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

II. THE CASE WHEN MOLECULES CAN BE ADSORBED ON TO BOTH OF C AND P CRYSTAL SITES THROUGH CARBOXYL AND BASIC GROUPS

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SUMMARY

The theory of hydroxyapatite chromatography when the amount of macromolecules loaded is small is extended to the general case when the adsorption of molecules on to both C and P crystal sites is taken into consideration. By using this theory, the chromatography of several proteins is discussed. It is probable that in the case of basic proteins and tropocollagen, virtually all molecules are adsorbed on to a single type of crystal site. On the other hand, in the case of β-lactoglobulin A, the molecules appear to be adsorbed on to both types of site.

INTRODUCTION

In Part I[1], the relationship between the elution molarity, m_{elu}, and the experimental parameter, s, which includes the column length, L, and the slope of the gradient of competing ions, g, was investigated when a linear molarity gradient is applied and when the amount of macromolecules loaded is small. This relationship, however, is valid only when all molecules are adsorbed on to a single type of adsorption site and when they are eluted by a single type of competing ion, this being a condition for the validity of eqn. 12 in Part I. Now, it is possible to rewrite as a more general expression eqn. 12 from Part I:

$$s = \int_{m_{\mathrm{in}}}^{m_{\mathrm{elu}}} \frac{B}{1-B} \cdot \mathrm{d}m \qquad (1)^*$$

which is valid independently of the manner of adsorption and elution of macromolecules. In this paper, the experimental parameter B is calculated taking into account the fact that there are two different sites on the crystal surfaces and that there are two different competing ions (see the Introduction in Part I) and using the values of several parameters estimated in Appendix I in Part I. The experimental results presented in an earlier paper[2] are used in this discussion.

* If one substitutes eqn. 1 from Part I into eqn. 1, eqn. 12 in Part I can be obtained.

Subscripts 1, 2 and 3 are assigned to competing phosphate ion, competing cation and macromolecule, respectively; subscripts a and b represent acidic (*i.e.*, carboxyl) and basic groups of macromolecules; and subscripts C′ and P′ represent C and P sites (see Part I) of the adsorbent. The other symbols are the same as those used in earlier papers[1,3–8] unless otherwise specified.

THEORETICAL

General equations

The equilibrium between the adsorption phase and the solution in the column section is expressed as

$$\mu_1 = \mu^0{}_1 \tag{2}$$

$$\mu_2 = \mu^0{}_2 \tag{3}$$

$$\mu_3 = \mu^0{}_3 \tag{4}$$

Introducing absolute activities, the chemical potentials in the solution can be written as

$$\mu^0{}_1 = kT \log \lambda_1 \tag{5}$$

$$\mu^0{}_2 = kT \log \lambda_2 \tag{6}$$

$$\mu^0{}_3 = kT \log \lambda_3 \tag{7}$$

The chemical potentials in the adsorbed phase can be written by using the Helmholtz free energy of the adsorption state, *i.e.*,

$$F = U - TS \tag{8}$$

as

$$\mu_1 = \left(\frac{\partial F}{\partial n_1}\right)_{T,n_2,n_3} = \frac{\partial U}{\partial n_1} - T\frac{\partial S}{\partial n_1} \tag{9}$$

$$\mu_2 = \left(\frac{\partial F}{\partial n_2}\right)_{T,n_3,n_1} = \frac{\partial U}{\partial n_2} - T\frac{\partial S}{\partial n_2} \tag{10}$$

$$\mu_3 = \left(\frac{\partial F}{\partial n_3}\right)_{T,n_1,n_2} = \frac{\partial U}{\partial n_3} - T\frac{\partial S}{\partial n_3} \tag{11}$$

We have assumed that cations and anions in the buffer behave independently, which is possible as the concentrations of these ions in the buffer are usually so high when there is desorption of macromolecules that the ratio between them is not influenced by the reaction with the adsorbent.

Energy terms

For the total energy of adsorption in the column section, U, if the mutual interactions among adsorbed molecules are neglected, we can write:

$$U = -n_1\varepsilon_1 - n_2\varepsilon_2 - n_{3C'}x_a\varepsilon_a - n_{3P'}x_b\varepsilon_b \tag{12}$$

in which $n_{3C'}$ and $n_{3P'}$ are the numbers of macromolecules adsorbed through carboxyl and basic groups on C and P sites, respectively, and x_a and x_b are the numbers of groups that actually react in these cases. Eqn. 12 can be rewritten by using the total number of the adsorbed macromolecules, *i.e.*,

$$n_3 = n_{3C'} + n_{3P'} \tag{13}$$

as

$$U = -n_1\varepsilon_1 - n_2\varepsilon_2 - n_3x_b\varepsilon_b - n_{3C'}(x_a\varepsilon_a - x_b\varepsilon_b) \tag{12a}$$

and we have

$$\frac{\partial U}{\partial n_1} = -\varepsilon_1 \tag{14}$$

$$\frac{\partial U}{\partial n_2} = -\varepsilon_2 \tag{15}$$

$$\frac{\partial U}{\partial n_3} = -x_b\varepsilon_b - (x_a\varepsilon_a - x_b\varepsilon_b)\frac{\partial n_{3C'}}{\partial n_3} \tag{16}$$

Entropy terms

The total entropy on the crystal surface in the column section, S, can be expressed as

$$S = k\log(\Omega_{C'}\Omega_{P'}) \tag{17}$$

where $\Omega_{C'}$ and $\Omega_{P'}$ are the numbers of possible distributions of macromolecules plus competing ions on C and P sites of HA, respectively. $\Omega_{C'}$ and $\Omega_{P'}$ can be written as

$$\Omega_{C'} = \Omega_{3C'}\Omega_1 \tag{18}$$

and

$$\Omega_{P'} = \Omega_{3P'}\Omega_2 \tag{19}$$

where $\Omega_{3C'}$ and $\Omega_{3P'}$ are the numbers of possible distributions of macromolecules adsorbed through carboxyl and basic groups on C and P sites, respectively, and Ω_1 and Ω_2 are the numbers of possible distributions of phosphate ions and cations on C and P sites, respectively, when macromolecules have already been adsorbed. Following the procedure used in an earlier paper[3], Ω_1 and Ω_2 can be written as

$$\Omega_1 = \frac{(n_{0C'} - x'_{C'}n_{3C'})!}{(n_{0C'} - n_1 - x'_{C'}n_{3C'})!n_1!} \tag{20}$$

and

$$\Omega_2 = \frac{(n_{0P'} - x'_{P'} n_{3P'})!}{(n_{0P'} - n_2 - x'_{P'} n_{3P'})! n_2!} \tag{21}^*$$

where $n_{0C'}$ and $n_{0P'}$ are the total numbers of C and P sites of HA, respectively, in the column section and $x'_{C'}$ and $x'_{P'}$ are the numbers of C and P sites, respectively, where phosphate ions and cations cannot be adsorbed owing to the presence of an adsorbed macromolecule.

As now we limit ourselves to a small density of molecules on the crystal surfaces, the probability that a randomly added new molecule is not superposed on other molecules already adsorbed[3] is virtually unity. It can also be considered that the number of possible orientations of each molecule is equal to the coordination number of the adsorption sites of HA[1]. Therefore, instead of eqn. 25 in ref. 3, we can have

$$\frac{\mathrm{d}\log\Omega_{3C'}}{\mathrm{d}n_{3C'}} = \log(x'_{C'} z_{C'} \sigma_{C'}) - \log\left(\frac{x'_{C'} n_{3C'}}{n_{0C'}}\right) \tag{22}$$

and

$$\frac{\mathrm{d}\log\Omega_{3P'}}{\mathrm{d}n_{3P'}} = \log(x'_{P'} z_{P'} \sigma_{P'}) - \log\left(\frac{x'_{P'} n_{3P'}}{n_{0P'}}\right) \tag{23}$$

where $\sigma_{C'}$ and $\sigma_{P'}$ are the symmetry factors of macromolecules on C and P sites, respectively (they are usually equal to unity[1]). Now, using Stirling's approximation and the approximation that the proportion of crystal surfaces occupied by macromolecules is negligibly small, we can derive the following expressions:

$$\frac{1}{k} \cdot \frac{\partial S}{\partial n_1} = \frac{\partial \log\Omega_1}{\partial n_1} = \log\left(\frac{1-\Theta_1}{\Theta_1}\right) \tag{24}$$

$$\frac{1}{k} \cdot \frac{\partial S}{\partial n_2} = \frac{\partial \log\Omega_2}{\partial n_2} = \log\left(\frac{1-\Theta_2}{\Theta_2}\right) \tag{25}$$

$$\begin{aligned}\frac{1}{k} \cdot \frac{\partial S}{\partial n_3} &= \frac{\partial \log\Omega_{3C'}}{\partial n_3} + \frac{\partial \log\Omega_{3P'}}{\partial n_3} + \frac{\partial \log\Omega_1}{\partial n_3} + \frac{\partial \log\Omega_2}{\partial n_3} \\ &= \frac{\partial\Theta_{3C'}}{\partial\dot{\Theta}_3} \cdot \log\left(\frac{(1-\Theta_1)^{x'_{C'}}}{(1-\Theta_2)^{x'_{P'}}}\right) + \log(1-\Theta_2)^{x'_{P'}} \\ &\quad + \log(x'_{P'} z_{P'} \sigma_{P'}) - \log\{\kappa(\dot{\Theta}_3 - \Theta_{3C'})\} \\ &\quad + \frac{\partial\Theta_{3C'}}{\partial\dot{\Theta}_3} \cdot \log\left(\frac{\kappa(\dot{\Theta}_3 - \Theta_{3C'})}{\Theta_{3C'}} \cdot \frac{x'_{C'} z_{C'} \sigma_{C'}}{x'_{P'} z_{P'} \sigma_{P'}}\right)\end{aligned} \tag{26}$$

* For the estimation of Ω_1 and Ω_2, the effect of the possible orientations of each ion on the adsorption site is not taken into consideration (see Appendix I in Part I).

where

$$\Theta_1 = \frac{n_1}{n_{0C'}} \tag{27}$$

$$\Theta_2 = \frac{n_2}{n_{0P'}} \tag{28}$$

$$\Theta_{3C'} = \frac{x'_{C'} n_{3C'}}{n_{0C'}} \tag{29}$$

$$\Theta_{3P'} = \frac{x'_{P'} n_{3P'}}{n_{0P'}} \tag{30*}$$

$$\dot{\Theta}_3 = \frac{x'_{C'} n_3}{n_{0C'}} \tag{31}$$

and

$$\kappa = \frac{x'_{P'}}{x'_{C'}} \cdot \frac{n_{0C'}}{n_{0P'}} \tag{32}$$

$\Theta_{3C'}$ as a function of Θ_1, Θ_2 and $\dot{\Theta}_3$

It is evident that the thermodynamic state of the column section is determined if n_1, n_2 and n_3 or Θ_1, Θ_2 and $\dot{\Theta}_3$ are known. In eqn. 26, the entropy term for macromolecules has been expressed by using Θ_1, Θ_2, $\dot{\Theta}_3$ and $\Theta_{3C'}$. Here, we express $\Theta_{3C'}$ as a function of Θ_1, Θ_2 and $\dot{\Theta}_3$. This function could be determined by the condition of the minimum Helmholtz free energy in the adsorption state:

$$(F(n_{3C'}))_{n_1,n_2,n_3} = \text{minimum} \tag{33}$$

or by the relationship

$$\left(\frac{\partial F}{\partial n_{3C'}}\right)_{n_1,n_2,n_3} = 0 \tag{34}$$

The left-hand side of eqn. 34 can be rewritten, by using Stirling's approximation and the approximation that the proportion of the crystal surface occupied by macromolecules is negligibly small, as

$$\frac{\partial F}{\partial n_{3C'}} = -(x_a\varepsilon_a - x_b\varepsilon_b) - kT\log\left(\frac{(1-\Theta_1)^{x'_{C'}}}{(1-\Theta_2)^{x'_{P'}}} \cdot \frac{\kappa(\dot{\Theta}_3 - \Theta_{3C'})}{\Theta_{3C'}} \cdot \frac{x'_{C'} z_{C'} \sigma_{C'}}{x'_{P'} z_{P'} \sigma_{P'}}\right) \tag{35}$$

From eqns. 34 and 35, the function $\Theta_{3C'}$ is calculated as

$$\Theta_{3C'} = r\dot{\Theta}_3 \tag{36}$$

* This equation, which is unnecessary here, will be useful later.

in which

$$r = \frac{1}{1 + \frac{1}{\kappa'} \cdot \frac{(1-\Theta_2)^{x'_{P'}}}{(1-\Theta_1)^{x'_{C'}}} \cdot \exp\left(\frac{x_b \varepsilon_b - x_a \varepsilon_a}{kT}\right)} \tag{37}$$

and

$$\kappa' = \kappa \cdot \frac{x'_{C'} z_{C'} \sigma_{C'}}{x'_{P'} z_{P'} \sigma_{P'}} = \frac{n_{0C'} z_{C'} \sigma_{C'}}{n_{0P'} z_{P'} \sigma_{P'}} \tag{38}$$

In eqn. 36 or 37, r has to satisfy the inequalities

$$0 \leqslant r \leqslant 1 \tag{39}$$

When $r = 1$, all macromolecules are adsorbed on to C sites through carboxyl groups; when $r = 0$, they are all adsorbed on to P sites through basic groups.

Adsorption isotherm

Introducing the parameters

$$\Lambda_1 = \lambda_1 \, e^{\varepsilon_1/kT} \tag{40}$$

and

$$\Lambda_2 = \lambda_2 \, e^{\varepsilon_2/kT} \tag{41}$$

it can be first calculated from eqns. 2, 5, 9, 14 and 24 and from eqns. 3, 6, 10, 15 and 25, respectively, that

$$1 - \Theta_1 = \frac{1}{1 + \Lambda_1} \tag{42}$$

and

$$1 - \Theta_2 = \frac{1}{1 + \Lambda_2} \tag{43}$$

Using eqns. 42 and 43, eqn. 37 can be rewritten as

$$r = \frac{1}{1 + \frac{1}{\kappa'} \cdot \frac{(1+\Lambda_1)^{x'_{C'}}}{(1+\Lambda_2)^{x'_{P'}}} \cdot \exp\left(\frac{x_b \varepsilon_b - x_a \varepsilon_a}{kT}\right)} \tag{37a}$$

and also using eqns. 4, 7 ,11, 16 and 26, the adsorption isotherm of the macromolecule is calculated as

$$\dot{\Theta}_3 = R \lambda_3 \tag{44}$$

in which

$$R = e^{\frac{1}{kT}\{r x_a \varepsilon_a + (1-r) x_b \varepsilon_b\}} \cdot \{r^r (1-r)^{1-r}\}^{-1} \cdot (1+\Lambda_1)^{-r x'_{C'}} \cdot (1+\Lambda_2)^{-(1-r) x'_{P'}} \cdot (x'_{C'} z_{C'} \sigma_{C'})^r \cdot (x'_{P'} z_{P'} \sigma_{P'})^{1-r} \cdot \kappa^{-(1-r)} \quad (45)$$

Experimental parameter B

The parameter B is defined by eqn. 27 in ref. 3 or by

$$B = \frac{m_3 \delta V}{\delta N_3} \quad (46)$$

On the other hand, we have

$$\delta N_3 = \frac{\Theta_{3C'}}{x'_{C'}} \cdot \delta A_{C'} + \frac{\Theta_{3P'}}{x'_{P'}} \cdot \delta A_{P'} + m_3 \delta V \quad (47)$$

As it can be considered that $\delta A_{C'}$ and $\delta A_{P'}$ are approximately proportional to $n_{0C'}$ and $n_{0P'}$, respectively, we can write

$$\kappa \approx \frac{\delta A_{C'}/x'_{C'}}{\delta A_{P'}/x'_{P'}} \quad (48)$$

By using eqns. 13 and 29–32, one can also have

$$\Theta_{3P'} = \kappa(\dot{\Theta}_3 - \Theta_{3C'}) \quad (49)$$

and from eqns. 47, 48 and 49

$$\delta N_3 = \dot{\Theta}_3 \frac{\delta A_{C'}}{x'_{C'}} + m_3 \delta V \quad (50)$$

is obtained. Therefore, using Γ_3 defined by eqn. 29 in ref. 3:

$$\Gamma_3 = \frac{\lambda_3}{m_3} \quad (51)$$

and also using eqn. 45, B can be expressed finally as

$$B = \frac{1}{1 + R^*} \quad (52)$$

where

$$R^* = \frac{R}{x'_{C'}} \cdot \frac{\delta A_{C'}}{\delta V} \cdot \Gamma_3 = R' \left(\frac{x'_{C'} z_{C'} \kappa}{x'_{P'} z_{P'}} \right)^{r-1} \cdot \beta_{C'} = R' \left(\frac{x'_{C'} z_{C'} \kappa}{x'_{P'} z_{P'}} \right)^{r} \cdot \beta_{P'} \quad (53)$$

$$R' = e^{\frac{1}{kT}\{rx_a\varepsilon_a + (1-r)x_b\varepsilon_b\}} \cdot r^{-r} \cdot (1-r)^{-(1-r)} \cdot \sigma_{C'}{}^{r} \cdot \sigma_{P'}{}^{(1-r)} \cdot (1+\Lambda_1)^{-rx'_{C'}} \cdot (1+\Lambda_2)^{-(1-r)x'_{P'}} \quad (54)$$

$$\beta_{C'} = \Gamma_3 z_{C'} \frac{\delta A_{C'}}{\delta V} \quad (55)$$

and

$$\beta_{P'} = \Gamma_3 z_{P'} \frac{\delta A_{P'}}{\delta V} \quad (56)$$

It is easy to show that when $r=1$, $R^* = R'\beta_{C'} = \beta_{C'}\sigma_{C'}e^{x_a\varepsilon_a/kT}\ (\Lambda_1+1)^{-x'_{C'}}$ and that when $r=0$, $R^* = R'\beta_{P'} = \beta_{P'}\ \sigma_{P'}e^{x_b\varepsilon_b/kT}\ (\Lambda_2+1)^{-x'_{P'}}$. In these cases, eqn. 52 is identical with eqn. 1 in Part I[1].

RELATION TO EXPERIMENTS

Some qualitative criteria

In Table I in ref. 9 the elution molarities of several globular proteins with different isoelectric points when elution is carried out using either a gradient of potassium phosphate buffer or a gradient of potassium chloride are shown*. In all experiments, the amount of molecules loaded is small and the column is about 20 × 1 cm. The slope of the gradient, grad (see ref. 2), unfortunately is not constant; it was of the order of 3 M/l relative to potassium ions in most instances. In order to compare the theory developed above with the experimental results, we have to calculate the elution molarities of several model molecules for those experimental conditions which give a value of about —3.28 for log $s_{(K^+)}$. The calculation is carried out for two cases when the gradient is made by using phosphate buffer consisting of equimolar amounts of mono- and dipotassium phosphate, which is usually called "phosphate buffer of pH 6.8" and when it is made by using potassium chloride. In the latter case, the ion that interferes in the elution of macromolecules is potassium (see ref. 9 and Appendix I in ref. 5). For the calculation, it has been assumed that the molarities of both potassium and phosphate ions are proportional to their activities. For several of the parameters, we have used values evaluated in Appendix I in Part I. It has been assumed also that $x'_{C'}$ is equal to $x'_{P'}$, which is a reasonable assumption (see Appendix I in Part I). The model molecules chosen have values of x' equal to 10, 20 and 30, the first one of which could represent a molecule similar to lysozyme (mol. wt. = 14,000)[1]. It can be also considered that the basic proteins in Table I in ref. 9 could be represented by model molecules with $x' \approx 10$–20 and that acidic proteins in the same Table could be represented by those with $x' \approx 20$–30**. When

* In Table I in ref. 9 the results obtained by using calcium chloride instead of potassium chloride are also given. We do not, however, refer to these results, as Ca^{2+} ions appear to cause a secondary effect, *viz.*, the binding of carboxyl groups on to C sites[9].

** Molecular weights of the proteins in Table I in ref. 9 are as follows: lysozyme, 14,000 (ref. 10); cytochrome *c*, 12,000 (ref. 10); ribonuclease, 17,000 (ref. 11); α-chymotrypsin, 21,600 (ref. 11); spleen acid deoxyribonuclease, 38,000 (ref. 12); spleen acid exonuclease, estimated as about 60,000 from the data in ref. 13; myoglobin, 17,000 (ref. 10); snail acid deoxyribonuclease, 30,000 (ref. 14); pancreatic deoxyribonuclease, 31,000 (ref. 15); bovine serum alubumin, 65,400 (ref. 11); and pepsin, 32,700 (ref. 16).

$x'=10$, the elution molarities are calculated for different molecules satisfying the relationships $x_a+x_b=6$; $0\leqslant x_a\leqslant 6$ and $x_b=0$; and $x_a=0$ and $0\leqslant x_b\leqslant 6$, respectively. The value of x_b for lysozyme could be estimated to be about 4 assuming that virtually all molecules are adsorbed on to P sites through basic groups (see Part I; for the validity of this approximation, see below). As the total number of acidic groups is about half the number of basic groups[10], $x_a=2$ and $x_b=4$ could represent lysozyme provided that both groups are distributed homogeneously on the molecular surface. When $x'=20$, the calculation is carried out for three cases: $x_a+x_b=12$; $0\leqslant x_a\leqslant 12$ and $x_b=0$; and $x_a=0$ and $0\leqslant x_b\leqslant 12$. When $x'=30$, the calculation is carried out for $x_a+x_b=18$; $0\leqslant x_a\leqslant 18$ and $x_b=0$; and $x_a=0$ and $0\leqslant x_b\leqslant 18$. Figs. 1, 2 and 3 show the results. In Figs. 2 and 3, parts (a) and (b) are the cases when the gradients are made by using phosphate buffer and potassium chloride, respectively, and the values of the elution molarities always relate to the potassium ions in the solvent. In Fig. 1, the results of the calculation do not depend on the gradient that has been used owing to the fact that the chromatography depends uniquely on basic groups of molecules, because the adsorption energy on a carboxyl group (estimated[1,6] to be about 0.5 kcal/mole) is small and because the total energy of the adsorption of a molecule, provided that it is adsorbed through carboxyl groups, is too small for the retention of the molecule on the column. It is therefore reasonable to consider that small basic proteins such as lysozyme and cytochrome c are adsorbed only on

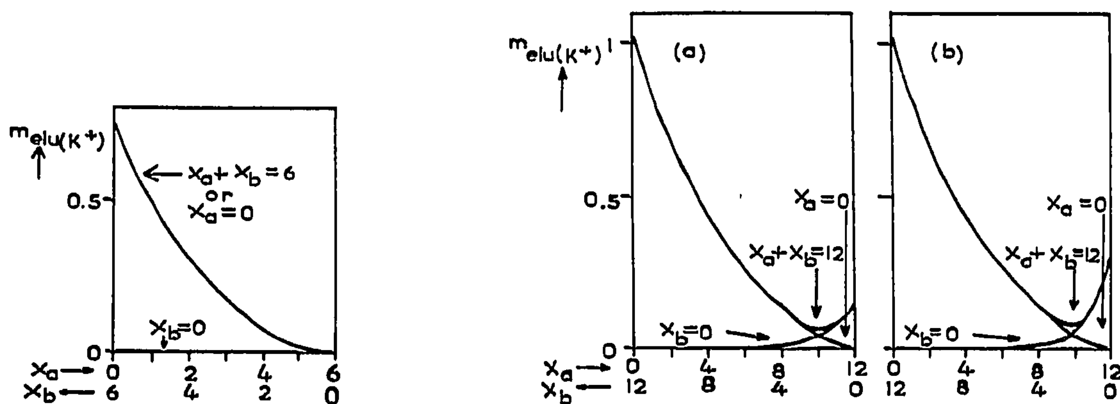

Fig. 1. $m_{elu(K^+)}$ as a function of x_a or x_b when $x'_{C'}=x'_{P'}=10$ and $\log s_{(K^+)}=-3.28$. Calculations were carried out for two cases when the elution gradients were made by using potassium phosphate buffer of pH 6.8 and potassium chloride, which gave essentially the same results. Calculations were also made for three cases when x_a and x_b vary, satisfying the relationship $x_a+x_b=6$; when $x_b=0$ and x_a varies from 0 to 6; and when $x_a=0$ and x_b varies from 0 to 6. The results of the calculations for the first and last cases are the same and in the second case the value of $m_{elu(K^+)}$ is always virtually zero.

Fig. 2. As Fig. 1; $x'_{C'}=x'_{P'}=20$ and parts (a) and (b) show the cases when the elution gradients are made by using phosphate buffer of pH 6.8 and potassium chloride, respectively. Calculations were carried out for three cases when x_a and x_b vary, satisfying the relationship $x_a+x_b=12$; when $x_b=0$ and x_a varies from 0 to 12; and when $x_a=0$ and x_b varies from 0 to 12. It can be seen that the curves for the first and second cases coincide if x_a is sufficiently large and that those for the first and last cases coincide if x_b is sufficiently large. It can be seen also that the elution molarity when the gradient is made by using potassium chloride is high if x_a is large.

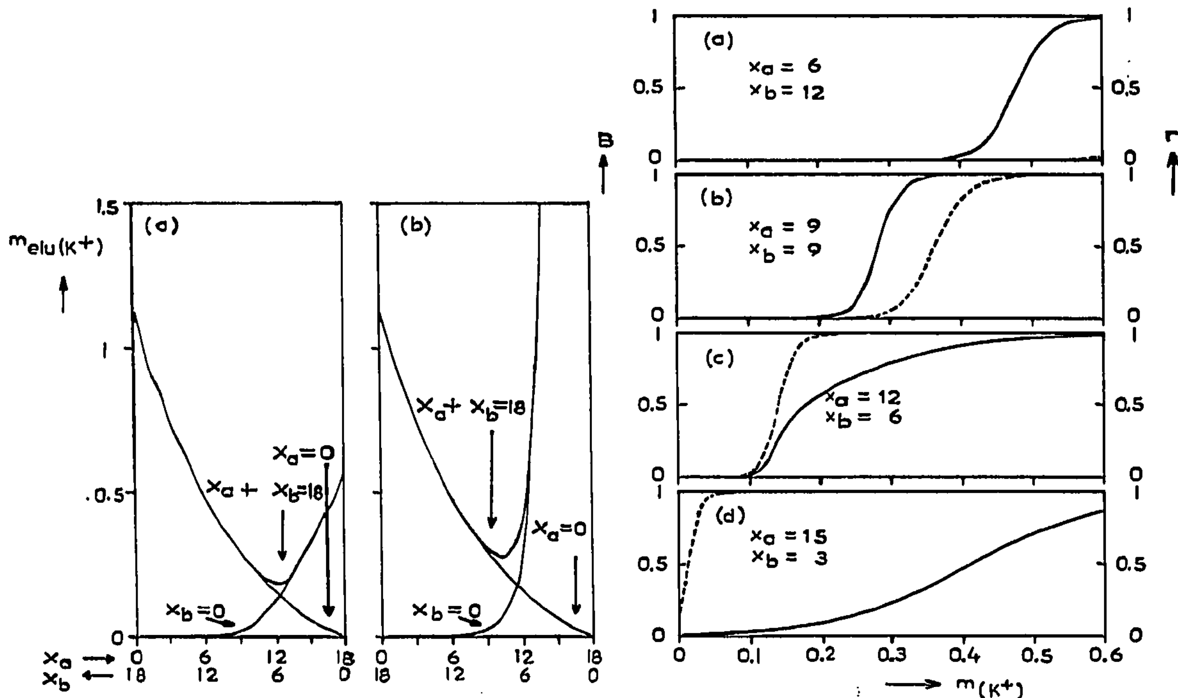

Fig. 3. As Fig. 2; $x'_{C'} = x'_{P'} = 30$ and calculations were carried out for three cases when x_a and x_b vary, satisfying the relationship $x_a + x_b = 18$; when $x_b = 0$ and x_a varies from 0 to 18; and when $x_a = 0$ and x_b varies from 0 to 18. It can be seen that the elution molarity when the gradient is made by using potassium chloride is extremely high if x_a is sufficiently large.

Fig. 4. B (solid line) and r (broken line) as functions of $m_{(K^+)}$ in phosphate buffer of pH 6.8 when $x'_{C'} = x'_{P'} = 30$ and when the relationship $x_a + x_b = 18$ is satisfied.

to P sites. We can expect that acidic proteins with dimensions similar to those of lysozyme are not, in many instances, retained on the columns. Unfortunately, however, we have no experimental data for these types of proteins. When a molecule is sufficiently large and the number of carboxyl groups that can react is large, the manner of adsorption depends on the ratio between x_a and x_b. It can be seen in Figs. 2 and 3 that if the value of x_a/x_b is sufficiently small, the behaviour of the molecule is the same as when $x_a = 0$. If the value of x_a/x_b is sufficiently large, it is the same as when $x_b = 0$. The range of values of x_a/x_b in which molecules can be adsorbed through both carboxyl and basic groups is rather small. When $x' = 30$, the maximum value of x_a/x_b at which a molecule behaves as if it has only basic groups is about unity. When x' is smaller, this value becomes larger. In Fig. 4 are shown both B and r as functions of the molarity of potassium ions in phosphate buffer of pH 6.8 for the case when $x' = 30$ and when $x_a + x_b = 18$. It can be seen that when $x_a = 6$ and $x_b = 12$, r is virtually zero in the region of $m_{(K^+)}$ where there is the transition of B. When $x_a = 15$ and $x_b = 3$, r is unity and in intermediate cases, or when $1 \leqslant x_a/x_b \leqslant 2$, r varies from 0 to 1 in the same region of $m_{(K^+)}$.

We now discuss the experimental results of Table I in ref. 9 by using the above theoretical results. It can be seen in that Table that in the case of basic proteins (lysozyme, cytochrome *c*, ribonuclease, α-chymotrypsin and spleen acid deoxyribonuclease), the elution molarity concerning potassium chloride in the solution of potassium chloride plus a small amount of phosphate buffer of pH 6.8 is always twice the molarity concerning phosphate in the pure phosphate buffer of pH 6.8 (and 1.5 times the molarity in the same buffer of pH 5.8). If phosphate ions do not interfere with the elution of macromolecules* and if both the pH and the activity coefficient of K^+ are the same in the potassium chloride solution and the phosphate buffer of pH 6.8, the elution molarity in potassium chloride solution has to be equal to that concerning K^+ in the phosphate buffer of pH 6.8 and 1.5 times (instead of twice) the value concerning phosphate in the same buffer. However, as the ratio between the elution molarities of potassium chloride solution and phosphate buffer is constant for basic proteins with isoelectric points between 8 and 11 and as this relationship breaks down abruptly when the isoelectric point becomes less than neutral (see Table I in ref. 9), it is improbable that 2/1.5 or 1.3 times the difference in the elution molarities for basic proteins is due to the fact that phosphate ions partially interefere in the elution of macromolecules. In order to explain the constant ratio that occurs, independent of the isoelectric points, one is obliged to assume that the elution is related only to the basic groups in the molecule. It is probable, as has been suggested by Bernardi *et al.*[9], that the 1.3 times discrepancy is due to the slight differences in the activity coefficient of K^+ and in the pH between two solutions. The results of our calculations explain reasonably well why a constant ratio between elution molarities is realized only when proteins are basic (see below).

As already mentioned, one could consider that all of the acidic proteins in Table I in ref. 9 (snail acid deoxyribonuclease, pancreatic deoxyribonuclease, bovine serum alubumin and pepsin), the isoelectric points of which are between 1 and 6, have x' values between about 20 and 30. Figs. 2 and 3 explain qualitatively why the constant ratio of the elution molarity that occurs for basic proteins breaks down and why the elution molarity increases in potassium chloride solution for acidic proteins. It can be seen from Table I in ref. 9 that myoglobin, with an isoelectric point at pH 7 and with dimensions similar to those of lysozyme, behaves in a similar manner to acidic proteins. It can be suggested that a large proportion of the carboxyl groups can react with C sites of HA in the case of this molecule, as in the case of β-lactoglobulin A (see below).

In Part I, it was shown that the chromatographic behaviour of tropocollagen can be described neglecting the effect of basic groups and that the value of x' is probably about 300 and the value of x_a for the species which appears in the highest part of the chromatogram has been estimated to be about 22. It is known that the total number of carboxyl groups in tropocollagen is about equal to the number of basic groups[17]. Fig. 5 shows the elution molarities concerning Na^+ derived from the phosphate buffer (see below) of model molecules with the same dimensions as those of tropocollagen, *i.e.*, with values of $x'_{C'}$ and $x'_{P'}$ equal to 300. Figs. 5a, 5b, 5c and 5d are the cases when 0, 0.05, 0.1 and 0.15 M sodium chloride, respectively, is present during the whole process of the chromatography. In all instances, it has

* It is shown experimentally that Cl^- ions do not interfere in the elution of macromolecules (see ref. 9 and Appendix I in ref. 5).

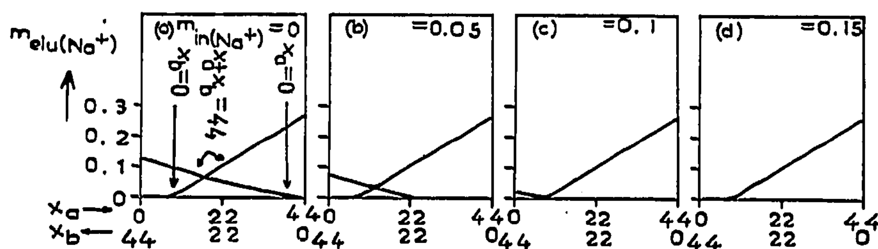

Fig. 5. $m_{elu(Na^+)}$ as a function of x_a or x_b when $x'_{C'}=x'_{P'}=300$ and when the elution is made by using the gradient of sodium phosphate buffer of pH 6.8 and by using the condition log $s_{(Na^+)}=-3.28$. Parts (b), (c) and (d) are the cases when the sodium chloride concentration is 0.05, 0.1 and 0.15 *M*, respectively. It should be noted that $m_{elu(Na^+)}$ and $s_{(Na^+)}$ concern sodium ions derived from the buffer. Calculations were carried out for three cases when x_a and x_b vary, satisfying the relationship $x_a+x_b=44$; when $x_b=0$ and x_a varies from 0 to 44; and when $x_a=0$ and x_b varies from 0 to 44.

been assumed that the gradient is made by using sodium phosphate buffer of pH 6.8 and that log $s_{(Na^+)}=-3.28$, where the subscript (Na^+) indicates the sodium ions derived from the buffer. It has been also assumed that Na^+ behaves in a similar manner to K^+, that the activity of Na^+ is proportional to its molarity and that Cl^- does not interfere in the elution of macromolecules*. In each part of Fig. 5, the calculation has been carried out for three cases: $x_a+x_b=44$; $0\leqslant x_a\leqslant 44$ and $x_b=0$; and $x_a=0$ and $0\leqslant x_b\leqslant 44$. It can be seen in Fig. 5 that when $x_a=x_b=22$, which could represent the case of tropocollagen species appearing at the highest part of the chromatogram, the elution molarity is always the same as that realized in practice, provided that the molecule has only acidic groups. If the concentration of sodium chloride is 0.15 *M*, which is the actual experimental condition, all model molecules behave as if they have no basic groups. This suggests that virtually all the species of tropocollagen behave in a similar manner to molecules with no basic groups. In Part I, however, it has been mentioned that some molecular species are probably adsorbed through basic groups only in the initial state, where the density of molecules on the crystal surfaces is high. As suggested in Appendix II in Part I, a more precise estimation of the experimental parameters is necessary for the justification of the above hypothesis.

Analysis of the chromatography of β-lactoglobulin A

In Part I, assuming that β-lactoglobulin A is adsorbed only on to P sites through basic groups or that it is adsorbed only on to C sites through carboxyl groups, the values of x' and log q were estimated. Using the first assumption, $x'_{P'}=10$ and log $q=9.5$ and by using the values of several parameters estimated in Appendix I in Part I, a value of $x_b=4.2$ can be obtained. On the second assumption, one obtains $x'_{C'}=57$, log $q=6.6$ and $x_a=18.5$. It is reasonable, however, to consider that the true value of x' is intermediate, *i.e.*, about 20 (see Fig. 3c in Part I). Here, assuming that $x'_{C'}=x'_{P'}=20$ and that the activities of both phosphate and potassium ions are proportional to their molarities, and taking into account the fact that β-lactoglobulin A can be adsorbed on both of P and C sites, we can estimate the

* Here, we consider Na^+ instead of K^+, because in the experiments with tropocollagen cations that exist in solution are Na^+ ions[2].

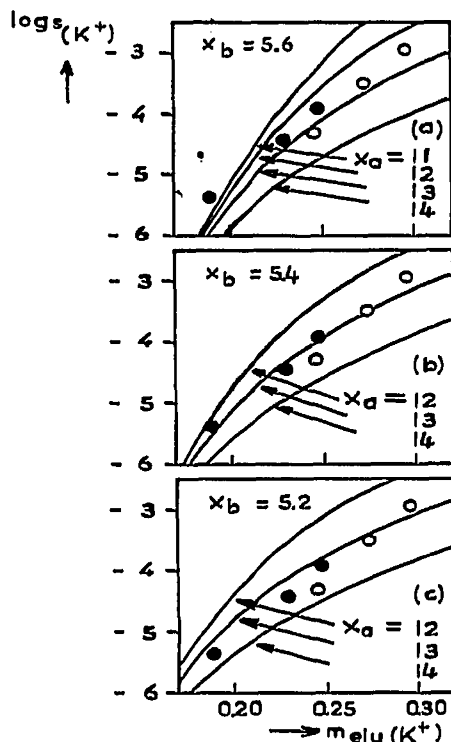

Fig. 6. Plots, on to the (log $s_{(K^+)}$, $m_{elu(K^+)}$) plane, of the experimental results of the chromatography of β-lactoglobulin A given in Fig. 1C in ref. 2 and of theoretical curves calculated assuming that $x'_{C'} = x'_{P'} = 20$ for several values of x_b and x_a. The symbols correspond to those in Fig. 1C in ref. 2. It can be seen that the best fit between the experimental and theoretical results is obtained when $x_b = 5.4$ and $x_a = 13$.

values of x_a and x_b. Fig. 6 shows the plots, on to the ($m_{elu(K^+)}$, log $s_{(K^+)}$) plane, of the experimental results in Fig. 1C in ref. 2 and of theoretical curves calculated by using eqn. 1. In Figs. 6a, 6b and 6c, it has been assumed that $x_b = 5.6$, 5.4 and 5.2, respectively, and for each value of x_b, several values of x_a have been assumed. It can be seen that the best fit between the experimental and theoretical results is obtained when $x_b = 5.4$ and $x_a = 13$ and we have $\xi_b = 0.27$ and $\xi_a = 0.65$.

It is known that the isoelectric point of β-lactoglobulin is 5.1–5.2 (ref. 18), and that the total number of carboxyl groups is between 24 and 78 and that of basic groups is 40 (ref. 10). It is reasonable to obtain a ξ_b value smaller than that for lysozyme and cytochrome *c* (see Table AI in Appendix I in Part I), as β-lactoglobulin A is acidic. The fact that ξ_a is more than twice ξ_b and that it is larger than any ξ values estimated so far (see Table AI in Appendix I in Part I) suggests that the distribution of carboxyl groups on the molecular surface is such that they can react with a larger proportion of the C sites of HA than is the case with other proteins. This may explain why β-lactoglobulin A is eluted at a particularly high molarity compared with the other acidic proteins in Table I in ref. 9*. It should be noted finally

* From Fig. 6, one can estimate that the value of $m_{elu(K^+)}$ when log $s_{(K^+)} = -3.28$ is about 0.29. Therefore, the value of the elution molarity concerning phosphate ions is about 0.2, which is particularly high in comparison with the values of 0.03–0.1 for acidic proteins given in Table I in ref. 9.

that the value of x_a for β-lactoglobulin A (13) is comparable with the value for tropocollagen (22), while the value of x' for the former molecule (20) is only 0.07 times the value for the latter (300). It can be seen, in general, that the values of ξ for small globular molecules are much higher than the value for the rod-like tropocollagen molecule (see Table AI in Appendix I in Part I). As mentioned in Appendix I in Part I, this could be explained either by the fact that the total number of active groups is small in the case of a small molecule, which produces a large deviation of the adsorption mode of the molecule, or that the structure of a globular molecule is more flexible than the structure of rod-like tropocollagen.

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All calculations were performed on the CII 10070 computer of the Faculty of Sciences, University of Paris.

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