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Further Analysis of Poly-L-lysine Chromatogram on Hydroxyapatite Column

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Abstract

Earlier the multipeak chromatogram of low-molecular weight poly-L-lysine obtained by Bernardi on a hydroxyapatite column was analyzed on the basis of a chromatographic theory. Chromatography was carried out in the presence of a linear molarity gradient of sodium phosphate buffer at pH 6.8. In the present paper, on the basis of a recently developed new theory, a peak in the multipeak chromatogram is analyzed again in detail. By taking into account an entropy effect occurring due to the variation in number of geometrical configurations of a molecule adsorbed on the crystal surface of hydroxyapatite, the width in the experimental peak can be satisfactorily explained.

INTRODUCTION

Recently, through a series of papers (*1–6*), a theory of gradient chromatography on hydroxyapatite (HA) columns was developed with small sample loads. Thus it can be deduced (*1*) that chromatography occurs on the basis of a competition mechanism between sample molecules with adsorption groups (ε-amino groups in the case of poly-L-lysine; see below) and particular ions from the buffer (sodium ions in the experiment in Fig. 1) for adsorption onto crystal sites on the HA surfaces (P sites in the experiment in Fig. 1). Molarity, m, of competing ions increases linearly with an increase in elution volume, V, with linear gradient chromatography. When dealing with small sample loads (as in the experiment in Fig. 1), the chromatographic behavior of any single component in the mixture is independent of the other components (*1*). The chromatographic process virtually is a quasi-static process. A thermodynamic equilibrium is locally realized within any

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elementary volume, δV, in the column at any instant t, and longitudinal diffusion in the column is carried out. This is caused essentially only by the heterogeneity in the flow rate of the solution occurring within each vertical section of the column (*1*). The chromatogram, $f_s(m)$, for a molecular component under consideration in the mixture can approximately be represented as

$$f_s(m) = \frac{1}{\sqrt{2\pi}\sigma(s)} e^{-[m-\mu(s)]^2/2[\sigma(s)]^2} \tag{1}$$

where

$$\mu(s) = \frac{1}{\varphi'}\{[(x'+1)\varphi' qs + (\varphi' m_{\text{in}} + 1)^{x'+1}]^{1/(x'+1)} - 1\} \tag{2}$$

$$\sigma(s) = \sqrt{2g\Theta_0 s}\{1 + q[\varphi'\mu(s) + 1]^{-x'}\} \tag{3}$$

$$s = gL \tag{4}$$

and

$$q = \beta\tau e^{x\epsilon/kT} \tag{5}$$

(cf. Eqs. 3, 8–11, and 13 in Ref. *4*). Physical meanings of the symbols involved in Eqs (1)–(5) are:

m = molarity of competing (sodium) ions in the chromatogram; the chromatogram, f_s, is represented as a function of m (which increases linearly with an increase in elution volume V; in Fig 1, m is plotted on the right-hand ordinate axis).

m_{in} = initial molarity of competing ions introduced at the top of the column.

φ' = positive constant representing the property of competing ions assuming that molarity is proportional to activity (for this assumption, see below).

L = length of the column.

g = positive constant representing the slope of the molarity gradient of competing ions in the column; this is expressed as an increase in mean ion molarity (in solution within a column section) per unit column length, measured from the bottom to the top.

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Θ_0 = positive constant with a dimension of length that measures the longitudinal diffusion in the column.

β = positive constant representing the property of the column.

x' = average number (in equilibrium state) of adsorbing (P) sites of HA on which the adsorption of competing (sodium) ions is impossible due to the presence of an adsorbed molecule; x', therefore, represents the effective dimensions of the sample molecule.

x = average number (in equilibrium state) of functional (ε-amino) groups per molecule that react with sites of HA.

$-\varepsilon$ ($\varepsilon > 0$) = adsorption energy of a functional (ε-amino) group of the molecule on to one of the sites of HA. $-x\varepsilon$, therefore, represents the energy per molecule on the HA surface.

τ = the number of effective geometrical configuration(s) of a molecule on the crystal surface (in the equilibrium state). Therefore,

$$Q \equiv -kT(\ln q - \ln \beta) = -x\varepsilon - kT \ln \tau$$

represents the free energy per molecule on the HA surface (neglecting a solvent effect).

The point of argument in the present paper is concerned with the parameter τ (see below). It can be deduced that low molecular weight poly-L-lysine molecules such as those used in the experiment in Fig. 1 have highly stretched conformations, and that a molecule is adsorbed onto a single array of P sites on the HA crystal surface by conserving the extended molecular conformation (except for very small molecules; see below). Under this situation, x' should be virtually independent of the molecular configuration on the crystal surface, and τ can be considered to be related only to the distribution of the ε-amino groups on the molecular surface (cf. Ref. *1*, Appendix I). It can also be considered that a molecule on the crystal surface is a canonical system. This means that x and $\ln \tau$ can be written as

$$x = \sum_j x_j g(x_j) \tag{6}$$

and

$$\ln \tau = -\sum_j g(x_j) \ln g(x_j) \tag{7}$$

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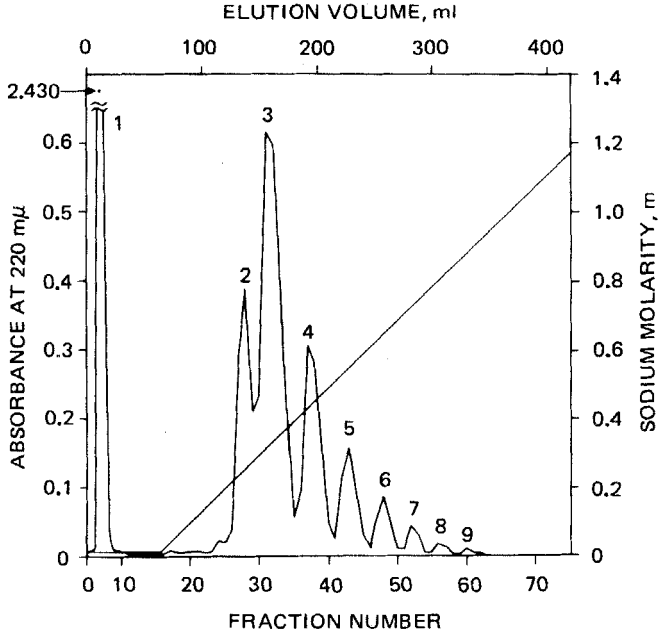

FIG. 1. Experimental chromatogram at room temperature of a poly-L-lysine·HBr sample with molecular weights ranging between 1500 and 8000 daltons (degrees of polymerization 7–38) obtained by Bernardi on a HA column (with length $L = 22$ cm) in the presence of a linear molarity gradient of sodium ions (with slope $g = 0.00207\ M/\text{cm}$), the initial molarity, m_{in}, being 0.015 M. For details, see Ref. *9* (Reproduced from Fig. 1 in Ref. *9*, or, with modifications, from the original figure, Fig. 5a, in Ref. *8*.)

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respectively, where

$$g(x_j) = \frac{e^{x_j \varepsilon/kT}}{\sum_j e^{x_j \varepsilon/kT}} \tag{8}$$

In eqs. (6)–(8), j and x_j represent a configuration of the molecule on the crystal surface and the number of ε-amino groups that react with P sites of HA when the jth-type configuration is being realized, respectively [cf. Eqs. A2–A4 in Ref. *7*, Appendix]. Provided ε is large, Eq. (6) reduces to

$$x = \max_j (x_j) \tag{9}$$

and τ now represents the number of possible configuration(s) of the molecule on the crystal surface that can be realized by using the maximum possible number of ε-amino groups that react with P sites.

Earlier (*8*) a multipeak chromatogram by Bernardi of a synthetic poly-L-lysine · HBr sample was obtained on a HA column in the presence of a linear molarity gradient of sodium ions (Fig. 1). The molecular weights of the sample range between 1500 and 8000 daltons (degrees of polymerization 7–38), and it can be assumed that the poly-L-lysine is an assembly of a number ($\approx$22) of components with a statistical distribution for chain lengths (*9*).

In Ref. *9*, on the basis of a chromatographic theory (see below) combined with both stereochemical data of poly-L-lysine and crystallographic data of HA, an analysis was made of the poly-L-lysine chromatogram obtained by Bernardi (Fig. 1). When Ref. *9* was written, however, the chromatographic theory was in an early stage of development, and only Eq. (2) had been derived on the basis of a primitive consideration on the chromatographic mechanism; Eq. (2) represents the elution molarity, μ, at the mean part of a chromatographic peak as a function of the experimental parameter s (Eq. 4). Recently, this theory was expanded to the theory in Refs. *1–6* where Eqs. (1)–(3) are involved. Thus, from the analysis in Ref. *9*, it can be deduced that poly-L-lysine with a highly stretched conformation (*10, 11*) is adsorbed, conserving the extended molecular conformation (except for very small molecules; see below), onto the ($\vec{a}$, $\vec{b}$) crystal surface of HA (*12–14*) where adsorbing sites (constructed with six oxygen ions belonging to three crystal phosphates, respectively; called P sites) are arranged hexagonally with a minimal interdistance of 9.42 Å. It is highly probable (*9*) that, except for very small molecules (presumably composed of less than 17 amino residues), there are two arrays of ε-amino groups of the side-chains that are arranged with a minimum distance of 7.23 Å along the highly stretched chain of poly-L-lysine. Every four ε-amino groups on one of the two arrays i.e., every eight ε-amino groups of the whole molecule, react with every three P sites on an array of P sites that are arranged with a minimum interdistance of 9.42 Å (*9*).

The multipeak chromatogram in Fig. 1 can be assumed to occur as a result of a stepwise variation in the number of ε-amino groups per molecule that are available for the reaction with P sites. The number, x, of ε-amino groups per molecule that react with P sites can be deduced to be 1, 2, . . . , 9 for molecules that are involved in Peaks 1, 2, . . . , 9, respectively (*9*). In this deduction, however, a tacit assumption is involved that the energy of adsorption, ε, for an ε-amino group is high enough for Eq. (6) to virtually

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coincide with Eq. (9). The validity of this assumption will be confirmed later. It can now be considered that in a peak N in Fig. 1 are involved molecules with degrees of polymerization ranging between $8N$ - 7 and $8N$, since every eight ε-amino groups of the molecule interact with P sites (see above). On the other hand, from the molecular weight, it can be estimated that the degrees of polymerization of the poly-L-lysine sample in the chromatogram in Fig. 1 range between 7 and 38 (see above). This means that the number of ε-amino groups per molecule that interact with P sites or the value of the parameter x lies between 7/8 and 38/8, i.e., about 1 and 5. These x values should correspond to the x values for molecules involved in Peaks 1–5 in Fig. 1. It can be seen in Fig. 1 that Peaks 1–5, in fact, occupy most of the chromatograms. This is consistent with the manner of adsorption of poly-L-lysine on the HA surface that has been deduced above (for details, see Ref. *9*).

From the intervals between the neighboring peaks in the multipeak chromatogram (Fig. 1), the energy of adsorption, ε, onto a P site for an ε-amino group of poly-L-lysine can be estimated to be 2–2.2 kcal/mol (*9*). Since the experiment was performed at room temperature (*8*), which can be assumed to be close to 25°C, it can be estimated that the value of the Boltzmann factor, $e^{\varepsilon/kT}$, is 29–41. This would mean that the probability that poly-L-lysine is adsorbed in the energetically most stable configuration(s) (by using the maximum possible number of ε-amino groups) is approximately 29–41 times as high as the probability that it is adsorbed in the other configurations; Eq. (6) virtually coincides with Eq. (9). In the experimental analysis in the present paper (Experimental Analysis of Peak 3 in Figure 1 Section), a most reasonable value, 2.2 kcal/mol, of ε will be used (*9*). Associated with this value, it has been estimated (*9*): $\varphi' = 9(M^{-1})$, $\langle x' \rangle = 2x$ and $\beta\langle\langle\tau\rangle\rangle \approx 0.049$ (see Eqs. 2, 3, and 5), where $\langle\ \rangle$ and $\langle\langle\ \rangle\rangle$ mean the average within a chromatographic peak (where x is constant) and the average concerning all molecules that are involved in Peaks 3–9 in Fig. 1, respectively. (In Ref. *9*, τ was written as σ, and the symbol $\langle\ \rangle$ or $\langle\langle\ \rangle\rangle$ was not applied. For φ', see Appendix IV in Ref. *9* where φ' is represented as a dimensionless quantity. φ' should have a dimension of M^{-1}, however.)

The experimental analysis in Ref. *9* (the result of which has been mentioned above) was carried out only on the basis of Eq. (2) (see above), from which no information on the width of the chromatographic peak is obtained. Further, it simply was assumed (*9*) (a) that the change in elution molarity due to the variation in τ should be much smaller than the change due to the variation in x; the τ value should be virtually constant, at least for all molecules that are involved in Peaks 3–9 in Fig. 1. And (b), the effect of the variation in x' within a peak (where x is constant) should also be negligible

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(*9*). In the present paper the analysis of Peak 3 in Fig. 1 is carried out again, taking into account both τ and x' effects. It can be confirmed that the change in μ due to the variation in τ should, in fact, be much smaller than the change due to the variation in x. However, a finite τ effect can be expected to occur. Thus it can be deduced that, due to variations in both τ and x' among eight components with the same x value that are involved within Peak 3 in Fig 1, the width of Peak 3 should be slightly larger than the width that should occur, provided only a single component is involved in it. Taking into account both τ and x' effects, and on the basis of Eq. (3) [or a more precise expression of $\sigma(s)$ obtained directly from both Eqs. 36′ and 34 in Ref. *1*, or Eqs. 62 and 73 in Ref. *3*], the width of Peak 3 in Fig. 1 is estimated. A good fit with the experiment is obtained.

Finally, in contrast to most usual cases when chromatograms appear in a range between about 0.05 and 0.3 *M* of competing ions (see, for instance, Refs. *8, 15, 16*), the chromatogram of poly-L-lysine is extending up to about 0.9 *M* (Fig. 1). As a result, it is unreasonable to apply the assumption of the constant φ' value (see above) to the analysis of the total chromatogram in Fig. 1. In Ref. *9* by means of an extrapolation method, account was taken of the change in φ' occurring with a change in m. For the present analysis for τ, the assumption of the constant φ' value is necessary. However, as far as Peak 3 is concerned, which appears at about 0.3 *M* of sodium ions (see Fig. 1), this assumption can be considered to be a good approximation (see Ref. *9*, Appendix IV). Analyses of Peaks 4–9 that appear at higher molarities were not performed. The analyses of Peaks 1 and 2 were not performed since the manners of adsorption of very small molecules (presumably with degrees of polymerization less than 17, see above) that are involved in Peaks 1 and 2 can be deduced to be different from the adsorption manners for larger molecules involved in the other peaks (*9*).

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EXPERIMENTAL ANALYSIS OF PEAK 3 IN FIGURE 1

It can be deduced that, in Peak 3 in Fig. 1, eight molecular components are involved that are composed of 17–24 amino residues. From the analysis on the basis of Eq. (2), it can be estimated that $x = 3$ and that $\langle x' \rangle = 6$ (Introduction Section). Since poly-L-lysine with a stretched conformation can be deduced to be adsorbed on an array of P sites (with its main axis parallel to the array of P sites) by using the maximum possible number of ε-amino groups (Introduction Section), τ should be represented as

$$\tau = k \tag{10}$$

for the $(8i + k)$-mer molecule, where $i = 0, 1, 2, \ldots$ and $k = 1, 2, \ldots, 8$

(*9*). Therefore, the eight components in Peak 3 in Fig. 1 with 17, 18, . . . , 24 amino residues should have the values 1, 2, . . . , 8 of τ, respectively. On the basis of an approximation that x' should increase linearly with an increase in length of the polypeptide chain, it can be estimated that $x' = 5.125, 5.375, \ldots, 6.875$ for the respective molecular components in Peak 3 in Fig. 1. It can also be assumed that β (Eq. 5) should take a value such that the average elution molarity of the eight components be essentially equal to the hypothetical elution molarity occurring, provided that all eight molecules have the same values, 6 and 0.049, of x' and $\beta\tau$, respectively (cf. Introduction Section). From this assumption it can be estimated that $\beta = 0.014$. This value of β can be considered to be in accordance, within the limit of experimental error, with the value 0.0246 estimated in Ref. *9*. (For the range in the fluctuation in the experimental value of $\beta\tau$, see Ref. *9*.)

The eight points in Fig. 2 represent mean elution molarities for the eight components with $\tau = 1, 2, \ldots, 8$ that presumably are involved in Peak 3 in Fig. 1, calculated from Eq. (2) on the basis of the considerations that have

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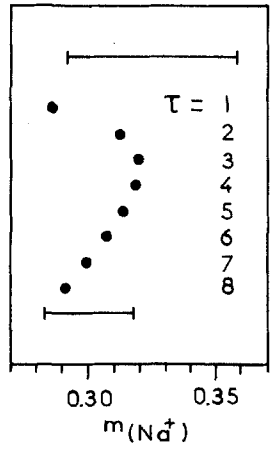

FIG. 2. Eight points: Mean elution sodium molarities for the eight components with $\tau = 1, 2, \ldots, 8$ that presumably are involved in Peak 3 in Fig. 1, calculated from Eq. (2). Lower horizontal line: Theoretical width of the region inserted between the lower and upper limits of the standard deviation (the width of which is twice the standard deviation) of Peak 3 in Fig 1, calculated from Eq. (3) assuming that all molecules involved in this peak have the same values of both x' and $\beta\tau$. It can be shown that the width of the peak of a single component with any value of τ should be almost equal to the width of the lower horizontal line. Upper horizontal line: Range of sodium molarities over which appears the width at half of the maximum height of Peak 3 in Fig. 1, which represents approximately twice the standard deviation of the peak. It can be concluded that the sum of the peaks of the components with eight different τ values should, in fact, have a total width that is essentially equal to the width of the upper horizontal line or the width in Peak 3 in the experimental chromatogram in Fig 1. For details, see text.

been mentioned above. For the calculation, the values 9 M^{-1}, 2.2 kcal/mol, 0.015 M, 22 cm, and 0.00207 M/cm have been used for φ', ε, m_{in}, L, and g, respectively. (For φ' and ε, see Introduction Section; for the other parameters, see the legend of Fig. 1) It can be seen in Fig. 2 that the elution molarity of the single component increases first with an increase of τ but then decreases. This is due to the fact that the elution molarity of poly-L-lysine with the same x value is governed by two factors, τ and x'. τ increases with an increase of x' (see above), and the elution molarity increases with an increase of τ but decreases with an increase of x'.

The lower horizontal line in Fig. 2 represents the theoretical width of the region inserted between the lower and upper limits of the standard deviation (the width of which is twice the standard deviation) of Peak 3 in Fig. 1, calculated from Eq. (3) assuming that all molecules involved in this peak have the same values, 6 and 0.049, of x' and $\beta\tau$, respectively. For the diffusion parameter Θ_0, a best value, 0.3 cm, was used (*2*). (Essentially the same result can be obtained if, instead of Eq. 3, both Eqs. 36′ and 34 in Ref. *1*, or Eqs. 62 and 73 in Ref. *3* are used; cf Introduction Section.) It can be shown that the width of the chromatographic peak of a single component changes only slightly with a change in elution molarity. This means that the width of the peak of a component with any value of τ shown in Fig. 2 should be almost equal to the width of the lower horizontal line in Fig. 2. The upper horizontal line in Fig. 2 illustrates the range of the sodium molarities over which the width at half of the maximum height of Peak 3 in Fig. 1 appears. This represents approximately twice the standard deviation of the peak. It can now be concluded from Fig. 2 that the sum of the peaks of the components with eight different τ values should, in fact, have a total width that is essentially equal to the width of the upper horizontal line in Fig. 2 or the width in Peak 3 in the experimental chromatogram in Fig. 1. Of course, taking into account the fluctuation in standard deviations in the experimental peaks as can be seen in Fig. 1 in Ref. *2*, an exceptionally close fit between the theoretical and experimental results seen in Fig. 2 would be rather fortuitous. Finally, the slight difference between the theoretical and experimental mean elution molarities of Peak 3, also seen in Fig. 2, can be assumed to be in the range of the fluctuation in elution molarities in the experimental chromatogram.

DISCUSSION

The present work is the second trial of interpreting an aspect of the experimental chromatogram in terms of an entropy factor for the variation in number of geometrical configurations of a molecule on the crystal surface of HA. The first trial was performed in an earlier paper (*17*) on behalf of the

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multipeak experimental chromatogram of collagen. By combining the entropical interpretation with the microheterogeneous collagen model, a good fit was obtained between the experimental result and the theoretical prediction (*17*). In Ref. *17*, common features between the poly-L-lysine and collagen chromatograms were also discussed.

The third possibility that might be interpretable in terms of the entropy factor can be seen in the experiment of DNA carried out by Wilson and Thomas (*18*). Thus it can be observed (*18*) that the elution molarity of double-stranded DNA on a HA column (where a linear molarity gradient of the phosphate buffer is applied) increases slowly with an increase in molecular weight or the number of nucleotide pairs involved in the molecule (hereafter, this number is written briefly as N.P.). When N.P. lies between about 20 and 1000, the elution molarity increases almost linearly with a decrease in the reciprocal of N.P. (*18*). When N.P. exceeds the order of magnitude of 1000, however, the elution molarity increases much more rapidly with a decrease in the reciprocal of N.P., and the linear relationship occurring when N.P. $\lesssim$ 1000 is broken (*18*). For instance, for T7 bacteriophage DNA with about 4×10^4 of N.P., the actual elution molarity is 0.02–0.04 *M* higher than the elution molarity obtained by extrapolation of the linear part of the elution molarity versus 1/N.P. plot (*18*). It can be deduced (*1*, *15*) that DNA (and collagen also) is adsorbed on other crystal sites (called C sites) than P sites that are arranged on different crystal surfaces from those where P sites exist, and that the desorption of DNA (or collagen) occurs due to competition with phosphate ions from the buffer. On the basis of Eq. (2) it can be shown that, provided τ is essentially constant, the elution molarity of DNA should, in fact, increase almost linearly with a decrease of 1/N.P. in a way similar to that observed experimentally. It can be suggested that DNA with N.P. larger than the order of magnitude of 1000 takes a conformation close to a random coil, and that it is adsorbed on the HA surface by using only a small part of the total structure, conserving the random coil conformation in solution. On the contrary, it is probable that DNA with N.P. smaller than the order of magnitude of 1000 is adsorbed on the crystal surface by using the total molecular structure or a considerable part of it. As a result, the number of possible conformations of DNA might be smaller on the crystal surface than in solution, and the entropy might be lost when adsorption occurs. This might explain the reason why an abnormal feature is observed in the elution molarity versus 1/N.P. plot when N.P. $\gtrsim$ 1000. A slight increase in the elution molarity occurring with high molecular weight DNA was also observed by Bernardi (*15*). It should be added that, in Ref. *18*, a slight influence of cations that exists in the buffer upon the DNA elution molarity is shown; the DNA elution molarity depends slightly upon the type of cation (*18*). A secondary cation effect was also observed by Bernardi (*15*); some wider cation effects are argued by Martinson (*19*).

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