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# Gradient Hydroxyapatite Chromatography with Small Sample Loads. III. Fundamental Differential Equation for Gradient Chromatography

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# Gradient Hydroxyapatite Chromatography with Small Sample Loads. III. Fundamental Differential Equation for Gradient Chromatography

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**Abstract**

In contrast to stepwise chromatography, with gradient chromatography, it is impossible in principle for a chromatographic process to be described on the basis of a continuity equation for the actual molecular flux occurring on the column itself. It is the abstract molecular flux occurring on the molarity gradient of competing ions that is fundamental. From this point of view it is not the column but rather the molarity gradient of the ions that is fixed; both the sample molecules and the "gradient" which is defined as an assembly of longitudinal positions on the column migrate on the molarity gradient. A continuity equation for the abstract flux is proposed. It is confirmed that the theoretical chromatogram obtained in an earlier paper is a solution of this equation.

## INTRODUCTION

When dealing with hydroxyapatite (HA) chromatography, the sample initially adsorbed at the top of the column forming a narrow band is eluted from the column by increasing the molarity of competing ions of the buffered solvent stepwise or gradually. The molecular elution is carried out by competition with particular ions from the buffer for adsorbing sites on the HA crystal surfaces (*1*). A linear molarity gradient is often applied.

Due to the additive property of flux, it can be stated in general that the longitudinal diffusion in the column is contributed from two types of diffusion: (1) diffusion due to heterogeneity in the flow rate of the solution within a vertical section of the column. Without investigating the hydrodynamic mechanism, it can simply be assumed that heterogeneity in the flow rate occurs due to heterogeneity in the interspaces among HA crystals

packed in the column. (2) Thermodynamic diffusion; this is defined in this paper (and in Ref. *1*) as any diffusion occurring provided the flow rate of the solution is homogeneous within any vertical section of the column. Now, with gradient chromatography on HA columns, virtually no deformation of the chromatogram nor the change in elution molarity occurs when the flow rate is changed. An increase in the width of the chromatographic peak does occur, however, with an increase in the column length when it is long enough, demonstrating the existence of longitudinal molecular diffusion in the column (*1*). These experimental facts can be explained by introducing the following three assumptions. (a) The rate of increase in the width of the molecular band due to thermodynamic diffusion is negligibly small in comparison with the rate provoked by heterogeneity in the flow rate; the ratio of the rate of increase in the band width due to heterogeneity in the flow rate to the mean flow rate is constant. (b) Even concerning the longitudinal diffusion of competing ions, the effect of thermodynamic diffusion is negligible in comparison with the effect of diffusion due to heterogeneity in the flow rate; this means that the longitudinal diffusion of both sample molecules and competing ions occurs essentially in parallel in the column and is caused by heterogeneity in the flow rate. (c) The chromatographic process is virtually a quasi-static process. Within any elementary volume in the column, thermodynamic equilibrium is locally attained at any instant of the chromatographic process (for details, see Ref. *1*). As both Assumptions (a) and (b) are necessary conditions for Assumption (c), we shall call the chromatographic process occurring under all these assumptions a quasi-static process. Owing to Assumptions (a) and (b), the chromatogram for molecules eluted out of a column with length $L$ can be calculated as concentration $C$ of molecules in the interstitial liquid at position $L$ on the hypothetical column with an infinite length. This is because these assumptions mean that the flows of sample molecules and competing ions that proceed backward on the column are both negligible (*1*).

In an earlier paper (*1*) a theory of linear gradient HA chromatography was developed by using the following method. The column was divided into a number of parallel hypothetical columns with diameters of the order of magnitude of the interdistances among HA crystals being packed. The effect of heterogeneity in the flow rate should be negligible in each microcolumn. A distribution law of the flow among different microcolumns was assumed, and the total chromatogram was represented as a sum of chromatograms for the respective microcolumns. The equations representing the total chromatogram derived in Ref. *1* were confirmed experimentally (*2*). In Appendix II in Ref. *1*, it was suggested that, with gradient chromatography, the chromatogram be given as a solution of the continuity equation for an abstract flux of molecules migrating on the molarity gradient of competing ions, in constrast

with the chromatogram in stepwise chromatography which is given as a solution of the continuity equation for the actual flux of molecules migrating on the column itself. In the present paper the abstract continuity equation is explored. It is confirmed that the theoretical chromatogram obtained in Ref. *1* is a solution of this equation.

The theoretical conclusions reached in Ref. *1* are briefly summarized in Appendix I of Ref. *2*.

## THEORETICAL

### Actual Flux J and Abstract Flux J*



Let us consider an actual flow of molecules migrating on a column. For each component of the sample mixture the amount of molecules should be conserved, at any instant $t$, within a vertical section at a longitudinal position $L'$ on the column following a continuity equation

$$\mathrm{div}_{L'}\mathbf{J} + \frac{\partial \Omega}{\partial t} = 0 \qquad (1)$$

where $\Omega$ represents the total density of molecules (of the component under consideration) in the interstices, including the crystal surfaces of HA, of the column sections. $\mathbf{J}$ is the corresponding flux. For convenience sake we hereafter represent the position $L'$ as a sum of interstitial volumes involved between the column top and the position under consideration. $L'$ will simply be called "distance" or "length."

For each component of the mixture the ratio $R_F$ of the mean migration rate of molecules to the mean migration rate of the solvent at column position $L'$ should be equal to the ratio $B$ of the amount of molecules in the interstitial liquid (i.e., the mobile phase) to the total amount in the column section at that position. This is a first principle of chromatography (*3*). $B$ is independent of the total amount of molecules in the column section if the amount of molecules is small because, in this situation, the linear section of the adsorption isotherm should be realized. With stepwise chromatography the migration of molecules on the column can, in fact, be described by using Eq. (1) (see Ref. *1*, 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). It should be pointed out that in this situation, in principle, it is impossible to describe causally the migration of molecules on the column by using Eq. (1) (even though the conservation of the amount of molecules in a column section can be represented by Eq. 1). This is because Eq. (1) gives a causal relationship between flux **J** and density $\Omega$ (or the total amount of molecules) in a given column section, whereas with gradient chromatography the factor $B$, being involved in **J**, can be determined only by *m independently of* $\Omega$ (see above). Even with gradient chromatography, however, the migration of molecules on the column should be describable by using a certain continuity equation because a conservation of the amount of molecules should still be predictable if the initial condition of chromatography is given. This leads to a consideration that, besides flux **J**, a certain flux (denoted by **J***) should exist. This flux, as a constituent of the new continuity equation, should play a fundamental role in gradient chromatography. In fact, what is necessary is the existence of a fundamental flux. *A priori*, we have no reason why it should be identical with the actual flux **J**.

The existence of flux **J*** can be suggested from another consideration and the experimental confirmation thereof. Thus it can be considered that, in the first stage of the development process in gradient chromatography, the gradient of competing ions simply passes through the position of the band of sample molecules adsorbed at the top of the column. When molarity $m$ of the ions attains some value at that position, the desorption of molecules would begin and molecules would begin to migrate gradually. The migration rate would increase, and it would approach the migration rate of the ions. This rate should be virtually equal to the migration rate of the solvent (water) (cf. Remark 1 below). In other words, the molarity of the ions at which the molecular band appears should increase with time $t$. This suggests the existence of flux **J*** migrating upward along the gradient of the ions (cf. Remark 2 below). Experimentally, it can be observed that the elution molarity of sample molecules increases, in general, with an increase in column length (Figs. 1 and 2 in Ref. *4*, which are partially reproduced in Fig. A1 in Appendix II of Ref. *2*; cf. Remark 3 below). This demonstrates that the molecular band, in fact, migrates upward along the gradient.

It can further be suggested that flux **J*** should fulfill the following continuity equation:

$$\mathrm{div}_m \mathbf{J}^* + \frac{\partial C}{\partial t} = 0 \tag{2}$$

where

$$C = B\Omega \tag{3}$$

represents the density or the concentration of molecules in the interstitial liquid in a column section. The flux with density $C$, but not with density $\Omega$, has been suggested because density $\Omega$ is concerned with molecules in both the mobile and stationary phases whereas molarity $m$ is an intensive quantity. This represents the "force" that drives molecules out of the crystal surface through a competition mechanism (see Appendices I and II of Ref. *1*). The concept of the co-existence of two phases in the locale of a flux would have a physical meaning only when the locale belongs in the actual extensive space. When dealing with a flux with density $C$, the concept of the co-existence of different phases does not appear. (The existence of the fundamental flux **J*** with density $C$ in gradient chromatography can also be suggested from a consideration made in Appendix II of Ref. *1*) Proofs that flux **J*** should fulfill Eq. (2) will be given in the sections entitled "Derivation of Flux **J** from Flux **J***," "Derivation of Flux **J*** from Flux **J**," and "General Method of Finding the Fundamental Flux in Any Type of Chromatography." A general method of finding the fundamental flux for any type of chromatography will be given in the last-mentioned section.

*Remark 1.* It can be observed 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 the Theoretical Section of Ref. 5). This means that, even though the delay of the gradient occurs immediately after the gradient has been introduced because of the adsorption of ions, any part of the gradient migrates 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 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 the Theoretical Section of Ref. 5). Therefore $m$ should be virtually independent of the adsorption and desorption phenomena of sample molecules in the column. This has been confirmed experimentally (*4*).

*Remark 2.* It can be considered that competing ions are locally distributed within a column section (Introduction). It is therefore necessary to specify the meaning of the molarity gradient. Thus we define $m$ as the mean molarity of the ions within a column section. The molarity gradient is defined as the gradient obtained by connecting $m$ values occurring within respective column sections. This is linear in linear gradient chromatography (cf. the section entitled "Microscopical Point of View: Longitudinal Diffusion of Competing Ions, the Form of Function $B(s, m)$, and a Solution of Eq. (17)".

*Remark 3.* In general, the larger the molecular dimensions the smaller is the increase in elution molarity occurring with an increase in column length (*4*). With a virus or large molecules of DNA the elution molarity is

essentially independent of the column length (*4*, *6*). Even in this case the concept of flux **J*** is valid. It can be considered that molecules with extremely large dimensions are completely adsorbed at the top of the column until the ion molarity attains a critical value $m^\circ$. When once this value is attained, however, molecules are completely desorbed, so that the migration rate of the molecules changes stepwise from zero to a rate that is equal to the migration rate of the solvent or the competing ions (cf. Remark 1). This explains the reason why the elution molarity is independent of the column length (cf. the section entitled "The Case of Molecules with Very Large Dimensions").

## Two Points of View on Gradient Chromatography

Here we specify the two points of view on gradient chromatography, the first on which Eq. (1) is based and the second on which Eq. (2) is based. We consider how the chromatogram $C$ [which should be represented as a function of $m$ (or elution volume $V$) for a column of length $L'$] can be related to the function $C(t, m)$ as a solution of Eq. (2). [It should be recalled that the chromatogram of the molecules eluted out of a column with length $L$ or $L'$ can be represented as concentration $C$ of the molecules in the interstitial liquid at longitudinal position $L$ or $L'$ on the hypothetical column with infinite length (see Introduction).]

Figure 1 summarizes these two points of view, where the abscissa $L'$ and the ordinate $m$ represent the general longitudinal column position and the mean (see Remark 2 in the preceding section) molarity of competing ions, respectively. The oblique straight line represents the linear molarity gradient of the ions with a slope $g'$ occurring at time $t$. The slope $g'$ is defined as positive in order for it to have a dimension of molarity/volume, representing the increase in $m$ from the bottom to the top of the column. $g'$ is independent of time $t$. $L_0'$ *and* $m_0$ show the column position which exists at the beginning of the gradient and the ion molarity at the top of the column, respectively. $m_{in}$ is the initial molarity of the ions introduced at the top ($L' = 0$) of the column at time 0. This can be considered to be equal to the molarity at the beginning ($L' = L_0'$) of the gradient at time $t$. [It is common practice that the sample dissolved in a buffer involving molarity $m_{in}$ of competing ions is initially loaded on the column; a narrow molecular band is formed at the top of the column. The column is rinsed with a certain volume of the same buffer. In this process the band usually stays at the same position. After rinsing, the gradient with initial molarity $m_{in}$ is applied; this instant is defined as time 0. It is possible that the linearity of the gradient is disturbed in the neighborhood of the beginning of the gradient due to diffusion; the linearity may also be disturbed at the beginning of the gradient due to the adsorption of the ions onto the HA surfaces if $m_{in}$ is small (cf. Remarks 1 and 2 in the section entitled

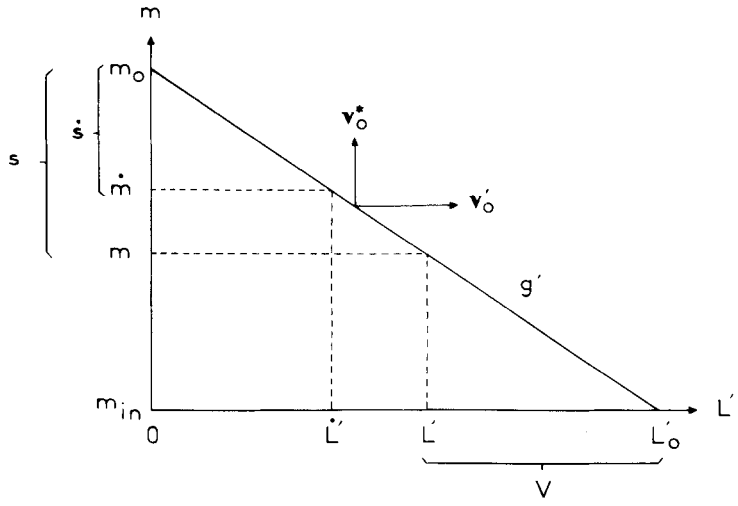


FIG. 1. Schematic representation of the molarity gradient of competing ions migrating on the column at time $t$. The abscissa $L'$ and the ordinate $m$ represent the longitudinal position on the column and the molarity of competing ions, respectively. The oblique straight line represents the linear molarity gradient of the ions with a constant slope $g'$; this migrates on the column, or in the $L'$ direction on the $(L', m)$ plane, with a velocity $v_0'$. However, it also is possible to consider that the oblique straight line represents the "gradient" of the column position $L'$ with a slope $1/g'$; this can be considered to migrate along the molarity gradient of the ions, or in the $m$ direction on the $(L', m)$ plane, with velocity $v_0^*$. These are the first and the second point of view on gradient chromatography, respectively. (For details, see text.) This figure is used also in the Appendix. In this instance, $L'$ represents the position, at time $t$, of a given particle migrating in any way on the column; $\dot{m}$ is the molarity of the ions at column position $L'$. (For details, see Appendix.)



"Actual Flux **J** and Abstract Flux **J***"). To be precise, $L_0'$ should therefore be defined as the column position at which the molarity of the ions for the hypothetical gradient, which is obtained by extrapolation of the linear part of the gradient, is equal to $m_{in}$.]

In the first point of view, it is the column itself that is fixed. Both the molarity gradient and the band of sample molecules migrate in the $L'$ direction on the $(L', m)$ plane. We shall call $\mathbf{v}_0'$ the migration velocity of the gradient and $\mathbf{v}'$ the mean migration velocity of sample molecules observed at a given longitudinal position $L'$ on the column (see Fig. 1). Both $\mathbf{v}_0'$ and $\mathbf{v}'$ are expressed in units of volume/time. Let us introduce a concept of elution volume (denoted by $V$) occurring at the position $L'$ on the column. This can be defined as

$$V = L_0' - L' = \frac{1}{g'}(m - m_{in}) \tag{4}$$

(see Fig. 1), where both $L'$ and $m_{in}$ are constant, and both $L_0'$ and $m$ increase with time $t$, the latter being defined as the ion molarity at the given column position $L'$. Therefore $V$ increases with time $t$. Now, it is possible to write

$$| \mathbf{v}_0' | = dV/dt \tag{5}$$

Introducing a new flux $\mathbf{j}$, defined as

$$\mathbf{j} = \mathbf{J}/| \mathbf{v}_0' | \tag{6}$$

and using Eq. (5), Eq. (1) can be rewritten as

$$\mathrm{div}_{L'}\, \mathbf{j} + \frac{\partial \Omega}{\partial V} = 0 \tag{7}$$

In the second point of view, it is the molarity gradient of competing ions that is fixed. Both the column and the band of sample molecules migrate in the $m$ direction on the $(L', m)$ plane. It can be considered that the oblique straight line in Fig. 1 represents the linear "gradient" of column position $L'$ (with slope $1/g'$) rather than the linear molarity gradient of the ions (with slope $g'$). We shall call $\mathbf{v}_0^*$ the migration velocity of the "gradient" along the molarity gradient, and $\mathbf{v}^*$ the mean migration velocity of sample molecules observed at a given position $m$ on the molarity gradient (see Fig. 1; for a detailed definition of $\mathbf{v}^*$, see the section "Specification of Flux $\mathbf{J}^*$ in Quasi-Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography"). Both $\mathbf{v}_0^*$ and $\mathbf{v}^*$ are expressed in units of molarity/time. Here it is possible to find a parameter $s$ that corresponds to the elution volume $V$ in the first point of view, occurring at a given position $m$ on the molarity gradient. This can be defined as

$$s = m_0 - m = g'L' \tag{8}$$

(see Fig. 1). In Eq. (8), $m$ is constant and both $m_0$ and $L'$ increase with time $t$, the latter now being defined as the longitudinal position on the column at which the molarity of the ions is always equal to $m$. Therefore, $s$ increases with time $t$. Corresponding to Eq. (5), it is possible to write

$$| \mathbf{v}_0^* | = ds/dt \tag{9}$$

Introducing a new flux $\mathbf{j}^*$, defined as

$$\mathbf{j}^* = \mathbf{J}^*/| \mathbf{v}_0^* | \tag{10}$$

and using Eq. (9), Eq. (2) can be rewritten as

$$\mathrm{div}_m\, \mathbf{j}^* + \frac{\partial C}{\partial s} = 0 \qquad (11)$$

Now, corresponding to the fact that, in Eq. (2), $(\partial C/\partial t)_m\, dt$ represents the change in molecular density $C$ at a given position $m$ on the molarity gradient occurring when the time proceeds from $t$ to $t + dt$, in Eq. (11) $(\partial C/\partial s)_m\, ds$ represents the change of $C$ at position $m$ on the gradient occurring when the value of the parameter $s$ increases from $s$ to $s + ds$ with time $t$. This latter is caused by a change in the longitudinal column position (where the molarity of the ions is always equal to $m$) from $L'$ to $L' + dL'$ occurring with time $t$ (see Fig. 1). Corresponding to the fact that, in Eq. (2), $(\partial C/\partial m)_t\, dm$ represents the change of $C$ at time $t$ occurring between position $m$ and $m + dm$ on the molarity gradient, in Eq. (11) $(\partial C/\partial m)_s\, dm$ represents the change of $C$ at time $t$ when the parameter $s$ takes a value $s$, occurring between position $m$ and $m + dm$ on the gradient. It is possible, however, to interpret the quantities appearing in Eq. (11) as based on the first point of view. Thus $(\partial C/\partial s)_m\, ds$ in Eq. (11) can represent the change of $C$ at time $t$ when the molarity of the ions at position $L'$ on the column is equal to $m$ (or when the elution volume at position $L'$ is $V$), occurring between positions $L'$ and $L' + dL'$ on the column which correspond, respectively, to the value $s$ and $s + ds$ of the parameter $s$ through Eq. (8) (the relationship between the extreme left- and the extreme right-hand side of the equation; see Fig. 1). $(\partial C/\partial m)_s\, dm$ in Eq. (11) can represent the change of $C$ at a given position $L'$ on the column to which corresponds a value $s$ of the parameter $s$ (through Eq. 8) occurring when the molarity of the ions increases from $m$ to $m + dm$ (or the elution volume increases from $V$ to $V + dV$) with time $t$ (see Fig. 1). Hence it can be considered that the solution $C(s, m)$, of Eq. (11) can represent a chromatogram for a column of length $L'$ (which corresponds to a value $s$ of the parameter $s$) as a function of molarity $m$ of competing ions, or as a function of elution volume $V$. This can be estimated through Eq. (4) (the relationship between the extreme left- and the extreme right-hand side of the equation).

It should be noted, however, that the form of flux $\mathbf{J}^*$ is not yet specified. Since a chromatogram is produced by the actual flow of molecules issuing from the bottom of the column, in order for the function $C(s, m)$ to represent a chromatogram it is necessary that flux $\mathbf{J}^*$ be defined such that it coincides, at any instant $t$, with the actual flux $\mathbf{J}$ at the bottom of the column (see the following section).

## Specification of Flux J* in Quasi-Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography

For the purpose of specifying the abstract flux (**J*** or **j***) with density $C$, let us consider an apparent flux with density $\Omega$ also migrating upward along the molarity gradient. This flux plays an intermediate role of relating the abstract flux to the actual flux (**J** or **j**) with density $\Omega$. Thus the mean migration velocity $\mathbf{v}'$ of the actual flux observed at a given position $L'$ on the column can be represented as

$$\mathbf{v}' = R_F \mathbf{v}_0' = B\mathbf{v}_0' \tag{12}$$

(see Fig. 1). The apparent flux is defined as a flux (with density $\Omega$) that migrates concomitantly upward along the gradient with a mean velocity

$$\hat{\mathbf{v}} = (1 - R_F)\mathbf{v}_0^* = (1 - B)\mathbf{v}_0^* \tag{13}$$

Since, actually, a position $L'$ on the column faces directly a position $m$ on the molarity gradient, it is evident that any molecule as an element of the actual molecular flow coincides with the molecule as an element of the apparent flow existing at the corresponding position $m$ on the gradient. It is generally impossible, however, that the position of any molecule in the apparent flow occurring on the molarity gradient coincides with the position of the same molecule as the element of the abstract flow occurring on the same gradient. This is because the densities of the two fluxes are different. In order to have a one-to-one correspondence between the molecules in the abstract flow and the molecules in the apparent or the actual flow, and in order for the function $C(s, m)$ as a solution of Eq. (11) to have a physical meaning of the chromatogram, it is necessary that any molecules as elements of the three flows coincide instantly at the bottom of the column (see the preceding section). For this purpose, denoting by $L'$ the position at the bottom of the column with length $L'$, and by $m$ the corresponding position on the gradient (the first point of view; see the preceding section), let us define the abstract flow such that the part $\Omega\, dL'$ of the actual flow existing between position $L'$ and $L' - dL'$ on the column, or the part $\Omega\, dm$ of the molecular distribution $\Omega$ of the apparent flow existing between positions $m$ and $m + dm$ on the gradient, coincides, at any instant $t$, with the part $C\, dm/B$ of the molecular distribution $C$ of the abstract flow existing between positions $m$ and $m - dm/B$ on the same gradient. The position $m$ migrates on the gradient with time $t$. Two conditions for the coincidence of the three fluxes at the bottom of the column are explored below. As a whole, these will make a sufficient condition.

The first condition would be that the mean migration velocity $\mathbf{v}^*$ of the

abstract flow to be equal to $\hat{v}/B$ or $(1 - B)\,v_0^*/B$ (see Eq. 13) at any position on the molarity gradient, or that the mean flux be equal to $[(1 - B)v_0^*/B]C$. Here, the following remark is useful: It is possible to consider that any position $L'$ on the actual column is the bottom of the subcolumn involved between the position $L'$ and the column top, so that every time the position $L'$ is given on the column, both the subcolumn and the corresponding abstract flow can be defined. (It should be emphasized, however, that these are definitions based on the first point of view in gradient chromatography; cf. Remark 1 below.) Let us consider a subcolumn that just involves the band of molecules initially formed at the top of the column (see the Introduction). In a first stage of the development process when molecules are completely adsorbed on the crystal surfaces, the state of $B = 0$ is realized in the subcolumn. Now, denoting by $\Delta L'$ the actual longitudinal distance from the bottom of the subcolumn to the position of a given molecule in the subcolumn, and by $\Delta m$ the corresponding distance on the molarity gradient (expressed in units of molarity), then, in the apparent flow, the same molecule should exist at position $m + \Delta m$ on the gradient, $m$ being the position at the bottom of the subcolumn. In the abstract flow, however, the molecule should exist on the other side of the position $m$ the distance $\left| \int_m^{m-\Delta m} dm/B \right|$ apart from this position, or it should exist at position $m + \int_m^{m-\Delta m} dm/B$. $B$, in general, is a function of $m$ with a minimum limiting value zero that decreases monotonically with a decrease of $m$. Therefore, when $B = 0$ at the position of the molecule under consideration in the apparent flow, then, in the abstract flow the value of $B$ for the corresponding molecule should also be zero. This means that the position of the molecule in the abstract flow is $m + \int_m^{m-\Delta m} dm/B = -\infty$ (cf. Remark 2 below). This hypothesis explains the actual situation where the molecule cannot be eluted out of the subcolumn if it is completely adsorbed on the crystal surface. In order for the molecule to be eluted out of the subcolumn when $B$ increases to have a finite value (due to increase in molarity of the ions), it is necessary that, when $B = 0$, the molecule already be migrating with an infinite velocity at a position infinitely apart from the position $m$. This is the physical interpretation for the fact that, in the abstract flow, when $B = 0$, then $|\mathbf{v}^*| (= (1 - B)|\mathbf{v}_0^*|/B) = \infty$. As $C = 0$ when $B = 0$ (Eq. 3), the mean flux $[(1 - B)\mathbf{v}_0^*/B]C$ [which can be rewritten as $(1 - B)\mathbf{v}_0^*\Omega$; see Eq. 3] has a finite value $\mathbf{v}_0^*\Omega$ even when $|\mathbf{v}^*| = \infty$.

Let us explore the second condition (see above). Due to the additive property of flux, the total flux $\mathbf{J}^*$ can, in general, be represented as a sum of the mean flux (see above) and the flux due to molecular diffusion. With a quasi-static chromatographic process occurring under Assumptions (a)–(c) in the Introduction Section, the actual and the apparent fluxes due to

diffusion should be proportional to $-\text{grad}_L\ \Omega$ and $-\text{grad}_m\ \Omega$ at any position on the column and the gradient, respectively (cf. the explanation of Eq. 1 in Ref. *1*). In order for the total abstract flux to coincide always with the actual or the apparent flux at the bottom of the column, it is both sufficient and necessary that the abstract flux due to diffusion also be proportional to $-\text{grad}_m\ \Omega$ or $-\text{grad}_m\ (C/B)$ at least at the bottom of the column.

The actual and the apparent fluxes due to diffusion should also be proportional to $B$ at any position on the column and the gradient, respectively, because these fluxes (with density $\Omega$) should be proportional to the mean migration rate, due to diffusion, of molecules in a column section, and this latter should be proportional to the ratio $B$ of the amount of molecules existing in the mobile phase to the total amount in the column section. Hence the actual and apparent fluxes should finally be proportional to $-B\ \text{grad}_L\ \Omega$ and $-B\ \text{grad}_m\ \Omega$ at any position on the column and the gradient, respectively.



The situation is different with the abstract flow. We show below that the abstract flux due to diffusion should be inversely proportional to $B$. In the above it was mentioned that, when a molecule actually exists at a position on the gradient the distance $\Delta m$ apart from the position $m$ at the bottom of the column (measuring in units of molarity), then, in the abstract flow, the same molecule should exist at a position on the other side of the position $m$ the distance $|\int_m^{m-\Delta m} dm/B|$ apart from this position. This means that the actual distance (denoted by $d'm$) from this molecule to any other molecule existing in the neighborhood of this molecule should correspond to the distance $d'm/B$ in the abstract flow. It follows from this that, in the abstract flow, the relative mean migration rate, due to diffusion, between any pair of molecules existing in an elementary domain at any position on the gradient should be inversely proportional to $B$. Hence the abstract flux due to diffusion should finally be proportional to $-(1/B)\ \text{grad}_m\ (C/B)$, at least at the bottom of the column. For this purpose it is sufficient that this flux is proportional to $-(1/B)\ \text{grad}_m\ (C/B)$ at any position on the gradient. This is the second condition for the coincidence of the three fluxes at the bottom of the column.

The total flux $\mathbf{J}^*$ can now be represented as

$$\mathbf{J}^* = \frac{1 - B(t,m)}{B(t,m)}\mathbf{v}_0^* C - \frac{D^*}{B(t,m)}\ \text{grad}_m \frac{C}{B(t,m)} \qquad (14)$$

where $D^*$, with dimensions of molarity$^2$/time, is proportional to $|\mathbf{v}_0^*|$. [This, or the constancy of $\Xi$ (see below), can be confirmed by using a method similar to that used to show the constancy of the parameter $\theta$ in Appendix III of Ref. *1*.] The reason why $B$ is considered as a function of not only $m$ but

also of time $t$ will be mentioned later. Introducing a proportionality constant

$$\Xi = \frac{D^*}{|\mathbf{v}_0^*|} = \frac{D^*}{ds/dt} \tag{15}$$

(see Eq. 9), the flux $\mathbf{j}^*$ (Eq. 10) can be represented as

$$\mathbf{j}^* = \frac{\overrightarrow{1 - B(s, m)}}{B(s, m)} C - \frac{\Xi}{B(s, m)} \operatorname{grad}_m \frac{C}{B(s, m)} \tag{16}$$

where the arrow shows that the term under the arrow is a vector. $B(s, m)$ simply shows that $B$ is a function of $s$ and $m$ (see below), and it does not mean that the form of the function $B(s, m)$ is identical witht he form of $B(t, m)$. By substituting Eq. (16) into Eq. (11), the fundamental differential equation for gradient chromatography:

$$\operatorname{div}_m \left[ \frac{\overrightarrow{1 - B(s, m)}}{B(s, m)} C - \frac{\Xi}{B(s, m)} \operatorname{grad}_m \frac{C}{B(s, m)} \right] + \frac{\partial C}{\partial s} = 0 \tag{17}$$

is obtained. (Proofs that Eq. 17 represents the fundamental equation for gradient chromatography will be given in the section entitled "Derivation of Flux **J** from Flux **J***," "Derivation of Flux **J*** from Flux **J**," and "General Method of Finding the Fundamental Flux in Any Type of Chromatography.")

Based on the second point of view on gradient chromatography (see the preceding section), let us consider a column section migrating on the column with the same velocity as that of the molarity gradient. Within this column section the mean molarity $m$ of the ions is kept constant. However, the width in the distribution in molarity around the mean value $m$ increases with time $t$ due to diffusion. This is the reason why $B$ (which depends upon not only $m$ but also on the distribution in molarity around $m$) is considered as a function of both $t$ and $m$ (Eq. 14). When the position $L'$ of the column section under consideration migrates toward the bottom of the column, the value of the parameter $s$ increases (Eq. 8). Therefore, $s$ increases with time $t$, and B can be considered as a function of $s$ and $m$ (Eqs. 16 and 17). The form of the function $B(t, m)$ or $B(s, m)$ can be determined by introducing the assumption of parallel longitudinal diffusions of both sample molecules and competing ions in the column [Assumption (b) in the Introduction Section; see the section entitled "Microscopical Point of View: Longitudinal Diffusion of Competing Ions, the Form of Function $B(s, m)$, and a Solution of Eq. (17)"].

*Remark 1.* Equation (17) has been derived on the basis of a hypothesis that the abstract molecular flux should coincide with the actual flux at the

bottom of the column. Since the bottom of the column is a fixed longitudinal position on the column, this hypothesis belongs in the first point of view on gradient chromatography (see the section entitled "Two Points of View on Gradient Chromatography"). When once it is derived, however, Eq. (17) belongs purely in the second point of view. As a result, for any subcolumn defined as part of the actual column, the chromatographic behavior of molecules can be described by using the same equation, Eq. (17) (see below). However, the behavior based on the second point of view has only some abstract meaning. It is only by transferring from the second to the first point of view that the abstract meaning can be translated into a physical meaning for the chromatogram. It is in the process of this translation that the meaning of the column length (i.e., a fixed longitudinal position at the bottom of the column) is given to the quantity $s/g'$ (see Eq. 8), $s$ being a variable involved in Eq. (17). It also is possible to give $s/g'$ the meaning of any fixed longitudinal position on the column, or the meaning of the length of the subcolumn involved between this position and the column top. It was mentioned earlier in this section that every time a position $L'$ is given on the column, both the subcolumn and the corresponding abstract flow can be defined. It should be emphasized, however, that these are definitions made after the translation has been achieved. The abstract flow occurring without the translation is uniquely represented by a unique equation, Eq. (17). In the latter half of the preceding section, the process of translation was mathematically pursued.

*Remark 2.* It was mentioned that when $B = 0$, then, in the abstract flow, the molecule should exist at a position $m = -\infty$ on the molarity gradient. This is based on the first point of view [cf. the section entitled "Microscopical Point of View: Longitudinal Diffusion of Competing Ions, the Form of Function $B(s, m)$, and a Solution of Eq. (17)"]. Actually, however, the minimum value of $m$ is $m_{in}$ ($\geq 0$). It is therefore necessary that, in defining the abstract flow, the quantity $m$ be defined even when it takes negative values. This definition should be made at the same time as the definition of the parameter $B$ or $B_\lambda$, which is a function of $m$ or $m_\lambda$. For this problem, see section entitled "The Form of Function $B_\lambda(m_\lambda)$ and the Normalized Property of the Solution (Eq. 62) of Eq. (17)."

## Derivation of Flux J from Flux J*

In an extreme case when there is no molecular diffusion in the abstract flow or when $\Xi \to 0$, Eq. (17) reduces to

$$\mathrm{div}_m \left[ \overrightarrow{\frac{1 - B(m)}{B(m)} C} \right] + \frac{\partial C}{\partial s} = 0 \tag{18}$$

where $B$ can be assumed to be a function of only $m$ because, in the situation where there is no molecular diffusion, there should also be no diffusion of the ions. Denoting by $\chi$ the absolute value of the flux appearing in Eq. (18), or writing

$$\chi = \frac{1 - B(m)}{B(m)} C = (1 - B(m))\Omega \tag{19}$$

it is possible to give $\chi$ a physical meaning of the mean density of molecules on the crystal surfaces at position $L'$ on the column. The position $L'$ is determined when the value of $s$ is given (Eq. 8). By using Eq. (19), Eq. (18) can be rewritten as

$$\frac{\partial}{\partial s}\left[\frac{B(m)}{1 - B(m)}\chi\right] + \frac{\partial \chi}{\partial m} = 0 \tag{20}$$

Based on the first point of view (see the section entitled "Two Points of View on Gradient Chromatography"), it can be considered that $(\partial\chi/\partial m)_s\, dm$ represents the change in density $\chi$ at position $L'$ on the column (corresponding to a given $s$ value), occurring when the molarity of the ions increases from $m$ to $m + dm$ or when the elution volume increases from $V$ to $V + dV$ with time $t$ (Eq. 4; see Fig. 1). On the other hand, $(\partial C/\partial s)_m\, ds$ or

$$\left[\partial\left(\frac{B(m)}{1 - B(m)}\chi\right)/\partial s\right]_m ds$$

would represent the change in flux

$$\frac{\overrightarrow{B(m)}}{1 - B(m)}\chi$$

with density $\chi$ and migration velocity

$$\frac{\overrightarrow{B(m)}}{1 - B(m)}$$

at time $t$ when the ion molarity at position $L'$ is $m$ or the elution volume at that position is $V$, occurring between positions $L'$ and $L' + dL'$. These positions correspond to the values $s$ and $s + ds$ of the parameter $s$, respectively. Under these considerations, Eq. (20) (i.e., Eq. 18) can still be rewritten by using Eqs. (4) and (8):

$$\mathrm{div}_{L'}\left[\frac{\overrightarrow{B(m)}}{1-B(m)}\chi\right]+\frac{\partial\chi}{\partial V}=0 \tag{21}$$

Equation (21) can give chromatograms for subcolumns involved between any longitudinal column positions ($L'$) and the column top, respectively (see the section entitled "Specification of Flux **J*** in Quasi-Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography"). In other words, Eq. (21) can give not only a chromatogram for a column with a given length $L'$ but also a distribution, at time $t$, of molecules on the column occurring, provided there is no longitudinal molecular diffusion.

Nevertheless, Eq. (21) (derived from Eq. 17) does not represent the continuity equation for the actual molecular flow on the column; viz., it does not express the conservation of the amount of molecules within the interstices, including the crystal surfaces, of the column section. This can be confirmed by the following consideration. The flux in Eq. (21) can be rewritten as

$$\frac{\overrightarrow{B(m)}}{1-B(m)}\chi=\overrightarrow{B(m)}\Omega \tag{22}$$

(see Eq. 19). By using Eqs. (6), (12), and (22), it is easy to show that the flux in Eq. (21) is identical with flux **j** (Eq. 7) provided there is no longitudinal diffusion in the column, whereas in Eq. (21) it is the molecular density $\chi$ on the crystal surfaces and not the total density $\Omega$ in the column section that changes with an increase in elution volume $V$ (the second term on the left-hand side of Eq. 21; compare Eq. 21 with Eq. 7). Thus Eq. (21) shows that, in the ideal state of no longitudinal diffusion in the column, the chromatography is carried out independently of the amount of molecules existing in the interstitial liquid; the proportion of molecules adsorbed on the crystal surfaces (within the molecular band) decreases monotonically with time $t$, caused simply by a gradual increase in ion molarity $m$. Therefore the migration of the molecular band on the column is carried out due to an increase of $m$. This is done independently of the amount of molecules in the interstitial liquid.

In order for chromatography to occur independently of the amount of molecules in the interstitial liquid, it logically is necessary that the behavior of molecules in the interstitial liquid be independent of the interaction with crystal surfaces. This means that, in the absence of heterogeneity in the flow rate ("Introduction" section), Fick's second law:

$$D'_{\mathrm{therm}}\Delta_{L'}C=\partial C/\partial t \tag{23}$$

should be realized in the interstitial liquid, where $D'_{\text{therm}}$, with dimensions of volume$^2$/time, represents the thermodynamic diffusion constant concerning the $L'$ direction on the column, $L'$ being represented in units of volume. Introducing a parameter

$$\theta_{\text{therm}} = \frac{D'_{\text{therm}}}{|\,v_0'|} = \frac{D'_{\text{therm}}}{dV/dt} \tag{24}$$

with dimensions of volume, Eq. (23) can be rewritten as

$$\theta_{\text{therm}} \Delta_L C = \partial C/\partial V \tag{25}$$

By using Eqs. (21), (22), and (25) and writing $B(L', m)$ instead of $B(m)$ [since $B$, in general, depends upon both $s$ and $m$ (see the immediately preceding section), and $s$ can be considered as a function of $L'$ (Eq. 8)], the change in total density $\Omega$ occurring with an increase in elution volume $V$ in the absence of heterogeneity in the flow rate can be written as

$$\frac{\partial \Omega}{\partial V} = \frac{\partial \chi}{\partial V} + \frac{\partial C}{\partial V} = -\text{div}_{L'}\,[\overrightarrow{B(L', m)}\Omega] + \theta_{\text{therm}} \Delta_L C \tag{26}$$

or, with rearrangement

$$div_L\,[\overrightarrow{B(L', m)}\Omega - \theta_{\text{therm}}\,\text{grad}_L\,C] + \frac{\partial \Omega}{\partial V} = 0 \tag{26'}$$

is obtained. It can be considered that the first and the second terms within the divergence term in Eq. (26') represent the mean flux and the flux due to thermodynamic diffusion in the actual molecular flow occurring on the column itself, respectively. Therefore, Eq. (26') should be valid even when $\partial \chi/\partial V$ and $\partial C/\partial V$ are not independent of one another, representing the general continuity equation for the actual molecular flux in the absence of heterogeneity in the flow rate. In its presence, it is necessary to add to the flux in Eq. (26') a flux due to diffusion occurring caused by flow heterogeneity. In the immediately preceding section it was shown that this flux should be proportional to $-B\,\text{grad}_{L'}\,\Omega$. Thus, introducing a positive proportionality constant, $\theta$, with dimensions of volume

$$\text{div}_{L'}\,[\overrightarrow{B(L', m)}\Omega - \theta_{\text{therm}}\,\text{grad}_{L'}\,C - \theta B(L', m)\,\text{grad}_{L'}\,\Omega] + \frac{\partial \Omega}{\partial V} = 0 \tag{27}$$

is obtained. In a quasi-static chromatographic process where the effect of thermodynamic longitudinal diffusion is neglibible in comparison with the

effect of diffusion due to heterogeneity in the flow rate [Assumption (a) in the "Introduction"], the relationship

$$\theta_{\text{therm}} \operatorname{grad}_L C \ll \theta B(L', m) \operatorname{grad}_L \Omega \tag{28}$$

should be fulfilled; Eq. (27) reduces to

$$\operatorname{div}_L [\overrightarrow{B(L', m)}\Omega - \theta B(L', m) \operatorname{grad}_L \Omega] + \frac{\partial \Omega}{\partial V} = 0 \tag{29}$$

By comparing Eq. (29) with Eq. (7), the expression of flux $\mathbf{j}$,

$$\mathbf{j} = \overrightarrow{B(L', m)}\Omega - \theta B(L', m) \operatorname{grad}_L \Omega \tag{30}$$

is obtained. Let us introduce a diffusion coefficient $D'$ for diffusion due to heterogeneity in the flow rate. This has dimensions of volume$^2$/time and is proportional to $|\mathbf{v}_0'|$, fulfilling the relationship

$$\theta = \frac{D'}{|\mathbf{v}_0'|} = \frac{D'}{dV/dt} \tag{31}$$

(cf. Eq. A37 in Appendix III of Ref. *1*). Corresponding to Eq. (28), the relationship

$$D'_{\text{therm}} \operatorname{grad}_{L'} C \ll D' B(L', m) \operatorname{grad}_{L'} \Omega \tag{32}$$

is fulfilled. By using Eq. (31), Eq. (29) can be rewritten as Eq. (1) where

$$\mathbf{J} = B(L', m)\mathbf{v}_0'\Omega - D' B(L', m) \operatorname{grad}_{L'} \Omega \tag{33}$$

Equations (1) and (33) are equivalent to Eq. (4) in Ref. *1*.

Finally, in an extreme case when the adsorption of sample molecules does not occur at all on the crystal surfaces during the chromatographic process, or when $B = 1$ and $C = \Omega$ (see Eq. 3), the coincidence of the three fluxes at the bottom of the column (see the immediately preceding section) leads to the relationship

$$\theta \operatorname{grad}_{L'} \Omega = \Xi \operatorname{grad}_m \Omega = \frac{\Xi}{g'} \operatorname{grad}_{L'} \Omega \tag{34}$$

from which the general relationship between the parameters $\Xi$ (Eq. 15) and $\theta$ (Eq. 31):

$$\Xi = g'\theta \tag{35}$$

is obtained.

## Derivation of Flux J* from Flux J

Following the argument in the immediately preceding section in the other direction, Eq. (18) can be derived starting from Eqs. (1) and (33). This process is argued in detail in Appendix II in Ref. *1*. The flux **j*** can be obtained by adding a flux due to diffusion to the flux in Eq. (18). This should be done in such a way that flux **j*** coincides with flux **j** at the bottom of the column. This process is equivalent to the process of finding the second coincidence condition of the three fluxes at the bottom of the column (see the section entitled "Specification of Flux **J*** in Quasi-Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography"). Flux **j*** can be transformed into flux **J*** through Eq. (10).

It has now been shown that both fluxes **J*** and **J** are derivable from each other. Under Assumptions (a)–(c) in the "Introduction" section, the expression of the abstract flux **J** (Eq. 33) *a priori* is true. This means that the expression of the actual flux **J*** (Eq. 14) is also true; Eq. (17) should, in fact, describe the chromatographic process in gradient chromatography.



## General Method of Finding the Fundamental Flux in Any Type of Chromatography†

Flux **J*** or **j*** is not the only one that is conceivable on the molarity gradient; the apparent flux with density $\Omega$ is conceivable (see the section entitled "Specification of Flux **J*** in Quasi-Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography"). This flux, denoted by $\hat{\mathbf{j}}$, can be represented as

$$\hat{\mathbf{j}} = [\overrightarrow{1 - B(s, m)}]\Omega - \Xi B(s, m)\ \mathrm{grad}_m\ \Omega \tag{36}$$

fulfilling a continuity equation

$$\mathrm{div}_m\ \hat{\mathbf{j}} + \frac{\partial \Omega}{\partial s} = 0 \tag{37}$$

We consider below a general method of judging what type of flux is the fundamental one for any type of chromatography.

For this purpose it is sufficient to investigate the property of the continuity equation concerning the mean flux, or the equation in which the value of the parameter $\theta$ or $\Xi$ is zero. This is because the flux due to diffusion is conceivable independently of whether or not it is chromatographically fundamental (see the section entitled "Specification of Flux **J*** in Quasi-

† The argument made in this section partially overlaps the argument made in the Theoretical Section of Ref 5.

Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography"). The mean flux represents a flux concerning the mean part, with an infinitesimal width, of the molecular band migrating on the column (or the molarity gradient of competing ions). In this section the effect of diffusion is always canceled out and the relationship

$$R_F = B \tag{38}$$

is fulfilled. As Eq. (38) represents the causality itself in chromatography, if Eq. (38) is fulfilled on the column, then the chromatographic process should in general be represented by using a continuity equation concerning a certain flux (cf. the section entitled "Actual Flux **J** and Abstract Flux **J***"). Therefore, arguing in the other direction, if Eq. (38) can be derived on the basis of the continuity equation for the given mean flux under a boundary condition such that the molecular band migrating on the column or the molarity gradient should have an infinitesimal width (at a given instant), then the flux under consideration should be chromatographically fundamental.

By using this method, let us first confirm the fact that flux **j** is fundamental with stepwise chromatography (see the section entitled "Actual Flux **J** and Abstract Flux **J***"). When $\theta \to 0$, the continuity equation for flux **j** (Eq. 29) reduces to

$$\frac{\partial}{\partial L'}(B(m)\Omega) + \frac{\partial \Omega}{\partial V} = 0 \tag{39}$$

where $m$ or $B(m)$ is constant. Therefore, Eq. (39) can be rewritten as

$$\left[\frac{\partial L'}{\partial V}\right]_{\Omega} = -\frac{\partial \Omega / \partial V}{\partial \Omega / \partial L'} = B(m) \tag{39'}$$

On the basis of Eq. (39'), it is easy to show that, if the molecular band (with density $\Omega$) has an infinitesimal width at any instant when the elution volume $V$ is given (at a position $L'$ on the column; this is the boundary condition for Eq. 39'), then the band should maintain the infinitesimal width during the whole process of chromatography. Writing $\bar{L}'$ for the position of the infinitesimal band, it also is easy to derive

$$d\bar{L}'/dV = B(\bar{m}) \tag{40}$$

where $\bar{m}$ represents the molarity of the ions at which the molecular band with infinitesimal width exists. In this instance, $\bar{m}$ is constant. The left-hand side of Eq. (40) is the definition of $R_F$. Hence Eq. (38) has been obtained.

Let us examine flux **j*** for gradient chromatography. When $\Xi \to 0$, the continuity equation for flux **j*** (Eq. 17) reduces to Eq. (18), which can be rewritten as Eq. (20) or as

$$\left[\frac{\partial s}{\partial m}\right]_{\chi} = -\frac{\partial \chi/\partial m}{\partial \chi/\partial s} = \frac{B(m)}{1 - B(m)} \tag{20'}$$

On the basis of Eq. (20'), it can be shown that, if the molecular band (with density $\chi$ occurring on the crystal surfaces) has an infinitesimal width at any instant when molarity $m$ is given [at a position $L'$ ($= s/g'$; see Eq. 8) on the column], then the band should maintain the infinitesimal width during the whole process of chromatography. Defining $\bar{s}$ as

$$\bar{s} = g'\bar{L}' \tag{41}$$

the relationship

$$\frac{d\bar{s}}{d\bar{m}} = \frac{B(\bar{m})}{1 - B(\bar{m})} \tag{42}$$

can be derived. On the other hand, we have a general relationship

$$\frac{d\bar{s}}{d\bar{m}} = \frac{d\bar{L}'/dV}{1 - d\bar{L}'/dV} \tag{43}$$

the derivation of which is made in the Appendix. By substituting Eq. (43) into Eq. (42), Eq. (40) can be obtained. Hence, recalling that $d\bar{L}'/dV$ represents $R_F$, Eq. (38) can be derived.

Let us examine the apparent flux $\hat{\mathbf{j}}$ for gradient chromatography. When $\Xi \to 0$, we can derive, from Eqs. (36) and (37), the relationship

$$\frac{\partial}{\partial s}\left[\frac{1}{1 - B(m)}\chi\right] + \frac{\partial \chi}{\partial m} = 0 \tag{44}$$

or

$$\left[\frac{\partial s}{\partial m}\right]_{\chi} = -\frac{\partial \chi/\partial m}{\partial \chi/\partial s} = \frac{1}{1 - B(m)} \tag{44'}$$

from which

$$\frac{d\bar{s}}{d\bar{m}} = \frac{1}{1 - B(\bar{m})} \tag{45}$$

is obtained. As Eq. (45) is different from Eq. (42), it can be concluded that Eq. (38) cannot be derived from Eq. (44).

Finally, let us confirm the fact that flux **j** cannot be fundamental with gradient chromatography. When $\theta \to 0$, the continuity equation for flux **j** (Eq. 29) reduces to Eq. (39). In this instance, however, $m$ is not constant. By using Eqs. (4) and (8), Eq. (39) can be rewritten as

$$\frac{\partial}{\partial s}(B(m)\Omega) + \frac{\partial \Omega}{\partial m} = 0 \tag{46}$$

or as

$$\left[\frac{\partial s}{\partial m}\right]_{\Omega} = -\frac{\partial \Omega/\partial m}{\partial \Omega/\partial s} = B(m) \tag{46'}$$

from which

$$d\bar{s}/d\bar{m} = B(\bar{m}) \tag{47}$$

is obtained. As, again, Eq. (47) is different from Eq. (42), Eq. (38) cannot be derived from Eq. (46) or (39).

## Microscopical Point of View: Longitudinal Diffusion of Competing Ions, The Form of Function $B(s, m)$, and a Solution of Eq. (17)

In order for the shape of the theoretical chromatogram to be calculated from Eq. (17), it is necessary that both the form of the function $B(s, m)$ and the initial chromatographic condition be given. The form of $B(s, m)$, however, depends upon not only the initial condition itself of chromatography but also the distribution of competing ions within each vertical section of the column due to longitudinal diffusion. With a quasi-static chromatographic process, the longitudinal diffusion of the ions is carried out essentially in parallel with the diffusion of the sample molecules (Assumption (b) in the "Introduction" section). In the argument below, the following four processes, which are related to one another, will proceed at the same time: (1) the derivation of the distribution law of the ions within a vertical column section, (2) the introduction of the initial condition for Eq. (17); it is assumed that the molecules are initially adsorbed at the top of the column, forming a band with an infinitesimal width, (3) the determination of the form of the function $B(s, m)$, and (4) the solution of Eq. (17).

In order to consider the general relationship between the distribution of sample molecules and that of competing ions in a column, a microscopical

point of view is useful. Thus, let us divide the column into a number of parallel microcolumns (called column $\lambda$) with infinitesimal diameters (to be precise, diameters of the order of magnitude of the interdistances among HA crystals being packed; see Ref. *1*). We characterize column $\lambda$ in such a way that the volume of the solution that flows into any column $\lambda$ is the same within any unit time interval (*1*). It is possible to give $\lambda$ a meaning of the (bi-dimensional) coordinate that indicates a position on the surface of a vertical column section. We define $d\lambda$ as the ratio of the volume of the solution that flows into a column $\lambda$ to the volume that flows into the total column, fulfilling the relationship

$$\int d\lambda = 1 \tag{48}$$

Due to this definition, the total macroscopical area of any vertical column section is unity if it is measured by using the coordinate $\lambda$. Now, based on the second point of view of gradient chromatography (see the section entitled "Two Points of View on Gradient Chromatography"), let us consider a local molarity $m_\lambda$ of competing ions at position $\lambda$ on the surface of a column section where the mean molarity of the ions is always equal to $m$. The probability density (denoted by $\upsilon$) of the occurrence of molarity $m_\lambda$ changes with time $t$ or with an increase in the value of the parameter $s$. With a lapse of time the longitudinal position $L'$ on the column with constant $m$ value migrates toward the bottom of the column, and the value of $s$, being proportional to $L'$ (Eq. 8), increases. For any vertical section of the column, the relationship

$$\upsilon \, dm_\lambda = d\lambda \tag{49}$$

is fulfilled. We now show that, according to the assumption of parallel longitudinal diffusions of the molecules and the ions (Assumption (b) in the "Introduction" section), $\upsilon$ should fulfill the differential equation

$$\Xi \frac{\partial^2 \upsilon}{\partial m_\lambda^2} = \frac{\partial \upsilon}{\partial s} \tag{50}$$

under the initial condition

$$\upsilon_{s=0} = \delta(m_\lambda - m) \tag{51}$$

Thus, within the section (with an infinitesimal width) at the top of the column, the molarity of the ions which have just been introduced into the column should be homogeneous. Their molarity should be equal to the mean

molarity $m$. It can be considered that these ions constitute a part of the molarity gradient. This part has an infinitesimal width when it exists at the top of the column. With the lapse of time the position $m$ on the gradient migrates toward the bottom of the column, and the ions under consideration are diffused around the same position $m$ on the gradient. The gradient itself is always kept constant because the diffusion of the ions under consideration is canceled out by the diffusion of the other ions. It is evident that, if a physical meaning of the general position on the gradient is given to $m_\lambda$, then Eq. (50) (or the equation obtained by replacing $s$ and $\Xi$ in Eq. 50 with time $t$ and diffusion coefficient $D^*$, respectively; see Eq. 15) represents the movement of the ions under consideration on the molarity gradient. Due to the linearity of the gradient, however, if within a part $d\lambda$ of the surface of a column section, a change $dm_\lambda$ in molarity of the ions under consideration occurs, then this should be compensated by the change $-dm_\lambda$ in molarity of the other ions. This means that Eq. (50) can also represent the distribution in molarity of the ions in general occurring on the surface of a column section, thus giving $m_\lambda$ a physical meaning of the local molarity of the ions on the surface of a column section. Under the condition given by Eq. (51), Eq. (50) has a solution

$$\upsilon = \frac{1}{\sqrt{4\pi\Xi s}} e^{-(m_\lambda - m)^2/4\Xi s} \tag{52}$$

which represents the distribution law of the ions within a column section.

In general, it is possible to represent the macroscopical parameters $m$, $\Omega$, and $C$ occurring within a column section in terms of the corresponding microscopical parameters $m_\lambda$, $\Omega_\lambda$, and $C_\lambda$:

$$m = \int m_\lambda \, d\lambda = \frac{1}{\sqrt{4\pi\Xi s}} \int m_\lambda e^{-(m_\lambda - m)^2/4\Xi s} \, dm_\lambda \tag{53}$$

$$\Omega = \int \Omega_\lambda d\lambda = \frac{1}{\sqrt{4\pi\Xi s}} \int \Omega_\lambda e^{-(m_\lambda - m)^2/4\Xi s} \, dm_\lambda \tag{54}$$

and

$$C = \int C_\lambda \, d\lambda = \frac{1}{\sqrt{4\pi\Xi s}} \int C_\lambda e^{-(m_\lambda - m)^2/4\Xi s} \, dm_\lambda \tag{55}$$

where the extreme right-hand terms have been obtained by using Eqs. (49) and (52). Calling $B_\lambda$ the partition of sample molecules in solution occurring locally in an infinitesimal part $d\lambda$ of the column section, we can write

$$C_\lambda = B_\lambda \Omega_\lambda \tag{56}$$

where, with small sample loads, $B_\lambda$ is a function of only $m_\lambda$, which increases monotonically with an increase of $m_\lambda$, tending to unity when $m_\lambda$ approaches to infinity. The form of the function $B_\lambda(m_\lambda)$ will be given in the following section (Eqs. 75 and 76). $B_\lambda$ is related to the macroscopical parameter $B$ by the general relationship

$$B = \frac{1}{\Omega}\int B_\lambda \Omega_\lambda d\lambda = \frac{1}{\Omega}\frac{1}{\sqrt{4\pi\Xi s}}\int B_\lambda \Omega_\lambda e^{-(m_\lambda - m)^2/4\Xi s}\, dm_\lambda \tag{57}$$

and $C$ (Eq. 55) can be represented by using $\Omega_\lambda$ and $B_\lambda$ as

$$C = \frac{1}{\sqrt{4\pi\Xi s}}\int B_\lambda \Omega_\lambda e^{-(m_\lambda - m)^2/4\Xi s}\, dm_\lambda \tag{58}$$

From the first equality in Eq. (55), Eq. (56), and the first equality in Eq. (57), the general macroscopical relationship between $C$ and $\Omega$ (Eq. 3) can be derived.

In agreement with the experiment ("Introduction" section), let us assume that a molecular band with an infinitesimal width is formed initially at the top of the column. According to the assumption of parallel longitudinal diffusions of the molecules and the ions, however, the band should broaden to have a finite width when the migration of the band begins (cf. Eq. 52). This means that, except for the section at the top of the column, within any column section existing between position $L'$ and $L' + dL'$, when the mean molarity of the ions is between $m$ and $m + dm$, the sample molecules should appear only in some infinitesimal local regions where molarity of the ions is also extended in an infinitesimal range. Let this molarity range be between $\bar{m}_\lambda$ and $\bar{m}_\lambda + dm_\lambda$. Then we evidently have

$$\Omega_\lambda = \delta(m_\lambda - \bar{m}_\lambda) \tag{59}$$

where $\Omega_\lambda$ is normalized in order for the chromatogram $C$ finally to be normalized (see the following section). Since $L'$ is proportional to $s$ (Eq. 8), Eq. (59) is fulfilled for any column section being defined when both $s$ and $m$ are given.

Actually, due to thermodynamic longitudinal diffusion, the whole region within the column section is occupied by molecules. The molecules form a band with a finite width in the longitudinal direction on the column (see above). Nevertheless, Eq. (59) (obtained under the assumption of no thermodynamic longitudinal diffusion) should be valid for the final result of the calculation of the chromatogram because, with a quasi-static process, the

effect of thermodynamic diffusion is canceled out in the interior of the molecular band.

Substituting Eq. (59) into Eqs. (54), (57), and (58), and writing $m_\lambda$ instead of $\bar{m}_\lambda$, we obtain

$$\Omega = \frac{1}{\sqrt{4\pi\Xi s}} e^{-(m_\lambda - m)^2/4\Xi s} \tag{60}$$

$$B(s, m) = B_\lambda(m_\lambda) \tag{61}$$

and

$$C = \frac{1}{\sqrt{4\pi\Xi s}} e^{-(m_\lambda - m)^2/4\Xi s} B_\lambda(m_\lambda) \tag{62}$$

respectively. It can be considered that $m_\lambda$ is a function of both $s$ and $m$ (see below), and that $\Omega$ and $C$ in Eqs. (60) and (62) have the forms

$$\Omega = \Omega(s, m_\lambda(s, m)) \tag{63}$$

and

$$C = C(s, \mathrm{m}_\lambda(s, m)) \tag{64}$$

respectively. The reason why both $\Omega$ and $C$ have the forms of Eqs. (63) and (64), instead of the forms $\Omega(s, m, m_\lambda(s, m))$ and $C(s, m, m_\lambda(s, m))$, respectively, will be understood later.

Let us calculate the function $m_\lambda(s, m)$. This will determine the form of the function $B(s, m)$ because the function $B_\lambda(m_\lambda)$ is given (Eqs. 75 and 76; see Eq. 61). The slope of the molarity gradient on any microcolumn $\lambda$ is the same if it is expressed in units of molarity/volume, and it can be written as $g'/\delta\lambda$. (We here write $\delta\lambda$ instead of $d\lambda$ in order to represent simply an infinitesimal part of the column section.) This is because the volume of the solution that flows into any microcolumn is the same within a unit time interval (see above). Writing $\bar{L}_\lambda'\ \delta\lambda$ as the longitudinal position of the infinitesimal molecular band (in the absence of thermodynamic longitudinal diffusion) on a column $\lambda$ expressed as a sum of interstitial volumes involved between this position and the column top, and defining $\bar{s}_\lambda$ as

$$\bar{s}_\lambda = \frac{g'}{\delta\lambda} \bar{L}_\lambda'\ \delta\lambda = g' \bar{L}_\lambda' \tag{65}$$

it is evident that $\bar{s}_\lambda$ is related to the local molarity $\bar{m}_\lambda$ at the position of the band by the relationship

$$\bar{s}_\lambda = m_0 - \bar{m}_\lambda \tag{66}$$

This is because the molarity of the ions at the column top should be equal to $m_0$ for any column $\lambda$ (cf. Eq. 8). On the other hand, calling $(L_0')_\lambda\ \delta\lambda$ the position at the beginning of the gradient on the column $\lambda$ (for some remarks on the beginning of the gradient, see p. 822),we have a general relationship

$$\frac{d\bar{s}_\lambda}{d\bar{m}_\lambda} = \frac{d\bar{L}_\lambda'/d(L_0')_\lambda}{1 - d\bar{L}_\lambda'/d(L_0')_\lambda} \tag{67}$$

the derivation of which is made in the Appendix. As $(L_0')_\lambda\ \delta\lambda$ is equal to (or, depending upon the definition of the elution volume, it is different only by a constant value from) the local elution volume $V_\lambda\ \delta\lambda$, we also have

$$\frac{d\bar{L}_\lambda'}{d(L_0')_\lambda} = \frac{d\bar{L}_\lambda'}{dV_\lambda} = (R_F)_\lambda = B_\lambda \tag{68}$$

where $(R_F)_\lambda$ represents the local $R_F$ for the infinitesimal molecular band on the column $\lambda$ being equal to $B_\lambda$. By substituting Eq. (68) into Eq. (67), and writing $\bar{m}_\lambda$ simply as $m_\lambda$ (because in Eqs. (60)–(64), $\bar{m}_\lambda$ was written as $m_\lambda$):

$$\frac{d\bar{s}_\lambda}{dm_\lambda} = \frac{B_\lambda(m_\lambda)}{1 - B_\lambda(m_\lambda)} \tag{69}$$

is obtained. Taking into account the fact that at the top of the column (where $\bar{s}_\lambda = 0$) the relationship $m_\lambda = m_{in}$ is fulfilled, Eq. (69) can be integrated to give

$$\bar{s}_\lambda = r(m_\lambda) \tag{70}$$

where

$$r(m_\lambda) = \int_{m_{in}}^{m_\lambda} \frac{B_\lambda(m_\lambda)}{1 - B_\lambda(m_\lambda)}\, dm_\lambda \tag{71}$$

By substituting Eq. (70) into Eq. (66), and writing $m_\lambda$ instead of $\bar{m}_\lambda$,

$$r(m_\lambda) = m_0 - m_\lambda \tag{72}$$

is obtained. Now, by eliminating $m_0$ between Eq. (72) and the left-hand side equation in Eq. (8), we have

$$m = m_\lambda + r(m_\lambda) - s \tag{73}$$

which represents the function $m_\lambda(s, m)$ in an implicit form. Hence the function $B(s, m)$ has been determined (see above). It should be noted that, due to Eq. (73), the term $m_\lambda - m$ appearing in the exponential parts of both Eqs. (60) and (62) can be replaced by the term $s - r(m_\lambda)$. This is the reason why Eqs. (60) and (62) have forms represented by Eqs. (63) and (64), respectively.

It can be considered that Eq. (62) is a solution of Eq. (17). Equation (62) has the form of Eq. (64), and the functions $B(s, m)$ and $m_\lambda(s, m)$ are given by Eqs. (61) and (73), respectively. This can be directly confirmed by substituting Eq. (62) into Eq. (17) (although the calculation is somewhat laborious). At time 0 at the top ($L' = 0$) of the column, $m_\lambda$ is equal to $m_{in}$ for any microcolumn $\lambda$. At the column top the relationship $s = 0$ is fulfilled (Eq. 8). This means that Eq. (60) tends to a delta-function at time 0 at the top of the column, giving

$$\lim_{\substack{s \to +0 \\ m_\lambda \to m_{in}}} \Omega = \delta(m - m_{in}) \tag{74}$$

Equation (74) shows that a band of molecules with an infinitesimal width is formed initially at the column top, being compatible with a fundamental assumption (Point 2 in the first paragraph of this section). It can be considered that Eq. (62) (with both Eqs. 61 and 73) is a solution of Eq. (17) obtained under the initial condition given by Eq. (74).

Equation (17) itself belongs in the second point of view on gradient chromatography (see the section entitled "Two Points of View on Gradient Chromatography"; see Remark 1 in the section entitled "Specification of Flux **J*** in Quasi-Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography"). It can be considered that Eq. (74) also belongs in the second point of view because, from this point of view, when $t \to +0$, then $s \to +0$ (see the section entitled "Two Points of View on Gradient Chromatography"). Equation (74) shows that, at time 0, a molecular band with an infinitesimal width is formed at a given position, $m = m_{in}$, on the molarity gradient. This is not incompatible with the consideration made in the section entitled "Specification of Flux **J*** in Quasi-Static Chromatographic Process; Fundamental Differential Equation for Gradient Chromatography" that, in the initial state of $B_\lambda$ or $B = 0$ at the top of the column, the molecules should exist at position $m = -\infty$ on the molarity gradient, since this latter is a picture captured after the transfer from the second to the first point of view has been achieved (see Remarks 1 and 2 in the section entitled "Specification of Flux **J*** in Quasi-Stati Chromato-

graphic Process; Fundamental Differential Equation for Gradient Chromatography").

Equation (62) represents, with Eq. (73), a chromatogram, as a function of ion molarity $m$, obtained when the parameter $s$ is given (i.e., when both length $L'$ of the column and slope $g'$ of the molarity gradient are given; see Eq. 8). $m_\lambda$ is an intermediate parameter. Both Eqs. (62) and (73) were derived in an earlier paper (*1*) by using a different method [Eqs. 36 (or 36') and 34 in Ref. *1* where somewhat different expressions are used]. These equations have been confirmed experimentally (*2*).

*Remark.* In Fig. 1 in Ref. *2* the standard deviations $\sigma_{(K^-)}$ of both theoretical and experimental chromatographic peaks of lysozyme are shown as functions of length $L$ (being proportional to $L'$) of the column for three different slopes $g'$ of the molarity gradient. It can be seen in this figure that $\sigma_{(K^+)}$, in general, decreases rapidly with an increase of $L$ when $L$ is small, but that $\sigma_{(K^+)}$ increases slowly after the first rapid decrease. Figure A1 in Appendix II of Ref. *2* shows that, when $L$ is small, the elution molarity (at the center of gravity of the peak) is also small. This means that the smaller the column length, the smaller the ion molarity within the vertical section at the bottom of the column occurring when the sample molecules co-exist in this section. The aspect of Fig. 1 in Ref. *2* can explicitly be understood from the structure of Eq. (17). Thus, in this equation, the diffusive part of the abstract molecular flux is represented by the term $(\Xi/B)\ \mathrm{grad}_m\ (C/B)$. The abstract flux coincides with the actual flux at the bottom of the column. Since the factor $C/B$ within the gradient term in the diffusive part of the abstract flux can be written simply as $\Omega$ (see Eq. 3), representing the total molecular density within the column section, it is not this factor but rather the factor $1/B$ in the exterior of the gradient term that depends markedly upon the value of $B$. On the other hand, $B$ decreases with a decrease in molarity of the ions co-existing with the molecules. This means that the smaller the length $L$ of the column, the smaller is the $B$ value for molecules existing at the bottom of the column (see above). Therefore, when $L$ is extremely small, a considerable longitudinal molecular diffusion should occur at the bottom of the column. This explains the reason why $\sigma_{(K^+)}$ increases rapidly with a decrease of $L$ when $L$ is small. The fact that $\sigma_{(K^+)}$ increases slowly with an increase of $L$ when $L$ is large enough can be considered to be due to the fact that the $B$ value is close to the maximum value (equal to unity). This means that $B$ is almost constant. Therefore, the diffusion that occurs is essentially caused by the factor $-\mathrm{grad}_m\ C$ or $-\mathrm{grad}_L\ C$ (since $-\mathrm{grad}_L\ C$ is proportional to $-\mathrm{grad}_m\ C$). This means that $\sigma_{(K^+)}$ increases with an increase of $L$ because, the larger the length $L$ of the column, the larger is the volume of the solution that passes the interior of the total column.

## The Form of Function $B_\lambda(m_\lambda)$ and the Normalized Property of the Solution (Eq. 62) of Eq. (17)

With small sample loads, when the adsorption of molecules occurs onto a single type of crystal site, the function $B_\lambda(m_\lambda)$ (see Eq. 61) is given by both Eqs. (A1) and (A2) in Appendix I of Ref. *1*. These equations can be rewritten into a single equation as

$$B_\lambda(m_\lambda) = \frac{1}{1 + \beta\tau e^{x\varepsilon/kT}(\varphi' m_\lambda + 1)^{-x'}} \tag{75}$$

where $\varphi'$ and $\beta$ are positive constants representing the properties of the competing ions and the column, respectively. $x'$, $x$, $\varepsilon$, and $\tau$ are also positive constants representing the properties of the sample molecules. Thus $x'$ 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. $x$ 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 on one of the sites of HA, and $\tau$ is the number of effective geometrical configurations of a sample molecule on the crystal surface of HA (in the equilibrium state). (For details, see Appendix I of Ref. *1*.)

Equation (75) is valid, however, only when $0 \leq m_\lambda < \infty$, and it shows that $B_\lambda$ increases monotonically with an increase of $m_\lambda$, tending to unity when $m_\lambda$ approaches infinity. Experimentally, $m_\lambda$ moves between the initial value $m_{in}$ ($\geq 0$) and a value large enough for virtually all sample molecules to be eluted out of the column. Practically, only the case when molecules are initially retained at the top of the column is important since, unless this is the situation, it is unnecessary to apply the molarity gradient. Retained molecules can be characterized by the fact that $B_\lambda$ is almost equal to zero when $m_\lambda = m_{in}$. Now, the migration of molecules on the column is possible only when $B_\lambda$ is not close to zero, so that the molarity of the ions in the neighborhood of the molecular band migrating on the column should, in general, be much higher than $m_{in}$. Practically, this means that the function $B_\lambda(m_\lambda)$ (Eq. 75) is important only when $m_\lambda \gg m_{in}$ ($\geq 0$).

In the preceding section, in order to obtain the solution (Eq. 62) of Eq. (17), Eq. (52) was considered. Equation (52) had been introduced, however, under the tacit assumption that $m_\lambda$ can move in the whole range of $[-\infty, \infty]$, so that the function $B_\lambda(m_\lambda)$ is tacitly defined in the range $[-\infty, \infty]$ of $m_\lambda$. It is therefore necessary here to give a definition of $B_\lambda(m_\lambda)$ when $m_\lambda < 0$. Thus, let us define $B_\lambda(m_\lambda)$ when $m_\lambda < 0$, for instance, as

$$B_\lambda(m_\lambda) = \frac{1}{1 + \beta\tau e^{x\varepsilon/kT} e^{-x'\varphi' m_\lambda}} \tag{76}$$

$B_\lambda(m_\lambda)$ is now a differential and continuous function defined in the range $[-\infty, \infty]$ of $m_\lambda$; $B_\lambda(m_\lambda)$ increases monotonically with an increase of $m_\lambda$, tending to 0 and 1 when $m_\lambda$ approaches $-\infty$ and $\infty$, respectively. Actually, $B_\lambda(m_\lambda) \approx 0$ when $-\infty < m_\lambda \leq m_{in}$, however.

We now show that Eq. (62) is normalized with Eq. (73). Thus partially differentiating Eq. (73) with respect to $m$,

$$\left[\frac{\partial m_\lambda}{\partial m}\right]_s = \frac{1}{1 + dr(m_\lambda)/dm_\lambda} \tag{77}$$

is obtained, while, from Eq. (71), we have

$$B_\lambda(m_\lambda) = \frac{dr(m_\lambda)/dm_\lambda}{1 + dr(m_\lambda)/dm_\lambda} \tag{78}$$

By using Eqs. (77), (78), and (73), Eq. (62) can be rewritten as

$$C(s, m) \left[\frac{\partial m}{\partial m_\lambda}\right]_s dm_\lambda = \frac{1}{\sqrt{4\pi\Xi s}} e^{-(r-s)^2/4\Xi s}\, dr \tag{79}$$

which can be integrated, while keeping $s$ constant, to give

$$\int_{-\infty}^{\infty} C(s, m)\, dm = \int_{-\infty}^{\infty} \frac{1}{\sqrt{4\pi\Xi s}} e^{-(r-s)^2/4\Xi s}\, dr = 1 \tag{80}$$

Actually, when $m_\lambda \leq m_{in}$, then $B_\lambda \approx 0$ (see above). This means that when $m \leq m_{in}$, then $B \approx 0$ because when $m_\lambda \to m_{in}$, then $m \to m_{in}$ (see Eq. 61). Therefore, when $m \leq m_{in}$, then $C \approx 0$ (see Eq. 3). This means that, as a practical matter, the lower limit of the integral on the left-hand term of Eq. (80) can be replaced with $m_{in}$. Further, when $m = m_\lambda = m_{in}$, then $r = 0$ (Eq. 71). This means that the lower limit of the integral on the intermediate term of Eq. (80) can be replaced with 0.

## The Case of Molecules with Very Large Dimensions

From the physical meanings of the parameters $x'$ and $x$, it can be considered that to change the value of $x'$, while keeping the value of the parameter

$$\xi = x/x' \tag{81}$$

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 $\xi$, or when the molecule has infinite dimensions. In this instance, Eqs. (75) and (76) reduce to

$$\left.\begin{aligned} B_\lambda(m_\lambda) &= 0 \qquad \text{for } m_\lambda < m^\circ \\ \text{and} \\ B_\lambda(m_\lambda) &= 1 \qquad \text{for } m_\lambda \geq m^\circ \end{aligned}\right\} \tag{82}*$$

where

$$m^\circ = (e^{\xi\varepsilon/kT+(\ln\tau)/x'} - 1)/\varphi' \approx (e^{\xi\varepsilon/kT} - 1)/\varphi' \tag{83}$$

With molecules initially retained on the column (see the preceding section), the relationship

$$m^\circ > m_{\text{in}} \tag{84}$$

is fulfilled. It can be considered that molecules are eluted out of any microcolumn $\lambda$ [see the section entitled "Microscopical Point of View: Longitudinal Diffusion of Competing Ions, the Form of Function $B(s, m)$, and a Solution of Eq. (17)"] at the same molarity $m^\circ$ of the ions.

Now, when

$$m_\lambda \to m^\circ + 0 \tag{85}$$

Eq. (17) reduces to

$$\Xi \frac{\partial^2 C}{\partial m^2} = \frac{\partial C}{\partial s} \tag{86}$$

(cf. Eq. 61 and the second equation in Eq. 82). We show below that the situations occurring when $m_\lambda \neq m^\circ$ can be represented as a boundary condition for Eq. (86). Thus let us first examine the case when $m_\lambda < m^\circ$. It can be shown that, under both the initial chromatographic condition given by Eq. (74) and the condition of Eq. (84), the relationship

$$\lim_{s \to +0} \Omega(s, m) = \delta(m - m_\lambda) \tag{87}$$

*This equation simply shows that $B_\lambda$ increases stepwise from 0 to 1 with the increase of $m_\lambda$ at $m_\lambda = m^\circ$. It is only for convenience that the case of $B_\lambda = 1$ is involved within the case where $m_\lambda$ takes the critical value $m^\circ$.

is fulfilled for any value of $m_\lambda$ less than $m^\circ$. Thus, due to Eq. (78) and the first equation in Eq. (82), if $m_\lambda < m^\circ$, then $dr(m_\lambda)/dm_\lambda = 0$, whereas, by using Eq. (73), the term $m_\lambda - m$ in Eq. (60) can be replaced by $s - r(m_\lambda)$. The value of $s - r(m_\lambda)$ is independent of the value of $m_\lambda$ when $m_\lambda < m^\circ$ because $r(m_\lambda) =$ constant (see above). Therefore, when $m_\lambda < m^\circ$, the form of the function $\Omega(s, m)$ (Eq. 60) is independent of the value of the parameter $m_\lambda$ involved in it. We now have

$$\lim_{m_\lambda \to m^\circ - 0} \left[ \lim_{s \to +0} \Omega(s, m) \right] = \delta(m - m^\circ) \tag{88}$$

On the other hand, from Eq. (62) and the first equality in Eq. (82)

$$\lim_{\substack{(s \to +0) \\ m_\lambda \to m^\circ - 0}} C(s, m) = 0 \tag{89}$$

is obtained.

Let us examine the case when $m_\lambda > m^\circ$. Due to the physical meaning of $\Omega$, it can be assumed that the value of $\Omega$ changes continuously with a change in the value of $m_\lambda$. This means that

$$\lim_{m_\lambda \to m^\circ + 0} \left[ \lim_{s \to +0} \Omega(s, m) \right] = \delta(m - m^\circ) \tag{90}$$

(see Eq. 88). On the other hand, from Eqs. (3), (61), and the second equality in Eq. (82),

$$\lim_{m_\lambda \to m^\circ + 0} \Omega(s, m) = \lim_{m_\lambda \to m^\circ + 0} C(s, m) \tag{91}$$

is obtained, so that we have

$$\lim_{\substack{s \to +0 \\ m_\lambda \to m^\circ + 0}} C(s, m) = \delta(m - m^\circ) \tag{92}$$

Equations (89) and (92) can be rewritten into a single equation as

$$\lim_{\substack{s \to +0 \\ m_\lambda \to m^\circ}} C(s, m) = \delta(m - m^\circ) \tag{93}$$

Writing Eq. (93) simply as

$$\lim_{s \to +0} C(s, m) = \delta(m - m^\circ) \tag{93'}$$

it can be considered that Eq. (93') gives the boundary condition for Eq. (86). Under this condition, Eq. (86) has a solution

$$C = \frac{1}{\sqrt{4\pi\Xi s}} e^{-(m-m^{\circ})^2/4\Xi s} \tag{94}$$

Equation (94) can also be obtained if both Eq. (85) and the second equality in Eq. (82) are substituted into Eq. (62). It is evident that Eq. (94) fulfills Eq. (80).

Equation (94) represents a chromatogram for molecules with very large dimensions. In an earlier paper (*1*) this equation was derived by using a different method (Eq. 45 in Ref. *1*).

## APPENDIX

The purpose of this Appendix is to derive both Eqs. (43) and (67).

Let us consider a particle (or a molecule) migrating on the column at time $t$. We assume that a linear molarity gradient of the ions is migrating at the same time toward the bottom of the column while keeping its slope $g'$ constant. We show now that, calling $\dot{L}'$ the longitudinal position of the particle on the column, $L_0'$ the position at the beginning of the gradient, $\dot{m}$ the molarity of the ions at position $\dot{L}'$, $m_0$ the molarity of the ions at the top of the column, and $m_{in}$ the molarity of the ions at the beginning of the gradient (see Fig. 1: cf. p. 822), then the following relationship generally is fulfilled:

$$\frac{d\dot{s}}{d\dot{m}} = \frac{d\dot{L}'/dL_0'}{1 - d\dot{L}'/dL_0'} \tag{A1}$$

where

$$\dot{s} = m_0 - \dot{m} = g'\dot{L}' \tag{A2}$$

(see Fig 1). $\dot{L}'$, $L_0'$, $\dot{m}$, $m_0$, and $\dot{s}$ change with time $t$; $g'$ and $m_{in}$ are constant. In Fig. 1, we can find two general relationships

$$\frac{\dot{m} - m_{in}}{m_0 - m_{in}} = \frac{L_0' - \dot{L}'}{L_0'} \tag{A3}$$

and

$$m_0 - m_{in} = g'L_0' \tag{A4}$$

By substituting Eq. (A4) into Eq. (A3), and by differentiating with respect to time $t$, we obtain

$$\frac{1}{g'}\frac{d\dot{m}}{dt} = \frac{dL_0'}{dt} - \frac{d\dot{L}'}{dt} \tag{A5}$$

which can be rearranged to

$$g'\frac{d\dot{L}'}{d\dot{m}} = \frac{d\dot{L}'/dL_0'}{1 - d\dot{L}'/dL_0'} \tag{A6}$$

Equation (A6) gives the general relationship among $\dot{L}'$, $\dot{m}$, and $L_0'$. From Eqs. (A6) and(A2), Eq. (A1) can be derived.

It is evident that a relationship similar to that given by Eq. (A1) is fulfilled if we consider, instead of the migration of a particle, the migration of the mean part (with an infinitesimal width) of the molecular band (see the section entitled "General Method of Finding the Fundamental Flux in Any Type of Chromatography"). In this instance, $\dot{s}$, $\dot{m}$, and $\dot{L}'$ in Eq. (A1) can be replaced by $\bar{s}$, $\bar{m}$, and $\bar{L}'$, respectively. $L_0'$ in Eq. (A1) can be replaced by elution volume $V$ because $L_0'$, in general, is different from $V$ only by a constant value. Hence Eq. (43) can be derived.

It is also possible to consider, instead of the migration of the molecular band on the actual column, the migration of the band with inifinitesimal width on a microcolumn $\lambda$ [see the section entitled "Microscopical Point of View: Longitudinal Diffusion of Competing Ions, the Form of Function $B(\dot{s}, \dot{m})$, and a Solution of Eq. (17)]. In this instance, $\dot{s}$, $\dot{m}$, $\dot{L}'$, and $L_0'$ in Eq. (A1) can be replaced by $\bar{s}_\lambda$, $\bar{m}_\lambda$, $\bar{L}_\lambda'\,\delta\lambda$, and $(L_0')_\lambda\,\delta\lambda$, respectively, thus giving Eq. (67).



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