terms of the distribution in Ω (or c_{tot} in Refs. *29* and *30*) and not by the distribution in molecular concentration C in the mobile phase [or $c(o)$ in Refs. *29* and *30*] as a function of elution volume V (or time t). Under this assumption it is not Eq. (A35) or (A35′), but rather Eq. (A34) that gives a chromatogram (see Appendix III). Sørensen mentions (*29*) that this assumption is justified by the fact that "both the 'mobile' and the 'stationary' peak are eluted when the peaks suddenly reach the end of the bed of stationary phase, i.e., the end of the chromatographic column. The elution profile is therefore the sum of the two peaks." This statement is unreasonable because it is molecules in the mobile phase that actually migrate on the column. For any vertical column sections involving that existing at the bottom of the column, it is *through* the mobile phase that molecules are transported. This means that it is concentration C in the mobile phase within the last section at the bottom of the column, and not the total density Ω in that section, that represents the molecular concentration in solution that has just been eluted out of the column. Experimentally, it can be observed directly by using colored molecules like cytochrome *c* that the width in the molecular band migrating on the column, i.e., the volume of the solution over which the band within the column appears is generally smaller than the volume of the solution that is eluted out of the column and over which the chromatogram appears. This can be explained only by assuming that it is C (and not Ω with $\Omega \geqslant C$) that represents a chromatogram (cf. the "Introduction"). It is highly desired that the experimental analysis in Ref. *30* be reexamined, since it is designed in a very rigorous way.

APPENDIX I

In a canonical system δV in a column, the total Helmholtz free energy should be at a minimum (see the "Introduction"). The chemical potentials should be equal between solution and the adsorbed phase for molecules of any components of the mixture and for competing ions. When adsorption occurs onto a single type of crystal sites (see the "Introduction") and when the total density of sample molecules in δV is small, the partition

$B_{\delta V}$ for a given molecular component in solution (i.e., the ratio of the amount in solution to the total amount in δV) can be represented as a function of only molarity $m_{\delta V}$ of competing ions in solution. The function $B_{\delta V}(m_{\delta V})$ is given by Eq. (1) in Ref. *2*. This can be rewritten with a slight modification as

$$B_{\delta V}(m_{\delta V}) = \frac{1}{1 + q(\varphi' m_{\delta V} + 1)^{-x'}} \tag{A1}$$

where

$$q = \beta\tau e^{x\varepsilon/kT} \tag{A2}$$

In Eq. (A1) the term $\varphi' m_{\delta V}$ represents the "force" endowed with the competing ions which drives the sample molecules out of the crystal surfaces. The ions drive the molecules through a competition mechanism. [The "driving force" $\varphi' m_{\delta V}$ was written as Λ_2 or Λ in earlier papers (*2–6*). Λ is defined as the product of the absolute activity of the ions and the exponent of the adsorption energy of an ion onto a crystal site (where energy is defined as positive and expressed in units of kT).] It can be assumed (*5, 6*) that φ' is essentially positively constant throughout the chromatographic process. This means that "driving force" is virtually proportional to molarity $m_{\delta V}$. x' is the 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. In Eq. (A2), x is the average number (in the equilibrium state) of functional groups per molecule that react with sites of HA; $-\varepsilon$ ($\varepsilon > 0$) is the adsorption energy of a functional group of the molecule onto one of the sites of HA; T is the absolute temperature; k is the Boltzmann constant; and β and τ are positive constants related to the properties of the column and the sample molecules, respectively. Thus, neglecting a solvent effect, τ represents the number of effective geometrical configuration(s) of a sample molecule on the crystal surface (in the equilibrium state) and is related, in general, to both the distribution of functional groups on the molecular surface and the flexibility (or the rigidity) of the molecular structure. [It can, therefore, be considered that, in Eq. (A2), $Q \equiv -kT(\ln q - \ln \beta) = -x\varepsilon - kT \ln \tau$ represents the free energy per molecule on the HA surface (neglecting the solvent effect), whereas $-x\varepsilon$ represents the energy per molecule on the HA surface.] In the special case where the flexibility (or the rigidity) of the molecular structure is the same in both solution and the adsorbed state, which should be true at least with rigid or native molecules, τ should be related only to the distribution of the adsorption groups on the molecular surface. Therefore, provided ε is large, τ should represent the number of

energetically most stable geometrical configuration(s) of the molecule on the crystal surface.

In the competition model the energetical interaction between the sample molecule and the competing ion is not taken into consideration. In some instance, however, it can be assumed that the apparent x' value is changed due to this interaction (*32*).

APPENDIX II

On the basis of Eq. (1) we here derive two differential equations representing idealized chromatographic processes that should occur in the absence of any type of longitudinal diffusion in the column. These equations are valid with stepwise and gradient chromatographies, respectively.

As a first step in this procedure, let us consider an intermediate case where only thermodynamic diffusion survives. In this instance, Eq. (1) reduces to

$$\mathrm{div}_L\,(\mathbf{v}\Omega - D_{\mathrm{therm}}\,\mathrm{grad}_L\,C) + \frac{\partial\Omega}{\partial t} = 0 \tag{A3}$$

It is possible to rewrite Eq. (A3) into two different forms:

$$D_{\mathrm{therm}}\frac{\partial^2 C}{\partial L^2} = \frac{\partial(|\mathbf{v}|\Omega)}{\partial L} + \frac{\partial\Omega}{\partial t} \tag{A3'}$$

and

$$\frac{\partial\left(|\mathbf{v}|\dfrac{1}{1-B}\chi\right)}{\partial L} + \frac{\partial C}{\partial t} - D_{\mathrm{therm}}\frac{\partial^2 C}{\partial L^2} + \frac{\partial\chi}{\partial t} = 0 \tag{A3''}$$

where

$$\chi = (1 - B)\Omega \tag{A4}$$

represents the mean molecular density at time t on the crystal surface in a vertical column section at position L. By using Eq. (2) it is easy to derive the general relationship occurring among three types of molecular densities:

$$\Omega = C + \chi \tag{A5}$$

In order for the ideal cases of no longitudinal diffusion to be attained from this intermediate case, let us examine the following two assumptions: (a) that the longitudinal diffusion of molecules does not occur in solution in the interstices of the column, and (b) that the longitudinal diffusion of molecules in solution is carried out independently of the interaction with crystal surfaces. In general, it is reasonable to assume that the diffusion cannot occur on the crystal surfaces.

Assumption (a) can be written simply as

$$D_{\text{therm}} = 0 \tag{A6}$$

If Eq. (A6) is substituted into Eq. (A3′), and both Eq. (20) and the relationship

$$|\mathbf{v}| = \frac{1}{\alpha}\frac{dV}{dt}B \tag{A7}$$

(where α is a macroscopic constant representing the pore volume per unit length of the column and V is the elution volume) are applied, then

$$\frac{\partial(B\Omega)}{\partial L'} + \frac{\partial \Omega}{\partial V} = 0 \tag{A8}$$

is obtained. [It should be noted that, by its definition (Eq. 20), L' represents the total interstitial volumes involved between the top and a position L of the column.] This is DeVault's equation in classical theories of adsorption chromatography (*16*) (see below). Equation (A8) still conserves the property as the continuity equation for the molecular flux on the column, the first and the second terms on the left-hand side representing the divergence of the flux and the time change of the density of the flux, respectively; time t is transformed into elution volume V, however. Equation (A8) should represent a chromatographic process occurring provided there is no longitudinal diffusion in the column.

Assumption (b) means that the distribution of molecules in solution follows Fick's second law, which can be represented as

$$\frac{\partial C}{\partial t} - D_{\text{therm}}\frac{\partial^2 C}{\partial L^2} = 0 \tag{A9}$$

If Eq. (A9) is substituted into Eq. (A3″) and both Eqs. (20) and (A7) are applied, then

$$\frac{\partial\left(\frac{B}{1-B}\chi\right)}{\partial L'} + \frac{\partial \chi}{\partial V} = 0 \tag{A10}$$

is obtained. This is Wilson's equation in classical theories of adsorption chromatography (*15*) (see below). Equation (A10) means that, provided the diffusion of molecules in the interstitial liquid of the column occurs independently (Eq. A9), then the chromatographic mechanism is also independent of the molecules in solution, except for the fact that the divergence of the mean flux or the mean migration rate of molecules on the column (the first term on the left-hand side of Eq. A10) is governed by the partition B of molecules in solution, so that the chromatography is carried out independently of the longitudinal diffusion of molecules in the

column, following Eq. (A10). Let us now introduce assumption (a) or Eq. (A6), which leads to the conclusion that, provided there is no longitudinal molecular diffusion in the column, the chromatography is carried out following Eq. (A10). In fact, the only way in which the chromatography can be carried out without the effect of longitudinal molecular diffusion in the column should be that the mechanism of chromatography is independent of the molecules in solution.

We now have two different differential equations, Eqs. (A8) and (A10), that should represent the idealized chromatographic processes occurring in the absence of any longitudinal diffusion in the column. Whether or not these equations are valid depends upon whether or not they are self-consistent with the physical meanings that are involved in them under the given experimental conditions.

Let us first examine Eq. (A10) for the case of stepwise chromatography. Under this experimental condition, Eq. (A10) is self-inconsistent with the physical meaning of the independence of the chromatographic mechanism from the molecules in solution (see above). In fact, the second term on the left-hand side of Eq. (A10) is still related to the interstices of the column; the situation where the change $\partial\chi$, with time, of the density or the amount of molecules on the crystal surface is directly related to the change ∂V, also with time, of the elution volume, i.e., the dimensions of the interstitial volume of the column, but that it is independent of the amount of molecules partitioned in the interstices evidently is contradictory.

Let us examine Eq. (A10) for the case of linear gradient chromatography. In this instance, molarity m of competing ions in a given section of the column increases linearly with increase in elution volume V, or we can write

$$dm/dV = g' \tag{A11}$$

where g' is a positive constant representing the slope of the molarity gradient of competing ions (expressed in units of molarity per volume). Therefore, introducing a parameter

$$s = g'L' = gL \tag{A12}*$$

which has a dimension of molarity, Eq. (A10) can be transformed into

$$\frac{\partial\left(\frac{B}{1 - B\chi}\right)}{\partial s} + \frac{\partial\chi}{\partial m} = 0 \tag{A13}$$

*g ($= \alpha g'$) is a positive constant representing the slope of the molarity gradient of competing ions on the column, expressed as the increase in molarity per unit length of the column (measured from the bottom to the top).

where B is a function of only m (cf. Eq. A1 or A24). In gradient chromatography, it is Eq. (A13) rather than Eq. (A10) that has a fundamental physical meaning. We show now that Eq. (A13) is self-consistent with the physical meaning which is involved in it, that the chromatographic mechanism is independent of molecules in solution. The first term on the left-hand side of Eq. (A13) conserves the physical meaning of the divergence of the flux of molecules on the column in a sense such that the flux is concerned with an abstract flow of molecules that move along a type of molarity gradient of competing ions, since s has a dimension of molarity. The second term is also related only to molarity of competing ions; molarity m originates in time t in the fundamental equation, Eq. (A3). However, the molarity of competing ions is an intensive quantity [representing the "force" that drives sample molecules out of the crystal surface (to be precise, the quantity being proportional to the "force"; see Appendix I)], so that the second term in Eq. (A13) is no longer related to the interstices themselves of the column with an extensive property. Therefore the chromatographic mechanism should be independent of molecules in solution except for the fact that the migration rate of molecules along the "molarity gradient" of the ions is governed by the partition B of molecules in solution (the first term on the left-hand side of Eq. A13). However, B is a function of molarity m of the ions. B involves a parameter β that is related to interstices of the column [see Eq. A1 (i.e., Eq. A24) and Eq. A2]. However, β is constant throughout the chromatographic process, so that β does not play any role in the above argument.

It should be noted that, although Eq. (A13) represents the chromatographic process, it no longer represents the continuity equation for the actual molecular flux in the column. Thus Eq. (A13) or Eq. (A10) does not represent the conservation of the amount of molecules in the interstices, including the crystal surfaces, of the column section, because Eq. (A13) is no longer concerned with molecules in the interstitial liquid in the column (see above). Nevertheless, Eq. (A13) conserves the property of the continity equation for an abstract flux of molecules migrating along a "molarity gradient" of competing ions (i.e., the gradient of the quantity s; see above). However, introducing a new parameter C defined as

$$C = \frac{B}{1 - B}\chi \tag{A14}$$

Eq. (A13) can be rewritten as

$$\frac{\partial\left(\frac{1 - B}{B}C\right)}{\partial m} + \frac{\partial C}{\partial s} = 0 \tag{A13'}$$

From the similarity of the form of Eq. (A13′) to that of Eq. (A13), it is possible to consider that C also represents some "density," and that the first and the second terms on the left-hand side of Eq. (A13′) represent the divergence of the flux along the gradient of the parameter m, i.e., the molarity gradient in the ordinary sense and the change in "density" C occurring with a change in the parameter s, respectively. s is now considered to change with time t. Here it is possible to give C a physical meaning of concentration of molecules in the interstitial liquid of the column, since C defined by Eq. (A14) is mathematically identical with C defined by both Eqs. (2) and (A4). By extending Eq. (A13′) to an equation in which account is taken of the longitudinal diffusion in the column, the fundamental differential equation for gradient chromatography can be obtained. This is Eq. (17) in Ref. *8* which is reproduced as Eq. (A41). This equation concerns the abstract molecular flux migrating along the gradient of molarity m of competing ions (see above; cf. the "Introduction").

Let us examine Eq. (A8) for the case of stepwise chromatography. Equation (8) has been derived by using Eq. (A6), which simply states that longitudinal molecular diffusion does not occur in the interstices of the column. [For the derivation of Eq. A10 or A13 also, the assumption of no longitudinal molecular diffusion in the interstices of the column (Eq. A6) *apparently* is used. However, this assumption was introduced after Eq. A9 had been introduced (see above). As a result, Eq. A6 does not mathematically participate in the derivation of Eq. A10. As far as Eq. A10 is concerned, the condition of no longitudinal diffusion in the column can be derived even without the assumption of no longitudinal diffusion *in the interstices* of the column. This condition is derived through the logic that the state of no longitudinal diffusion should be achieved provided the chromatographic mechanism is independent of the molecules in the interstices of the column since, on the crystal surfaces, there is no diffusion. This mechanism spontaneously precludes the possibility of the existence of longitudinal molecular diffusion in the column.] Now the statement of no longitudinal molecular diffusion in the interstices of the column is *a priori* inconsistent with the fundamental assumption of chromatography that the mobile and stationary phases coexist on the column. This is because the coexistence of the two phases is achieved through molecular diffusion itself, and it is physically impossible to separate the longitudinal diffusion from the total diffusion and to fix only the former (without destroying the chromatographic mechanism). It should be noted, however, that if the migrating band of molecules on the column has an infinite width, the effect of the longitudinal diffusion should be canceled out in the interior of the band, and Eq. (A6) should apparently be fulfilled. It would

therefore be possible to imagine that the molecular band under consideration is part of the band with infinite width; the migration of this part can be described by Eq. (A8). [At least with the quasi-static process (see the "Introduction") it is possible to consider that the actual column is part of the imaginary column with infinite length; cf. Appendix III]. The problem is now whether or not the assumption of the existence of a molecular band with infinite width is self-consistent with Eq. (A8). It can be shown on the basis of Eq. (A8) that, at least when B is constant (which is the case with small sample loads), then the width of the band should be maintained constant during the chromatographic process. This enables us to assume, during the whole process of chromatography, a band with a width that is larger than any given constant finite value, i.e., the band with an infinite width.

Finally, let us examine Eq. (A8) for the case of gradient chromatography. In this instance, Eq. (A8) can be transformed into

$$\frac{\partial(B\Omega)}{\partial s} + \frac{\partial \Omega}{\partial m} = 0 \tag{A15}$$

On the basis of Eq. (A15) it can be shown that, at least when B is a function of only m and when B increases monotonically with an increase of m (Eq. A1 or A24 fulfills these two conditions), then the width of the band should decrease monotonically toward zero with the chromatographic process. Therefore it is impossible to assume the existence of the band with a width that is larger than any fixed finite value during the chromatographic process.

Hence it can be concluded that, provided there is no longitudinal diffusion in the column, it is DeVault's equation, Eq. (A8), that is valid in stepwise chromatography, whereas it is a Wilson-type equation, Eq. (A13) or (A13′), that is valid in gradient chromatography. A direct mathematical proof for this statement is given in both the Theoretical section in Ref. *7* and the Theoretical section in Ref. *8*.

As B is constant with stepwise chromatography with small sample loads (see above), introducing a parameter

$$W = BV \tag{A16}$$

Eq. (A8) can be rewritten more simply as

$$\frac{\partial \Omega}{\partial L'} + \frac{\partial \Omega}{\partial W} = 0 \tag{A8′}$$

With small loads when the band with an infinitesimal width is formed initially (i.e., when V or $W = 0$) at the top ($L' = 0$) of the column, the initial condition for Eq. (A8′) can be written, by using a delta-function, as

$$\Omega_{W=0} = \delta(L') \tag{A17}$$

Under this condition, Eq. (A8′) has a solution

$$\Omega = \delta(L' - W) \tag{A18}$$

Due to the property of the delta-function, Eq. (A18) only shows symbolically that the band with a very small width is formed at a longitudinal position L' or L (see Eq. 20) on the column at which the relationship

$$L' = W \tag{A19}$$

$$L' = BV \tag{A19′}$$

or

$$L = BV_0 \tag{A19″}$$

is fulfilled (for Eq. A19″, see Eq. 48). In other words, a sharp chromatographic peak is obtained at reduced elution volume

$$V_0 = L/B \tag{A19‴}$$

by using a column with length L.

As B is a function of m with gradient chromatography with small loads (see above), introducing a function

$$r(m) = \int_{m_{in}}^{m} \frac{B}{1 - B}\, dm \tag{A20}$$

where m_{in} represents the initial value of m at the beginning of the molarity gradient, Eq. (A13) can be rewritten more simply as

$$\frac{\partial \chi}{\partial s} + \frac{\partial \chi}{\partial r} = 0 \tag{A13″}$$

With small loads when the band with an infinitesimal width is formed initially at the top of the column [where $L' = 0$, i.e., $s = 0$ (Eq. A12) and $m = m_{in}$ (Eq. A20)], the initial condition for Eq. (A13″) can be written as

$$\chi_{r=0} = \delta(s) \tag{A21}$$

Under this condition, Eq. (A13″) has a solution

$$\chi = \delta(s - r) \tag{A22}$$

This means that the band of molecules with a very small width is formed at a longitudinal position L' or L on the column at which the relationship

$$s = r(m) \tag{A23}$$

is fulfilled. The partition $B_{\delta V}$ of sample molecules in solution in system δV is given by Eq. (A1). In the absence of longitudinal diffusion in the

column, it is sufficient to consider a column section instead of δV; $B_{\delta V}$ and $m_{\delta V}$ in Eq. (A1) can be replaced with B and m, respectively. Hence we have

$$B(m) = \frac{1}{1 + q(\varphi' m + 1)^{-x'}} \tag{A24}$$

By using Eqs. (A24) and (A20), Eq. (A23) can be rewritten as

$$m = \frac{1}{\varphi'}\{[(x' + 1)\varphi' qs + (\varphi' m_{in} + 1)^{x'+1}]^{1/(x'+1)} - 1\} \tag{A25}$$

Equation (A25) gives, as a function of s, the molarity m of competing ions at which the sharp chromatographic peak is eluted out of the column.

Equation (A25) is the fundamental equation that is used in earlier works (*2*, *5*, *6*) which originally was derived as Eq. (15) in Ref. *2* by using a different method. Even without awaiting the theory in which the longitudinal molecular diffusion is taken into consideration, it is reasonable to assume that the elution molarity of a mean part of the actual chromatographic peak can be represented by Eq. (A25). This has been verified experimentally [*2*, *6* (Appendix IV), *7* (Theoretical section *F*); see also Appendix II in Ref. *9*].

Historically, it is Eq. (A10) that was derived for the first time by Wilson (*15*) for the purpose of describing the elution process in stepwise chromatography. The method of derivation is different from that shown in this Appendix (or originally in Ref. *7*), and the equation is expressed somewhat differently in Ref. *15*. In Ref. *15* it is simply assumed (a) that an instantaneous equilibrium is attained between the solution and the adsorbed material on the column, and (b) that the effect of the longitudinal diffusion in the column is negligible. It is also assumed that the interstitial volume per unit length of the column is negligible. Under this assumption, Eq. (A10) might represent a continuity equation for the actual molecular flux in the column. DeVault (*16*) modified Wilson's equation (Eq. A10) in order for it to behave as the continuity equation (for the actual flux) even when the interstitial volume of the column has a finite value; this is Eq. (A8). In Ref. *16*, Eq. (8) is expressed somewhat differently however. These theories, with a further theory developed by Weiss on the basis of Wilson's equation (*17*), are categorized as classical theories of adsorption chromatography. However, all these theories are valid only for stepwise chromatography.

APPENDIX III

When dealing with stepwise chromatography, the longitudinal dis-

tribution of molecules on a column or the shape of the molecular band migrating on the column can be represented as a sum of molecular bands migrating on respective microcolumns and as a function of elution volume V or the parameter W (Eq. A16). In parallel with Steps 1–3 in the "Theoretical" section, let us here calculate (1′) *the contribution of the band of molecules migrating on a column λ to the total band on the actual column as a function of local elution volume $V_\lambda\,\delta\lambda$ on the column λ or the parameter*

$$W_\lambda = BV_\lambda \tag{A26}$$

for each λ by assuming that there is no thermodynamic longitudinal diffusion, (2′) *the distribution of the flow among different microcolumns*, and (3′) *the relationship between the local elution volume $V_\lambda\,\delta\lambda$, i.e., the parameter W_λ and the actual elution volume V, i.e., the parameter W*. Since W_λ can be considered as a function of L' (see below), the macroscopic molecular density Ω on the actual column can be represented as a function of L' as

$$\Omega = \int_0^\infty \Omega_\lambda \frac{dW_\lambda(L')}{dL'}\,\vartheta(L'_\lambda)\,dL'_\lambda \tag{A27}$$

where Ω_λ represents the molecular density in the interstices, including the crystal surfaces, on the column λ. L', L'_λ, and $\vartheta(L'_\lambda)$ are defined by Eqs. (20), (19), and (21), respectively. [W_λ is different from V_λ only by a constant factor B (Eq. A26). V_λ can be considered as a function of L'_λ because, in a column λ, the elution volume $V_\lambda\,\delta\lambda$ should depend upon the apparent total interstitial volumes $L'_\lambda\,\delta\lambda$. L'_λ is a function of L (Eq. 19), and L can be considered as a function of L' (Eq. 20). Therefore, W_λ can be considered as a function of L'. The physical meaning of Eq. (A27) can be understood from analogy with the meaning of Eq. (5).]

Let us perform the calculation of Ω_λ (Step 1′). Through a procedure similar to the derivation of Eq. (A8), but with some attention to the fact that the pore volume per unit length of the column λ depends microscopically upon the longitudinal position, DeVault's equation, representing the idealized elution process occurring on a column λ provided there is no thermodynamic longitudinal diffusion,

$$\frac{\partial(B_\lambda\Omega_\lambda)}{\partial L'_\lambda} + \frac{\partial\Omega_\lambda}{\partial V_\lambda} = 0 \tag{A28}$$

can be derived. It can be assumed that B_λ is virtually independent of the microscopic variation in pore volume per unit length of the column λ [see the section entitled "Step 1: Calculation of $f_\lambda(m_\lambda, s_\lambda)$"]; this means that B_λ in Eq. (A28) can be replaced by the macroscopic parameter B (Eq. 2), which is independent of both L'_λ and V_λ. Hence, by using Eq. (A26), Eq. (A28) can be rewritten as

$$\frac{\partial \Omega_\lambda}{\partial L'_\lambda} + \frac{\partial \Omega_\lambda}{\partial W_\lambda} = 0 \tag{A29}$$

which can be compared with Eq. (A8′).

With small sample loads when the band of molecules with an infinitesimal width is formed initially at the top of the column, or under the initial condition

$$[\Omega_\lambda]_{W_\lambda = 0} = \delta(L'_\lambda) \tag{A30}$$

Eq. (A29) has a normalized solution

$$\Omega_\lambda = \delta(L'_\lambda - W_\lambda) \tag{A31}$$

(cf. Eqs. A17 and A18). Equation (A31) represents the contribution of the molecular band on a column λ to the total band on the actual column as a function of W_λ when L'_λ is given.

The calculation of $\vartheta(L'_\lambda)$ (Step 2′) has already been done (see Eq. 21). Now, if both Eqs. (A31) and (21) are substituted into Eq. (A27),

$$\Omega = \frac{1}{\sqrt{4\pi\theta L'}} e^{-[W_\lambda(L') - L']^2/4\theta L'} \frac{dW_\lambda(L')}{dL'} \tag{A32}$$

is obtained.

Let us perform the calculation in Step (3′). Instead of directly calculating the function $W_\lambda(L', W)$, it is sufficient to give a proof for the following relationship:

$$\begin{aligned}
&\frac{1}{\sqrt{4\pi\theta L'}} e^{-(W_\lambda - L')^2/4\theta L'} \, dW_\lambda \qquad (\equiv \vartheta(W_\lambda)\, dW_\lambda) \\
&= \frac{1}{\sqrt{4\pi\theta L'}} e^{-(L'_\lambda - L')^2/4\theta L'} \, dL'_\lambda \qquad (\equiv \vartheta(L'_\lambda)\, dL'_\lambda) \\
&= \frac{1}{\sqrt{4\pi\theta W}} e^{-(L'_\lambda - W)^2/4\theta W} \, dL'_\lambda \\
&= \frac{1}{\sqrt{4\pi\theta W}} e^{-(L' - W)^2/4\theta W} \, dL'
\end{aligned} \tag{A33}$$

The equality between the extreme left- and the extreme right-hand side in Eq. (A33) gives the function $W_\lambda(L', W)$ in an implicit form. Now, let us give a proof for Eq. (A33). Thus, first, $W_\lambda \, \delta\lambda$ has a physical meaning of a sum of interstitial volumes involved between the top and a position of the column λ *through which the molecular band with an infinitesimal width passes during the whole process of chromatography*. This is evidently equal to the sum $L'_\lambda \, \delta\lambda$ of the interstitial volumes involved between the top and this position of the column λ. Thus the first equality in Eq. (A33) has been obtained. Second, due to its definition, $\vartheta(L'_\lambda)\, dL'_\lambda$ can represent a

probability that, when the center of the actual molecular band is at position L on the column or the position at which the "distance" from the column top is equal to L' (measuring as a sum of interstitial volumes), then a molecule exists at positions where the "distance" to the position L are between $|L'_\lambda - L'|$ and $|L'_\lambda - L'| + dL'_\lambda/2$. $L'_\lambda - L' > 0$ and $L'_\lambda - L' < 0$ represent the cases when the position of the molecule is closer and less close to the column top than the position L, respectively. This is because, on a column λ, the total interstitial volumes involved between the position L and the position of the molecule under consideration should be equal to $(L'_\lambda - L')\,\delta\lambda$ and $(L' - L'_\lambda)\,\delta\lambda$, neglecting the infinitesimal fluctuation $(dL'_\lambda/2)\,\delta\lambda$, respectively. The probabilities of the occurrence of both these volumes (equal to $|L'_\lambda - L'|\,\delta\lambda$) should be equal to the probabilities of the appearance of a molecule at the two positions on the actual column where the "distance" to the position L are equal to $|L'_\lambda - L'|$, respectively. Third, it is possible to give W a physical meaning of the sum of interstitial volumes involved between the top and a longitudinal position of the actual column *through which a molecular band with an infinitesimal width passes during the whole process of chromatography provided there is no longitudinal diffusion* because, under this hypothetical condition, V_λ should be equal to V, and Eqs. (A16) and (A26) show that W_λ is equal to W. As molecules at the center of the band should apparently stay at the same position as in the absence of longitudinal diffusion, W can also represent the sum of interstitial volumes involved between the center of the band, i.e., the position L defined in the second step of the consideration, and the column top. This means that the parameter L' involved in the function $\vartheta(L'_\lambda)$ (see Eq. 21) can be replaced with W. Thus the second equality in Eq. (A33) has been obtained. Lastly, it is possible to denote by L' the sum of interstitial volumes involved between the column top and the position of any part of the actual molecular band. By using this new notation, the factor $L'_\lambda - W$ [or the factor $L'_\lambda - L'$ in the function $\vartheta(L'_\lambda)$, given the physical meaning in the second step of the consideration] can be replaced with $-(L' - W)$. The minus sign is necessary because, when $L'_\lambda > L'$ and $L'_\lambda < L'$ (in the old expression), the "position" L' (in the new expression) should be closer and less close to the column top than the "position" W, respectively, the symbol W being common to both the old and new expressions. However, the value of ϑ does not change if the factor $-(L' - W)$ is replaced with $L' - W$. This means that the factors L'_λ and dL'_λ appearing in the third term in Eq. (A33) can be replaced directly with L' and dL', respectively. Thus the last equality in Eq. (A33) has been obtained.

If the equality between the extreme left- and the extreme right-hand side in Eq. (A33) is substituted into the right-hand side of Eq. (A32), then

$$\Omega = \frac{1}{\sqrt{4\pi\theta W}} e^{-(L'-W)^2/4\theta W} \tag{A34}$$

is obtained, which can be compared with Eq. (A18). It is evident that, at a limit of $\theta \to 0$, Eq. (A34) reduces to Eq. (A18). It can be seen in Eq. (A34) that, if Ω is considered as a function of L', Ω represents a Gaussian distribution. W represents a sum of interstitial volumes involved between the column top and the longitudinal position at which the center of the molecular band exists (see above). The theoretical chromatogram for a column of length L (denoted by f_L) can now be represented as the concentration C ($\equiv f_L$) of molecules in the interstitial liquid within the last section at the bottom of the column of length L, and as a function of elution volume V as

$$f_L(V) = \sqrt{\frac{B}{4\pi\theta V}} e^{-(\alpha L - BV)^2/4\theta BV} \tag{A35}$$

It is often more convenient to represent the chromatogram in terms of V_0 (Eq. 48) rather than of V. In this instance, writing f_L° instead of f_L, we have

$$f_L^\circ(V_0) = \sqrt{\frac{B}{4\pi\theta_0 V_0}} e^{-(L - BV_0)^2/4\theta_0 BV_0} \tag{A35'}$$

When the column has a macroscopic length, both f_L and f_L° are normalized such that

$$\int_0^\infty f_L(V)\, dV = \int_0^\infty f^\circ{}_L(V_0)\, dV_0 = 1 \tag{A36}$$

Equation (A34) can also be obtained as a solution of the continuity equation for the molecular flux on the column (Eq. 4), or the continuity equation represented by using, instead of time t, elution volume V as a variable. Thus by introducing the parameter

$$\theta = \frac{\alpha D}{\frac{1}{\alpha}\frac{dV}{dt}} \tag{A37}$$

and by using Eqs. (20) and (A7), Eq. (4) can be rewritten as

$$\mathrm{div}_{L'}\,(\vec{B}\Omega - \theta B\, \mathrm{grad}_{L'}\,\Omega) + \frac{\partial\Omega}{\partial V} = 0 \tag{A38}$$

where the arrow shows that the term under the arrow is a vector.

We confirm below the fact that θ (Eq. A37) is constant. This means that, in Eq. (A38), time t is not involved as the parameter. This latter is necessary in order for Eq. (A38) to be compatible with the experimental fact that

both the shape of the chromatogram and the elution molarity are independent of the flow rate (see the "Introduction"). An equivalence of the argument below has already been made in Ref. 7. Thus it is possible to give α (see Eq. A7) a physical meaning of the total area, projected on the surface of the vertical column section, of the interspaces among HA crystals being packed, i.e., the total area for the part of a column section through which the solution can pass in the chromatographic process. α is macroscopically constant. Fick's first law shows that the amount of molecules, dQ/dt, that pass through the area α by diffusion (caused by the microscopical heterogeneity in α) per unit length of time should be proportional to the density gradient, $-\text{grad}_L\,\Omega$, with a proportionality constant αBD. [Fick's first law usually is stated in such a way that the amount of molecules that pass through a unit area by diffusion in unit interval of time, $(dQ/dt)/\alpha$, or the molecular flux should be proportional to the density gradient, $-\text{grad}_L\,\Omega$, with a proportionality constant BD. This statement is equivalent to the statement made above. Cf. the explanation of the symbol D in Eq. 1]. The quantity dQ/dt should also be proportional to the mean flow rate $|v|$ of molecules (see Eq. A7), because the variation in the flow rate (by which the diffusion under consideration occurs) around the mean flow rate of molecules should be proportional to the mean flow rate (at least in the range of the flow rate examined experimentally; see the "Introduction"). This means that the proportionality constant in Fick's law or the quantity αBD should be proportional to the mean flow rate $|v|$ or that the quantity αD should be proportional to the mean flow rate $(dV/dt)/\alpha$ of the solvent, thus giving θ a physical meaning of the proportionality constant between αD and $(dV/dt)/\alpha$. It will be understood later that the parameter θ defined by Eq. (A37) is identical with θ introduced in Eq. (21).

By introducing the parameter W (Eq. A16), Eq. (A38) can be rewritten as

$$\theta \frac{\partial^2 \Omega}{\partial L'^2} = \frac{\partial \Omega}{\partial L'} + \frac{\partial \Omega}{\partial W} \tag{A39}$$

In order to find the initial condition for Eq. (A39), the following consideration is necessary: With small sample loads when a narrow band is formed initially at the top ($L = 0$) of the column, this initial state could be replaced approximately with a hypothetical state in which a band with an infinitesimal width is formed at a position ($L = 0$) on the column with an infinite length. This column extends even in the minus direction beyond the origin. This approximation would be justified by the fact that the actual longitudinal diffusion of molecules in the column is essentially due to the heterogeneity in the flow rate, so that there should be hardly any flow of molecules that proceeds backward on the column. The mathemati-

cal calculation on the basis of this approximation might predict, however, that a minor part of the molecular band extends over the column top immediately after the development of molecules has begun. Therefore it would be at least after the distance between the center of the molecular band and the column top has reached a macroscopic value that the correspondence between the theory and the experiment can be expected. It can be assumed that, in this situation, the shape of the theoretical peak receives only a slight influence from the approximation of the initial state of the molecular band (see above). To calculate the chromatogram with a very short column, another approximation would be required. (For this calculation, a type of initial condition such as applied in Ref. *4* or in Appendix II in Ref. *7* might be useful.) The concentration of molecules in the interstitial liquid at position L on a column with infinite length should be virtually equal to the concentration in solution that has just been eluted out of the column with finite length L. This is because the probability of the occurrence of the flow that proceeds backward on the column should be negligible (see above). Hence the theoretical chromatogram for a column of length L can be represented by the concentration of molecules in the mobile phase at position L on the hypothetical column with infinite length as a function of elution volume V.

Now, when a narrow band of molecules is formed initially at the top, $L = 0$, of the column, the initial condition for Eq. (A39) can be written, by using a delta-function, as

$$\Omega_{W=0} = \delta(L') \tag{A40}$$

Under the condition of Eq. (A40), Eq. (A39) has a solution given by Eq. (A34).

The fact that the same equation, Eq. (A34), can be obtained by using two different methods demonstrates that the two methods are equivalent; the diffusion parameter θ defined by Eq. (A37) is identical to θ introduced in Eq. (21).

In Ref. *8* it is shown that Eqs. (36) and (34) or Eqs. (36′) and (34) that represent the chromatogram in gradient chromatography are given as a solution of the continuity equation for an abstract molecular flux (cf. Appendix II):

$$\operatorname{div}_m\left[\frac{\overrightarrow{1 - B(s, m)}}{B(s, m)} C - \frac{\Xi}{B(s, m)} \operatorname{grad}_m \frac{C}{B(s, m)}\right] + \frac{\partial C}{\partial s} = 0 \tag{A41}$$

under the initial condition given by

$$\lim_{\substack{s \to +0 \\ m_\lambda \to m_{1n}}} \Omega = \delta(m - m_{in}) \tag{A42}$$

where

$$\Xi = g'\theta = g\theta_0 \tag{A43}$$

Equation (A41) (corresponding to Eq. A38 in stepwise chromatography) can be compared with Eq. (A13') (corresponding to Eq. A8 in stepwise chromatography). (For details, see Ref. *8*.)

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