CHROM. 8912

THEORY OF CHROMATOGRAPHY OF RIGID MOLECULES ON HYDROXYAPATITE COLUMNS WITH SMALL LOADS

III. THEORY ON THE BASIS OF THE CLASSICAL THEORY OF ADSORPTION CHROMATOGRAPHY FOR THE CASE WHEN MOLECULES ARE ADSORBED ON TO A SINGLE TYPE OF CRYSTAL SITE

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(First received July 21st, 1975; revised manuscript received December 22nd, 1975)

SUMMARY

Hydroxyapatite chromatography with small loads carried out with a linear gradient of competing ions has been investigated theoretically on the basis of the classical theory of adsorption chromatography for the case when molecules are adsorbed on to a single type of crystal site. By using this result, the experimental chromatography of lysozyme has been re-examined and the limit of the resolving power of the hydroxyapatite column is discussed.

INTRODUCTION

In our previous papers[1,2], a theory of hydroxyapatite (HA) chromatography with small loads was developed for the case when the elution is carried out with a linear molarity gradient of competing ions. It was shown that the elution molarity of sample molecule can generally be expressed as a function of a parameter s, defined as the product of the column length, L, and the slope, g, of the gradient of competing ions. The experimental results for several proteins are explained reasonably well by this theory and the values of parameters such as the number of crystal sites covered by an adsorbed molecule and the adsorption energy per molecule were estimated through the analysis of experimental data[1,2]. This theory is based on the assumptions of (a) instantaneous equilibrium of the adsorbed phase and solution and (b) no longitudinal diffusion of molecules on the column, as in the classical theory (see below). However, it also involves the further assumption that the width of the band of the moving solute on the column is very small, which means that no information about the shape of the chromatographic peak can be obtained through the theory.

On the other hand, a general differential equation that describes the development of solute on the column on the basis of only assumptions (a) and (b) was given over 35 years ago by Wilson[3]. Subsequently, a theory of adsorption chromatography based on this equation and a slightly modified version[4] was further developed by

De Vault[4], Weiss[5] and others for the case when the elution is carried out with a solvent with a constant composition. Recently, this classical theory was reconsidered by Kawasaki for the case of small loads and it was shown that the general asymmetrical shape of the experimental chromatogram observed in stepwise elution can be explained satisfactorily if a boundary condition for the differential equation given in the classical work is modified (see Appendix).

In this paper, HA chromatography with small loads when the elution is carried out with a linear gradient of competing ions and when molecules are adsorbed on to a single type of crystal site[1] is considered on the basis of the classical equation and the experimental result obtained for lysozyme in earlier work[6] is discussed.

THEORETICAL

It is possible to write the differential equation governing the chromatographic process on the column given by Wilson[3] and modified by De Vault[4], for the case of a single molecular species, as

$$\frac{\partial C}{\partial L} + \left(\frac{\partial C}{\partial V} + \frac{\partial \chi}{\partial V}\right) \cdot \alpha = 0 \tag{1}$$

where χ is the proportion of the effective surface of HA occupied by adsorbed solute, being equal to unity in the saturated state, 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., $\alpha = \delta V/\delta L$; and C is the concentration of solute in solution or mobile phase, as a function of V and L, defined as

$$C = \frac{B}{1 - B} \cdot \chi \tag{2}$$

where B is the ratio of the amount of solute in solution to the total amount in a column section. It should be noted that eqn. 1 is valid even for each component in a mixture when the total load is small, as the density of molecules on the column must be small.

In the case when the elution is carried out with a linear activity gradient of competing ions, it is covenient to rewrite eqn. 1 by using the following parameters:

$$\Lambda = \lambda_2 \cdot e^{\varepsilon_\lambda/kT} \tag{3*}$$

$$G = \alpha \cdot \frac{d\Lambda}{dV} \tag{4}$$

and

$$S = G \cdot L \tag{5}$$

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* In an earlier paper[1], Λ was written as Λ_2.

where λ_2 is the absolute activity of competing ions; $-\varepsilon_2 (\varepsilon_2 > 0)$ is the adsorption energy of a competing ion to one of the adsorption sites of HA; and G is a constant representing the slope (per unit length of the column) of the activity gradient of competing ions. It should be noted that, if the activity of competing ions is proportional to the molarity, m, then Λ can be written by using a proportionality constant φ' (see eqn. 14 in ref. 1) as

$$\Lambda = \varphi' \cdot m \tag{6}$$

and that G and S are related to the parameters g and s in the earlier paper[1] by

$$G = \varphi' \cdot g \tag{7}$$

and

$$S = \varphi' \cdot s \tag{8}$$

Now, eqn. 1 can be rewritten as

$$\frac{\partial C}{\partial S} + \frac{\partial C}{\partial \Lambda} + \frac{\partial \chi}{\partial \Lambda} = 0 \tag{9}$$

The parameter B (see eqn. 2) is related to Λ by

$$\frac{B}{1 - B} = q^{-1} \cdot (\Lambda + 1)^{x'} \tag{10}$$

(see eqn. 1 in ref. 1) where x' is the number of sites of HA on which competing ions cannot be adsorbed owing to the presence of an adsorbed macromolecule and

$$\log q = \frac{x\varepsilon_3}{kT} + \log (\beta_3 \sigma) \tag{11}$$

where x is the number of adsorption groups per macromolecule that can react with sites of HA; $-\varepsilon_3 (\varepsilon_3 > 0)$ is the adsorption energy of an adsorption group of macromolecule to one of the sites of HA; and σ and β_3 are constants related to the symmetry of the molecule and to the properties of the column, respectively. Therefore, eqn. 9 becomes

$$\frac{\partial C}{\partial S} + \left(1 + q \cdot (\Lambda + 1)^{-x'}\right) \cdot \frac{\partial C}{\partial \Lambda} = qx' \cdot (\Lambda + 1)^{-x'-1} \cdot C \tag{12}$$

which is a linear first-order partial differential equation for C as a function of S and Λ. In order to solve eqn. 12, the corresponding characteristic curve

$$dS = \frac{d\Lambda}{1 + q \cdot (\Lambda + 1)^{-x'}} = \frac{dC}{qx' \cdot (\Lambda + 1)^{-x'-1} \cdot C} \tag{13}$$

has to be considered. Integrations of the left- and the right-hand side equalities in eqn. 13 give

$$K_1 = \frac{1}{x'} \cdot \log \{1 + q^{-1} \cdot (\Lambda + 1)^{x'}\} - S \tag{14}$$

and

$$\begin{aligned} K_2 &= C \cdot [1 + q \cdot (\Lambda + 1)^{-x'}] \\ &= \chi \cdot [1 + q^{-1} \cdot (\Lambda + 1)^{x'}] \end{aligned} \tag{15}$$

respectively, where K_1 and K_2 are integration constants and an approximation

$$(\Lambda^n + 1)^{x'} \approx (\Lambda^n + 1)^{x'+1} \tag{16}$$

has been used for the calculation of eqn. 14. Eqn. 16 is a good approximation when Λ or m (see eqn. (6) is small. Now, the general solution for eqn. 12 can be expressed as

$$F\left(\frac{1}{x'} \cdot \log \Omega - S, \chi\Omega\right) = 0 \tag{17}$$

in which

$$\Omega = \frac{\chi + C}{\chi} = 1 + q^{-1} \cdot (\Lambda + 1)^{x'} \tag{18}$$

is the ratio of the total amount of macromolecules to that adsorbed in a column section and F is any function. Eqn. 17 can be rearranged to

$$\chi = \frac{1}{\Omega} \cdot P\left(\frac{1}{x'} \cdot \log \Omega - S\right) \tag{17'}$$

in which P is an arbitrary function.

Now, in order for the function P to be determined, boundary conditions for eqn. 17′ have to be considered. It is evident that the width of the chromatogram is much larger than the column length, both when the slope of the gradient of competing ions is extremely small and when the column is extremely short or, in general, when the value of the parameter S (see eqn. 5) is very small. In this case, the total interstitial volume of the column is much smaller than the total volume of the eluent in which macromolecules are involved, which means that the loss of macromolecules from the column when a solution with a volume the same as that of the column interstices is eluted is virtually equal to the total amount of macromolecules that exist in the interstitial liquid of the column. Therefore, we can write

$$-\left(\frac{dC}{d\,[V/(\alpha L^\circ)]} + \frac{d\chi}{d\,[V/(\alpha L^\circ)]}\right) = C \tag{19}$$

where L° is the length of the column and $V/(\alpha L^\circ)$ is the elution volume measured in such units that the total interstitial volume of the column is equal to unity[*]. Eqn. 19 can be rewritten by using Λ instead of V and by introducing the parameter

$$\delta S = GL^\circ \tag{20}$$

as

$$-\left(\frac{dC}{d\Lambda} + \frac{d\chi}{d\Lambda}\right) = \frac{C}{\delta S} \tag{21}$$

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[*] It should be noted that the physical meaning of eqn. 19 is similar to that for eqn. 1 in ref. 7. As V in eqn. 1 in ref. 7 means $V/(\alpha L^\circ)$ in this paper, eqn. 19 can be rewritten in terms of the symbols in ref. 7, by using eqn. 2, as

$$-\left[\frac{d\left(\frac{B}{1-B} \cdot \chi\right)}{dV} + \frac{d\chi}{dV}\right] = \frac{B}{1-B} \cdot \chi \tag{19a}$$

The integration of eqn. 21, with the approximation (cf., eqn. 16)

$$(A + 1)^{x'-1} \approx (A + 1)^{x'} \tag{22}$$

gives

$$\chi = \chi^* \cdot \Omega^{-\left(1 + \frac{1}{x' \cdot \delta S}\right)} \tag{23}$$

where χ^* is the initial value of χ and for the initial value, Ω^*, of Ω it has been assumed that

$$\Omega^* = 1 + q^{-1} \cdot (A^* + 1)^{x'} \approx 1 + q^{-1} \approx 1 \tag{24}$$

A^* being the initial value of A. Eqn. 24 is a reasonable assumption as the concentration of the competing ions is usually small (or virtually zero) in the initial state, i.e., $A^* \ll 1$ and as, in the case of a "retained" molecule, its adsorption energy must be so large that virtually all molecules are in the adsorbed state in the absence of competing ions, i.e., $q^{-1} \ll 1$ (see eqn. 11 and also ref. 1). It can be tested that eqn. 23 fulfils in the range of the approximations in eqns. 22 and 24 a conservation condition:

$$\int_0^\infty C\,dV = \Omega^* \chi^* \alpha L^0 \tag{25}$$

where it is evident that the right-hand side of eqn. 25 shows the total amount of the sample loaded.

It can be considered that eqn. 23 is a boundary condition that eqn. 17 or 17′ has to satisfy when $S \to 0$. Another condition is that

$$\chi = 0 \tag{26}$$

when $A = A^*$, i.e., $\Omega = \Omega^* = 1$ (see eqn. 24), and when $L > 0^*$ or, more generally, $S > 0$. Under these conditions, eqn. 17′ becomes

$$\left.\begin{array}{ll} \chi = \dfrac{\chi^*}{\Omega} \cdot \exp\left\{-\dfrac{1}{\delta S} \cdot \left(\dfrac{1}{x'} \cdot \log\Omega - S\right)\right\} & \left(S < \dfrac{1}{x'} \cdot \log\Omega\right) \\[3mm] \chi = 0 & \left(\cdot S > \dfrac{1}{x'} \cdot \log\Omega\right) \end{array}\right\} \tag{27}$$

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which, however, is different from eqn. 1 in ref. 7, i.e., the first term of the left-hand side of eqn. 19a is lacking in eqn. 1 in ref. 7. As this term expresses the change in the amount of macromolecules in solution, it can be said that eqn. 1 in ref. 7 and eqn. 19a (or eqn. 19) correspond to the continuity equation proposed by Wilson (eqn. 3 in ref. 3) and that modified by De Vault (eqn. 1 in ref. 4), respectively. However, eqn. 19a reduces to eqn. 1 in ref. 7 when the absolute value of the adsorption energy per macromolecule [i.e., $x\varepsilon_3$ or $x'\xi\varepsilon_3$ (see ref. 8)] is infinity, which is the case in ref. 7. This can be understood by the fact that, when $x'\xi\varepsilon_3 = \infty$, the chromatography is carried out independently of the parameter β_3 (see eqn. 11). which is proportional to the ratio of the total effective surface area of HA crystals to the interstitial volume in the column section (see eqn. 31 in ref. 8). Thus, the chromatography is carried out only due to the decrease in the adsorption capacity (denoted by χ' in refs. 7 and 8) of HA crystals with the increase in A (proportional to y in refs. 7 and 8) and the excess of macromolecules that cannot be adsorbed on to the crystal surfaces is in solution (see refs. 7 and 8), which means that the chromatography depends only on the change in the amount of the adsorbed molecules and that the first term of the left-hand side of eqn. 19a is unnecessary.

* $L > 0$ can be written more precisely as $L > L^\circ$. However, L° should be virtually equal to zero in the case of small loads.

or

$$C = \chi^* \cdot \frac{\Omega - 1}{\Omega} \cdot \exp\left\{ - \frac{1}{\delta S} \cdot \left(\frac{1}{x'} \cdot \log \Omega - S \right) \right\} \quad \left(S < \frac{1}{x'} \cdot \log \Omega \right)$$
$$C = 0 \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \left(S > \frac{1}{x'} \cdot \log \Omega \right) \tag{27'}$$

which gives the theoretical chromatogram (see Figs. 2 and 3). It can be verified that eqn. 27' fulfils another conservation condition:

$$\int_{-\infty}^{\infty} \Omega \chi \alpha dL = \Omega^* \chi^* \alpha L^0 \tag{28}$$

where it should be recalled that the right-hand side of eqn. 28 is the total amount of the sample loaded and $\Omega \chi \alpha$ on the left-hand side shows the total amount of solute per unit length of the column. It should be noted that the integration of eqn. 28 is carried out for a fixed value of V, and therefore of Λ (see eqn. 4), i.e., Ω (see eqn. 18), and that the range of the integration has been taken as $(-\infty, \infty)$ instead of $(0, \infty)$ because, in the latter calculation, the amount of solute that is still in the top column section L° is not integrated.

As eqn. 27' is a discontinuity solution to eqn. 12, it is important to discuss the relationship between the S and Λ values, $\dot{S}$ and $\dot{\Lambda}$, at which there is the discontinuity of the C value. This relationship is given by

$$\dot{S} = \frac{1}{x'} \cdot \log \dot{\Omega} \tag{29}$$

(see eqn. 27'), where

$$\dot{\Omega} = 1 + q^{-1} \cdot (\dot{\Lambda} + 1)^{x'} \tag{30}$$

If $q^{-1} \cdot (\dot{\Lambda} + 1)^{x'}$ is small, eqn. 29 reduces to

$$\dot{S} = \frac{1}{qx'} (\dot{\Lambda} + 1)^{x'} \tag{31}$$

Further, if the elution molarity, m_{elu}, is defined as the molarity of competing ions at which the discontinuous part of the chromatographic peak, i.e., the sharp leading boundary of it (see Figs. 2 and 3) appears, we have

$$\dot{\Lambda} = \varphi' m_{elu} \tag{32}$$

(see eqn. 6) and, writing the s value (see eqn. 8) that corresponds to the $\dot{S}$ value simply as s, eqn. 16 in the earlier paper[1] can be obtained.

RELATION TO EXPERIMENTS

The points in Fig. 1 are a reproduction of Fig. 6 in an earlier paper[6], namely, the experimental plots of the reduced standard deviation (to column diameter = 1 cm),
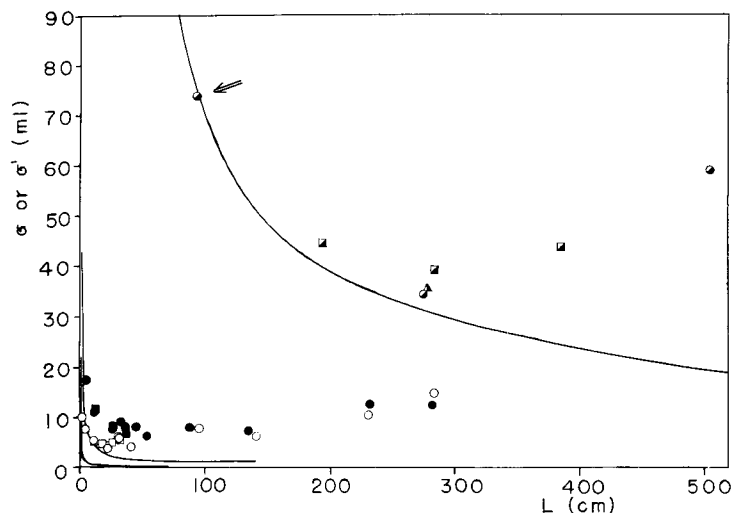
Fig. 1. Points reproduced from Fig. 6 in ref. 6, *i.e.*, the experimental plots of the reduced standard deviation (to column diameter $= 1$ cm), σ (ml), of the chromatographic peak of lysozyme as a function of the column length, L (cm), for three different values of the slope, $g_{(K^+)}$, of the molarity gradient of competing ions, *i.e.*, potassium ions in the buffer. Values of $g_{(K^+)}$: $\bigcirc$ and $\square$, 1.2–$1.4 \cdot 10^{-3}$ M/cm; $\bullet$ and $\blacksquare$, 4.0–$4.9 \cdot 10^{-4}$ M/cm; $\blacktriangle$, $\mathbb{O}$ and $\blacksquare$, 3.5–$3.8 \cdot 10^{-5}$ M/cm. The point indicated by an arrow corresponds to the experimental chromatogram in Fig. 2. The three curves are theoretical curves calculated through eqn. 27' assuming that $\delta S = 0.005$, corresponding to three experimental values of $g_{(K^+)}$. To simplify the calculation, the half-width, σ', of the chromatographic peak at half of the maximum height has been considered instead of σ. These two values must be approximately equal.

σ(ml), of the chromatographic peak of lysozyme with small loads as a function of the column length, L (cm), for three different values of the slope of the molarity gradient of competing ions. The curves in Fig. 1 are the theoretical curves that correspond to the three slopes of the gradient calculated through eqn. 27'. In order to simplify the calculation, the half-width, σ', of the chromatographic peak at half of the maximum height has been considered instead of σ. These two values must be approximately equal. For the calculation of the curves, it was taken into account that lysozyme is adsorbed only on to P crystal sites, that it competes only with potassium ions in the phosphate buffer, that $x' = 10$, that $\log q = 6.4$, that $\varphi' = 5.0$ and that the ratio of the interstitial volume of the column section to the total packed crystal volume is 0.8 (see refs. 1 and 9), and $\delta S = 0.005$ (see eqn. 27') was assumed in order to have a best fit with the experiment (see Discussion). It can be seen in Fig. 1 that the theory explains fairly well the facts that, when the slope of the gradient is steep, σ decreases rapidly in the range of short column lengths, that the decrease stops at short column lengths and that the decrease in σ is less rapid when the gradient is small. The increase in σ that takes place after having first decreased remains unexplained, however. It may be due, at least partially, to the longitudinal thermodynamic diffusion of macromolecules on the column (which is not taken into account in the theory). As however, the shape of the experimental chromatogram is virtually independent of the flow-rate, the increase in σ may mainly be due to the heterogeneity in the flow-rate in different parts of a column section (see Discussion).

The solid and the broken lines in Fig. 2 are the experimental chromatogram corresponding to the point indicated by an arrow in Fig. 1 and a theoretical chromatogram calculated through eqn. 27′ as a function of the molarity of potassium ions in the buffer, respectively. For the calculation of the theoretical curve, it was assumed that $\delta S = 0.005$, $\varphi' = 5.0$, $x' = 10$, $\log q = 6.4$ and $\dot{S}/\varphi' = gL = 3.818 \cdot 10^{-3}$, where $3.818 \cdot 10^{-3}$ is the value for the experimental point indicated by the arrow in Fig. 1.
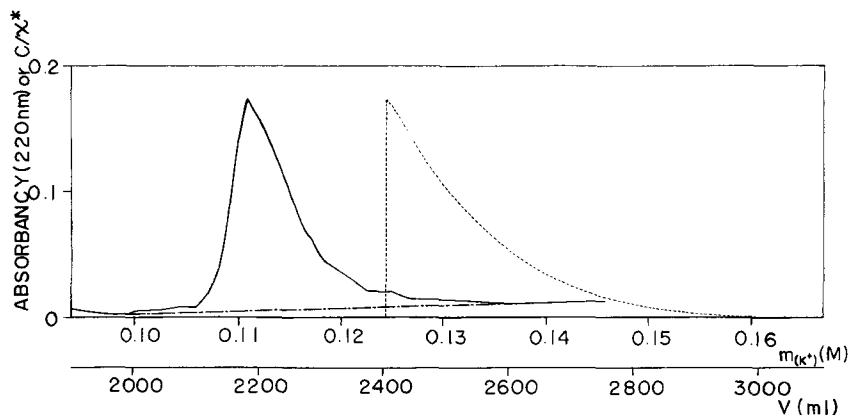

Fig. 2. Plots of the experimental chromatogram corresponding to the point indicated by an arrow in Fig. 1 (solid line) and the theoretical chromatogram (broken line) calculated through eqn. 27′ as a function of the molarity of potassium ions. The actual reduced elution volume (to column diameter $= 1$ cm) for the experimental chromatogram is also shown on the abscissa. The value of σ in Fig. 1 for the experimental chromatogram has been calculated assuming that the base line is represented by the line $-\cdot-\cdot-\cdot$.

The theoretical chromatogram is drawn so that the maximum height would be about equal to that for the experimental peak. It can be seen in Fig. 2 that the shape of the experimental chromatogram, with considerable tailing, is fairly well explained by the theory. However, the leading boundary of the experimental chromatogram is less sharp than in the theoretical case, which may also be due mainly to heterogeneity in the flow-rate and slightly to the longitudinal thermodynamic diffusion of molecules (see Discussion). It can also be seen in Fig. 2 that the elution of the theoretical peak is slightly delayed, which can be considered to be due to the approximation of eqn. 16. In fact, the mean part of the theoretical chromatogram has to be eluted at 0.11 M
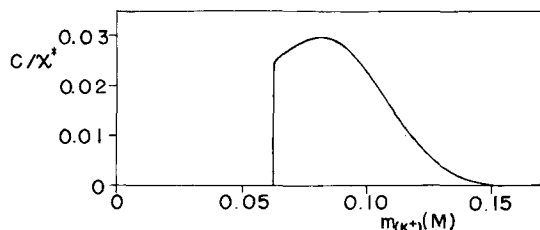

Fig. 3. Example of the calculation of the theoretical chromatogram through eqn. 27′ when $\dot{S}/\varphi'$ is very small.

according to a more precise approximation (eqn. 15 in ref. 1; see also Discussion). It should be noted, however, that the difference in the elution molarities between the experimental and the theoretical chromatograms is in the range of the experimental error (see Fig. 2 in ref. 1).

Fig. 3 shows another example of the theoretical chromatogram when the value of $\dot{S}/\varphi'$ is very small ($\dot{S}/\varphi' = 5 \cdot 10^{-4}$). In this case, the chromatogram has a maximum height at its centre.

DISCUSSION

As mentioned in Relation to experiments, the theory does not explain why the width, σ, of the experimental chromatogram increases after having first decreased with the increase in the column length, L (see Fig. 1). It should be recalled, however, that the theory developed in ref. 1 explains reasonably well the experimental relationship between the elution molarity, m_{elu}, and the parameter s for all points in Fig. 1 (see Fig. 2 in ref. 1). This theory involves the assumption that the width of the chromatogram is negligibly small (see Introduction), but it describes the relationship between m_{elu} and s more precisely (i.e., without using eqn. 16) than the present theory. As the other assumptions involved in the theory in ref. 1 (instantaneous equilibrium and no longitudinal diffusion, see Introduction) are also the basis of the present theory, there must actually exist a factor that has not been taken into account in the present theory and that concerns only the shape or the width of the chromatographic peak. As the shape of the actual chromatogram when the column is long is generally rather symmetrical[10], this factor must broaden the chromatographic peak symmetrically. The possibility that the broadening of σ is due mainly to the longitudinal thermodynamic diffusion of molecules can be discounted as the shape of the chromatographic peak is virtually independent of the flow-rate. It can be suggested that the increase in σ is due mainly to the heterogeneity in the flow-rate in different parts of the column section, as the preparation of HA crystals is usually very heterogeneous (see Figs. A1 and A2 in Appendix II in ref. 11) and it is difficult to pack the crystals homogeneously enough in the column. According to this hypothesis, the fit between theory and experiment must be satisfactory when the column is short. The good fit may be obtained also because of the increase in the precision of the approximation of eqn. 16 when the column is short, as molecules are eluted at a small value of m (see Fig. 2 in ref. 1), i.e. Λ (see eqn. 6) when s is small or when the column is short*.

Let us recall again that one of the basic assumptions of the theory is that the longitudinal diffusion of macromolecules is negligible. Although this assumption seems valid as the shape of the chromatographic peak is hardly influenced by a change in the flow-rate, it has no validity if the interior of a thin enough section of the column is considered. This means that the fundamental equation, eqn. 1 or

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* As eqn. 16 does not give a good approximation when the column is long, one cannot eliminate the possibility that the theoretical value σ' does not increase with an increase in L because of the rough approximation of eqn. 16. Because, however, the activity of competing ions is generally higher in the rear part of the chromatographic peak than in the front part, it is impossible that the R_F value (see ref. 1) in the front part of the peak is larger than the value in the rear part. This means that an increase in σ' with increase in L is impossible.

eqn. 9, has no validity in the interior of the thin column section. It is evidently impossible for a thin column section to be divided into a large number of sub-sections through which the transport of molecules is carried out smoothly, as the movement of molecules in the thin section must essentially be random, caused primarily by molecular diffusion, even though molecules are transported, on average, in the direction of the flow. Similarly, if the slope of the gradient of competing ions is small, *i.e.*, the difference in concentration of the ions at the top and the bottom of the column is always small enough, then the concentration of macromolecules must essentially be independent of the position on the column. In this case, if there is a slight difference in molecular concentrations between the top and the bottom of the column, provided there is no molecular diffusion, there must be no difference in molecular concentration because of molecular diffusion. It should be emphasized, however, that the assumption of no longitudinal diffusion is still valid when the width of the chromatographic peak is large, as the effect of molecular diffusion is offset in the interior of the chromatographic peak. Finally, it should be pointed out that another basic assumption, *i.e.*, equilibrium between the adsorbed phase and solution, is reasonable, again because of the lack of dependence of the shape of the chromatogram on the flow-rate, but that the equilibrium is possible only as a result of molecular diffusion.

Let us consider chromatography with small loads. In this case, the width $L°$ (see eqn. 20) of the initial band of macromolecules on the top of the column must be very small, so the movement of molecules in the interior of the top section, $L°$, must essentially be random. Further, the value of $L°$ must be independent of the load when it is small enough, as it is almost impossible to decrease $L°$ to a value less than some minimal value because of molecular diffusion (perhaps occurring mainly in the loading process). Fig. 4 in ref. 6 gives experimental support to this statement, as it shows that the width, σ, of the experimental chromatogram approaches a finite value when the load tends to zero. It is evident that the width of the chromatogram is determined by $L°$ and that the width decreases, in general, with a decrease in $L°$*. It is also evident that the effect of the interaction between macromolecules is negligible for a small enough load. On the other hand, if G is smaller than some minimal value, the desorption of molecules in the interior of the initial band must be carried out independent of the position on the band, also because of molecular diffusion; it has already been pointed out that the concentration of macromolecules must be independent of the position on the column if G is small enough. Hence the reason why the parameter δS (see eqn. 20) is a finite constant for a small enough load can be understood (see Fig. 1). It can be said that δS is a parameter that indicates the limit of the resolving power of the column.

APPENDIX

In connection with the theory developed in the text, it is of interest to reconsider

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* It should be noted that the heterogeneity in the flow-rate in different parts of the column section gives the same effect as the longitudinal thermodynamic diffusion of molecules. It is perhaps this type of "diffusion" rather than the thermodynamic diffusion that determines mainly the value of $L°$, as the width of the experimental chromatogram is virtually independent of the flow-rate or the time used for the chromatography.

the classical Wilson, De Vault and Weiss theory[3-5] (which can be applied to general adsorption chromatography when the elution is carried out with a solvent with a constant composition) for the case of small loads. We shall show that a boundary condition for the fundamental equation, *i.e.*, eqn. 1 or eqn. A1 given by these authors, cannot be applied to the case of small loads and that, if it is modified, an asymmetrical shape of the chromatogram with a sharp leading boundary and considerable tailing is obtained, independent of the type of adsorption isotherm for the solute, as only the initial slope of this isotherm is of importance in the case of small loads.

The fundamental equation (eqn. 1) can be written for convenience as

$$\frac{\partial C}{\partial L} + \alpha \cdot \frac{\partial C}{\partial V} + \frac{\partial Q}{\partial V} = 0 \tag{A1}$$

in which C is now the amount of solute per unit volume of solution and Q is the amount of solute adsorbed per unit length of the column. Writing the adsorption isotherm of a solute on the adsorbent as $I(C)$, Q can be expressed as

$$Q = MI(C) \tag{A2}$$

where M is a positive constant. Eqn. A1 can be rewritten as

$$\frac{\partial C}{\partial L} + [\alpha + MI'(C)] \cdot \frac{\partial C}{\partial V} = 0 \tag{A3}$$

where I' is the first derivative of I. The general solution for eqn. A3 when the composition of the solvent is constant is given by

$$C = \Phi\{V - L \cdot [\alpha + MI'(C)]\} \tag{A4}$$

in which Φ is an arbitrary function.

Now, let us assume that a very small amount of the sample solution is loaded on the column and that a band with a small width, δL, of adsorbed molecules is formed at the top of the column. It can be considered that the movement of molecules in the interior of the band δL is random in any chromatographic process, as δL is small (see text), and that the amount, $\delta\omega$, of molecules on the top of the column decreases according to the equation

$$-\frac{d(\delta\omega)}{dV} = C \tag{A5}$$

As

$$\delta\omega = C\alpha\delta L + Q\delta L = [\alpha C + MI(C)]\delta L$$

eqn. A5 can easily be integrated when

$$I(C) = \zeta C \tag{A6}$$

where ζ is a positive constant and

$$C = C^* \exp\left[-\frac{1}{(\alpha + M\zeta) \cdot \delta L} \cdot V\right] \tag{A7}$$

is obtained, in which C^* is the initial concentration of solute in solution. The assumption of a linear adsorption isotherm (eqn. A6) is reasonable in the case of small loads because the density of the initial band must be small owing to molecular diffusion. It is also reasonable to consider that δL has a finite value virtually independent of load if it is small enough (see text)*. It can be tested that eqn. A7 fulfils a conservation condition

$$\int_0^\infty C\mathrm{d}V = \delta\omega^*$$

in which

$$\delta\omega^* = [\alpha C^* + MI(C^*)]\cdot\delta L$$

is the total amount of the sample loaded.

Eqn. A7 can be considered as a boundary condition that eqn. A4 has to satisfy when $L = 0$ and $V > 0$. Another condition is that

$$C = 0 \tag{A8}$$

when $V = 0$ and $L > 0$. Under these conditions, eqn. A4 becomes

$$\left.\begin{array}{ll} C = C^* \exp\left\{-\dfrac{1}{(\alpha + M\zeta)\cdot\delta L}\,[V - (\alpha + M\zeta)\cdot L]\right\} [V > (\alpha + M\zeta)\cdot L] \\ C = 0 \hspace{4cm} [V < (\alpha + M\zeta)\cdot L] \end{array}\right\} \tag{A9}$$

where it should be noted that eqn. A9 has been derived following a principle such that the initial step of the development is a spreading of the initial band (see eqn. A5) and, therefore, that the continuity equation (eqn. A1 or A3) can be applied immediately after the spreading has begun, as eqn. A1 is valid only when the band of molecules is broad (see text)**. It can be verified that eqn. A9 fulfils another conservation condition:

$$\int_{-\infty}^\infty (\alpha + M\zeta)\cdot C\mathrm{d}L = \delta\omega^*$$

in which $(\alpha + M\zeta)\cdot C$ is the total amount of solute per unit length of the column and the integration is carried out for a fixed value of V. The range of the integration has been taken as $(-\infty, \infty)$ instead of $(0, \infty)$ because, in the latter calculation, the amount of solute that is still in the top section of the column, δL, is not integrated. Fig. A1 is the plot of C versus V, which gives the theoretical chromatogram available for a column of length L and explains reasonably well the general shape of the experimental chromatogram.

The only difference between the present and the classical theory concerns a boundary condition for eqn. A4. In the classical theory, a boundary condition such

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* The value of δL must also be independent of the flow-rate, as the shape or the width of the experimental chromatogram is independent of the flow-rate (cf. footnote on p. 280).
** It should be noted that the situation is the same if a column with the moving band of solute with a large width is connected to the top of the column under consideration, in place of the initial band.
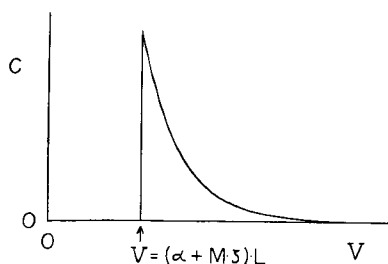
Fig. A1. Representation (in arbitrary units) of a theoretical chromatogram for a column of length L in the case of small loads. The chromatogram moves on the abscissa with a change in the L value. The shape of the chromatogram, however, is independent of L.

that $C = 0$ has been applied instead of eqn. A7 when $L = 0$ and $V > 0$. It is important to see that the classical boundary condition is valid only if eqn. A1 or A3 can be applied in the interior of the initial band, i.e., if the section δL can be divided into a large number of sub-sections through which the transport of solute is carried out smoothly, which, however, is impossible if the value of δL is small enough (see text).

According to the classical theory, if the adsorption isotherm of the solute is linear and if the elution is carried out by using the same buffer as the solvent of the sample solution, the shape of the chromatogram has to be a rectangle with the same width as the initial band. In ref. 3, it is also mentioned that a rectanglar chromatogram with the same width as the initial band has to be realized even when the elution is carried out by using a new solvent. This statement is not true, as point 1 of eqn. 10 in ref. 3 is not correct when a new solvent is used. It is evident that point 1 of eqn. 10 in ref. 3 cannot express a state such that the solvent in the upper half of the initial band is new and that in the lower half it is old, while in the case of infinitesimal loads, the shape of the chromatogram does not depend on whether the buffer is changed or not for the elution.

Finally, it should be added that there are several kinetic theories that explain the spreading of the molecular zone on the column or the tailing of the chromatogram in the case of a linear adsorption isotherm[12,13]. These theories cannot be applied, however, at least to HA chromatography in which the shape of the chromatogram is virtually independent of the flow-rate.

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