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THEORY OF CHROMATOGRAPHY OF ROD-LIKE MACROMOLECULES ON HYDROXYAPATITE COLUMNS*

IX. GENERAL METHOD FOR THE ANALYTICAL CALCULATION OF CHROMATOGRAMS FOR MIXTURES OF MOLECULES WITH THE SAME DIMENSIONS AND THE SAME SHAPE

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SUMMARY

A theory for the general analytical calculations of chromatogram for mixtures of rod-like molecules with the same dimensions and the same shape is proposed for the case when the elution is carried out with a linear molarity gradient of competing ions. Most of the theory is valid, however, for molecules other than those with rod-like shapes. If the adsorption occurs on C crystal sites of hydroxyapatite, in many instances all of the theory is valid for molecules of any shape. The theory is based on the classical theory of adsorption chromatography, but the physical meanings of the basic assumptions in the classical theory are necessarily reconsidered. It is shown that the differential equation originally proposed by Wilson for the description of the chromatographic process on the column is generally valid in gradient elution, assuming that there is no longitudinal diffusion of molecules on the column, while the equation that was modified by DeVault is not valid except for some extreme cases. In stepwise elution, however, it is DeVault's equation and not Wilson's equation that is generally valid.

INTRODUCTION

The classical theory of adsorption chromatography was developed nearly 40 years ago by Wilson[1], DeVault[2] and Weiss[3] on the basis of the assumptions (a) that thermodynamic equilibrium between the adsorbed phase and solution is attained instantaneously and (b) that the longitudinal diffusion of molecules on the column is negligible. These assumptions would be justified, at least in hydroxyapatite (HA)

* The term "rigid rod-like macromolecules" has been reduced to "rod-like macromolecules" in the title, as it was shown in an earlier paper[5] that the theory can be extended to the case of molecules with flexible structures.

chromatography, by the fact that the width of the chromatographic peak is usually large and that no virtually deformation of the chromatogram or change in the elution molarity is observed when the flow-rate is varied. In fact, virtually the same chromatogram is observed if the flow-rate varies between 1 and 0.1 ml/min with a column with a diameter of 1 cm, which is common practice. It is necessary, however, to reconsider the physical meanings of these assumptions (see Theoretical, section *E* and *F*).

In earlier papers[4–8] a theory of chromatography on HA columns with small loads was developed for the case when the elution is carried out with a linear molarity gradient of competing ions (see below). This theory is based on the classical assumptions (a) and (b) above, and a further assumption (c) that sample molecules and particular ions from the buffer compete for adsorbing sites that are arranged on the crystal surface of HA. (For the reasoning behind this assumption, see, for instance, Introduction in ref. 8 and Appendix I in ref. 11.) Two types of adsorbing sites, called C and P sites, exist on different crystal surfaces of HA[4,5,7,8]. In many instances, however, the adsorption of molecules occurs on virtually only one of these crystal surfaces[4,5], which is the case treated in this paper. With small loads, mutual interactions among molecules are negligible, and the chromatography of a component in the mixture is carried out independently of the coexistence of the other components. It has been shown[4,5] that the elution molarity can reasonably be expressed as a function of the product, s, of the slope, g, of the molarity gradient of competing ions and the length, L, of the column, and a theory for the analytical calculation of the (mean) elution molarity of the chromatographic peak was proposed. On the basis of this theory, several experiments were analysed in order to check quantitatively the parallelism between the theoretical and experimental results, and several experimental parameters such as the number, x', of adsorbing sites of HA covered by an adsorbed molecule, the interaction energy, ε, between an adsorbing site of HA and an adsorbed functional group of the molecule, etc., were estimated[4,5,7,8]. In earlier papers[7,8], both the distribution and the structure of adsorbing sites on the surface of HA were explored on the basis of crystallographic data. Reasonable results obtained so far verify the validities of both the theory itself and the basic assumptions thereof (see above). A theory on the basis of the classical theory[1–3] was proposed[6] with which some information on the width of the chromatographic peak can be obtained. The relationship between this theory and that developed earlier[4,5] (see above) was also discussed[6].

In earlier papers[9–16] in this series, a theory of chromatography for mixtures of rod-like macromolecules was developed also for a linear gradient of competing ions and on the basis of assumptions (a), (b) and (c) above, taking into account the fact that there are repulsive interactions among molecules adsorbed side by side on the crystal surface of HA. Owing to these interactions, the total chromatogram of the mixture is deformed and tends to be a right-angled triangle as a whole, beginning abruptly and finishing gradually with considerable tailing[9–16]; this explains reasonably well the general shape of the experimental chromatogram. It was also shown[15,16] that this deformation of the chromatogram should be associated with a high chromatographic resolution among molecules with very slight structual differences. In this step, however, the calculation of theoretical chromatograms for mixtures of any types of (rod-like) molecules can be performed only numerically by using several ap-

proximations[15]. A theory for the analytical calculation of chromatograms was proposed[16] for the case when all molecules have the same large dimensions and the same shape. In ref. 16, it is mentioned that the theory is valid only when the slope of the gradient of competing ions is small. In Appendix I of this paper, it will be shown, however, that the theory in ref. 16 is valid almost independently of the slope of the gradient.

In this paper, a theory for the analytical calculation of chromatograms for mixtures of rod-like molecules with the same dimensions, and the same shape but with any dimensions, is proposed for the case when the elution is carried out with a linear molarity gradient of competing ions. Most of the theory is valid, however, for molecules other than those with rod-like shapes (see Discussion). Further, in many instances all of the theory can be considered to be valid for molecules with any shapes if the adsorption occurs on C sites of HA (see Discussion; for C sites, see ref. 7). The present theory is based on the classical theory[1-3], but the physical meanings of assumptions (a) and (b) in the classical theory (see above) are necessarily reconsidered. It is shown that the differential equation originally given by Wilson[1] for the description of the chromatographic process on the column is generally valid for gradient elution, assuming that there is no longitudinal diffusion of molecules on the column, while the equation that was modified by DeVault[2] is not valid except for some extreme cases. In stepwise elution, however, it is DeVault's equation and not Wilson's equation that is generally valid. In Appendix I, some properties of HA chromatography when molecules have very large dimensions are discussed. In Appendices I and II, relationship of the present theory with theories developed in earlier papers[6,16] are considered.

THEORETICAL

(A) Wilson's equation and DeVault's equation

A general partial differential equation that would describe the development of a solute on the column in adsorption chromatography was given originally by Wilson[1] on the basis of assumptions (a) and (b) in Introduction. The equation proposed by Wilson, which involves simultaneous equations for a mixture of molecular species 1, 2, ..., ϱ', ..., ϱ, can be written as

$$\frac{\partial C_{(\varrho')}}{\partial L} + \alpha \cdot \frac{\partial \chi_{(\varrho')}}{\partial V} = 0 \tag{1}$$

($\varrho' = 1, 2, \ldots, \varrho$) in which $\chi_{(\varrho')}$ is the molecular density of species ϱ' on the adsorbent, *i.e.*, the proportion of the effective surface of HA occupied by adsorbed molecules of species ϱ', being unity when the surface is saturated only with species ϱ', as a function of the elution volume, V, and position, L, on the column expressed as the distance from the top of the column; α^* is the pore volume per unit length of the column, *i.e.*

$$\alpha = \frac{\delta V}{\delta L} \tag{2}$$

* α is written as ν in earlier papers[7,8].

and $C_{(\varrho')}$ is the concentration of species ϱ' in solution or in the mobile phase, as a function of V and L, defined as

$$C_{(\varrho')} = \frac{B_{(\varrho')}}{1 - B_{(\varrho')}} \cdot \chi_{(\varrho')} \tag{3}$$

where $B_{(\varrho')}$ is the ratio of the amount of species ϱ' in solution or in the interstitial liquid to the total amount of the same species in a column section. The quantity $\partial C_{(\varrho')}/\partial L \cdot \delta L$ therefore represents the difference, at time t or at an elution volume V, of the concentration $C_{(\varrho')}$ between the position $L - \mathrm{d}L$ and L on the column, and $\partial \chi_{(\varrho')}/\partial V \cdot \delta V$ represents the change occurring between time t and $t + \mathrm{d}t$ or between the elution volume V and $V + \mathrm{d}V$, of the density $\chi_{(\varrho')}$ at position L on the column. A general physical meaning of eqn. 1 will be reconsidered in section *E*. It appears that Wilson himself considers simply that eqn. 1 represents a form of the continuity equation of the flow when the pore volume of the column is negligibly small (see ref. 1 and below). It is evident, however, that the first term on the left-hand side of eqn. 1 corresponds to the divergence term in the general continuity equation of the flow (*cf.*, eqn. 39). The second term, which would correspond to the term expressing the change, with time, of the density of the fluid in the general continuity equation, would have to describe, therefore, the change, with time or the elution volume, of the molecular density or the concentration of species ϱ' in a section of the column, *i.e.*, the change in the amount of species ϱ' per unit volume of the column interstices, including the surface itself of the adsorbent, or the crystal surface in the case of HA. In eqn. 1, however, this term expresses only the change in the molecular density on the crystal surface. DeVault[2] modified Wilson's equation in order for it always to behave as the continuity equation of the flow, which can be written as

$$\frac{\partial C_{(\varrho')}}{\partial L} + \alpha \cdot \left[\frac{\partial C_{(\varrho')}}{\partial V} + \frac{\partial \chi_{(\varrho')}}{\partial V}\right] = 0 \tag{4}$$

($\varrho' = 1, 2, \ldots, \varrho$). It can be seen that the second term on the left-hand side of eqn. 4 now expresses the change in the molecular density in the column interstices, including the crystal surface. It should be noted that DeVault's equation (eqn. 4) reduces to Wilson's equation (eqn. 1) and that Wilson's equation acquires the property of the continuity equation of the flow (1) when the chromatography is carried out with a low R_F value, *i.e.*, when the probability that molecules are in solution is low during the development process in chromatography, which is possible provided that the pore volume, α, of the column is small, and/or that the adsorption energy per molecule is high, and (2) when the mechanism itself of the chromatography is virtually independent of the molecules in solution (see section *C*). When α tends to zero, the term $\alpha \cdot \partial C_{(\varrho')}/\partial V$ in eqn. 4 tends to zero, but the term $\alpha \cdot \partial \chi_{(\varrho')}/\partial V$ does not tend to zero, because this term can be rewritten as $\partial[(1 - B_{(\varrho')})/B_{(\varrho')} \cdot C_{(\varrho')}\alpha]/\partial V$ and $B_{(\varrho')}$ tends to zero when α tends to zero; this means that eqn. 4 reduces to eqn. 1.

In linear gradient elution, it is convenient to transform the variables V and L in eqns. 1 and 4, respectively, into the molarity, m, of the eluent (molarity of competing ions in HA chromatography) and the parameter s, which was introduced earlier[4] and is defined as

$$s = gL \tag{5}$$

where g represents the slope of the gradient of (competing) ions expressed as the change in molarity per unit length of the column, *i.e.*

$$g = -\frac{dm}{dL} = \alpha \cdot \frac{dm}{dV} \tag{6}$$

By using these new variables, eqns. 1 and 4 can be rewritten, respectively, as

$$\frac{\partial C_{(\varrho')}}{\partial s} + \frac{\partial \chi_{(\varrho')}}{\partial m} = 0 \tag{7}$$

($\varrho' = 1, 2, \ldots, \varrho$) and

$$\frac{\partial C_{(\varrho')}}{\partial s} + \frac{\partial C_{(\varrho')}}{\partial m} + \frac{\partial \chi_{(\varrho')}}{\partial m} = 0 \tag{8}$$

($\varrho' = 1, 2, \ldots, \varrho$). Eqns. 1–8 are valid for general adsorption chromatography.

(B) Hydroxyapatite chromatography

In HA chromatography, assuming that the activity of competing ions is proportional to molarity (it was shown that this is a reasonable assumption at least when the competing ions are Na^+ and the molarity of the ions is less than the order of 0.2 M; ref. 8) and by taking into account the mutual interactions among molecules adsorbed on the crystal surface, the parameter $B_{(\varrho')}$ or $B_{(\varrho')}/1 - B_{(\varrho')}$ can be expressed as[9,10]

$$\frac{B_{(\varrho')}}{1 - B_{(\varrho')}} = \frac{1}{q_{(\varrho')}} \cdot (\varphi' m + 1)^{x'} H(\chi) \tag{9}$$

and eqn. 3 can be rewritten as

$$C_{(\varrho')} = \frac{1}{q_{(\varrho')}} \cdot (\varphi' m + 1)^{x'} H(\chi) \chi_{(\varrho')} \tag{10}$$

where

$$\chi = \sum_{\varrho''=1}^{\varrho} \chi_{(\varrho'')} \tag{11}$$

and

$$q_{(\varrho')} = \beta \sigma_{(\varrho')} e^{x_{(\varrho')} \varepsilon / kT} \tag{12}$$

In eqns. 9 and 10, φ' is the proportionality constant between the "activity", A, of competing ions and the molarity, m (refs. 4–8); x' is the number of sites of HA on which competing ions cannot be adsorbed owing to the presence of an adsorbed macromolecule, where we omit the suffix ϱ' because only the case when all molecules have the same dimensions and the same shape is treated in this paper; $H(\chi)$ expresses the mutual interactions of macromolecules on the crystal surface of HA (see below). In eqn. 11, χ represents the proportion of the effective surface of HA occupied by adsorbed molecules of all species 1, 2, ..., ϱ, being unity when the surface

is saturated with these molecules. In eqn. 12, $-\varepsilon\,(\varepsilon > 0)$ is the adsorption energy of an adsorption group of the macromolecule on to one of the sites of HA; $x_{(\varrho')}$ the average number (concerning time) of adsorption groups per molecule that react with sites of HA, because the adsorption of each molecule must follows a Boltzmann distribution[9]. Similarly the parameter x' should represent the average quantity. With rod-like molecules, however, the adsorption by using a lateral surface, parallel to the main axis of the rod, must be energetically favoured, and the molecule must always be adsorbed by using this surface. Therefore, the value of x' must be virtually constant. $-x_{(\varrho')}\varepsilon$ therefore represents the average adsorption energy per molecule, which was written as $u_{(\varrho')}$ in earlier papers[9–16]; $\sigma_{(\varrho')}$ is related to some properties of the molecule, including the symmetry, the distribution of the adsorption groups on the molecular surface and the flexibility of the molecular structure[8]; and β represents the property of the column defined by eqn. 31 in ref. 9 or eqn. 42 in ref. 10, which can be written as

$$\beta = \Gamma z' \frac{\delta A}{\delta V} \tag{13}$$

where Γ is a constant that is effectively independent of the type of molecules (see refs. 9 and 10, in which Γ is written as Γ_3); z' is the number of possible orientations of each molecule on the crystal surface of HA, which should be virtually equal to the coordination number, z, of the adsorbing sites on the crystal surface when these sites are C sites*; and δA and δV are the total effective crystal surfaces and the interstitial volume in the column section, respectively. β is related, therefore, to α (see eqn. 2) by the relationship

$$\alpha\beta = \Gamma z' \frac{\delta A}{\delta L} \tag{14}$$

With rod-like molecules, the function $H(\chi)$ (see eqns 9 and 10) can be written[9,10] as

$$H(\chi) = \frac{e^{\frac{\hat{E}}{kT}\sqrt{\chi}}}{1-\chi} \tag{15}$$

where $\hat{E}$ [which was written as $-u_{(1)}\Xi$ in refs. 9–16, where Ξ represents the interaction energy per molecule (see below) measured in units of the adsorption energy, $u_{(1)}$, of molecular species 1, when all molecules have the same dimensions and the same shape] is the interaction energy for one of molecules with others on the crystal surface, provided that one of the two sides of the rod is brought into contact with (or, more precisely, keeps the minimum distance from) other molecules that are adsorbed side by side in the same orientation and that the distribution of molecules on the crystal surface follows the Bragg–Williams approximation[9]; with the usual repulsive interactions, $\hat{E}$ is defined as positive[9,10]. The formula of the numerator on

* The value of z for the C site is 2 (ref. 7). It is presumable that this value is virtually equal to the value of z' when the density of molecules on the crustal surface is small. Even with a high molecular density, z' must be equal to 2, because the molecules with elongated shapes must be adsorbed parallel to one another (see Appendix I in ref. 9).

the right-hand side of eqn. 15 can be considered to be different if the shape of the molecule differs from that of a rod. The denominator on the right-hand side of eqn. 15 is an approximate expression of the probability [denoted by $p(\chi)$ in earlier papers[9–16]] that, when a new molecule is added at random to the crystal surface, a proportion χ of which is already occupied by molecules, it is not superimposed on the already adsorbed molecules. It should be noted that $H(\chi)$ tends to unity when χ tends to zero or with small loads, and that each equation in the simultaneous equations, eqns. 7 and 8, becomes independent of the another. However, if there are no energetic interactions among adsorbed molecules or if $\hat{E} = 0$, then $H(\chi)$ is not always unity, and each equation in eqns. 7 and 8 is not independent owing to the denominator on the right-hand side of eqn. 15. In other words, this term would represent geometrical interactions among molecules on the crystal surface, which, however, are not very important in the chromatography; this can be understood by the fact that, in the development process in chromatography, in which χ is not very close to unity (it is evident that, in order for the migration of the band of molecules to occur on the column, the value of χ should be less than unity), the function $B_{(\varrho')}(m)$ is virtually independent of the probability $p(\chi)$[9,10], and this is also the reason why the theoretical chromatogram can be calculated with sufficient exactness by using the approximate expression $1 - \chi$ for the function $p(\chi)$ (see above).

(C) The case when $x' \gg 1$

It can be understood, from the physical meanings of parameters x' and $x_{(\varrho')}$ (see section *B*), that to change the value of x', while keeping the value of the parameter

$$\xi_{(\varrho')} = \frac{x_{(\varrho')}}{x'} \tag{16}$$

constant, corresponds to considering homologous molecules with different dimensions or different lengths. We show now that, if $x' \gg 1$ [and if $\xi_{(\varrho')}$ is constant], DeVault's equation (eqn. 8) reduces to Wilson's equation (eqn. 7) in gradient elution: by using eqns. 10, 12, 15 and 16, we obtain, for the ratio of the concentration of species ϱ' in solution to the density on the crystal surface, the relationship

$$\frac{C_{(\varrho')}}{\chi_{(\varrho')}} = \left\{ \frac{Z_{(\varrho')}}{[\beta\sigma_{(\varrho')} \cdot (1 - \chi)]^{1/x'}} \right\}^{x'} \tag{17}$$

in which $Z_{(\varrho')}$ is the parameter introduced as eqn. 48 in an earlier paper[10], *i.e.*

$$Z_{(\varrho')} = \frac{(\varphi' m + 1) e^{\frac{\hat{E}}{x'} \cdot \frac{1}{kT} \cdot \sqrt{\chi}}}{e^{\xi_{(\varrho')} \varepsilon / kT}} \tag{18}$$

where it can be considered that $\hat{E} = O(x')$, because both $\hat{E}$ and x' should be proportional to the length of the rod-like molecule. As χ is not very close to unity in the development process in chromatography (see section *B*) and as $x' \gg 1$ (see

above), the term $[\beta\sigma_{(\varrho')} \cdot (1 - \chi)]^{1/x'}$ on the right-hand side of eqn. 17 is virtually unity, and eqn. 17 reduces to

$$\frac{C_{(\varrho')}}{\chi_{(\varrho')}} \approx Z_{(\varrho')}{}^{x'} \tag{19}$$

which shows the value of $C_{(\varrho')}/\chi_{(\varrho')}$ makes a sharp transition, with increase in $Z_{(\varrho')}$, from 0 to ∞ at $Z_{(\varrho')} = 1$. This means that, when $Z_{(\varrho')} < 1$, $C_{(\varrho')} \ll \chi_{(\varrho')}$ and that the second term on the left-hand side of eqn. 8 is negligible (compared with the third term). Thus, eqn. 8 reduces to eqn. 7. When $Z_{(\varrho')} = 1$, it can be seen from eqn. 18 that χ decreases monotonously with increase in m, which is carried out independently of both the concentration and the amount of molecules in solution or the interstitial liquid on the column. This means that the change, with change in m, in the total amount of molecules in the section of the column is due only to the change in the amount on the crystal surfaces. Thus, eqn. 7 again holds. Finally, when $Z_{(\varrho')} > 1$, then $C_{(\varrho')} \gg \chi_{(\varrho')}$, which means that virtually all molecules in the column section are in solution. In this instance, the flow equation of molecules can be written simply as

$$\frac{\partial C_{(\varrho')}}{\partial s} + \frac{\partial C_{(\varrho')}}{\partial m} = 0 \tag{20}$$

($\varrho' = 1, 2, \ldots, \varrho$), which can be considered as a boundary condition that both eqns. 7 and 8 should fulfil spontaneously when $Z_{(\varrho')} \to 1\text{–}0$. Hence, provided that $x' \gg 1$, DeVault's equation (eqn. 8) always reduces to Wilson's equation (eqn. 7). The validity of Wilson's equation when x' has any value is considered in section *E*.

(D) Solution of Wilson's equation in gradient elution

We now solve Wilson's equation (eqn. 7) for gradient elution on an HA column: eqn. 7 can be rewritten by using eqn. 10 (and eqn. 11) as

$$q_{(\varrho')}{}^{-1} \cdot (\varphi' m + 1)^{x'} \cdot \left[H(\chi) \cdot \frac{\partial \chi_{(\varrho')}}{\partial s} + \chi_{(\varrho')} \cdot \frac{\mathrm{d}H(\chi)}{\mathrm{d}\chi} \cdot \sum_{\varrho''=1}^{\varrho} \frac{\partial \chi_{(\varrho'')}}{\partial s}\right] + \frac{\partial \chi_{(\varrho')}}{\partial m} = 0 \tag{21}$$

($\varrho' = 1, 2, \ldots, \varrho$), which are simultaneous linear first-order partial differential equations for $\chi_{(1)}, \chi_{(2)}, \ldots, \chi_{(\varrho)}$ as functions of s and m. Let us find solutions for eqn. 21 that fulfil a boundary condition with the physical meaning. This condition is that, in the development process of the chromatography, there is no inflow of sample molecules at the top of the column. In other words, when $L = 0$ or when $s = 0$ (see eqn. 5) (and when $V > 0$ or when $m > m_{\mathrm{in}}$, where m_{in} is the initial value of m), then

$$C_{(\varrho')} = 0 \tag{22}$$

($\varrho' = 1, 2, \ldots, \varrho$). This means that, when $s \to 0$, the first term on the left-hnad side of eqn. 7 becomes

$$\lim_{s\to 0} \frac{\partial C_{(\varrho')}}{\partial s} = \lim_{\substack{s\to 0 \\ \Delta s\to 0}} \frac{C_{(\varrho')}(s + \Delta s, m) - C_{(\varrho')}(s, m)}{\Delta s}$$

$$= \frac{C_{(\varrho')}(\delta s, m) - C_{(\varrho')}(0, m)}{\delta s}$$

$$= \frac{C_{(\varrho')}(\delta s, m)}{\delta s} \tag{23}$$

where

$$\delta s = \lim_{\Delta s\to 0} \Delta s \tag{24}$$

Therefore, with eqn. 10, eqn. 7 becomes

$$-\frac{\mathrm{d}\chi_{(\varrho')}}{\mathrm{d}m} = \frac{1}{q_{(\varrho')}\delta s} \cdot (\varphi' m + 1)^{x'} \cdot H(\chi)\chi_{(\varrho')} \tag{25}$$

($\varrho' = 1, 2, \ldots, \varrho$), which are simultaneous differential equations for $\chi_{(1)}, \chi_{(2)}, \ldots, \chi_{(\varrho)}$ as functions of only m. If an equation for species ϱ'' in eqn. 25 is divided by another for species ϱ', then

$$\frac{\mathrm{d}\log \chi_{(\varrho'')}}{\mathrm{d}\log \chi_{(\varrho')}} = \frac{q_{(\varrho')}}{q_{(\varrho'')}} \tag{26}$$

($\varrho', \varrho'' = 1, 2, \ldots, \varrho$) is obtained, which can easily be integrated to give

$$\chi_{(\varrho'')} = \chi^*_{(\varrho'')}\left[\frac{\chi_{(\varrho')}}{\chi^*_{(\varrho')}}\right]^{q_{(\varrho')}/q_{(\varrho'')}} \tag{27}$$

($\varrho', \varrho'' = 1, 2, \ldots, \varrho$) where we define $\chi^*_{(\varrho')}$ and $\chi^*_{(\varrho'')}$ as the initial values of $\chi_{(\varrho')}$ and $\chi_{(\varrho'')}$, respectively, when the development process of the chromatography begins (for a further interpretation for $\chi^*_{(\varrho')}$ and $\chi^*_{(\varrho'')}$, see below). Eqn. 11 can now be written as

$$\chi = \sum_{\varrho''=1}^{\varrho} \chi^*_{(\varrho'')}\left[\frac{\chi_{(\varrho')}}{\chi^*_{(\varrho')}}\right]^{q_{(\varrho')}/q_{(\varrho'')}} \tag{28}$$

It is important to see, in eqns. 27 and 28, that the density on the crystal surface for any species ϱ'', $\chi_{(\varrho'')}$, can be expressed in terms of the density for a given species ϱ', $\chi_{(\varrho')}$ (eqn. 27), and that the density for all species, χ, can be expressed as a function of the density of the given species ϱ' (eqn. 28). It can be considered that eqns. 27 and 28 (which have been derived from eqn. 22) are an expression of the boundary condition at the top of the column or when $s \to 0$, which can be applied directly to eqn. 21. It should be noted that coefficients of any terms in eqn. 21 do

not involve the variable s and that the shape of eqn. 21 is independent of the values of s. Hence, by substituting eqns. 27 and 28 into eqn. 21 and by introducing a new function

$$Y_{(\varrho')}(\chi_{(\varrho')}) = H(\chi)\chi_{(\varrho')} \tag{29}$$

eqn. 21 can be rewritten as

$$q_{(\varrho')}^{-1} \cdot (\varphi' m + 1)^{x'} \cdot \frac{\mathrm{d}Y_{(\varrho')}(\chi_{(\varrho')})}{\mathrm{d}\chi_{(\varrho')}} \cdot \frac{\partial \chi_{(\varrho')}}{\partial s} + \frac{\partial \chi_{(\varrho')}}{\partial m} = 0 \tag{30}$$

($\varrho' = 1, 2, \ldots, \varrho$). Each equation in eqn. 30 is now concerned only with species ϱ' and is independent of the others; this can easily be solved, giving the general solution

$$\mathrm{F}\left\{\int^{m} (\varphi' m + 1)^{x'} \mathrm{d}m - q_{(\varrho')} s \left[\frac{\mathrm{d}Y_{(\varrho')}(\chi_{(\varrho')})}{\mathrm{d}\chi_{(\varrho')}}\right]^{-1}, \chi_{(\varrho')}\right\} = 0 \tag{31}$$

or

$$\int^{m} (\varphi' m + 1)^{x'} \mathrm{d}m = q_{(\varrho')} s \left[\frac{\mathrm{d}Y_{(\varrho')}(\chi_{(\varrho')})}{\mathrm{d}\chi_{(\varrho')}}\right]^{-1} + \mathrm{Q}(\chi_{(\varrho')}) \tag{31'}$$

where F and Q are any functions.

In usual practice, the width of the chromatogram is much larger than the width of the initial band of molecules at the top of the columns. Therefore, it would be a good approximation to consider that the initial band has an infinitesimal width δL ($= \delta s/g$) (see above). Upon introducing this approximation, the following remarks are necessary, however: it can now be considered that, in eqns. 27 and 28, the parameters $\chi^*_{(\varrho')}$ (or $\chi^*_{(\varrho'')}$) and $\chi_{(\varrho')}$ (or $\chi_{(\varrho'')}$) represent proportions of the HA effective surfaces on the top δL of the column that are occupied by species ϱ' (or ϱ''), in the initial state of the chromatography and in the course of the development process, respectively. The same physical meanings should be conserved for these parameters in eqn. 30, where it should be recalled that the parameters $\chi^*_{(1)}$, $\chi^*_{(2)}$, ..., $\chi^*_{(\varrho)}$ are involved in the function $\mathrm{d}Y_{(\varrho')}/\mathrm{d}\chi_{(\varrho')}$. Thus, in eqn. 30, $\chi^*_{(\varrho')}$ should represent the proportion of the HA effective surfaces on the whole column that is occupied initially by species ϱ', or the initial mean density on the whole column. In order for $\chi^*_{(\varrho')}$ to have this physical meaning, calling $\chi^{**}_{(\varrho')}$ [$0 < \chi^{**}_{(\varrho')} < 1$] the density of species ϱ' in the interior of the actual initial band, and ΔL the width of this band, $\chi^*_{(\varrho')}$ in eqn. 30 should fulfil the relationship

$$\chi^*_{(\varrho')} = \frac{\Delta L}{L_T} \cdot \chi^{**}_{(\varrho')}$$

where L_T is the total length of the column and, for the practical calculation of the chromatogram, the approximation

$$\frac{\Delta L}{L_T} \cdot \chi^{**}_{(\varrho')} \approx \frac{\delta L}{L_T} \cdot \lim_{\chi^{**}_{(\varrho')} \to \infty} \chi^{**}_{(\varrho')}$$

is used (see above); this means that the parameter $\chi^*_{(\varrho')}$ in eqn. 30 should have not only the intensive property (see above) but also the extensive meaning of the loaded

amount of species ϱ', expressed in units such that $\chi^*_{(\varrho')} = 1$ provided that the whole column is initially saturated only with species ϱ'. In fact, the amount of molecules in solution is negligible compared with that of molecules adsorbed on the crystal surfaces in the interior of the initial band, in the case of the usual retained molecules, because this is the reason why molecules are retained on the column. The case of non-retained molecules is not important in practice, because, it is unnecessary to apply the molarity gradient of competing ions for the elution of molecules from the column. Further, the quantity $\chi^*_{(\varrho')}$ or $\chi_{(\varrho')}$ at the top (ΔL or δL) of the column decreases when the development process begins and this decrease must corresponds to the term $-\partial\chi_{(\varrho')}$ in eqn. 30; this means that the quantity $\chi_{(\varrho')}$ must also have an extensive property. Thus, in eqn. 30, the quantity $-\partial\chi_{(\varrho')}/\partial m \cdot \partial m$ should represent the decrease in the amount of species ϱ' between the position $L - \mathrm{d}L$ and L (where $L = s/g$; eqn. 5) on the column when the molarity of competing ions increases from m to $m + \mathrm{d}m$, or the amount passing through the position L within the time of the infinitesimal increase, $\mathrm{d}m$, in the molarity.

Similarly, the quantity $\chi_{(\varrho')}/L_T \cdot \mathrm{d}L$ shows the amount of species ϱ' adsorbed, at time t, between the position $L - \mathrm{d}L$ and L on the column. The factor L_T in the denominator of the term $\chi_{(\varrho')}/L_T \cdot \mathrm{d}L$ is necessary in order for $\chi_{(\varrho')}$ to be expressed in the same units as $\chi^*_{(\varrho')}$ (see above). On the other hand, the fundamental equation, eqn. 7, was derived (see section *A*) by giving to $\chi_{(\varrho')}$ an intensive physical meaning of the density of molecules on the crystal surface (which is, of course, consistent with the physical meaning of this parameter in eqns. 27 and 28; see above). Hence, the quantity $\chi_{(\varrho')}$ in eqn. 30 should also conserve both the extensive and the intensive properties; this is possible because eqn. 30 has a linear form.

We must now find a boundary condition that eqn. 31 or 31′ has to fulfil: eqn. 5 shows that when the slope, g, of the gradient of competing ions approaches zero, the value of s also tends to zero. In this instance, molecules must elute at the initial molarity, m_{in}, even though an extremely large volume of the eluent would be necessary. This situation can be considered as the boundary condition, which can be written as

$$m = m_{in} \tag{32}$$

when $s = 0$. Under this condition, eqn. 31′ reduces to

$$\int_{m_{in}}^{m} (\varphi' m + 1)^{x'} \mathrm{d}m = q_{(\varrho')} s \left[\frac{\mathrm{d}Y_{(\varrho')}(\chi_{(\varrho')})}{\mathrm{d}\chi_{(\varrho')}}\right]^{-1} \tag{33}$$

which can be rewritten, by integration and rearrangement, as

$$m = \frac{1}{\varphi'}\left[\left\{(x' + 1)\varphi' q_{(\varrho')} s \left[\frac{\mathrm{d}Y_{(\varrho')}(\chi_{(\varrho')})}{\mathrm{d}\chi_{(\varrho')}}\right]^{-1} + (\varphi' m_{in} + 1)^{x'+1}\right\}^{\frac{1}{x'+1}} - 1\right] \tag{33'}$$

Now, the amount of species ϱ' that elutes from a column of length L, when the molarity of competing ions increases from m to $m + \mathrm{d}m$, should be equal to the decrease in the amount between the position $L - \mathrm{d}L$ and L or the amount

passing through the bottom, L, on the column during the infinitesimal increase, dm, in the molarity, which, according to eqn. 30, should be $-[\partial\chi_{(\varrho')}/\partial m]_L dm$ (see above). This means that the contribution, $f_{(\varrho'),s}(m)$, of species ϱ' to the total chromatogram of the mixture, when both the length of the column and the slope of the molarity gradient are given, *i.e.*, when the parameter s is given, can be written as

$$f_{(\varrho'),s}(m) = -\left[\frac{\partial\chi_{(\varrho')}}{\partial m}\right]_s$$

$$= \frac{(\varphi' m + 1)^{x'}}{q_{(\varrho')} \cdot s} \cdot \left[\frac{dY_{(\varrho')}(\chi_{(\varrho')})}{d\chi_{(\varrho')}}\right]^2 \cdot \left[\frac{d^2 Y_{(\varrho')}(\chi_{(\varrho')})}{d\chi_{(\varrho')}^2}\right]^{-1} \tag{34}$$

where the extreme right-hand side was derived from eqn. 33'. Eqns. 34 and 33' describe $f_{(\varrho'),s}$ as a function of m by using $\chi_{(\varrho')}$ as an intermediate parameter. It is evident that eqn. 34 fulfils the conservation condition

$$\int_{m_{in}}^{\infty} f_{(\varrho'),s}(m)dm = \chi^*_{(\varrho')} \tag{35}$$

On the other hand, the quantity $\chi_{(\varrho')}/L \cdot dL$ shows the amount of species ϱ' adsorbed at time t, between the position $L - dL$ and L on a column of length L (see above), which can be rewritten as $\chi_{(\varrho')}/s \cdot dm$, because $dL = 1/\alpha \cdot dV = 1/g \cdot dm$ and $gL = s$ (see eqns. 2, 5 and 6). This means that the amount of molecules in solution in the last section of the column with thickness dL should be

$$\frac{\chi_{(\varrho')}}{L} \cdot dL \cdot \frac{B_{(\varrho')}}{1 - B_{(\varrho')}} = \frac{\chi_{(\varrho')}}{s} \cdot dm \cdot \frac{B_{(\varrho')}}{1 - B_{(\varrho')}}$$

$$= \frac{(\varphi' m + 1)^{x'}}{q_{(\varrho')} s} \cdot H(\chi)\chi_{(\varrho')} dm$$

$$= \frac{(\varphi' m + 1)^{x'}}{q_{(\varrho')} s} \cdot Y_{(\varrho')}(\chi_{(\varrho')}) dm$$

(see eqns. 3, 9 and 29), which must elute from the column between the molarity m and $m + dm$ of competing ions. Hence, $f_{(\varrho'),s}(m)$ can be written as

$$f_{(\varrho'),s}(m) = \frac{(\varphi' m + 1)^{x'}}{q_{(\varrho')} s} \cdot Y_{(\varrho')}(\chi_{(\varrho')}) \tag{36}$$

which, however, is different from eqn. 34; if eqn. 36 is substituted into the left-hand side of eqn. 35, its value becomes generally less than the value of the right-hand side. When $x' \gg 1$, however, the discrepancy between eqns. 34 and 36 is negligible, and eqn. 30 or 21, in fact, represents the continuity equation of the flow, because the relationship $\bar{E} = O(x')$ should hold (see section *C*), and eqns. 15, 28 and 29 show that the function $Y_{(\varrho')}(\chi_{(\varrho')})$ increases very rapidly with increase in $\chi_{(\varrho')}$ (when $x' \gg 1$), which means that

$$Y_{(\varrho')}(\chi_{(\varrho')}) \approx \frac{dY_{(\varrho')}(\chi_{(\varrho')})}{d\chi_{(\varrho')}} \approx \frac{d^2 Y_{(\varrho')}(\chi_{(\varrho')})}{d\chi_{(\varrho')}^2} \tag{37}$$

It is evident that both the first and the second derivatives of the function $Y_{(\varrho')}$ increase rapidly with increase in $\chi_{(\varrho')}$, when $Y_{(\varrho')}$ increases rapidly. In eqn. 37, the left-hand side and the intermediate terms can be obtained by integrating the intermediate and the right-hand side terms, respectively. As these terms increase rapidly with increase in $\chi_{(\varrho')}$ (see above), only the maximum values of the integrands are of importance for respective integrations; this is the reason why eqn. 37 is obtained (*cf.*, eqn. A3 in Appendix I).

Finally, with small loads when $H(\chi)$ tends to unity (see section *B*) and when $Y_{(\varrho')}(\chi_{(\varrho')})$ and $dY_{(\varrho')}(\chi_{(\varrho')}/d\chi_{(\varrho')}$ tend to $\chi_{(\varrho')}$ and unity, respectively (see eqn. 29, eqn. 33 or 33′ reduces to

$$\int_{m_{in}}^{m} (\varphi' m + 1)^{x'} dm = q_{(\varrho')} s \tag{38}$$

or

$$m = \frac{1}{\varphi'}\left[\left\{(x' + 1)\varphi' q_{(\varrho')} s + (\varphi' m_{in} + 1)^{x'+1}\right\}^{\frac{1}{x'+1}} - 1\right] \tag{38'}$$

which no longer involves the parameter $\chi_{(\varrho')}$. This means that the band of molecules on the column continues to have an infinitesimal width during the development process of the chromatography, and that the elution molarity, m, of the sharp band of species ϱ' is described by eqn. 38 or 38′, as a function of s, independent of the coexistences of the other species. It should be noted that eqns. 38 and 38′ are identical with eqns. 12 and 15, respectively, in an earlier paper[4], (in which m and $q_{(\varrho')}$ are written as m_{elu} and q), and that both eqns. 12 and 15 in ref. 4 were derived without using the assumption of $x' \gg 1$ (*cf.*, sections E and F).

(E) The case when x′ has any value

In this section we show that, in gradient elution, assuming that there is no longitudinal diffusion of molecules on the HA column, Wilson's equation is valid for molecules with any x' values or any dimensions, and that the result obtained in section *D* is valid for any molecules.

For this purpose, it is necessary to specify the physical meanings of both Wilson's and DeVault's equations, beginning with the general continuity equation of the flow of molecules on the column; this equation can be written as

$$\text{div}\,[\mathbf{v}_{(\varrho')}\Omega_{(\varrho')} - D\,\text{grad}\,C_{(\varrho')}] + \frac{\partial \Omega_{(\varrho')}}{\partial t} = 0 \tag{39}$$

($\varrho' = 1, 2, \ldots, \varrho$), where t is time: $\Omega_{(\varrho')}$ is the density or the amount of species ϱ' in the interstices, including the crystal surface, of the column section, which is defined as

$$\Omega_{(\varrho')} = C_{(\varrho')} + \chi_{(\varrho')} \tag{40}$$

and is related to the parameter $B_{(\varrho')}$ by the relationship

$$B_{(\varrho')} = \frac{C_{(\varrho')}}{\Omega_{(\varrho')}} \tag{41}$$

$v_{(\varrho')}$ is the mean velocity of the flow of molecules of species ϱ', or $v_{(\varrho')}\Omega_{(\varrho')}$ represents the mean flux of species ϱ' on the column; and D is the diffusion (or the dispersion) constant of molecules on the column, $v_{(\varrho')}\Omega_{(\varrho')} - D\mathrm{grad}\, C_{(\varrho')}$ representing, therefore, the total flux of species ϱ'. It should be noted that the definition of $\Omega_{(\varrho')}$ in this paper is different from that of $\Omega\,(= 1 + C/\chi)$ in an earlier paper[6].

We can show, at least in HA chromatography, that D does not necessarily bear the subscript ϱ' even when molecules are heterogeneous in dimensions and/or shape. Thus, the increase in the width of the chromatographic peak with an increase in the column length is observed in the gradient chromatography of lysozyme with small loads on the HA column[17]; this must be due to the diffusion of molecules in the interstitial liquid on the column, because the molarity or the activity of competing ions is higher in the rear than in the front part of the chromatographic peak in gradient elution, and it is impossible for the R_F value in the front part of the peak to be larger than the value in the rear part, unless there is molecular diffusion. This means that an increase in the width of the chromatographic peak with an increase in the column length is impossible without molecular diffusion[6], while the shape of the chromatogram is not deformed, nor does the elution position of molecules change when the flow-rate is varied (see Introduction). These two facts indicate that the dispersion of molecules on the column is not due to thermal diffusion but to the heterogeneity in the flow on the column[6], and that D is independent of the properties of the molecule; this leads to a further conclusion that D should be proportional to the volume of the solution or the solvent that elutes within unit time, $\mathrm{d}V(t)/\mathrm{d}t$, because, if $\mathrm{d}V(t)/\mathrm{d}t$ or the flow-rate of the solvent increases, the heterogeneity in the flow or the dispersion around the mean flow must increase proportionally. Hence, we obtain a relationship

$$D = D'\frac{\mathrm{d}V(t)}{\mathrm{d}t} \tag{42}$$

where D' is the proportionality constant.

In the quasi-static process of the flow, the assumption of which is justified by eqn. 42 (see below), it is reasonable to consider that the mean rate, $v_{(\varrho')}$, of the flow of species ϱ' is expressed as

$$v_{(\varrho')} = \frac{1}{\alpha} \cdot \frac{\mathrm{d}V(t)}{\mathrm{d}t} \cdot R_{F(\varrho')} = \frac{1}{\alpha} \cdot \frac{\mathrm{d}V(t)}{\mathrm{d}t} \cdot B_{(\varrho')} \tag{43}$$

where $R_{F(\varrho')}$ represents the mean R_F value for species ϱ' and $1/\alpha \cdot \mathrm{d}V(t)/\mathrm{d}t$ represents the flow-rate of the solvent at time t. Now, it is possible to rewrite eqn. 39, by using eqns. 40–43, as

$$\frac{\partial C_{(\varrho')}}{\partial L} + \alpha \cdot \left[\frac{\partial C_{(\varrho')}}{\partial V} - D' \cdot \frac{\partial^2 C_{(\varrho')}}{\partial L^2}\right] + \alpha \cdot \frac{\partial \chi_{(\varrho')}}{\partial V} = 0 \tag{44}$$

($\varrho' = 1, 2, \ldots, \varrho$). It is important to note that time, t, is not involved in eqn. 44, which is due to eqn. 42. In other words, it is eqn. 42 that justifies the treatment of HA chromatography on the basis of the equilibrium theory.

Now, in order to simplify eqn. 44, let us consider the following two assumptions: (1) that the diffusion of molecules in solution in the interstices of the column is negligible, and (2) that the diffusion of molecules in solution is carried out independently of the interaction with crystal surfaces. This assumption is similar to a Bragg–Williams approximation. It should be noted, however, that the dispersion of molecules in the interstitial liquid is not due to thermal diffusion but to heterogeneity in the flow (see above). Assumption (1) can be expressed as

$$D \text{ or } D' = 0 \tag{45}$$

If eqn. 45 is substituted into eqn. 44, DeVault's equation (eqn. 4) is obtained, which can be rewritten as

$$\frac{\partial[B_{(\varrho')}\Omega_{(\varrho')}]}{\partial L} + \alpha \cdot \frac{\partial \Omega_{(\varrho')}}{\partial V} = 0 \tag{46}$$

($\varrho' = 1, 2, \ldots, \varrho$). It should be noted that eqn. 46 still conserves the property as the general continuity equation of the flow (see section *A*).

Assumption (2) would mean that the distribution of molecules in solution follows Fick's second law, or we have

$$\frac{\partial C_{(\varrho')}}{\partial t} - D \cdot \frac{\partial^2 C_{(\varrho')}}{\partial L^2} = 0 \tag{47}$$

which can be rewritten, by using eqn. 42 as

$$\frac{\partial C_{(\varrho')}}{\partial V} - D' \cdot \frac{\partial^2 C_{(\varrho')}}{\partial L^2} = 0 \tag{47'}$$

If eqn. 47′ is substituted into eqn. 44, Wilson's equation (eqn. 1) is obtained, which can be rearranged, by using eqn. 3, to

$$\frac{\partial\left[\frac{B_{(\varrho')}}{1 - B_{(\varrho')}} \cdot \chi_{(\varrho')}\right]}{\partial L} + \alpha \cdot \frac{\partial \chi_{(\varrho')}}{\partial V} = 0 \tag{48}$$

($\varrho' = 1, 2, \ldots, \varrho$).

Eqn. 48 means that, provided the diffusion of molecules in the interstitial liquid on the column occurs independently (eqn. 47 or 47′), then the chromatographic mechanism is also independent of the molecules in solution, except for the fact that the mean flux or the mean rate of the migration of molecules on the column (the first term on the left-hand side of eqn. 48) is governed by the partition, $B_{(\varrho')}$, of the molecules in solution, so that the chromatography is carried out independently of the longitudunal diffusion of molecules on the column, following eqn. 48. Let us now introduce assumption (1) or eqn. 45, which leads to the conclusion, that provided there is no longitudinal diffusion of molecules on the column, the chromatogram

can be calculated by using eqn. 48. In fact, the only way in which the chromatography can be carried out without the effect of longitudinal molecular diffusion (although it cannot be performed exactly so in practice) is that the mechanism of the chromatography is independent of the molecules in solution. Here, it should be emphasized that assumptions (2) and (1), *i.e.*, eqns. 47 or 47′ and eqn. 45, have been introduced, in this order, only for the purpose of specifying the physical meaning of eqn. 48.

Eqn. 48 is, in fact, self-consistent with the conclusion, which is involved in itself, that the chromatographic mechanism is independent of molecules in the interstitial liquid on the column (see above), in gradient elution. In this instance, eqn. 48 is transformed into eqn. 7, or into

$$\frac{\partial\left[\frac{B_{(\varrho')}}{1-B_{(\varrho')}}\cdot\chi_{(\varrho')}\right]}{\partial s}+\frac{\partial\chi_{(\varrho')}}{\partial m}=0 \tag{49}$$

($\varrho' = 1, 2, \ldots, \varrho$), where the first term on the left-hand side still conserves the physical meaning of (the divergence of) the mean flux of molecules on the column [because $s = gL$ (eqn. 5) and g is constant], while the second term is now related only to the molarity of competing ions, as the force which drives molecules out of the crystal surface, and no longer to the interstices of the column. This can be compared with stepwise elution. In this instance, it can be considered that eqn. 48 is self-inconsistent with the physical meaning of the independence of the chromatographic mechanism from the molecules in solution, because the second term on the left-hand side of eqn. 48 is still related to the interstices of the column; the situation such that the change, $\partial\chi_{(\varrho')}$, with time, of the amount of molecules on the crystal surface is directly related to the change, ∂V, also with time, of the elution volume of the solvent, *i.e.*, to the dimensions of the interstitial volume of the column, but that it is independent of the amount of molecules partitioned in the interstices, evidently involves a logical inconsistency in the quasi-static treatment of the chromatographic process. A direct proof that Wilson's equation is not valid in stepwise elution will be given in section *F* for small loads, also with a direct proof that Wilson's equation, in fact, describes correctly the gradient elution process on the HA column, provided that the longitudinal diffusion of molecules on the column is negligible.

It should be emphasized that eqn. 49 does no longer represents the continuity equation of the flow, and that eqn. 49 does not express the conservation of the amount of molecules in the interstices, including the crystal surface, of the column section, because eqn. 49 is no longer concerned with molecules in the interstitial liquid on the column (see above). In spite of this fact, in order to derive eqn. 27 (or 28), which is a boundary condition for Wilson's equation, eqn. 21, when $s \to 0$, eqn. 22 has been used, which is concerned with molecules in solution or the interstitial liquid on the column. When $s \to 0$ or at the beginning of the chromatography, however, virtually all molecules in the column section are adsorbed on the crystal surfaces in the case of the usual retained molecules (see section *D*), or condition (1) in section *A* holds; this means that, when $s \to 0$, Wilson's equation coincides with DeVault's equation, and represents the continuity equation of the flow (see section

A). It should also be noted that, although eqn. 49 does not express the consevation of molecules in the section of the column (except in the extreme condition; see above), the conservation of molecules can be expressed, in the case of retained molecules (see above), by using the second term on the left-hand side of eqn. 49, as

$$\int_{m_{1a}}^{m} -\frac{\partial \chi_{(\varrho')}}{\partial m} \cdot \mathrm{d}m = \chi^{*}_{(\varrho')} \tag{50}$$

where $\chi^{*}_{(\varrho')}$ is the amount of molecules of species ϱ' loaded on the column, and $-\partial\chi_{(\varrho')}/\mathrm{d}m \cdot \mathrm{d}m$ represents the amount of molecules passing through a position L on the column when the molarity of competing ions increases from m to $m + \mathrm{d}m$ (*cf.*, section *D*). Eqn. 50 coincides with eqn. 35 if L represents the total length of the column, and if the function $f_{(\varrho'),s}(m)$ in eqn. 35 is defined by eqn. 34. Hence, the theoretical chromatogram for molecules with any values of x' can be calculated with eqn. 34, assuming that there is no longitudinal diffusion of molecules on the column. Eqn. 36 is not valid, however, except when $x' \gg 1$ (see section *D*), because eqn. 36 has been derived both on the basis of eqn. 49 and the assumption that it is molecules "in solution" in the last section of the column that elute from the column (see section *D*).

Finally, in order to understand better the physical meaning of eqn. 49, the following hypothesis would be useful: eqn. 49 or 48 describes the chromatographic process provided that there is no diffusion of molecules (see above). If there is no diffusion, eqn. 45 should hold, while we have always eqn. 44. Now, if eqn. 45 is substituted into eqn. 44, eqn. 46 is obtained, which is different from eqn. 48 or 49, the equation as the starting point of the logic. This inconsistency comes from the fact that eqn. 45 (unless introduced after eqn. 47 or 47′ has been introduced; see below) is already incompatible with eqn. 47, the basis of eqn. 44 (see above). In fact, eqn. 42 has been derived on the basis of the experimental conclusion that the thermal diffusion (denoted by D_t) of molecules is negligible compared with the diffusion (D) which is due to the heterogeneity in the flow on the column. In order for this relationship between D_t and D to be maintained even when D is infinitesimal, D_t should also be infinitesimal, which would preclude the existence itself of the solution, or, at least, the equilibrium between the adsorbed phase and solution, because it is the molecular diffusion (from which the longitudinal diffusion is inseparable) that maintains the equilibrium; this would mean that DeVault's equation, eqn. 46, is inconsistent with eqn. 42, the equation justifying the treatment of the chromatography on the basis of the equilibrium theory (for further discussions, see section *F-2*). It should be noted that eqn. 47 or 47′ used for the derivation of eqn. 48 or 49 is not incompatible, in the above sense, with eqn. 42. Further, eqn. 45 is no longer incompatible with eqn. 42 if it is introduced after eqn. 47 or 47′ has been introduced, because, in this instance, eqn. 45 is used only for the purpose of specifying the physical meaning of eqn. 49, which is no longer concerned with molecules in the interstitial liquid on the column (see above).

(F) Small loads: validities of Wilson's and DeVault's equations in gradient and stepwise elution

In this section, we show that when the elution of molecules is carried out

with a gradient of competing ions, Wilson's equation is valid for the description of the chromatographic process, but that DeVault's equation is not valid; in stepwise elution, it is DeVault's equation, and not Wilson's equation, that is valid. Here, we always consider small loads.

(1) Gradient elution. We examine first the validity of Wilson's equation. For this purpose, it is convenient to rewrite eqn. 49 as

$$\left(\frac{\partial s}{\partial m}\right)_{\chi_{(e')}} = -\frac{\partial \chi_{(e')}/\partial m}{\partial \chi_{(e')}/\partial s} = \frac{B_{(e')}}{1 - B_{(e')}} = q_{(e')}^{-1}(\varphi' m + 1)^{x'} \tag{51}$$

where it should be noted that $B_{(e')}/1 - B_{(e')}$ is independent of both s and $\chi_{(e')}$ with small loads; the extreme right-hand side of eqn. 51 has been obtained by using eqn. 9 taking into account the fact that $H(\chi) = 1$ with small loads (see section *B*). It can be considered that the extreme left-hand side of eqn. 51 represents the change of the position L of the part of the molecular band with density $\chi_{(e')}$ on the column, as a function of the molarity m of the competing ions, because $s = gL$ (eqn. 5) and the slope, g, of the gradient of the ions is constant. If the initial band of molecules on the top of the column has an infinitesimal width, the band maintains the infinitesimal width during the chromatographic process, because the R_F value in the front part of the band is, in general, smaller than the value in the rear part (see section *E*); this means that s no longer depends on $\chi_{(e')}$, and that eqn. 51 reduces to

$$\frac{\mathrm{d}s}{\mathrm{d}m} = \frac{B_{(e')}}{1 - B_{(e')}} = q_{(e')}^{-1}(\varphi' m + 1)^{x'} \tag{52}$$

which describes the change in the position of the infinitesimal band of molecules as a function of m. Eqn. 52 is also obtained if eqn. 38 is differentiated with respect to m.

Now, when adsorption chromatography can be described as a quasi-static process, it is possible to consider, in general, that the ratio, R_F, of the mean migration rate of the solute to that of the solvent is equal to the partition, $B_{(e')}$, of the solute in solution or the mobile phase, which is shown in eqn. 43 [where R_F is written as $R_{F(e')}$]. It can also be considered that the gradient of competing ions migrates with the same rate as that of the solvent, at least in HA chromatography. In fact, it is observed experimentally that the actual slope of the gradient is equal to the slope that should be realized provided that there is no adsorption of the ions on the crystal surface*; this means that, even though the delay of the gradient occurs immediately after the gradient is introduced because of the adsorption of the ions, any part of the gradient migrates with the same rate after the initial delay, and that this rate is equal to that realized, provided that there is no adsorption of the ions on the crystal surface because, in order for different parts of the gradient with different molarities, which should involve different proportions of the ions interacting with the crystal surface, to migrate with the same rate, there must apparently be no

* See, for instance, Fig. 1 in ref. 6 (or Figs. 2A and 6 in ref. 17), in which three series of experiments where the slopes, $g_{(K)^+}$, of the gradient, provided that there is no adsorption of the competing ions (K^+) should be $1.1775 \cdot 10^{-3}$, $4.239 \cdot 10^{-4}$ and $3.5325 \cdot 10^{-5}$ *M*/cm are shown. The experimentally observed values of $g_{(K^+)}$ are 1.2–1.4 · 10^{-3}, 4.0–4.9 · 10^{-4} and 3.5–3.8 · 10^{-5} *M*/cm, respectively, which are virtually identical with the theoretical values.

interaction of the ions with the crystal surface, which is possible only if the molarity of the ions is high enough, except just at the beginning of the gradient, for most proportions of the ions in the column section to be in solution *i.e.*, if a molarity, m', exists with such properties (1) that $0 < m' - m_{in} \ll m - m'$, where m is the elution molarity of the solute, and (2) that it is high enough for most proportions of the ions in the column section to be in solution (*cf.*, Discussion in ref. 7). Now, if the width of the initial band of molecules on the top of the column is very small, and if there is no longitudinal diffusion of molecules on the column, the width of the band should always be small during the development process of the chromatography (see above), and the mean rate of migration of molecules on the column should be equal to the rate of migration of the molecular band itself (see above). Hence, if a conclusion such that the ratio (which can also be defined as R_F; see above) of the rate of migration of the molecular band (with an infinitesimal width) to that of the gradient of competing ions is equal to the partition, $B_{(e')}$, of molecules in solution, is derived from eqn. 52, then it is proved that eqn. 52, *i.e.*, eqn. 49, describes correctly the chromatographic process, provided that there is no longitudinal diffusion of molecules on the column.

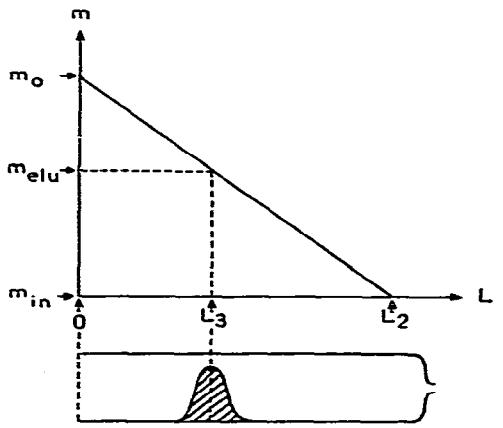

Fig. 1. Schematic representation of the development of molecules on the column when a linear molarity gradient of competing ions is applied. For details, see text. (Reproduced from ref. 4).

The above conclusion is, in fact, derived from eqn. 52 if eqns. 4–11 in an earlier paper[4] are followed (almost) in the other direction. Thus, calling L and m the coordinates that indicate the distance from the top of the column and the molarity of competing ions, respectively, L_3 and L_2 the distances from the top of the column of the molecular band and the beginning of the gradient, respectively, and m_{elu} and m_0 the values of m when $L = L_3$ and at the top of the column, respectively (see Fig. 1), then we evidently have

$$\frac{m_{elu} - m_{in}}{m_0 - m_{in}} = \frac{L_2 - L_3}{L_2} \tag{53}$$

$$m_0 - m_{in} = gL_2 \tag{54}$$

$$s = gL_3 \tag{55}$$

and

$$\frac{dL_3}{dL_2} = R_F \tag{56}$$

By substituting eqn. 54 into eqn. 53, and by differentiating with respect to time, t, we obtain

$$\frac{1}{g} \cdot \frac{dm_{elu}}{dt} = \frac{dL_2}{dt} - \frac{dL_3}{dt} \tag{57}$$

which can be rearranged to

$$\frac{1}{g} \cdot \frac{dm_{elu}}{dL_3} = \frac{dL_2}{dL_3} - 1 \tag{57'}$$

Eqn. 57′ becomes, with eqns. 55 and 56

$$\frac{dm_{elu}}{ds} = \frac{1}{R_F} - 1 \tag{58}$$

which can be rearranged to

$$\frac{ds}{dm_{elu}} = \frac{R_F}{1 - R_F} \tag{58'}$$

The physical meaning of m_{elu} in eqn. 58′ is identical with that of m in eqn. 52. Hence, it can be concluded, by comparing eqn. 58′ with eqn. 52, that

$$R_F = B_{(\varrho')} \tag{59}$$

and that Wilson's equation, eqns. 52 or 49, describes correctly the chromatographic process, provided that there is no longitudinal diffusion of molecules on the column.

We now examine, the validity of DeVault's equation. Eqn. 46 is transformed, in gradient elution, into

$$\frac{\partial[B_{(\varrho')}\Omega_{(\varrho')}]}{\partial s} + \frac{\partial \Omega_{(\varrho')}}{\partial m} = 0 \tag{60}$$

($\varrho' = 1, 2, \ldots, \varrho$). With small loads, $B_{(\varrho')}$ is independent of both s and $\Omega_{(\varrho')}$ and instead of eqns. 51 and 52, we have

$$\left(\frac{\partial s}{\partial m}\right)_{\Omega_{(\varrho')}} = -\frac{\partial \Omega_{(\varrho')}/\partial m}{\partial \Omega_{(\varrho')}/\partial s} = B_{(\varrho')} = \frac{1}{1 + q_{(\varrho')}(\varphi' m + 1)^{-x'}} \tag{61}$$

and

$$\frac{ds}{dm} = B_{(\varrho')} = \frac{1}{1 + q_{(\varrho')}(\varphi' m + 1)^{-x'}} \tag{62}$$

Therefore, by comparing eqn. 62 with eqn. 58′

$$\frac{R_F}{1 - R_F} = B_{(\varrho')} \tag{63}$$

or

$$R_F = \frac{B_{(\varrho')}}{1 + B_{(\varrho')}} \tag{63'}$$

is obtained, which is inconsistent with the basic equation for the equilibrium theory, eqn. 43, *i.e.*, eqn. 59. In fact, eqn. 63' shows that the value of R_F moves only in the range $0 \leqslant R_F \leqslant 0.5$, because $0 \leqslant B_{(\varrho')} \leqslant 1$, which, evidently, is an unreasonable conclusion (see also below).

The validity of Wilson's equation, eqn. 52 or 49, is, in fact, verified experimentally, because the validity of eqn. 38 or 38', which is obtained by integrating eqn. 52 and which is identical with eqn. 12 or 15 in ref. 4, is verified experimentally[4,7,8]. On the other hand, the fact that DeVault's equation is unreasonable is demonstrated clearly by the experiment with T_2 phage (presumably) with an extremely large value of x'; Fig. 1E in ref. 17 shows that the elution molarity, with small loads, for T_2 phage particles is independent of both the column length and the slope of the gradient of competing ions. It is evident that, if this result is plotted on an (m, $\log s$) plans (as in Figs. 2, 4 and 5 in ref. 4 and Figs. A7 and A8 in Appendix IV in ref. 8), then a straight line parallel to the ordinate or the $\log s$ axis is obtained, which indicates that $ds/dm = \infty$. Eqn. 62 shows, however, that the maximum value of ds/dm is unity, because the maximum value of $B_{(\varrho')}$ is unity, while, according to eqn. 52, ds/dm tends to infinity when $B_{(\varrho')}$ tends to unity. In earlier papers[9,10], it was shown that, when x' is large and when the amount of molecules in the column section is small, the value of $B_{(\varrho')}$, with an increase in the activity or the molarity m, makes a sharp transition from 0 to 1. Therefore, according to eqn. 52, and calling m^0 the value of m at which there is the sharp transition of $B_{(\varrho')}$, the value of ds/dm must also make a sharp transition from 0 to ∞ at $m = m^0$; this means that the value of s, which is obtained by integrating eqn. 52, is zero and infinity when $m < m^0$ and $m > m^0$, respectively, because $s = 0$ when $m = m_{\text{in}}$ (see section *D*) and, in order for this result of the integration to be obtained, the curve that is traced by the variables m and s must be such that $s = 0$ when $m < m^0$ and $m = m^0$ when $s > 0$. Therefore, the curve traced by the variables m and $\log s$ must be a straight line at $m = m^0$ parallel to the $\log s$ axis, and m^0 must represent the elution molarity of the solute. Hence, the validity of eqn. 52 has again been demonstrated.

Finally, it should be noted that eqn. 62 tends to eqn. 52 when $q_{(\varrho')}(\varphi' m + 1)^{-x'} \gg 1$ or when $B_{(\varrho')} \to 0$, *i.e.*, when condition (1) in section *A* holds, and that it is only in this condition that DeVault's equation is valid, unless $x' \gg 1$ [in an earlier paper[6], the gradient elution on the HA column is treated on the basis of DeVault's equation. This is done, however, only in the condition where DeVault's equation coincides with the Wilson's equation, *i.e.*, under condition (1) in section *A*; *cf.*, Appendix II]; if $x' \gg 1$, DeVault's equation always reduces to Wilson's equation [condition (2) in section *A*; see section *C*].

(2) Stepwise elution. We consider first Wilson's equation. Eqn. 48 can be rewritten as

$$\left(\frac{\partial L}{\partial V}\right)_{\chi(\varrho')} = -\frac{\partial \chi_{(\varrho')}/\partial V}{\partial \chi_{(\varrho')}/\partial L} = \frac{1}{\alpha} \cdot \frac{B_{(\varrho')}}{1 - B_{(\varrho')}} = \frac{1}{\alpha} \cdot q_{(\varrho')}^{-1} \cdot (\varphi' m + 1)^{x'} \tag{64}$$

because $B_{(\varrho')}/1 - B_{(\varrho')}$ is independent of both L and $\chi_{(\varrho')}$. Similarly, as in gradient elution (see subsection *I*), if the initial band of molecules on the top of the column has an infinitesimal width, the band continues to have this infinitesimal width during the development process of the chromatography, and eqn. 64 reduces to

$$\alpha \cdot \frac{dL}{dV} = \frac{B_{(\varrho')}}{1 - B_{(\varrho')}} = q_{(\varrho')}^{-1} \cdot (\varphi' m + 1)^{x'} \tag{65}$$

because the R_F value in the interior of the band should be constant. The extreme left-hand side of eqn. 65 represents the ratio of the rate of migration of the infinitesimal band of the solute, dL/dt to the rate of migration of the solvent, $1/\alpha \cdot dV/dt$. Therefore, we can write

$$R_F = \alpha \cdot \frac{dL}{dV} \tag{66}$$

and we have

$$R_F = \frac{B_{(\varrho')}}{1 - B_{(\varrho')}} \tag{67}$$

which is inconsistent with eqn. 43 or 59, and shows that R_F varies between 0 and ∞, because $0 \leqslant B_{(\varrho')} \leqslant 1$. This is an unreasonable conclusion. An explanation for the reason why Wilson's equation, eqn. 48, is not valid in stepwise elution was given in section *E*.

We now consider DeVault's equation. Eqn. 46 can be rewritten as

$$\left(\frac{\partial L}{\partial V}\right)_{\Omega_{(\varrho')}} = -\frac{\partial \Omega_{(\varrho')}/\partial V}{\partial \Omega_{(\varrho')}/\partial L} = \frac{1}{\alpha} \cdot B_{(\varrho')} = \frac{1}{\alpha} \cdot \frac{1}{1 + q_{(\varrho')}(\varphi' m + 1)^{-x'}} \tag{68}$$

because $B_{(\varrho')}$ is independent of both L and $\Omega_{(\varrho')}$. For an infinitesimal band, eqn. 68 reduces to

$$\alpha \cdot \frac{dL}{dV} = B_{(\varrho')} = \frac{1}{1 + q_{(\varrho')}(\varphi' m + 1)^{-x'}} \tag{69}$$

which, with eqn. 66, shows that the R_F is equal to the partition $B_{(\varrho')}$, or eqn. 59 is obtained; this is the condition for the quasi-static process of the chromatography (see above; *cf.*, eqn. 43). Hence, it can be concluded that the chromatographic process, provided that there is no londitudinal molecular diffusion, is correctly described by eqn. 69 or 46.

In section *E*, it was mentioned that the assumption, eqn. 45, which is necessary for the derivation of DeVault's equation, eqn. 46, is inconsistent with eqn. 42, the equation that justifies the quasi-static treatment of the chromatography and from which eqn. 44, the basis of DeVault's equation itself, is derived. In spite of this inconsistency, DeVault's equation describes correctly the stepwise elution process (see above); it is evident that, provided the migrating band of molecules has an infinite width, the effect of the longitudinal diffusion of molecules on the column is cancelled out in the interior of the band, and that eqn. 45 apparently holds. It would, therefore, be possible to imagine that the band with an infinitesimal width that has been considered above (or even the band with a finite width; *cf.*, Ap-

pendix II) is part of the band with an infinite width, and that the migration of this part can be described by DeVault's equation, eqn. 46. Similarly, it would be possible to consider that the actual column is part of the imaginary column with an infinite length. It is, in fact, possible to assume the band with an infinite width in stepwise chromatography, because the R_F value is constant in the interior of the band (at least for small loads) and the width of the band is maintained constant during the chromatographic process, provided that there is no longitudinal diffusion of molecules; this enables us to assume, during the whole process of the chromatography, the band with a width that is larger than any given constant value, *i.e.*, the band with an infinite width. In gradient elution, however, the width of the band decreases, in general, with the chromatographic process, provided that there is no longitudinal molecular diffusion, because the R_F value in the front part of the band is smaller than the value in the rear part (see section *E*). Therefore, it is only at the beginning of the elution process that we can assume the band with a width larger than any given value; this means that it is only near the beginning of the chromatographic process that DeVault's equation is valid as an approximation. Actually, the relationship $q_{(e^-)} \cdot (\varphi' m + 1)^{-x'} \gg 1$ holds near the beginning of the development process, for the usual retained molecules, and DeVault's equation coincides with Wilson's equation (see subsection *1*; *cf.*, Appendix II). It is evident that, also in stepwise elution, DeVault's equation coincides with Wilson's equation if $q_{(e^-)} \cdot (\varphi' m + 1)^{-x'} \gg 1$ or if $B_{(e^-)} \to 0$ [condition (1) in section A; *cf.*, eqns. 69 and 65].

RESULTS OF NUMERICAL CALCULATIONS OF THEORETICAL CHRIMATOGRAMS

These results will be given in a subsequent paper[18] together with results of the analysis of experimental chromatograms of tropocollagen. The results obtained when $x' \gg 1$ are almost the same as those obtained in an earlier paper[16] (see also Appendix I).

DISCUSSION

The fact that there is a longitudinal dispersion of molecules on the HA column, but that the chromatography is carried out independently of the flow-rate, indicates that the molecular dispersion is due to the heterogeneity in the flow, and that the effect of thermal diffusion is negligible (see Theoretical section, *E*). It can be considered, therefore, that the rate, relative to the mean rate, of the migration of the solute due to te heterogeneity in the flow, is, on average, much higher than the rate due to thermal diffusion if the two rates are measured over a range much wider than the mean interval among HA crystals on the column*; this must be due to the fact that the thermal diffusion of the solute is blocked by molecules of the solvent, but that the diffusion due to the heterogeneity in the flow is not interrupted. If the two rates are measured over a range comparable to the mean interval among HA crystals, however, the mean rate, relative to the mean flow, of the migration of the solute due to thermal diffusion must be much higher than the rate due to the heterogeneity in the flow, because the thermal motion of the solute must be

* "Range" = range which involves the system (as a part of the interior of the column) that is used to measure the two rates; "interval" = interdistance between neighbouring HA particles packed on the column.

blocked only slightly by molecules of the solvent in a small range. This molecular model of solution shows qualitatively that a virtually instantaneous equilibrium is attained between the adsorbed phase and solution, or that the classical assumption (a) in the Introduction is realized, justifying the quasi-static treatment of the chromatography; this can be compared with the method of the justification made under Theoretical, section E.

Another approximation that is used in the present theory is identical with the classical assumption (b) in the Introduction. It is important to note, however, that the processes through which this approximation, *i.e.*, the ideal state of no longitudinal molecular diffusion, is reached from the actual state of molecular diffusion are fundamentally different between the two systems (1) when time t is transformed into elution volume, V (stepwise chromatography) and (2) when time t is transformed into molarity, m, of competing ions (gradient chromatography) (see Theoretical, sections E and F).

It is interesting that the process of increasing the value of x' to infinity while keeping the value of $\xi_{(\varrho')}$ constant (see Theoretical, section C), resembles the process in which the quantum effect decreases with an increases in the dimensions of the system from a microscopic to a macroscopic scale. In fact, the value of $\xi_{(\varrho')}$ can change continuously when x' tends to infinity (see eqn. 16). Further, the number of possible orientations of a molecule on the crystal surface must approach infinity or the molecule can change its orientations continuously when x' tends to infinity and at least when the distribution of adsorption groups on the molecular surface is random. It should be recalled, however, that in earlier papers[4–16] we always assumed a finite number for the number of orientations of the molecule on the crystal surface, because it is actual molecules with finite dimensions that we consider, even though the approximation of $x' \to \infty$ is sometimes introduced for convenience.

Most conclusions in this paper obtained for rod-like molecules are valid for molecules with any shapes, because it is only the function $H(\chi)$ (eqn. 15) that depends on the molecular shape. Particularly when the adsorption of molecules occurs on C sites on the $(\vec{a}, \vec{c})$ and the $(\vec{b}, \vec{c})$ surfaces of the HA crystal[7], it can be considered that the function $H(\chi)$ obtained for rod-like molecules can be applied to molecules with any shapes, because the coordination number, z, for a C site is 2 (see Fig. 2 in ref. 7) and a molecule, with any shape, adsorbed on these crystal surfaces must interact, in many instances, with other molecules by using mainly both sides of the molecular surface, which is the general case of rod-like molecules[9].

In Appendix I, some properties of HA chromatography when $x' \gg 1$ and in gradient elution are discussed. In Appendices I and II, relationships of the present theory with theories developed in earlier papers[6,16] are considered.

APPENDIX I

Here we discuss some properties of HA chromatography when $x' \gg 1$, and consider the relationship of the present theory with the theory developed in an earlier paper[16].

Under the Theoretical, section E, it was mentioned that Wilson's equation, eqn. 49, in general does not represent the continuity equation of the flow. When $x' \gg 1$, however, eqn. 49, *i.e.*, eqn. 21, acquires the property of the continuity equation. In this instance, eqn. 25 also expresses the conservation of the amount of

molecules in the top section, δL (= $\delta s/g$), of the column. Now, by substituting eqn. 29 into eqn. 25, we obtain

$$-\frac{d\chi_{(\varrho')}}{dm} = \frac{1}{q_{(\varrho')} \cdot \delta s} \cdot (\varphi' m + 1)^{x'} \cdot Y_{(\varrho')}(\chi_{(\varrho')}) \qquad \text{(A1)}$$

($\varrho' = 1, 2, \ldots, \varrho$). Each equation in the simultaneous equations in eqn. A1 is now concerned only with molecular species ϱ', and is independent of the other equations; this can easily be integrated to give

$$\int_{m_{in}}^{m} (\varphi' m + 1)^{x'} dm = q_{(\varrho')} \cdot \delta s \cdot \int_{\chi_{(\varrho')}}^{\chi^*_{(\varrho')}} [Y_{(\varrho')}(\chi_{(\varrho')})]^{-1} \cdot d\chi_{(\varrho')} \qquad \text{(A2)}$$

where it should be noted that the integration of eqn. A1 has no physical meaning unless it expresses the conservation of the amount of molecules, or unless $x' \gg 1$ (see above). If x' is not large, eqn. A1 is not concerned with molecules in the interstitial liquid on the column, and it is only the change, $-d\chi_{(\varrho')}/dm$, itself of the amount of species ϱ' on the crystal surface, with increase in molarity m of competing ions, that has a physical meaning; in fact, the chromatogram $f_{(\varrho'),s}(m)$ can be calculated only by means of eqn. 34, *i.e.*, it can be expressed only in terms of the quantity $-(\partial\chi_{(\varrho')}/\partial m)_s$, and not by eqn. 36 (see Theoretical, section *E*). Under Theoretical, section *D*, it was mentioned that, when $x' \gg 1$, the function $Y_{(\varrho')}(\chi_{(\varrho')})$ increases very rapidly with increase in $\chi_{(\varrho')}$. Therefore, the integrand of the integral of the right-hand side of eqn. A2 or the quantity $[Y_{(\varrho')}(\chi_{(\varrho')})]^{-1}$ increases very rapidly with decrease in $\chi_{(\varrho')}$. Hence, only the maximum value of the integrand in the range $[\chi_{(\varrho')}, \chi^*_{(\varrho')}]$ is of importance for the integration, and we have

$$\int_{\chi_{(\varrho')}}^{\chi^*_{(\varrho')}} [Y_{(\varrho')}(\chi_{(\varrho')})]^{-1} d\chi_{(\varrho')} \approx [Y_{(\varrho')}(\chi_{(\varrho')})]^{-1} \qquad \text{(A3)}$$

As we have a similar relationship between the function $Y_{(\varrho')}(\chi_{(\varrho')})$ and its derivative, which is shown by eqn. 37, it is possible to rewrite eqn. A2 as

$$\int_{m_{in}}^{m} (\varphi' m + 1)^{x'} dm = q_{(\varrho')} \cdot \delta s \cdot \left[\frac{dY_{(\varrho')}(\chi_{(\varrho')})}{d\chi_{(\varrho')}}\right]^{-1} \qquad \text{(A4)}$$

Eqn. A4 can be considered as the boundary condition that eqn. 31′ has to fulfil when $s \to \delta s$ ($\to 0$) (see eqn. 24), under which eqn. 31′ becomes

$$\int_{m_{in}}^{m} (\varphi' m + 1)^{x'} dm = q_{(\varrho')} \cdot s \cdot \left[\frac{dY_{(\varrho')}(\chi_{(\varrho')})}{d\chi_{(\varrho')}}\right]^{-1} \qquad \text{(A5)}$$

which is the same as eqn. 33. It is important to note that eqns. A5 and A4 are identical except for the difference between s and δs; this means that the partial differential equation eqn. 21 or 30 reduces to the differential equation eqn. 25 or

A1, with only one variable m, when $x' \gg 1$; this leads to the conclusion that, when $x' \gg 1$ and at least when a band with a virtually infinitesimal width is formed initially on the top of the column (see Theoretical, section *D*), almost the same chromatogram should be obtained, independent of the value of s, *i.e.*, the column length, L, and/or the slope, g, of the molarity gradient of competing ions (see eqn. 5), provided that the amount of molecules loaded per unit packed volume of the crystals on the column is the same, and that there is no longitudinal diffusion of molecules on the column.

Under Theoretical, section *D*, we mentioned the process through which eqn. 25 had been derived. Eqn. 25 or A1 can also be derived through another process if $x' \gg 1$. Thus, when the slope, g, of the molarity gradient tends to an infinitesimal value, s tends to δs and the width of the chromatogram must become much larger than the total length of the column, *i.e.*, the total interstitial volume of the column must be much smaller than the total volume of the eluent in which macromolecules are involved (provided that there are repulsive interactions among molecules on the crystal surface; see Theoretical, section *B*). This means that the amount of molecules that exist in the interstitial liquid on the column, at time t, must be virtually equal to the loss of molecules from the column when a solution with the same volume as that of the column interstices is eluted, with an infinitesimal increase, dm, in the molarity of competing ions; this amount can be written as

$$\frac{(\varphi' m + 1)^{x'}}{q_{(e')}\delta s} \cdot Y_{(e')}(\chi_{(e')})\mathrm{d}m$$

which can be shown in a similar manner to the derivation of eqn. 36. Further, the loss of molecules from the column must be equal to the loss, $-\mathrm{d}\chi_{(e')}$, of adsorbed molecules from the crystal surfaces, because when $x' \gg 1$, the change, with change in the molarity, m, of competing ions, in the total amount of molecules in a section of the column is due only to the change in the amount on the crystal surfaces (see Theoretical, section *C*). Hence, eqn. A1 is obtained. It should be emphasized that this method for the derivation of eqn. A1 is valid only when $x' \gg 1$. It should also be noted that, unless $x' \gg 1$, eqn. A2 cannot be a simple boundary condition for eqn. 31', because we no longer have eqn. A3 or eqn. 37; in fact, unless $x' \gg 1$, the integration of eqn. A1, giving eqn. A2, has no physical meaning (see above).

Finally, the theory developed in an earlier paper[16] for the case when $x' \gg 1$ is based on the differential equation, eqn. A1, or eqn. 4 in ref. 16. The approximation, eqn. 7 in ref. 16, which is derived from the approximation

$$e^{\xi_{(e')}\varepsilon/kT} - 1 \approx \xi_{(e')} \cdot \varepsilon/kT \qquad \text{(A6)}$$

is also used. However, all of the results of the calculation of theoretical chromatograms obtained in ref. 16 are almost identical with those obtained on the basis of the present theory, when $x' \gg 1$.

APPENDIX II

In an earlier paper[6], a theory of HA chromatography with small loads, in which the effect of the longitudinal diffusion of molecules on the column was partially

taken into account, was developed for both gradient and stepwise elution (for the latter, see Appendix in ref. 6). Here, we consider the relationship of this theory with the present theory.

Wilson's equation, eqn. 49, which is generally valid in gradient elution provided that there is no longitudinal diffusion of molecules, can be rewritten, for small loads, by using eqn. 9 and by taking into account the fact that the relationship $H(\chi) = 1$ holds with small loads (see Theoretical, section *B*), as

$$q_{(\varrho')}{}^{-1}(\varphi' m + 1)^{x'} \cdot \frac{\partial \chi_{(\varrho')}}{\partial s} + \frac{\partial \chi_{(\varrho')}}{\partial m} = 0 \tag{A7}$$

the general solution of which can be written as

$$\chi_{(\varrho')} = \mathrm{P}\left[\frac{(\varphi' m + 1)^{x'+1} - (\varphi' m_{\mathrm{in}} + 1)^{x'+1}}{q_{(\varrho')}(x' + 1)\varphi'} - s\right] \tag{A8}$$

where P is any function. In ref. 6, it was mentioned, however, that if only the interior of a thin enough section of the column is considered as the system, eqn. A7 [in ref. 6, eqn. 60 here (*i.e.*, eqn. 9 or 12 in ref. 6) is considered instead of eqn. A7. These two equations are identical, however, near the beginning of the development process (see Theoretical, section *F-2*) or in the experimental condition which is treated in ref. 6 (see below)] or the differential equation that describes the development of molecules on the column provided that there is no longitudinal molecular diffusion is not valid, because it is the random motion of molecules that governs in the small region on the column. In fact, it is impossible for a thin section of the column to be divided into a large number of sub-sections through which the transport of molecules is carried out virtually in a single direction. With small loads, the initial band on the top of the column has a small width. However, when a molarity gradient of competing ions is applied, the difference in molarity between the two sides of the column top section initially occupied by molecules is also small; this means that the width of the molecular band broadens immediately after the development process begins (with retained molecules), and that eqn. A7 becomes valid[6]. Similarly, if the slope of the gradient of competing ions is very small, the differences in concentrations of both the ions and the sample molecules, in the interstices at the top and the bottom of the column itself, are always small during the chromatographic process, and the width of the chromatogram becomes much larger than the height of the column[6]. In fact, even if there is a slight difference in the molecular concentrations between the interstices at the top and the bottom of the column, provided that there is no molecular diffusion, there must be no difference in molecular concentration because of molecular diffusion, which means that eqn. A7 is also not valid under this extreme experimental condition. Further, it can be considered that the loss of molecules from the thin section at the top of the column in which molecules were initially adsorbed, and the loss of molecules from the column itself when the slope of the gradient of competing ions is very small, are virtually identical with the total amounts of molecules in the interstitial liquids in the column top section and in the column itself; this can be considered as the boundary condition that eqn. A8 has to fulfil when $s \to 0$ (and when $m > m_{\mathrm{in}}$),

because s tends to zero both when the column length, L, and the slope, g, of the molarity gradient of competing ions tend to zero (see eqn. 5), and the width of the chromatogram is much larger than the width of the initial band and the length of the column. In fact, Wilson's equation expresses the conservation of the amount of molecules in the column section when $s \to 0$ for retained molecules (see below).

Near the beginning of the development process or when the column is not so long, the relationship

$$q_{(e')}(\varphi' m + 1)^{-x'} \gg 1 \tag{A9}$$

should hold for retained molecules, and only a small proportion of molecules in the column section is in solution (see Theoretical, section F). Under this experimental condition, Wilson's equation, eqn. A7, represents the continuity equation of the flow (see Theoretical, section F), and the above boundary condition can be written as

$$-\frac{\mathrm{d}\chi_{(e')}}{\mathrm{d}[V/(\alpha L^0)]} = C_{(e')} \tag{A10}$$

where L^0 is the width of the column top section, or the total column length when g is small. Eqn. A10 can be rewritten by using the variable m, instead of V, and by introducing the parameter

$$\Delta s = gL^0 \tag{A11}$$

as

$$-\frac{\mathrm{d}\chi_{(e')}}{\mathrm{d}m} = \frac{C_{(e')}}{\Delta s} = \frac{B_{(e')}}{1 - B_{(e')}} \cdot \frac{\chi_{(e')}}{\Delta s} = q_{(e')}{}^{-1} \cdot (\varphi' m + 1)^{x'} \cdot \frac{\chi_{(e')}}{\Delta s} \tag{A12}$$

which can easily be integrated to give

$$\chi_{(e')} = \chi^*_{(e')} \cdot \exp\left[-\frac{1}{\Delta s} \cdot \frac{(\varphi' m + 1)^{x'+1} - (\varphi' m_{\mathrm{in}} + 1)^{x'+1}}{q_{(e')}(x' + 1)\varphi'} \right] \tag{A13}$$

where $\chi^*_{(e')}$ is the value of $\chi_{(e')}$ when $m = m_{\mathrm{in}}$; this represents the amount of molecules loaded on the column in the case of retained molecules (see Theoretical, section D).

Another boundary condition that eqn. A8 has to fulfil is that, when $m = m_{\mathrm{in}}$ and when $L > 0$, *i.e.* $s > 0$

$$\chi_{(e')} = 0 \tag{A14}$$

because it is only at the top of the column that molecules are adsorbed initially (see above), and the width of the initial band should be negligible compared with the total length of the column. Under the conditions in eqns. A13 and A14, eqn. A8 becomes

$$\left.\begin{aligned} \chi_{(\varrho')} &= \chi^{*}_{(\varrho')} \cdot \exp\left\{ -\frac{1}{\Delta s} \cdot \left[\frac{(\varphi' m + 1)^{x'+1} - (\varphi' m_{in} + 1)^{x'+1}}{q_{(\varrho')}(x' + 1)\varphi'} - s \right] \right\} \\ & \qquad \text{for } s < \frac{(\varphi' m + 1)^{x'+1} - (\varphi' m_{in} + 1)^{x'+1}}{q_{(\varrho')}(x' + 1)\varphi'} \\ \text{and} \\ \chi_{(\varrho')} &= 0 \qquad \text{for } s < \frac{(\varphi' m + 1)^{x'+1} - (\varphi' m_{in} + 1)^{x'+1}}{q_{(\varrho')}(x' + 1)\varphi'} \end{aligned}\right\} \quad \text{(A15)}$$

It can now be understood that eqn. 27 in ref. 6 is eqn. A15 itself, by noticing that the subscript ϱ' is omitted in eqn. 27 in ref. 6, by recalling the relationships between the symbols in ref. 6 (left-hand sides) and those in the present paper (right-hand sides):

$$S = \varphi' s \quad \text{(A16)}$$

$$\delta S = \varphi' \Delta s \quad \text{(A17)}$$

and

$$\Omega = 1 + q_{(\varrho')}^{-1}(\varphi' m + 1)^{x} \quad \text{(A18)}$$

and by using the approximations

$$q_{(\varrho')}^{-1}(\varphi' m + 1)^{x'} \ll 1 \quad \text{(A9')}$$

which is obtained from eqn. A9

$$\Omega \approx 1 \quad \text{(A19)}$$

for Ω in the denominator of the first equation in eqn. 27 in ref. 6

$$\log \Omega \approx q_{(\varrho')}^{-1}(\varphi' m + 1)^{x'} \quad \text{(A20)}$$

$$x' \approx x' + 1 \quad \text{(A21)}$$

and

$$(\varphi' m + 1)^{x'+1} \gg (\varphi' m_{in} + 1)^{x'+1} \quad \text{(A22)}$$

The theory in ref. 6 is based on DeVault's equation, eqn. 1, 9 or 12 in ref. 6, instead of Wilson's equation, eqn. A7. However, eqn. 16 in ref. 6, which is valid when A or m is small or near the beginning of the chromatographic process, reduces the result obtained from eqn. 12 in ref. 6 to that obtained from Wilson's equation. Similarly, eqn. 22 in ref. 6 reduces the result obtained from the boundary condition, eqn. 21 in ref. 6, to that obtained from the condition in eqn. A10 or

A12. All results obtained in ref. 6 and those obtained in this Appendix are valid only near the beginning of the chromatographic process (see above) or when the column is not so long; these explain satisfactorily, however, the decrease in the width of the experimental chromatogram (this occurs after the initial increase in the width of the band of molecules on the top of the column) with an increase in the column length (Fig. 1 in ref. 6), and the shape of the experimental chromatogram (Fig. 2 in ref. 6), observed for lysozyme, a molecule with a small value (4–8) of x' (see ref. 8), in a range of short column lengths, respectively.

Eqn. A15 is a discontinuity solution to eqn. A7, and the values of s and m at which there is a discontinuity of $\chi_{(\varrho')}$ fulfil the relationship given by eqn. 38′. Especially when $x' \to \infty$, eqn. A15 tends to a δ-function, which means that, when x' is large, the first decrease in the width of the chromatogram with an increase in the column length is very rapid; a small increase in the width of the chromatogram which follows this decrease, owing to the molecular diffusion on the column, must therefore begin when the column is very short. It should be noted that eqn. A15 tends to a δ-function also if Δs tends to zero.

In stepwise elution, it is DeVault's equation, eqn. 46, *i.e.*,

$$B_{(\varrho')} \cdot \frac{\partial \Omega_{(\varrho')}}{\partial L} + \alpha \cdot \frac{\partial \Omega_{(\varrho')}}{\partial V} = 0 \tag{A23}$$

that is valid (provided that there is no molecular diffusion). It should be noted again that $\Omega_{(\varrho')}$ is different from Ω in eqns. A18–A20; see note related to eqn. 40. $B_{(\varrho')}$ can be written as

$$B_{(\varrho')} = \frac{1}{1 + q_{(\varrho')}(\varphi' m + 1)^{-x'}} \tag{A24}$$

Eqn. A23 has the general solution

$$\Omega_{(\varrho')} = \mathrm{R}\left[\frac{B_{(\varrho')}}{\alpha} \cdot V - L\right] \tag{A25}$$

where R is any function. For small loads, the initial band on the top of the column must have a small width, ΔL, in which molecules move at random and where eqn. A23 cannot be applied (see above). The width of the band must broaden, however, immediately after the development process begins, if a low enough molarity of competing ions is used for the elution of molecules, and eqn. A23 becomes valid. If a high molarity of competing ions is used for the development of molecules, eqn. A23 is also valid. In this instance, eqn. A23 simply shows that the band of molecules with a small width migrates with an R_F value equal to $B_{(\varrho')}$ (≈ 1) (see below). In this instance, it can be considered that the loss of molecules from the section, ΔL, at the top of the column is virtually equal to the total amount of molecules in the interstitial liquid in this section; this can be considered as the boundary condition that eqn. A25 has to fulfil when $L \to 0$ (and when $V > 0$), which can be written as

$$-\frac{\mathrm{d}\Omega_{(\varrho')}}{\mathrm{d}[V/(\alpha \cdot \Delta L)]} = C_{(\varrho')} = B_{(\varrho')}\Omega_{(\varrho')} \tag{A26}$$

Eqn. A26 can easily be integrated to give

$$\Omega_{(\varrho')} = \Omega^{*}_{(\varrho')} \cdot \exp\left[-\frac{1}{\Delta L} \cdot \frac{B_{(\varrho')}}{\alpha} \cdot V \right] \tag{A27}$$

where $\Omega^{*}_{(\varrho')}$ is the value of $\Omega_{(\varrho')}$ when $V = 0$; this represents the amount of molecules loaded on the column. Another boundary condition that eqn. A25 has to fulfil is that when $V = 0$ and $L > 0$

$$\Omega_{(\varrho')} = 0 \tag{A28}$$

Under the conditions in eqns. A27 and A28, eqn. A25 becomes

$$\left.\begin{aligned} \Omega_{(\varrho')} &= \Omega^{*}_{(\varrho')} \cdot \exp\left\{ -\frac{1}{\Delta L} \cdot \left[\frac{B_{(\varrho')}}{\alpha} \cdot V - L \right] \right\} \\ &\text{for} \quad L < \frac{B_{(\varrho')}}{\alpha} \cdot V \end{aligned}\right\} \tag{A29}$$

and

$$\left.\begin{aligned} \Omega_{(\varrho')} &= 0 \\ &\text{for} \quad L > \frac{B_{(\varrho')}}{\alpha} \cdot V \end{aligned}\right\}$$

It is easy to understand that eqn. A9 in the Appendix in ref. 6 is eqn. A29 itself, because the concentrations or the amounts of molecules in solution, C and C^{*}, in eqn. A9 in ref. 6, can be transformed, by using eqn. 41, into $\Omega_{(\varrho')}$ and $\Omega^{*}_{(\varrho')}$, respectively, and δL and $M\xi$ in ref. 6 are related to ΔL, $B_{(\varrho')}$, α, etc., in this Appendix by the relationships

$$\delta L = \Delta L \tag{A30}$$

and

$$\begin{aligned} M\xi &= \alpha \cdot \frac{1 - B_{(\varrho')}}{B_{(\varrho')}} \\ &= \alpha q_{(\varrho')i} (\varphi' m + 1)^{-x'} \\ &= \left(\Gamma z' \cdot \frac{\delta A}{\delta L} \right) \left[\sigma_{(\varrho')} e^{x_{(\varrho')} \varepsilon / kT} \right] (\varphi' m + 1)^{-x'} \end{aligned} \tag{A31}$$

where, for the derivation of the extreme right-hand side term, eqns. 12 and 14 have been used. It should be noted that the terms in the first, second and third parentheses of this term represent the property of the column, the property of the molecule and the influence of competing ions on the adsorption of molecules on the HA surface, respectively. In the Appendix in ref. 6, it was mentioned that eqn. A9 in ref. 6 or eqn. A29 explains satisfactorily the general shape of the experimental chromatogram with tailing obtained in stepwise chromatography with small loads (see Fig. A1 in the Appendix in ref. 6). Eqn. A29 also shows, with eqn. A24, that, when the development is carried out with a high molarity of competing ions,

the value of $B_{(\varrho')}$ approaches unity and that the chromatogram becomes sharp. Particularly when x' is large, a transition in the value of $B_{(\varrho')}$ from 0 to 1, with an increase in m, occurs in a very small range of m (see Theoretical, section *F-1*). Therefore, it can be considered that, if the molarity of the solvent used for the chromatography is lower than the value of m^0 at which there is the transition of $B_{(\varrho')}$, the elution of molecules does not occur, whereas if the molarity of the eluent is higher than m^0, the elution must be carried out with an R_F value almost equal to unity, and a sharp chromatogram must be obtained. It should be noted that eqn. A29 tends to a δ-function, if ΔL tends to zero.

Finally, the above explanation of tailing of the chromatogram is different from both the classical explanation, in which the adsorption isotherm of molecules, the slope of the tangent of which decreases with an increase in the concentration of molecules in solution, is assumed[2,3], and the explanation on a kinetic basis as represented by Thomas[19] (see also ref. 20). The latter cannot be applied at least to HA chromatography, in which the shape of the chromatogram is virtually independent of the flow-rate (see Introduction). Further, in both theories, a boundary condition such that $C_{(\varrho')}$ or $\Omega_{(\varrho')} = 0$ is applied instead of eqn. A27 when $L = 0$ and $V > 0$, which is valid only if eqn. A23 can be applied in the interior of the initial band on the top of the column, *i.e.*, if the value of ΔL is large (see above and the Appendix in ref. 6). This condition should not, therefore, be applied when the initial band has a small width or with small loads (and especially in stepwise chromatography; see below), at least for the purpose of considering the shape of the chromatogram (see below). The situation is the same even if the flow on the column is highly homogeneous, because it must always be a random thermal motion of molecules that governs in a small enough region on the column. In this paper, however, this classical boundary condition was applied as eqn. 22 in gradient chromatography; this classical condition is valid in this instance because the width of the chromatogram decreases rapidly with an increase in the column length in the initial step of the chromatography (see above; see also Fig. 6 in ref. 17 and Fig. 1 in ref. 6). The effect of broadening of the band of molecules on the column, which occurs before this decrease and just at the beginning of the development process, therefore, does not appear unless the column is short. Further, the minimum length of the column necessary for this effect to appear decreases with increase in the value of x' (see above). This can be compared with stepwise chromatography, where the chromatogram continues to have a constant shape during the development process, provided that there is no effect of the longitudinal diffusion of molecules except initial broadening of the band. In the Theoretical, sections *F-2*, a band of molecules with an infinitesimal width was considered in order to show the validity of DeVault's equation in the stepwise chromatography; this is equivalent to considering, as a boundary condition, the classical condition $C_{(\varrho')}$ or $\Omega_{(\varrho')} = 0$ when $L = 0$ and $V < 0$. However, our purpose was to consider the rate of migration of molecules, R_F, which is the same in any part of the band. In this instance, to consider the infinitesimal band is equivalent to following the movement of a particular point in the interior of the band (see Theoretical, section *F-2*).

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