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

I. THE CASE WHEN VIRTUALLY ALL MOLECULES ARE ADSORBED ON TO A SINGLE TYPE OF CRYSTAL SITE THROUGH A SINGLE TYPE OF ADSORPTION GROUP

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SUMMARY

Assuming that macromolecules are adsorbed on to a single type of hydroxyapatite site through a single type of adsorption group, the relationship between the elution molarity and an experimental parameter that involves the column length and the slope of the gradient of competing ions has been investigated theoretically for the case when the linear molarity gradient is applied and when the amount of macromolecules loaded is small. By using this relationship, the chromatography of several proteins has been examined, and in the Appendix, parameters such as x and ξ for these proteins are roughly evaluated. The microheterogeneous model of tropocollagen proposed in earlier papers is also discussed.

INTRODUCTION

In earlier papers[1–6], the theory of the chromatography of rigid rod-like macromolecules has been developed. In this theory, the mutual interactions among adsorbed macromolecules were taken into consideration, but the assumption that the value of x', the number of sites on hydroxyapatite (HA) where competing ions cannot be adsorbed owing to the presence of an adsorbed macromolecule, is infinity has been involved. If the density of molecules on the crystal surfaces is small, the effect of the molecular interactions is negligible. A theory that is valid only when there are no molecular interactions but can be applied to molecules of any shape and dimensions has also been developed (see Appendix II in ref. 1). If the load of molecules is very small and if the width of the zone of molecules that have been initially adsorbed on the column is very small, the state of small molecular density will be realized during the chromatography (see below). In this paper, the relationship between the elution molarity, m_{elu}, and an experimental parameter, s, which involves the column length and the slope of the gradient of competing ions, is investigated theoretically for the case when a linear molarity gradient is applied and when the amount of macromolecules loaded is very small. Using this theory, we discuss the experimental

results obtained for several proteins in earlier work[7]. It should be noted that this theory, and also the theory developed earlier[1–6], are based on the assumption that molecules are adsorbed on to only a single type of adsorption site and that they are eluted by only a single type of competing ions. Bernardi *et al.*[8], however, showed that there are two different sites on the surface of HA crystals, the first (C sites) appearing to be responsible for the binding of acidic groups, carboxyl and phosphate, and the second (P sites) for the binding of basic groups and cations. It was also shown that acidic and neutral proteins appear to be eluted mainly by phosphate ions in the buffer and basic proteins by cations, which suggests that the former molecules are adsorbed mainly on to C sites through carboxyl groups and the latter on to P sites through basic groups (see also Appendix I in ref. 3). In the following paper[9], a theory in which account is taken of the existences of two different adsorption sites on the crystal surfaces is developed and the experimental results obtained earlier[7] are again discussed.

THEORETICAL

Let us consider the chromatography of a very small amount of molecules. A zone of adsorbed molecules with a very small width will be formed at the top of the column when the loading has been completed and will usually remain at the same position during the rinsing process which continues after the loading[1,10]. It is evident that the total amount of molecules in any column section is less than or equal to the maximum amount that can be adsorbed on the crystal surfaces of the section. This condition applies during the whole of the chromatography if the amount of molecules loaded is sufficiently small, and hence eqn. 39 in ref. 1 can be used for the expression of the experimental parameter B. In the case of gradient elution, the density of molecules on the crystal surfaces will decrease gradually with the movement of the molecular zone. If the amount of molecules loaded is very small and if the ratio of the width of the initial molecular zone to the total column length is very small, then the density will remain very small throughout the column during the chromatographic process and the effect of the mutual interactions among adsorbed molecules will therefore be negligible. Hence eqn. A32 in Appendix II in ref. 1 can be used for the expression of the parameter B. This equation can be written, with slight modifications, as

$$B = \frac{1}{1+q(\Lambda_2+1)^{-x'}} \tag{1}$$

where

$$\log q = \frac{x\varepsilon_3}{kT} + \log(\beta_3\sigma) \tag{2}$$

and

$$\Lambda_2 = \lambda_2 \, e^{\varepsilon_2/kT} \tag{3}^*$$

* For the definitions of the symbols, see ref. 1.

It should be recalled that the physical meaning of B is the ratio of the number of molecules in the mobile phase to the total number of molecules in a column section; x is the number of adsorption groups per macromolecule that can react with sites of HA; $-\varepsilon_2$ and $-\varepsilon_3$ ($\varepsilon_2, \varepsilon_3 > 0$) are the adsorption energies of a competing ion and of an adsorption group of a macromolecule to one of the adsorption sites of HA, respectively; λ_2 is the absolute activity of competing ions; and σ and β_3 are constants related to the symmetry of the molecule and to the properties of the column, respectively. It can be seen from eqns. 1–3 that B is independent of the concentration of macromolecules in the interstice of the column but depends uniquely on the activity of the competing ions. Hence, in the chromatography of a mixture of several components, each component is eluted independently. In Fig. 1, the elution

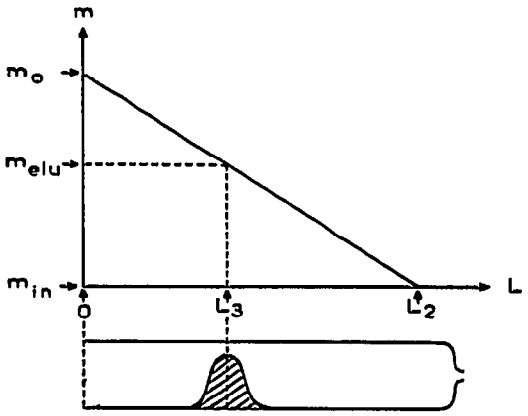

Fig. 1. Schematic representation of the development of macromolecules on the column when a linear molarity gradient is applied.

of macromolecules when the linear molarity gradient is applied is shown schematically; the abscissa L is the distance from the top of the column and the ordinate m is the molarity of the competing ions, and L_3 and L_2 are the distances from the top of the column of the mean part of the molecular zone and of the beginning of the molarity gradient, respectively. The fact that the concentration of the competing ions is not influenced by the interaction of the ions with the adsorbent means that the velocity of the beginning of the gradient is almost equal to the velocity of the solvent, provided that the ions are not adsorbed. Therefore, writing

$$R_F = \frac{dL_3}{dL_2} \tag{4}$$

R_F varies from 0 to 1 and the physical meaning of R_F is ratio between the actual mobility of the mean part of the molecular zone and the mobility of the mean part of the molecular zone realized provided that molecules are not adsorbed. We can consider that R_F is equal to B at the mean part of the molecular zone, which is evident from the physical meaning of this parameter. It should be noted that the velocity of the mean part of the molecular zone is equal to the mean velocity of the

macromolecules, because B is independent of the concentration of the molecules. We now have

$$R_F = B \tag{5}$$

From eqns. 1, 4 and 5

$$\frac{dL_2}{dL_3} = 1 + q\{A_2(m_{elu}) + 1\}^{-x'} \tag{6}$$

is obtained, where m_{elu} is the value of m when $L = L_3$ and can be defined as the elution molarity of macromolecules for a column of length L_3 (see Fig. 1). If we denote by m_0 the value of the molarity of competing ions at the top of the column and by m_{in} the same value in the initial buffer, the relationship

$$\frac{m_{elu} - m_{in}}{m_0 - m_{in}} = \frac{L_2 - L_3}{L_2} \tag{7}$$

is obtained (see Fig. 1). It is also evident that $m_0 - m_{in}$ is proportional to L_2, *i.e.*

$$m_0 - m_{in} = gL_2 \tag{8}$$

where g is a constant representing the slope of the gradient of competing ions. The value of m_{in} is virtually zero in most experiments. Using eqns. 7 and 8 and introducing the parameter

$$s = gL_3 \tag{9}$$

we have

$$\frac{dL_2}{dL_3} = \frac{dm_{elu}}{ds} + 1 \tag{10}$$

and from eqns. 6 and 10 the equation

$$\frac{dm_{elu}}{ds} = q\{A_2(m_{elu}) + 1\}^{-x'} \tag{11}$$

is obtained. In the case of "retained" molecules[10], it can be assumed that $m_{elu} = m_{in}$ when $s = 0$, as the initial molecular zone with an infinitesimal width remains at the top of the column before the gradient begins. This assumption is generally valid provided that the gradient begins as soon as the loading is completed. It follows from this assumption that

$$s = \frac{1}{q} \int_{m_{in}}^{m_{elu}} \{A_2(m) + 1\}^{x'} dm \tag{12}$$

As A_2 is, by definition (see eqn. 3), proportional to the activity of competing ions and as the activity can be written, using the activity coefficient γ, as γm, A_2 can be expressed, introducing a proportionality constant φ, as

$$A_2 = \varphi\gamma m \tag{13}$$

If the activity is proportional to the molarity, Λ_2 can be rewritten by using a new constant, φ', as

$$\Lambda_2 = \varphi' m \tag{14}$$

In this case, eqn. 12 becomes

$$s = \frac{1}{q} \cdot \frac{1}{(x'+1)\varphi'} \cdot \{(\varphi' m_{elu} + 1)^{x'+1} - (\varphi' m_{in} + 1)^{x'+1}\} \tag{15}$$

If $x' \gg 1$ and $(\varphi' m_{elu} + 1)^{x'+1} \gg (\varphi' m_{in} + 1)^{x'+1}$, eqn. 15 reduces to

$$s = \frac{1}{qx'\varphi'} \cdot (\varphi' m_{elu} + 1)^{x'} \tag{16}$$

Furthermore, if $\varphi' m_{elu}$ is small, but large enough for the relationship $(\varphi' m_{elu} + 1)^{x'} \gg (\varphi' m_{in} + 1)^{x'}$ to hold, then we have $\varphi' m_{elu} + 1 \approx e^{\varphi' m_{elu}}$ and eqn. 16 reduces further to

$$s = \frac{1}{qx'\varphi'} \cdot e^{x'\varphi' m_{elu}} \tag{17}$$

or

$$\log s = x'\varphi' m_{elu} - \log q - \log(x'\varphi') \tag{17a}$$

It should be noted that eqn. 12 can be rewritten as

$$\log s + \log q = \log \int_{m_{in}}^{m_{elu}} \{\Lambda_2(m) + 1\}^{x'} dm \tag{12a}$$

If the molecules are very large, both x' and x are also very large, so that the left-hand side of eqn. 12a is almost equal to log q (see eqn. 2) and m_{elu} is almost independent of s, *i.e.*, of the column length and the slope of the gradient.

ANALYSIS OF SEVERAL EXPERIMENTS

In the preceding section, the relationship between two experimental parameters, m_{elu} and s, was investigated theoretically and involves q, x' and φ (or φ') as constants, the first two concerning the properties of the macromolecule. In this section, these constants are evaluated for several proteins, namely lysozyme, cytochrome *c*, tropocollagen and β-lactoglobulin A by using the experimental data published in an earlier paper[7]. For lysozyme and tropocollagen, some other data are also used. In Figs. 1 and 2 in ref. 7, the elution molarity, m_{elu}, is plotted as a function of the column length and of the slope of the gradient. In the case of T_2 phage (see Fig. 1E in ref. 7), m_{elu} is independent of both the column length and the slope of the gradient, so that both x' and x are extremely large (see the preceding section). In this case, it is impossible to know the exact values of the constants. In Figs. 1 and 2 in ref. 7, m_{elu} is defined as the molarity of phosphate ions at which the centre of gravity of the protein peak is eluted. However, in this paper, we define m_{elu} of tropocollagen as the molarity at which the maximum height of the chromatogram is eluted (see below).

Lysozyme

As lysozyme is a basic protein (isoelectric point = 10.5–11.0, see ref. 11), we assume that the elution is caused only by potassium ions in the buffer. This assumption can be considered to be correct at least as a first approximation from the experimental point of view[8], and means that molecules are adsorbed on to P sites through basic groups (see Introduction). The validity of this assumption is considered again in the following paper[9]. The points in Fig. 2 are experimental plots of log $s_{(K^+)}$ *versus* $m_{elu(K^+)}$, where the subscript (K^+) indicates that these values concern potassium ions. All points except ▽ correspond to points in Fig. 2A in ref. 7. In Fig. 2a, the ratio

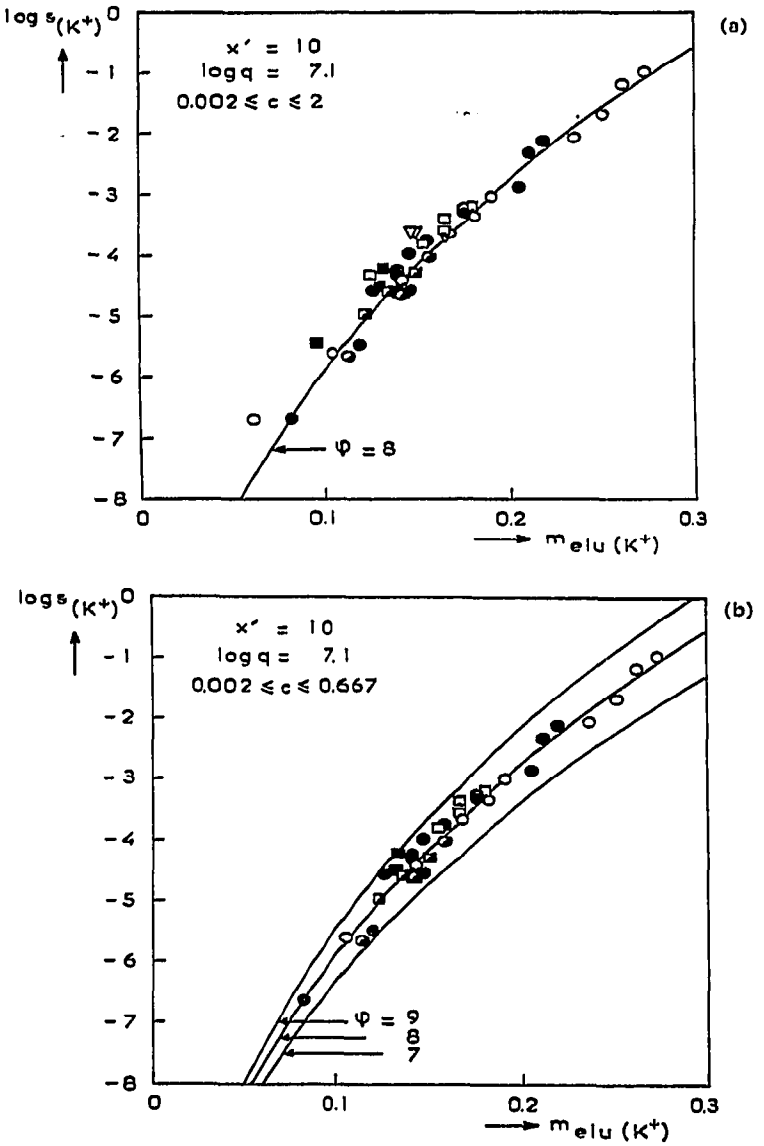

Fig. 2.

(henceforth called c) of the amount of the sample loaded (mg) to the column length (cm) is between 0.002 and 2. In Fig. 2b and 2c, the values of this parameter are between 0.002 and 0.667 and between 0.002 and 0.095, respectively. The curves in Fig. 2a and 2c (these are theoretical curves, see below) are the same as the intermediate curve in Fig. 2b. It can be seen that when c is less than 0.667, the experimental points are distributed about equally on both sides of the curves. When c increases, the points are distributed mainly on the left-hand side of the curve (see Fig. 2a), which means that when c is less than 0.667, the experimental points can be explained by assuming that an infinitesimal amount of molecules is loaded. In Fig. 2b, we

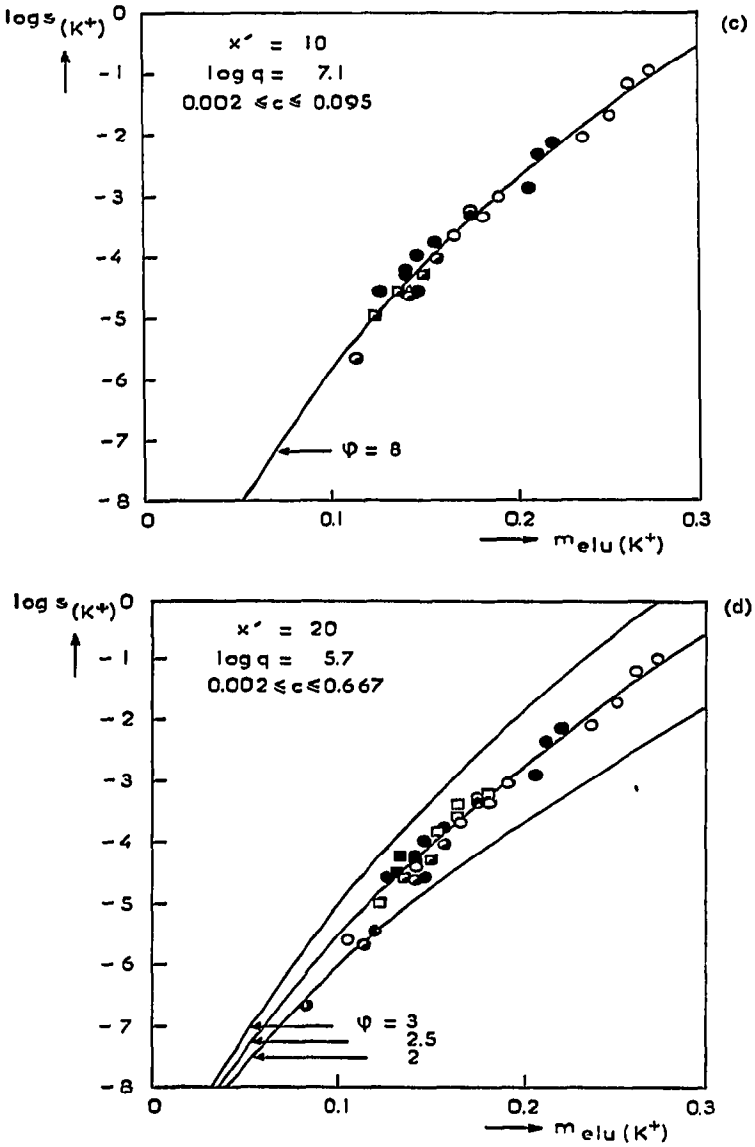

Fig. 2.

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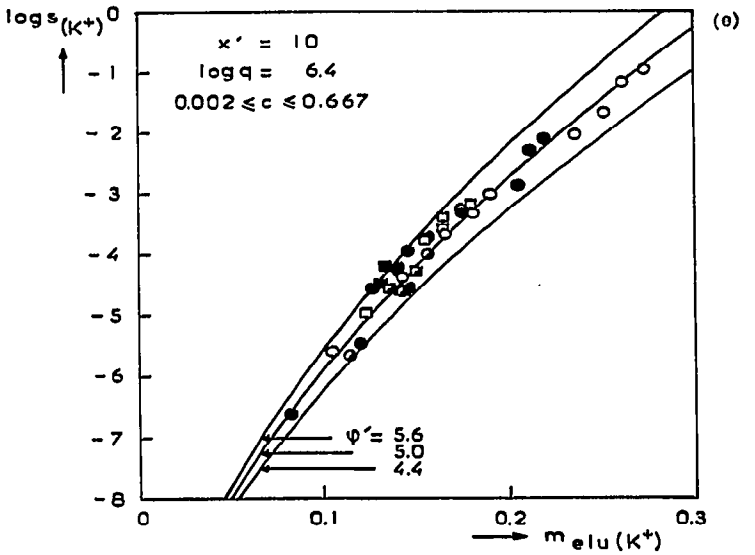

Fig. 2. (a) Plots of the experimental and theoretical results of the chromatography of lysozyme on to the (log $s_{(K^+)}$, $m_{elu(K^+)}$) plane. The symbols correspond to those in Fig. 2A in ref. 7, except for ▽. The values of the parameter c of any experimental point are between 0.002 and 2. The theoretical curve is the same as the intermediate one in (b). (b) As (a), $0.002 \leqslant c \leqslant 0.667$. Theoretical curves were calculated taking into account the dependence of the activity coefficient of potassium ions on their concentration. The intermediate curve calculated assuming that $x' = 10$, $\log q = 7.1$ and that $\varphi = 8$ shows a best fit with the experimental results. (c) As (a), $0.002 \leqslant c \leqslant 0.095$. (d) As (b), $x' = 20$. A good fit between the experimental and theoretical results cannot be obtained because a too high value was chosen for x'. (e) As (b), proportionality between the activity and the molarity of potassium ions has been assumed. It can be seen that the value of $\log q$ that gives the best fit between the experimental and theoretical results, *i.e.*, 6.4, is close to the value obtained by taking into account the effect of the concentration dependence of the activity coefficient of K^+, *i.e.*, 7.1 (see (b) and text).

have drawn three theoretical curves assuming that $x' = 10$ and using eqns. 12 and 13. The activity coefficient, γ, of potassium ions has been estimated by assuming that it is equal to the mean activity coefficient of potassium chloride, which could be expressed approximately as

$$\gamma \approx e^{-0.75\sqrt{m}} \tag{18}$$

when the concentration of potassium chloride is less than 0.3 M (ref. 12). The parameter g (see eqn. 8) has been estimated by using a value of 0.8 for the ratio of the interstitial volume of the column section to the total packed crystal volume[13]. It is evident from eqn. 12 that the theoretical curve moves vertically when $\log q$ changes. It is also evident that when φ increases, the slope of the curve increases. It can been seen from Fig. 2b that the best fit is obtained when $\log q = 7.1$ and when $\varphi = 8$. Good fits are also obtained between experiment and the theory if $5 < x' < 15$. The corresponding values of $\log q$ and φ are 12.7 and 90, respectively, when $x' = 5$, and 6.4 and 4, respectively, when $x' = 15$. If we assume that $x' = 20$, the theoretical curves are all less curved than the experimental result (Fig. 2d), while if x' is too small, the situation is reversed. In Fig. 2e, we have drawn theoretical curves assuming that $x' = 10$ and that the

activity of potassium ions is proportional to the molarity, and using eqn. 16. In order to obtain the best fit with the experimental results, log $q=6.4$ and $\varphi'=5$ should be chosen. It should be noted that the value of log q thus obtained is close to the value obtained by taking into account the dependence of the activity coefficient on the molarity, *i.e.*, 7.1.

In Appendix II in ref. 3, it was mentioned that cytochrome *c*, which is a basic protein like lysozyme, is adsorbed on the flat surfaces of the blade-like crystals of HA and that the adsorbing P sites are probably arranged hexagonally on the surface with a minimal distance of 9.432 Å (see also Appendix I). Fig. 3a shows schematically a molecule of lysozyme adsorbed on the flat crystal surface. In order to simplify the figure, it has been assumed that lysozyme is represented by a prolate spheroid of dimensions $45\times30\times30$ Å (ref. 14). It has been also assumed that the adsorption is such that the maximum molecular surface is brought into contact with the crystal, as this should be energetically favoured. It can be seen in Fig. 3a that the value of x' is about 10, which is in good agreement with the value $5<x'<15$ estimated above.

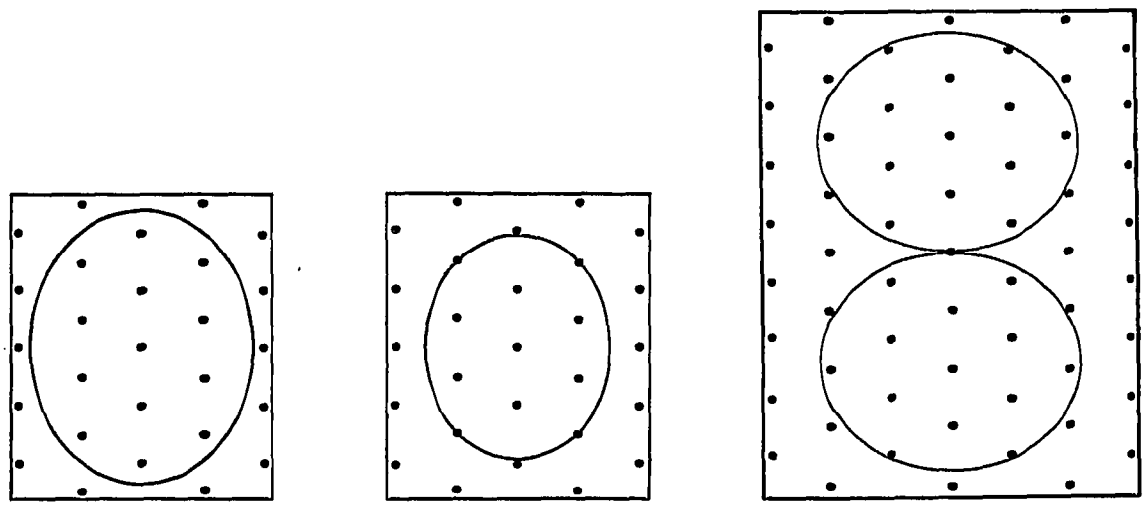

Fig. 3. (a) Schematic representation of the molecule of lysozyme adsorbed on P sites of HA. It has been assumed that lysozyme is represented by a prolate spheroid of dimensions $45\times30\times30$ Å and that crystal P sites are arranged hexagonally with a minimal interval of 9.432 Å. (b) As (a), in the case of cytochrome *c*. It has been assumed that cytochrome *c* is represented by a prolate spheroid of dimensions $37\times25\times25$ Å. (c) As (a), in the case of β-lactoglobulin A. It has been assumed that β-lactoglobulin A is represented by a series of two spheres each having a diameter of 36 Å.

Cytochrome c

In Fig. 4 are plotted the results for cytochrome *c* in the same manner as for Fig. 2. All points correspond to Fig. 1A in ref. 7 and have *c* values of less than 0.2. As cytochrome *c* is also a basic protein (isoelectric point = 9.8–10.1, ref. 15), it has been assumed that the elution is made only by potassium ions. The three curves are theoretical, calculated by using eqn. 18 for the expression of the activity coefficient of potassium ions and, as for lysozyme, using $\varphi=8$. In parts (a), (b) and (c) in Fig. 4, the value of x' has been chosen as 10, 8 and 6, respectively, and the corresponding

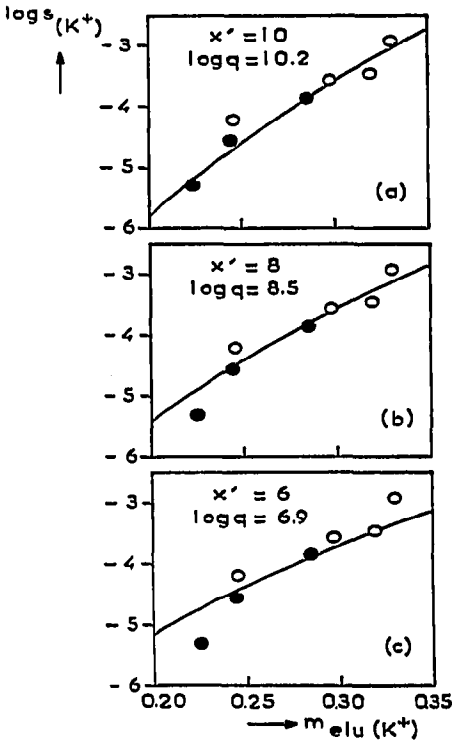

Fig. 4. Plots of the experimental and theoretical results of the chromatography of cytochrome *c* on to the (log $s_{(K^+)}$, $m_{elu(K^+)}$) plane. The symbols correspond to those in Fig. 1A in ref. 7. When x' is between 8 and 10, good fits between the experimental and theoretical results can be obtained.

values of log q have been determined in order to obtain the best fits. It can be seen that the slope of the theoretical curve is too small when $x'=6$, but when x' is between 8 and 10, good fits between the theoretical and experimental results are obtained and the value of log q is estimated to be between 8.5 and 10.2. As cytochrome *c* can be represented by a prolate spheroid of dimensions $37\times25\times25$ Å (ref. 16), the value of x' estimated above is reasonable (see Fig. 3b).

*Tropocollagen**

As the elution of tropocollagen is hardly influenced by sodium chloride or potassium chloride (see Appendix I in ref. 3), we can consider that the elution is caused by phosphate ions in the buffer and that molecules are adsorbed mainly on to C sites through carboxyl groups (see Introduction). The theoretical analysis is carried out on the data from 26 experiments, 16 of which correspond to 16 points in Fig. 2B in ref. 7. In this section, m_{elu} is defined as the phosphate molarity at which the maximum height of the chromatogram is eluted, because with tropocollagen the coordinate of the maximum height can often be measured more precisely than the centre of gravity of a peak, especially when the chromatogram shows several peaks.

* The isoelectric point of tropocollagen is about 7 (ref. 17).

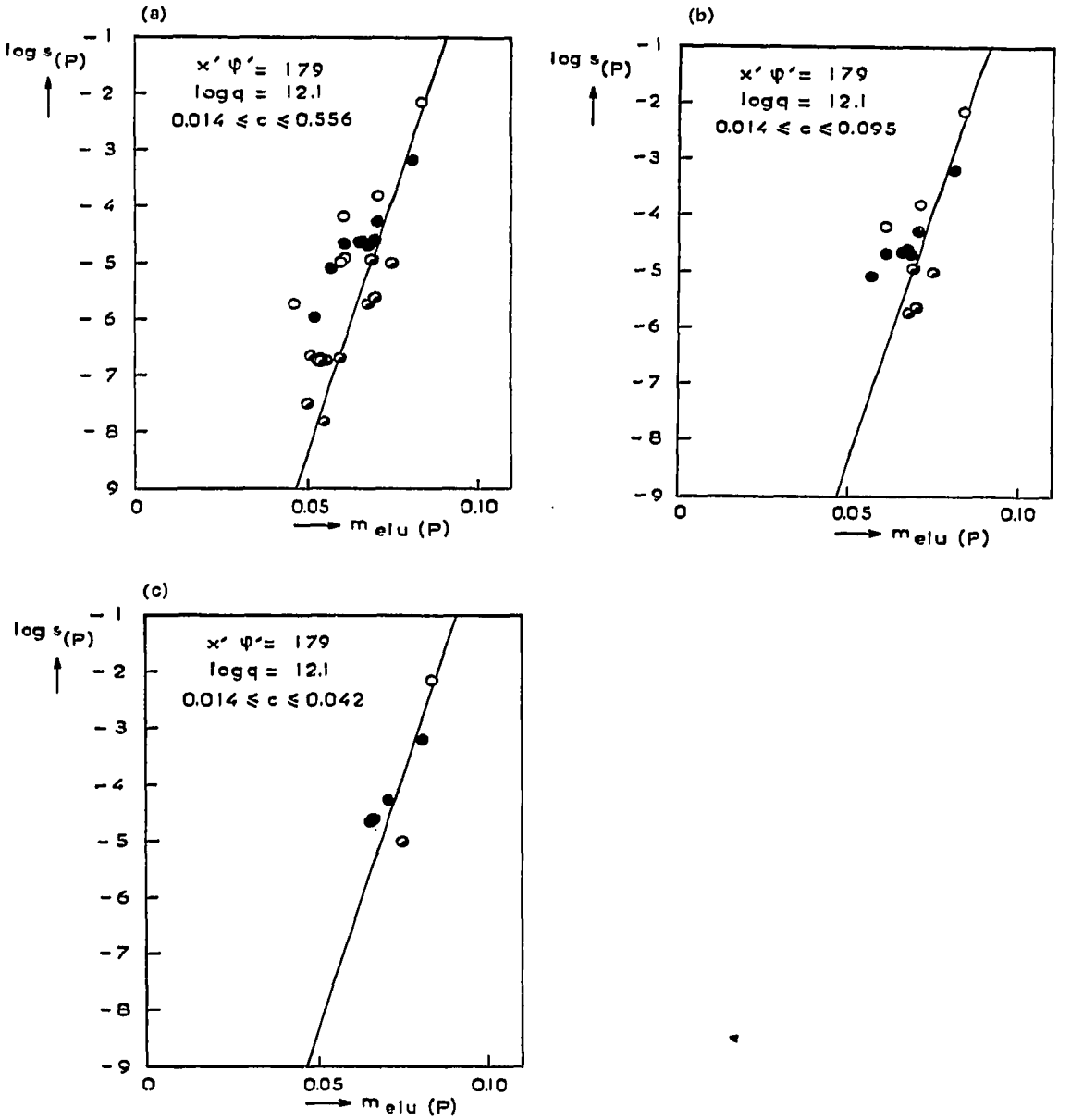

Fig. 5. (a) Plots, on to the (log $s_{(P)}$, $m_{elu(P)}$) plane, of the experimental result of the chromatography of tropocollagen and of the regression line for some of the experimental points shown in (c). The values of the parameter c of any experimental point are between 0.014 and 0.556. Symbols ○, ● and ◑ show the cases when the chromatography is carried out by using the slope, expressed as values reduced to a column of diameter 1 cm, of phosphate gradient, grad, that are equal to about 10^{-3} M/ml, $4 \cdot 10^{-4}$ M/ml and $4 \cdot 10^{-5}$ M/ml, respectively. Sixteen of the points are for the same experiments as in Fig. 2B in ref. 7. It can be considered that the regression line represents the theoretical curve and $x'\varphi'$ and log q can be evaluated from it. (b) As (a), $0.014 \leqslant c \leqslant 0.095$. (c) As (a), $0.014 \leqslant c \leqslant 0.042$.

The points in Fig. 5a, 5b and 5c are experimental plots of log $s_{(P)}$ *versus* $m_{elu(P)}$, where the subscript (P) indicates that the values concern phosphate ions. In Fig. 5a, 5b and 5c, the ranges of c values are $0.014 \leqslant c \leqslant 0.556$, $0.014 \leqslant c \leqslant 0.095$ and $0.014 \leqslant c \leqslant 0.042$, respectively. The straight line in each part of Fig. 5 is the regression line for m_{elu} on log s obtained by the least-squares method for six points in Fig. 5c. It can be seen that when c is greater than 0.095, the experimental points are distributed mainly on the left-hand side of the regression line, which means that the dependence of m_{elu} on c is greater for tropocollagen than for lysozyme (see Fig. 2a, 2b and 2c).

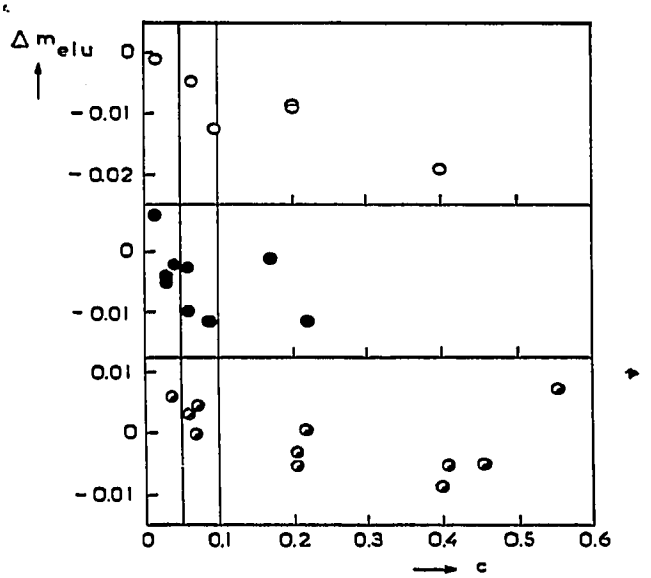

Fig. 6. Plots of Δm_{elu} as a function of c. The symbols correspond to those in Fig. 5. It is reasonable to assume that the value of Δm_{elu} extrapolated to $c=0$ is zero.

In Fig. 6 are plotted the differences, Δm_{elu}, between the experimental m_{elu} values and the values on the regression line at the same value of log $s_{(P)}$ as a function of c. The points obtained for the lowest values of c (*i.e.*, $c<0.05$) in Fig. 6 correspond to those in Fig. 5c that were used for the calculation of the regression line. It is reasonable to assume that the value of Δm_{elu} extrapolated to $c=0$ is zero, which means that the regression line can be used, provided that the amount of sample loaded is very small*. If we assume further that the activity of phosphate ions is proportional to the molarity, then eqn. 17a can be applied in order to relate log $s_{(P)}$ and $m_{elu(P)}$, because for tropocollagen x' is large and $\varphi' m_{elu}$ is small (see below). It should be noted that eqn. 17a shows a linear relationship between log $s_{(P)}$ and $m_{elu(P)}$ and that the regression line in each part of Fig. 5 can represent the theoretical curve for tropocollagen. This enables us to estimate values of log $q=12.1$ and $x'\varphi'_{(P)}=179$. In Appendix II in ref. 3, it was mentioned that C sites are probably arranged on the crystal surface with a

* In Figs. 2B and 1D in ref. 7, it has been shown that m_{elu} depends on the column length but not on the slope of the phosphate gradient. It can be shown that, even if this dependence does occur, it is too small to be detected in this case.

minimal interval of the order of 10 Å and that x' is of the order of several hundreds. If it is assumed that tropocollagen is adsorbed on a single array of C sites, which is probable (see Appendix II), then x' can be estimated to be about 300. Using this value, we obtain $\varphi' \approx 0.6$. It is easy to show that eqn. 15 or 16 gives essentially the same curve as eqn. 17 in the range of the experimental values of m_{elu} if both x' and φ' have values of the orders estimated above. This means that the estimation of x' by the method developed for lysozyme is impossible.

It would be interesting to estimate x' and log q when tropocollagen competes with cations. In this case also eqn. 17a can be used and the values $x' = 23.8$ and log $q = 30.0$ are obtained, assuming that $\varphi' = 5$ (see *Lysozyme*). As the tropocollagen molecule is much larger than those of basic proteins (tropocollagen is represented by a rod of about 15×3000 Å, ref. 18), the estimated value of x', which is only about twice the value obtained for basic proteins, is unreasonable. Therefore, we are obliged to assume that tropocollagen is adsorbed mainly through carboxyl groups.

Finally, it should be recalled that we obtained a value of log $q = 12.1$. As this value is of the same order as that calculated for basic proteins, and as the total number of carboxyl groups of tropocollagen, *i.e.*, about 280 (see ref. 19), is much larger than the number of basic groups of basic proteins, *i.e.*, about 20 (see ref. 20), it can be suggested, assuming that there is no great difference between values of log $(\beta_3 \sigma)$ for both types of adsorption (see also Appendix I), that the adsorption energy for a carboxyl group is much less than the value for a basic group. This fact could explain why the elution molarities of acidic proteins are generally low compared with the molarities of basic proteins[8,9].

*β-Lactoglobulin A**

Fig. 7a and 7b are plots of the experimental points of Fig. 1C in ref. 7, shown as $m_{elu(K^+)}$ *versus* log $_{(K^+)}$ and $m_{elu(P)}$ *versus* log $s_{(P)}$. The c values of all points are less than 0.2. In Fig. 7a and 7b, we have drawn the theoretical curves that show the best agreement with the experimental points. These curves were evaluated assuming that molecules are adsorbed on to P or C sites. For the calculation of the theoretical curve in Fig. 7a, eqn. 18 was used for the expression of the activity coefficient and it was assumed that $\varphi = 8$. For the curve in Fig. 7b, it was assumed that the activity is proportional to the molarity of phosphate ions and eqn. 17a was used. This equation can be considered to be valid as an approximate relationship even in the case of small macromolecules. In these calculations, the values of x' and log q obtained are $x' = 10$ and log $q = 9.5$, and $x' = 57$ and log $q = 6.6$. For the estimation of the latter value of x', it was assumed that $\varphi' = 0.6$ (see *Tropocollagen*). Fig. 3c represents schematically the molecule of β-lactoglobulin A adsorbed on P sites; we used as a model for the molecule two spheres each having a diameter of 36 Å (see ref. 22). It can be seen that the number of adsorption sites covered by a molecule is about twice the number corresponding to lysozyme and that the value of x' is about 20. This conclusion is valid even if the molecule is adsorbed on to C sites, as the minimal interval between neighbouring sites is probably about the same as for P sites (see Appendix II in ref. 3). This value is intermediate between those obtained by assuming that the molecule is

* The isoelectric point of β-lactoglubolin A is 5.1–5.2 (ref. 21).

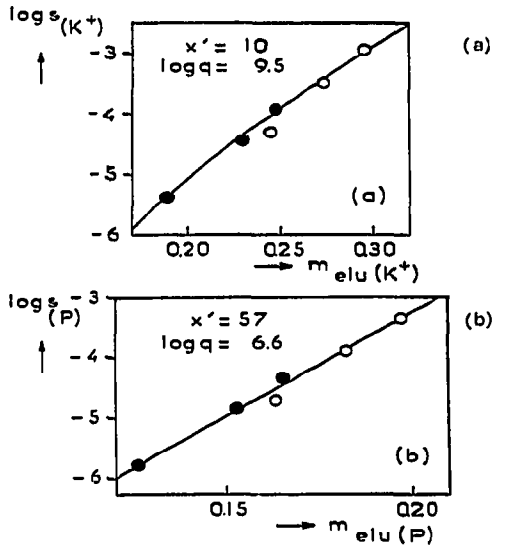

Fig. 7. (a) Plots, on to the (log $s_{(K^+)}$, $m_{elu(K^+)}$) plane, of the experimental result of the chromatography of β-lactoglobulin A in Fig. 1C in ref. 7 and of the theoretical curve that gives the best fit with the experimental results obtained by assuming that β-lactoglobulin A is adsorbed only on to P sites through basic groups. The symbols correspond to those in Fig. 1C in ref. 7. (b) As (a), with plots on to the (log $s_{(P)}$, $m_{elu(P)}$) plane assuming that β-lactoglobulin A is adsorbed only on to C sites through carboxyl groups.

adsorbed only on to P or C sites. In the following paper[9], it is shown that the experimental results in Fig. 7 can be explained by assuming that β-lactoglobulin can be adsorbed on to both P and C sites.

APPENDIX I

In this paper, $\log q$ has been estimated for several proteins. From its definition (see eqn. 2), $x\varepsilon_3/kT$ can be estimated if the value of $\log(\beta_3\sigma)$ is known. As all of the proteins considered have asymmetric shapes, the value of σ is unity. In this Appendix, we give a rough estimation of β_3 and of x and ξ.

Now, β_3 has been defined by eqn. 31 in ref. 1 and by eqn. 42 in ref. 2. When the density of the molecules on the crystal surfaces is small, the number of possible orientations of each molecule, z', can be replaced by the coordination number, z, of the adsorption sites on the crystal surface. In this case, eqn. 31 in ref. 1 (eqn. 42 in ref. 2) is valid for molecules of any shape and dimensions and can be written as

$$\beta_3 = \Gamma_3 z \cdot \frac{\delta A}{\delta V} \tag{A1}$$

where

$$\Gamma_3 = \frac{\lambda_3}{m_3} \tag{A2}$$

In eqn. A1, δA and δV are the total effective crystal surfaces and the interstitial volume in the column section, respectively. It should be noted that the value of δA can be different for P and C adsorption sites (see below). In eqn. A2, λ_3 and m_3 are the absolute activity and the concentration (number/volume) of macromolecules in the interstitial liquid. As the concentration of macromolecules is low, m_3 can be assumed to be equal to the activity, so that Γ_3 is effectively independent of the type of molecules.

Estimation of $\delta A/\delta V$

Adsorption on P sites. In Appendix II in ref. 3, it was mentioned that HA crystals have blade-like shapes and that the adsorption of cytochrome *c* on the flat surfaces of the crystals can be observed. On the other hand, it has been observed that about 14 mg of cytochrome *c* are adsorbed per millilitre of packed HA crystals equilibrated with 10^{-3}–10^{-2} *M* phosphate buffer (pH 6.8). When pure distilled water is used as the solvent, the amount of adsorbed molecules decreases slightly. In the case of lysozyme, the amount that can be adsorbed is about 10 mg/ml when equilibrated with 10^{-3}–10^{-2} *M* phosphate buffer and 2.5 mg/ml if pure distilled water is used as the solvent. Assuming that these molecules virtually saturate the crystal surfaces when the buffer concentration is between 10^{-3} and 10^{-2} *M* and that one molecule of cytochrome *c* (mol. wt. $1.2\cdot10^4$) occupies $37\times25=925$ Å^2 (see Fig. 3b) and one molecule of lysozyme (mol. wt. $1.4\cdot10^4$) occupies $45\times30=1350$ Å^2 (see Fig. 3a), then the total area of the crystal surfaces per millilitre of packed HA on which adsorption occurs can be estimated to be $6.5\cdot10^4$ cm^2 and $5.8\cdot10^4$ cm^2. These values are approximately equal. As, on the other hand, the interstice of the packed crystals occupies about 80% of the total volume[13], we obtain for the value of $\delta A/\delta V$

$$\frac{\delta A_{P'}}{\delta V}=\frac{6\cdot10^4}{0.8}=7.5\cdot10^4\ \text{cm}^{-1}=7.5\cdot10^{-4}\ \text{Å}^{-1} \qquad \text{(A3)}$$

where the subscript P′ indicates P sites; it should be noted that $\delta A/\delta V$ has the dimension of (length)$^{-1}$. In eqn. A1, however, the length has to be expressed in such a unit that the area of the elementary surface on HA that contains a single adsorption site is equal to unity. As P sites are very probably arranged hexagonally on the crystal surface and as the minimal interval between the neighbouring sites is 9.432 Å (see Fig. 3), it is easy to calculate the value of this unit as 8.78 Å and hence

$$\frac{\delta A_{P'}}{\delta V}=7.5\cdot10^{-4}\cdot8.78=6.6\cdot10^{-3}\ \text{unit}^{-1} \qquad \text{(A4)}$$

Now, assuming further that adsorption occurs on both faces of the crystal, the mean value of the thickness of the crystal can be estimated by using eqn. A3 to be about 670 Å.

Adsorption on C sites. We can consider that the most typical example of adsorption on C sites is the adsorption of DNA, because there are only acidic adsorption groups on the molecular surface. It has been shown by Bernardi[13] that about 10 A_{260} units or 0.5 mg of native DNA is adsorbed per millitre of packed HA crystals equilibrated with 10^{-3} *M* phosphate buffer. As the molecular weight of

DNA is about 700 per 3.4 Å and as its molecular diameter is about 20 Å, assuming that adsorbed molecules are parallel (see Appendix I in ref. 1) with an interval of 20 Å, one can estimate the total area of the adsorption surfaces of HA per millilitre of packed crystals to be about $3 \cdot 10^3$ cm^2, which is one twentieth of the value estimated for basic proteins. It follows from this estimation that

$$\frac{\delta A_{C'}}{\delta V} = 3 \cdot 10^{-4} \text{ unit}^{-1} \tag{A5}$$

where the subscript C′ indicates C sites and where it has been assumed that the value of the unit of eqn. A5 is the same as in eqn. A4. This assumption is reasonable as a first approximation, because the shape of the unit cell of HA is reasonably symmetrical (see Appendix II in ref. 3)*. As the effective surface is very small in the case of adsorption on C sites, it is likely that this type of adsorption occurs on the side faces of the crystals (see below). In this case, if a molecule is adsorbed perpendicular to the flat surfaces, it has to be adsorbed by using only one part of its length, because the molecules are usually much longer than the thickness of the crystal, *i.e.*, 670 Å. It is evident that the maximum possible number of molecules that can be adsorbed on the crystal surfaces in a column section is obtained if each molecule is adsorbed perpendicular to the flat crystal surfaces. If the total amount of molecules in the column section is large, one cannot exclude the possibility that the "perpendicular phase" is realized. However, as the concentration of molecules in the interstitial liquid is usually small, the "parallel phase", in which each molecule is energetically stable but where the maximum possible number of molecules that can be adsorbed is small, will be realized in the section of the column in the first step of the loading. In the second step, new molecules will flow into the section, but the total amount of molecules in the section will still be small, because the concentration of molecules in the solution is small. Even if the saturation state in the "parallel phase" has finally been reached on the crystal surfaces before the inflow of new molecules, the occurrence of the phase transition to the "perpendicular phase" caused by the inflow of molecules is improbable, because the amount of flowing molecules is always small. These molecules will remain in the solution and flow out in the next step. It should be noted that the "parallel phase" has been assumed for the calculation of eqn. A5**.

It is now necessary to estimate the ratio of the area of the side crystal faces to the area of the flat surfaces and to test whether this ratio is compatible with the

* If we assume that adsorption sites are arranged so as to make elementary rectangular cells of dimensions 6.881×9.432 Å^2 (see later), we can calculate essentially the same value for $\delta A/\delta V$.

** Using the assumption that the adsorption occurs on the side faces of the crystal, we can estimate the arrangement of the adsorption sites by using the same method as in Appendix II in ref. 3. It is now probable that the plane parallel to the $\vec{c}$ vector appears on the side faces. Therefore, it can be considered that the adsorption sites are arranged so as to make rectangular elementary cells, one of the sides of which has a length of $|\vec{c}|$ or 6.881 Å and is perpendicular to the flat crystal surfaces. If the row of the repeating units presenting the minimum interval between them on the flat crystal surfaces or a plane parallel to both $\vec{a}$ and $\vec{b}$ vectors appears on on the effective side faces, then the side of the elementary rectangle which is parallel to the flat crystal surfaces has a length equal to $|\vec{a}|$ or $|\vec{b}|$, *i.e.*, 9.432 Å, which seems the most probable value.

hypothesis that adsorption on C sites occurs on the side faces. We can consider that Fig. A2 in Appendix II in ref. 3 represents a typical photograph of HA crystals prepared for the chromatography, and it can be seen that many of crystals have a diameter of the order of 0.01 mm. On the other hand, as the thickness of the crystals has been estimated to be about 670 Å, the ratio between two different crystal surfaces can be estimated to be of the order of 10^{-2}, which is smaller than the value of 1/20 for the ratio of the adsorption capacities between DNA and basic proteins. However, as it is probable that the HA preparation contains small crystals that cannot be observed definitively, these two different ratios seem to be compatible. One cannot, of course, eliminate completely the possibility that DNA is adsorbed on the flat surfaces but that the adsorption capacity χ' (see ref. 1) is small because of the strong repulsive interaction between molecules. This model, however, does not seem probable, because the interaction energy would be extremely high.

Tropocollagen

It has been shown that about 2 mg of tropocollagen are adsorbed per millilitre of packed HA crystals equilibrated with 10^{-3} *M* phosphate buffer (pH 6.8) which is 0.15 *M* in sodium chloride and 1 *M* in urea. Assuming that a molecule (mol. wt. 300,000) occupies an area of 14×2800 Å^2 on the crystal surface, the adsorption surfaces of HA per millilitre of packed crystals can be estimated to be about $1.6 \cdot 10^4$ cm^2. This value is five times that for DNA and one quarter of that for basic proteins. Assuming that P and C sites are arranged on the flat surfaces and the side faces of HA, respectively, the adsorption capacity for tropocollagen could be explained by the hypothesis that molecules are adsorbed on both P and C sites through basic and carboxyl groups, respectively. On the other hand, we have mentioned that tropocollagen is adsorbed mainly on to C sites, because the elution of molecules is hardly influenced by sodium chloride and potassium chloride. The same conclusion has been reached in the analysis of the chromatography of tropocollagen. It should be noted, however, that the chromatography reflects mainly the state where the density of molecules on the crystal surfaces is small, because the development of molecules is possible only when the density is small. It will be in this state that molecules are adsorbed mainly on to C sites.

Finally, it should be also noted that basic proteins might be adsorbed on both P and C sites in the saturation state. If this is true, our calculation carried out on the assumption that all molecules are adsorbed on P sites in the case of basic proteins is valid, as the domain of C sites is much smaller than the domain of P sites.

Estimation of β_3

Assuming that P and C sites are on the flat surfaces and side faces of the crystals, respectively, the values of the coordination numbers of the adsorption sites can first be estimated to be

$$z_{P'} = 6 \tag{A6}$$

and

$$z_{C'} = 2 \tag{A7}^*$$

* It should be noted that in all calculations, the effect of the possible orientations of each competing ion on the adsorption site, which is related to the coordination number of the sites, has not been taken into consideration, as this effect will influence the result of the calculations only slightly.

Now, we can estimate Γ_3 and β_3 (see eqn. A1). Assuming that tropocollagen is adsorbed on a single array of C sites (when the density of the molecules on the crystal is small), it has been shown that

$$\varphi'_{(P)}=0.6 \tag{A8}$$

in which the subscript P indicates that the value concerns phosphate ions in the buffer and $\varphi'_{(P)}$ is defined by eqn. 14 or by

$$\varphi'_{(P)}=\frac{e^{\varepsilon_{(P)}/kT}\lambda_{(P)}}{m_{(P)}}=e^{\varepsilon_{(P)}/kT}\Gamma'_{(P)} \tag{A9}$$

In an earlier paper[4], assuming the "microheterogeneous model" for tropocollagen, the adsorption energy on a carboxyl group, ε_a (the subscript a represents an acidic group) has been estimated as

$$\varepsilon_a\approx 0.5 \text{ kcal/mole} \tag{A10}$$

or we have

$$e^{\varepsilon_a/kT}\approx 2.5 \tag{A10a}$$

On the other hand, it is known that the elution phosphate molarities of denatured DNA, poly(L-glutamic acid) and poly(L-aspartic acid) are about 0.12–0.14 M (ref. 13), 0.25 M and 0.4 M (ref. 23), respectively. We can consider that all these molecules exist as random coils under the experimental conditions. In DNA, there is one phosphate group per nucleotide, the molecular weight of which is about 350, and in poly(L-glutamic acid) and poly(L-aspartic acid) there is one carboxyl group per amino residue, the molecular weights of which are 130 and 150, respectively. Now, as the elution molarity is approximately proportional to the ratio between the numbers of adsorption groups of the molecule reacting with the adsorption sites of the crystal and of the crystal sites covered by the molecule, provided that the adsorption energies on phosphate and carboxyl groups are the same and as this ratio could be roughly proportional to the reciprocal of the molecular weight per residue that contains one adsorption group, it can be concluded that the adsorption energy on a carboxyl group is about equal to the energy on a phosphate group. It should be noted, on the other hand, that the phosphate buffer at pH 6.8 contains equal amounts of monovalent and divalent phosphate ions. We assume now that the adsorption energy on a divalent phosphate ion is twice the energy of a monovalent ion and that the energy on a phosphate ion of the buffer is, on average, 1.5 times the energy on a phosphate group of DNA or a carboxyl group of protein. We can now have

$$\varepsilon_{(P)}\approx 0.75 \tag{A11}$$

or

$$e^{\varepsilon(P)/kT}\approx 4 \tag{A11a}$$

and from eqns. Alla, A8 and A9, the value

$$\Gamma'_{(P)}\approx 0.15 \tag{A12}$$

is obtained. It should be noted that $m_{(P)}$ is expressed in terms of molarity and that that m_3 (see eqn. A2) has to be expressed in terms of number/(8.78 Å)^3, where 8.78 Å was the unit used in eqns. A4 and A5. It is easy to show that 1 M=0.407 (number/unit^3). Now, as the activity coefficient, γ, of the competing ions could be estimated to be roughly 0.75 when the molarity of the ions is close to the elution molarity[12] and as the activity coefficient of macromolecules could be considered to be virtually unity, because the concentration of macromolecules in the column interstice is usually low, Γ_3 can be roughly estimated as

$$\Gamma_3 \approx \Gamma'_{(P)} \cdot \tfrac{4}{3}/0.407 \approx 0.5 \tag{A13}$$

Hence, the value of β_3 in the cases of adsorption on P and C sites (denoted by $\beta_{P'}$ and $\beta_{C'}$, respectively) can be calculated, by substituting eqns. A4, A6 and A13 and eqns. A5, A7 and A13, respectively, into eqn. A1, as

$$\beta_{P'} \approx 2 \cdot 10^{-2} \tag{A14}$$

and

$$\beta_{C'} \approx 3 \cdot 10^{-4} \tag{A15}$$

or we have

$$\log \beta_{P'} \approx -3.9 \tag{A14a}$$

and

$$\log \beta_{C'} \approx -8.1 \tag{A15a}$$

Estimation of $x\varepsilon_3/kT$, x and ξ

In the third column of Table AI are given values of $x\varepsilon_3/kT$ estimated for lysozyme, cytochrome *c* and tropocollagen by using eqns. A14a, A15a and 2. Now,

TABLE AI

PARAMETERS FOR DIFFERENT PROTEINS

Protein	*Type of adsorption assumed*	$x\varepsilon_3/kT$	ε_3(*kcal/mole*)	x	x' *assumed*	ξ
Lysozyme	Basic groups, P sites	11.0	2	3.3	10	0.35
Cytochrome *c*	Basic groups, P sites	12.4	2	3.7	8	0.46
Tropocollagen	Carboxyl groups, C sites	20.2	0.5	22	300	0.073

we can estimate the adsorption energy on a basic group ε_b (the subscript b indicates a basic group) of a macromolecule. We now have the relationship

$$\varphi'_{(K^+)} = \frac{e^{\varepsilon(K^+)/kT}\lambda_{(K^+)}}{m_{(K^+)}} = e^{\varepsilon(K^+)/kT}\Gamma'_{(K^+)} \tag{A16}$$

for the potassium ions in the buffer. Therefore, assuming that

$$\Gamma'_{(K^+)} \approx \Gamma'_{(P)} \tag{A17}$$

and that the adsorption energy on a basic group is the same as the energy on a potassium ion, and using the value of $\varphi'_{(K^+)}$

$$\varphi'_{(K^+)} = 5 \tag{A18}$$

estimated in this paper, we can have

$$e^{\varepsilon_b/kT} \approx e^{\varepsilon_{(K^+)}/kT} \approx 30 \tag{A19}$$

or

$$\varepsilon_b \approx \varepsilon_{(K^+)} \approx 2 \text{ kcal/mole} \tag{A19a}$$

In the fifth column of Table AI are given the value of x for three proteins estimated by using eqn. A19a or eqn. A10. We have estimated the values of ξ (the last column) for three proteins using the values of x' given in the sixth column. It can be seen that the values of both x and ξ are larger for cytochrome c than for lysozyme, which can be explained by the fact that the total numbers of basic amino residues of cytochrome c and lysozyme are 24 and 18, respectively[20] and that the value of x' for cytochrome c is perhaps slightly smaller than the value for lysozyme (see Fig. 3a and 3b). It can also be seen that the values of ξ for both basic proteins are much larger than the value for tropocollagen. This could be explained at least partially by the fact that the proportion of basic amino residues to the total residues in basic proteins (0.14 and 0.23 for lysozyme and cytochrome c, respectively[20]) is larger than the proportion (0.09) of acidic residues in tropocollagen[19]. This difference, however, is not large enough to explain completely the difference in ξ values. It is probable that this difference can be mainly explained by the fact that a globular protein is more flexible (giving rise to a large value of h (ref. 3)) than tropocollagen. Moreover, it is known that the total number of active groups is small for small molecules; this produces, in general, large variations in the adsorption modes or in the number of groups that can react with adsorbing sites of HA. Therefore, there are large differences between the mean value and the maximum possible number which we have chosen as our parameter[3].

APPENDIX II

In Appendix I, the value of x for the molecular species of tropocollagen that appears in the main peak of the chromatogram was estimated to be about 22. This value, however, is larger than the value (about 13) calculated earlier[4]. In this Appendix, we want to explain the discrepancy between these x values. The manner of adsorption of tropocollagen on HA is also discussed.

In the earlier paper[4], we assumed, in order to calculate the theoretical chromatogram, that x' is infinite. Moreover, the total number, ν, of "elementary domains" was chosen in order to obtain the best fit with the experimental results. On the other hand, we have shown in Appendix II in ref. 1 that the transition in B values occurs (on average) at a smaller phosphate molarity if x' has a smaller value. This conclusion

is valid even when molecular interactions occur. One would therefore expect that the theoretical chromatogram of tropocollagen would appear at a smaller phosphate molarity if a finite x' value is used, which means that a smaller value of ν has also to be chosen in order to maintain the fit with the experimental values, because if ν decreases, x increases and the position of the peaks moves to the right (see Fig. 1 in ref. 3) or to a higher phosphate molarity. This explains qualitatively why the value of x estimated in Table AI in Appendix I is larger than the value in the earlier paper[4]. As the decrease in ν causes a slight increase in the total number of chromatographic peaks (see Fig. 1 in ref. 3), it can be suggested that π (see ref. 3) is of the order of 0.9 or slightly larger, because the number of peaks decreases if π increases (see Fig. 6 in ref. 3). Here, it should be recalled that the value of ε_a was estimated in ref. 4 by using the ν value which gives a best fit with the experimental values assuming that $x' = \infty$. It can be shown that the best value of ε_a changes only slightly if a smaller ν value and larger π value are chosen.

Now, we shall try to explain quantitatively the difference in the x values. In an earlier paper[1], we showed that the value of Λ_2 at which the chromatographic peak is eluted when there are no molecular interactions is equal to Λ°_2 ($=e^{\xi\varepsilon_a/kT}-1$) provided that $x' = \infty$. Therefore, denoting by $m^\circ_{(P)}$ the value of the phosphate molarity when $\Lambda_2 = \Lambda^\circ_2$, we have

$$m^\circ_{(P)} = \frac{\Lambda^\circ_{(P)}}{\varphi'_{(P)}} = \frac{e^{\xi\varepsilon_a/kT}-1}{\varphi'_{(P)}} \qquad \text{(A20)}$$

and the value of $m^\circ_{(P)}$ can be estimated by using eqns. A10 (or A10a) and A8 and a value of ξ (see Table AI) as 0.117. On the other hand, the experiments used for the comparison with theory in ref. 4 were run at a slope of the phosphate gradient of about $4 \cdot 10^{-5}$ M/ml (see Figs. 6, 7 and 8 in ref. 24) and correspond to points such as ◑ in Fig. 5 in this paper. It can be seen in Fig. 5 that these points exist in the range of phosphate molarities of 0.05–0.075 M, which means that the theoretical chromatogram has to be calculated (assuming that $x' = \infty$ and that there are no molecular interactions) such that the maximum height of the pattern is eluted at a phosphate molarity of 0.117 M instead of 0.05–0.075 M. It also means that the value of x for the highest peak has to be $13 \cdot 0.117/(0.05\text{–}0.075) = 20\text{–}30$ instead of 13. It can be seen that the range of values 20–30 is in good agreement with the value of 22 estimated in Table AI.

Now we can attempt to calculate the critical value, $m^\circ_{(K^+)}$, of the potassium molarity. This molarity can be expressed as

$$m^\circ_{(K^+)} = \frac{\Lambda^\circ_{(K^+)}}{\varphi'_{(K^+)}} = \frac{e^{\xi\varepsilon_b/kT}-1}{\varphi'_{(K^+)}} \qquad \text{(A21)}$$

As the total number of basic groups of tropocollagen is essentially equal to the number of carboxyl groups, using for ξ the value estimated in Table AI and also using eqns. A19 (or A19a) and A18, $m^\circ_{(K^+)}$ can be calculated to be 0.06, which is smaller than the value of the molarity of sodium chloride in the initial buffer, *i.e.*, 0.15 M (see refs. 7 and 24). This means that the adsorption of molecular species with ξ (concerning basic groups) equal to 0.073 on to P sites is generally impossible, provided that the adsorption energy on a sodium ion is equal to the energy on a

potassium ion and that the value of φ' is also the same for these two types of ions. In Appendix I, on the other hand, we mentioned that tropocollagen is probably adsorbed on to both C and P sites only in the initial stage of the chromatography. Here, it should be noted that some molecular species probably have higher ξ values. If we double the ξ value, we have $m^\circ_{(K^+)}=0.13$, which is close to 0.15. It seems, however, that even this value is slightly too small to explain the hypothesis that some molecules are initially adsorbed on to P sites. It should be noted, however, that all parameters in Appendix I have been evaluated only roughly.

Finally, let us recall that the parameter $\varphi'_{(P)}$ was evaluated on the assumption that tropocollagen is adsorbed on a single array of C sites when it is adsorbed through carboxyl groups. One cannot, however, exclude the alternative possibility that the molecules are adsorbed on two arrays of sites (see Appendix II in ref. 3). Using this assumption, one can evaluate x' to be 600 and $\varphi'_{(P)}$ to be 0.3. Following the same argument as in Appendix I, one obtains $\log \beta_{P'}=-4.6$ and $\log \beta_{C'}=-8.8$, which is essentially the same result as in Appendix I (see eqns. A14a and A15a). One can also calculate that $\varepsilon_b=2.5$ kcal/mole, and for tropocollagen, $x=23$, $\xi=0.038$ and $m^\circ_{(P)}=0.117$. If we assume further that $\varphi'_{(K^+)}=5$, then $m^\circ_{(K^+)}=0.03$ and $m^\circ_{(K^+)}=0.07$ are obtained for $\xi=0.038$ and $\xi=0.038\cdot 2=0.076$, respectively*. It can be seen that these values of $m^\circ_{(K^+)}$ are smaller than the values 0.06 and 0.13 obtained by assuming that tropocollagen is adsorbed on a single array of C sites when it is adsorbed through carboxyl groups. Therefore, it does not seem probable that molecules are adsorbed on more than a single array of C sites.

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* The possible number of arrays of sites on which a molecule is adsorbed in the case of adsorption on P sites is not considered, as this number does not seem to influence the value of ξ.

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