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Investigations on the Resolving Power of Hydroxyapatite Columns

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Synopsis

An investigation on the resolving power of hydroxyapatite columns is reported. The macromolecules chosen for this investigation have been five proteins endowed with a rigid structure and of different sizes: cytochrome c, lysozyme, β-lactoglobulin A, collagen, and T2 phage. The following points have been investigated in detail: (1) the dependence of the elution molarity upon column length and slope of the gradient; (2) the dependence of the elution molarity and the width of the protein peak upon the load and the presence of other chromatographic components; (3) the optimal conditions for the resolution of macromolecules having the same size or different sizes.

INTRODUCTION

Chromatography on hydroxyapatite (HA) columns is a very powerful technique for the separation of both proteins[1–3] and nucleic acid.[4–9] The present article reports work done in our laboratory on the resolving power of HA columns; to our knowledge, this very important problem has never been investigated before. The macromolecules chosen for this investigation are five proteins endowed with a rigid structure and having different sizes.

Since the mechanism of chromatography of proteins and nucleic acids is based on the competition between macromolecules and phosphate ions (or other competing ions) for the adsorbing sites on HA[3,4] the conclusions drawn for proteins in this article may be applicable for nucleic acids having a rigid structure.

Theoretical considerations related to certain points which could not be resolved experimentally will be given in the Appendix.

MATERIALS AND METHODS

The proteins used in the present work were cytochrome c (from horse heart; Sigma, St. Louis, Mo., type III), lysozyme (from egg white; Worthington, Freehold, N. J.), β-lactoglobulin A (a gift from Dr. S. N. Timasheff), collagen,[10] and T2 phage (prepared by chromatography on HA).

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HA was prepared according to Tiselius et al.[2] Elution was carried out by using a linear molarity gradient of potassium phosphate buffer (KP) of pH 6.8, except in the case of collagen where sodium phosphate buffer (NaP) of the same pH was used.[10] Molarity gradients were obtained, in most cases, with a Varigrad (Technicon, Chauncey, N.J.), the initial buffer being $0.001M$ in phosphate. Experiments were carried out at room temperature (in the case of collagen at 4°C) with the use of columns 0.5 or 1 cm in diameter; column lengths ranged from 1 to 505 cm; in the case of columns longer than 150 cm, several columns were used, connected with glass capillary tubing; the eluent flow was kept constant by using a Technicon (Chauncey, N.J.) or a Desaga (Heidelberg, Germany) peristaltic pump operated at 30–60 ml/hr for the 1-cm columns, and at a flow rate four times lower in the case of the 0.5-cm columns. The effect of flow rate was not investigated here since we know that it affects very little the chromatogram, at least in the flow rate range explored. Fractions, 3–5 ml, were collected by using Gilson (Madison, Wis.) or LKB (Stockholm, Sweden) fraction collectors. Yields were in all cases close to 100%.

Protein was determined by measuring the ultraviolet absorption of the fractions at 220 or 280 mμ, (lysozyme and β-lactoglobulin A), at 225 mμ, (collagen), at 415 or 425 mμ (cytochrome c), and at 260 mμ (T2 phage).

The elution molarity, m_{elu}, is defined as the phosphate molarity at which the center of gravity of the protein peak is eluted, the center of gravity being defined by $\overline{V} = \int VfdV/\int fdV$, where f is the distribution function of the peak and V the volume of the solution eluted from the column. In the case of collagen the molarity of elution has been determined at the center of gravity of the multipeak chromatogram.

The width of the peak has been calculated as its standard deviation:

$$\sigma = [\int (V - \overline{V})^2 fdV/\int fdV]^{\frac{1}{2}}$$

The slope of the gradient, *grad*, has been calculated as dm/dV, m being the molarity of the phosphate buffer.

The width of the peak, the slope of the gradient, and the load were in all cases normalized for a diameter of the columns equal to 1 cm by multiplying or dividing them by the square of the column diameter.

RESULTS AND DISCUSSION

Dependence of m_{elu} upon L and *grad*

The elution molarity m_{elu} of a macromolecule from a HA column depends upon the length of the column, L, the slope of the molarity gradient, *grad*, the load, and the presence of other chromatographic components. We consider first the dependence of m_{elu} of macromolecules of different sizes upon *grad* and L, when the load is small enough and when other chromatographic components are absent. Figure 1 shows the dependence of m_{elu} upon L and *grad* for cytochrome c, lysozyme, β-lactoglobulin A, collagen, and T2 phage.

We can see that in the case of small protein molecules (cytochrome c, MW = 12,500; lysozyme, MW = 14,300; β-lactoglobulin A, MW = 35,000) m_{elu} markedly depends upon both L and *grad*, (Fig. 1A, B, C). Among small protein molecules, different *grad* values cause about the same variation in m_{elu} for a given L value, provided that L is not too small (see note 1, Appendix). In the case of collagen (MW = 300,000) m_{elu} depends only upon L, but not upon *grad* (Fig. 1*D*). In the case of T2 phage particles (MW ≅ 2.6×10^8), m_{elu} is independent of both either L and *grad* (Fig. 1*E*).

Figure 2 shows the dependence of m_{elu} upon L and *grad* for lysozyme and collagen under a wider range of L and *grad* values, than those con-

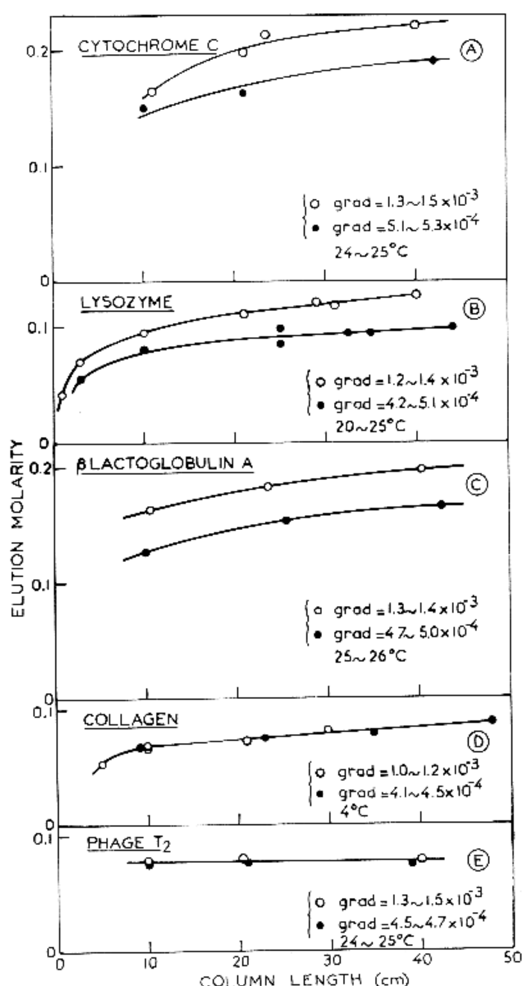

Fig. 1. Molarity of elution of (*A*) cytochrome c, (*B*) lysozyme, (*C*) β-lactoglobulin A, (*D*) collagen, (*E*) T2 phage particles as a function of L and *grad*. Load was 2 mg, except in the case of T2 phage where 1 A_{260} unit was loaded.

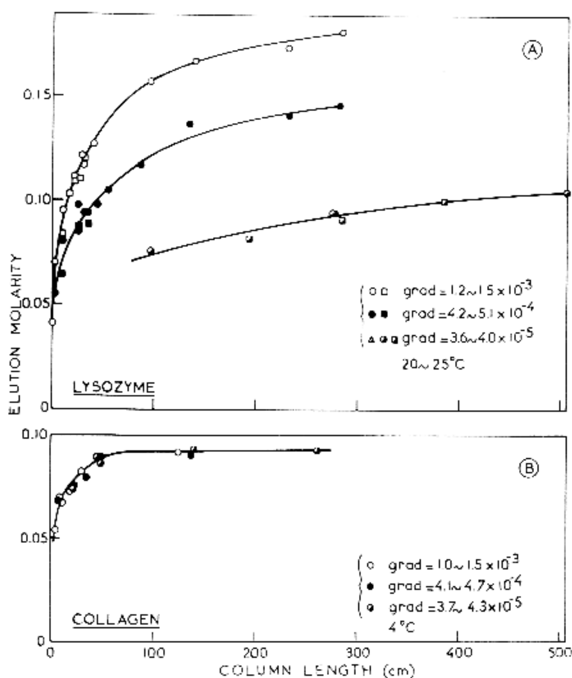

Fig. 2. Molarity of elution of lysozyme and collagen as a function of L and *grad*. A wider range of L and *grad* values were used than in to Fig. 1. Loads were (▲) 0.6 mg, (○, ●, ◑), 2 mg, (□, ■, ◪) 10 mg for lysozyme; the load was 2 mg for collagen. Values (◑) for collagen were obtained by extrapolation (see Fig. 5).

sidered in Figure 1. It is evident that for long enough columns (about 80 cm) the molarity of elution of collagen becomes independent of L. Such independence is not reached in the case of lysozyme for column lengths of 500 cm; the elution molarity of lysozyme is dependent upon L and *grad* under all experimental conditions explored.

The results presented in Figures 1 and 2 lead to the following conclusions.

In the chromatography of small proteins where m_{elu} shows a different behavior in its dependence upon L and *grad*, one can obtain the separation of the centers of gravity of the peaks by choosing appropriate *grad* and/or L values; the actual separation of the peaks will, however, depend also upon their widths.

In the case of the chromatography of proteins showing the same type of dependence of m_{elu} upon *grad* and L, the resolving power can be expressed as

$$\alpha = 1/\sigma\ (grad)$$

as far as m_{elu} and the width of the peak are not influenced by the presence of other components (see below); σ is the width of the peak calculated as its standard deviation, since the distance between the centers of gravity of each peak is inversely proportional to *grad*. This conclusion is self-

evident for the case of large proteins, whose elution molarities do not depend upon *grad*; in the case of small proteins for a given L value, different *grad* values will cause the same variation in the elution molarities as already mentioned above (see note 2, Appendix).

Dependence of m_{elu} and σ upon Sample Load and the Presence of Other Components

To find the optimal conditions for the separation of proteins, one should check preliminarily how m_{elu} and the width of the peak depend upon the load and the presence of other components. Figures 3 and 4 show these effects for small proteins. σ increases and m_{elu} decreases with increasing load, m_{elu} being only slightly dependent upon the load; the decrease of m_{elu}, when the load is 20 mg, is 16% of m_{elu} extrapolated to zero load in the case of cytochrome c (Fig. 3*A*), 7% in the case of lysozyme run under the same conditions (Fig. 3*B*), and 3% in the case of lysozyme run under different conditions (Fig. 3*C*). The increases in σ observed in the same experiments were 64% (Fig. 4*A*), 35% (Fig. 4*B*), and 45% (Fig. 4*C*), respectively. If two proteins are co-chromatographed, the m_{elu} of the lower-eluting component is decreased, whereas that of the higher eluting component remains the same; this displacement effect decreases with decreasing load. The separation expected by the displacement obtained with increasing load is offset by the concomitant increase in σ, this latter phenomenon being more important than the first one (see note 3, Appendix).

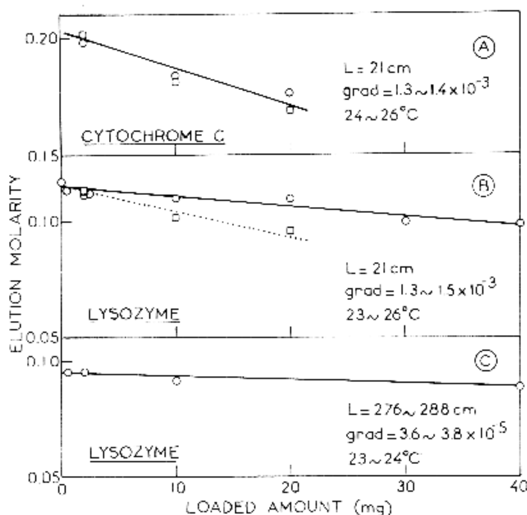

Fig. 3. Molarity of elution: (*A*) of cytochrome c as a function of the load in the absence (○) and in the presence (□) of the same amount of lysozyme; (*B*) of lysozyme as a function of load, in the absence (○) and in the presence (□) of the same amount of cytochrome c; (*C*) of lysozyme as a function of load in the absence of cytochrome c under experimental conditions different from those shown in *A* and *B*.

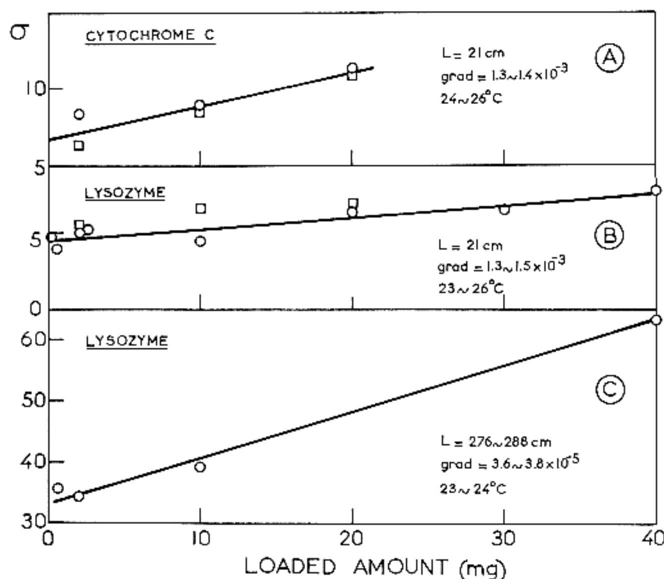

Fig. 4. Standard deviation of the peaks obtained in the experiments of Fig. 3.

Within experimental error, σ is not affected by the presence of another chromatographic component (see note 4, Appendix). If the load is small enough, both m_{elu} and σ become practically independent upon both load and presence of another component.

Figure 5 and Figures 5, 6, and 7 of the following paper[10] show the dependence of m_{elu} and width of the chromatogram of collagen upon load and coexistence of several components. They show that the same observations may be made, namely, (a) m_{elu} does not show a great dependence upon load; (b) lower eluting components are displaced to lower molarities if load is increased; at the same time, the width of the chromatogram increases; (*c*) if the load is small enough, the elution profiles obtained with different loads are practically identical.

The above results lead to the general conclusions that the resolution is essentially independent of load, at least under the present experimental conditions and, if the load is below a limit, the chromatogram of a mixture is identical with the sum of the independent chromatograms.

These conclusions justify the expression of the resolving power, α, under small load, given above.

Optimal Conditions for the Resolution of Macromolecules of Similar Size

As far as resolution is concerned, we should consider the average width of the peaks of the chromatographic components under investigation. Here, however, we will consider, instead, the width of the peaks of isolated proteins, by assuming that the chromatographic behavior of proteins belonging to the same molecular size class is similar (see note 5, Appendix).

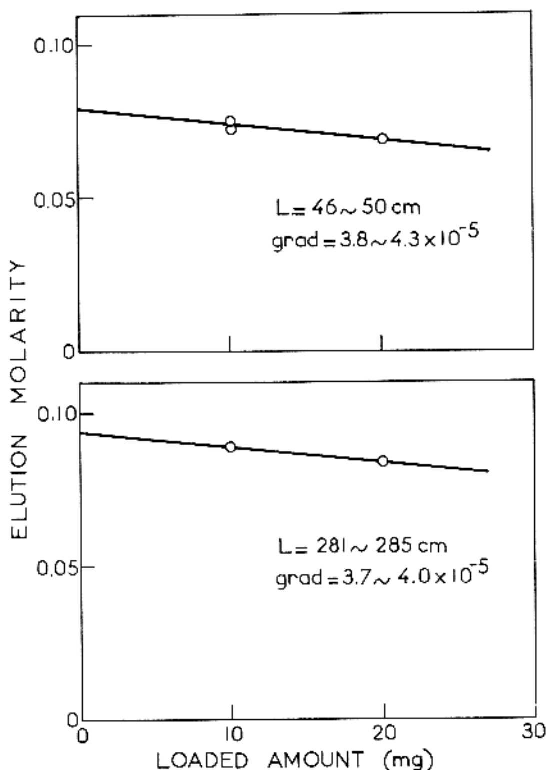

Fig. 5. Molarity of elution of collagen as a function of load.

Lysozyme. Figure 6 shows the dependence of σ upon L and *grad* when the load is small. It is evident that there is a value of L which gives σ a minimum value for each slope of the gradient, and that this value of L increases with decreasing *grad*.

Figure 7 shows which minimum value of σ is associated with the best resolution. Among the minima corresponding to different values of *grad*, those corresponding to lower values of *grad* give a better resolution. It is probable that the condition giving σ a minimum value, associated with a value of *grad* of 3.8×10^{-5}, is close to the best possible condition, since probably the extrapolation of $(1/\alpha)_{\sigma=\text{minimum}}$ to $grad = 0$ is finite and very close to the $(1/\alpha)_{\sigma=\text{minimum}}$ value corresponding to $grad = 3.8 \times 10^{-5}$.

Figure 8 shows chromatograms corresponding to the points marked by arrows in Figures 6 and 7; the increase in resolution in the order A, B, C is evident.

Collagen. In the case of collagen, as above, a better resolution is obtained by decreasing the slope of the gradient. Even if the slope of gradient is very shallow, it is not necessary to increase the column length very much to achieve good resolution (Fig. 6 of the following article[10]), since m_{elu} is almost constant ($R_f \simeq 1$) if the column is longer than about 80 cm; thus there are no interactions between macromolecules and the adsorbant.

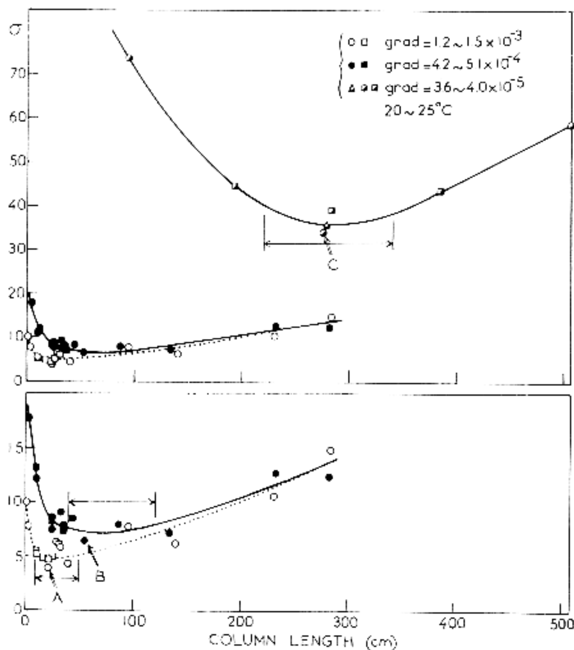

Fig. 6. Standard deviation of the lysozyme peak as a function of L and *grad*. The symbols of loads are same as in Fig. 2.

Phage T2. In the case of very large proteins, such as phage T2 particles, we can increase the resolving power by diminishing the slope of gradient, because the rate of increase of σ is smaller than the rate of decrease of the slope of gradient (Fig. 9) and since even a column length of 10 cm will be large enough to give an R_f value equal to 1 (Fig. 1*E*).

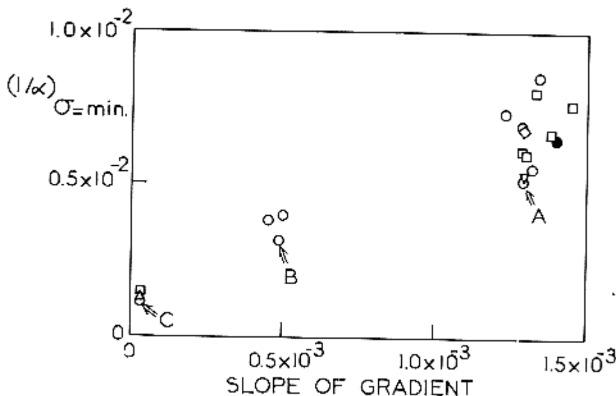

Fig. 7. Plot of $(1/\alpha)_{\sigma=\text{minimum}}$ vs. *grad*. The points of Fig. 6 lying in the regions indicated by the horizonal arrows were used, since it was assumed that minima were present in them. Loads were (●) 0.1 mg; (▽) 0.5 mg; (△) 0.6 mg; (○) 2 mg; (◇) 2.5 mg; (□) 10 mg.

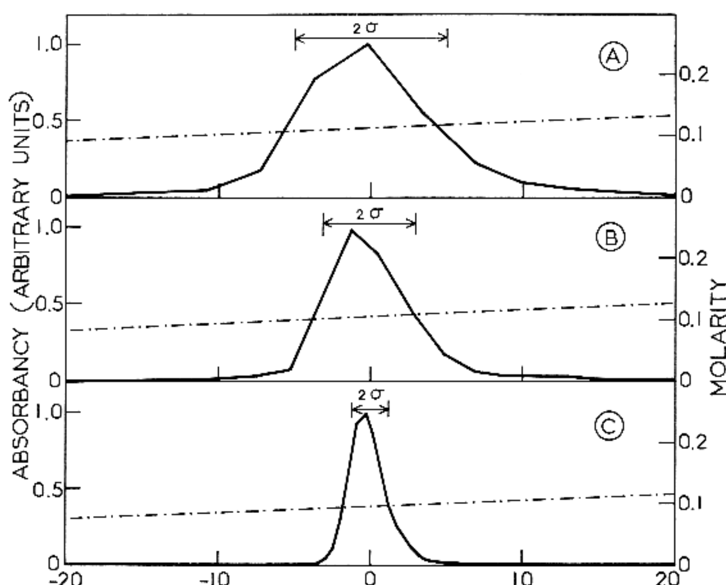

Fig. 8. Chromatograms of lysozyme corresponding to the points A, B, C, respectively, indicated by arrows in Figs. 6 and 7. The slopes of the gradients were traced in such a way as to be the same in the figure and the maxima of the peaks were normalized. The abscissa scale was centered on the center of gravity of the peaks.

Cytochrome c and β-lactoglobulin A. With cytochrome c and β-lactoglobulin A, we can also conclude that the resolving power increases with the decrease of the slope of the gradient for the same reason as for phage T2 (Fig. 10) but, as expected from the experiments with lysozyme, longer columns will be necessary to obtain a better resolution using lower slopes of gradient.

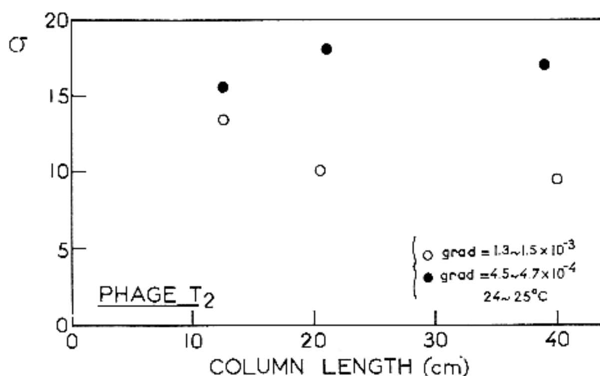

Fig. 9. Standard deviation of the T2 phage peak as a function of L and *grad*, the ratio of the slopes of molarity gradients is in the range 2.8–3.3. We can see that this value exceeds the ratio of σ for the corresponding points.

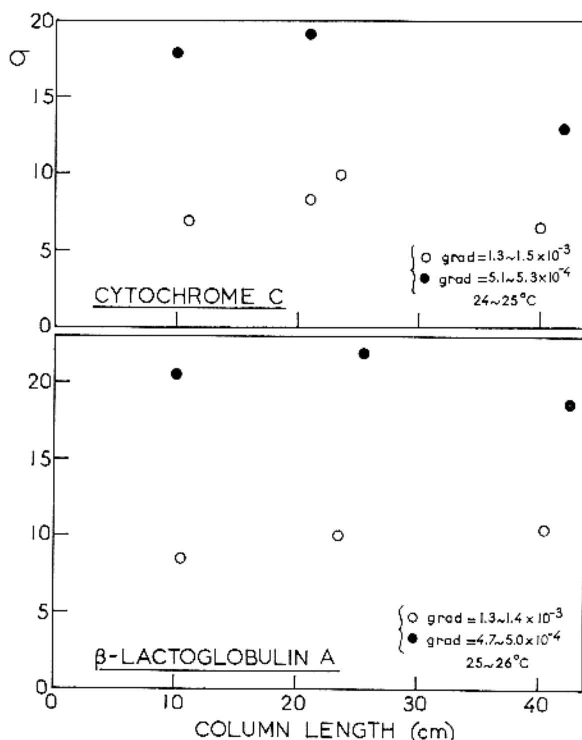

Fig. 10. Standard deviation of the cytochrome c and β-lactoglobulin A peaks as a function of L and *grad*; the ratios of the slopes of molarity gradients (*grad*) lie in the ranges 2.5–3.0 and 2.6–3.0, respectively. In both cases, the values exceed the ratios of σ for the corresponding points.

Optimum Conditions. Thus we reach the following conclusions. In general to obtain a better resolution, the slope of the gradient must be smaller. In the case of the separation of small proteins among themselves, the best column length corresponding to each slope of gradient increases with the decrease of the slope of gradient. It is probable that the association of a column length of ca. 3 m, and a *grad* of 3.8×10^{-5} is almost the best of all possible conditions. If macromolecules are larger, the column length can be shorter and good resolution can still be obtained. In fact, in the case of collagen about 80 cm is sufficient and in the case of phage T2, 10 cm is sufficient.

Optimal Conditions for the Resolution of Macromolecules of Different Sizes

The experimental results obtained so far lead to the following conclusions for the separation between small ($MW \simeq 10^4$) and large ($MW > 10^5$) macromolecules.

For the separation of large molecules with higher elution molarity from small molecules with lower elution molarity, the slope of gradient

must be low in order to reach a good resolution. In fact, the elution molarity of small molecules decreases as the slope of gradient decreases (Figs. 1 and 2), whereas ($1/\sigma$ *grad*), that is, α, increases with *grad* for both molecular species. If the large molecules are very large, the best column length will be that for small molecules, because the peak of the large component will not be influenced very much by longitudinal diffusion even after its R_f has reached a value of 1.

For the separation of large molecules with lower elution molarity and small ones with higher elution molarity, no general conclusion can be reached, since the two effects are opposed.

APPENDIX[11,12]

(1) We can expect that the variation of m_{elu} with *grad* is almost the same for a given L, among molecules with equal x', x' being the number of sites of the adsorbant covered by a macromolecule, and therefore a property related to molecular size, and that the molecular shape does not exert any influence, as long as L is large enough compared with the width of the initial zone of the loaded macromolecule.

(2) Displacements of m_{elu} between species with widely different sizes and shapes may be expected because of entropical reasons, but have not been found experimentally. These phenomena are different from those shown by macromolecules having about the same size but different elution molarities (see text).

(3) Figure 3 also shows that the decrease of m_{elu} with increase of load $-dm_{elu}/d$(load) is different in cytochrome c and lysozyme. Therefore we can also, in general, expect to improve the separation by increasing the load, although in Figure 3 this effect works so as to diminish it. Practically these phenomena cannot be used for improving the separation because of the small dependence of m_{elu} upon load.

Unless we assume interaction phenomena among macromolecules and/or very different molecular shapes, we cannot theoretically expect different $-dm_{elu}/d$(load) among macromolecules with equal x'.

(4) An increase in σ of the lower eluting component is predicted by the theory; this increase can be barely appreciated because of the experimental error in the estimation of σ.

(5) We can expect that the dependence of σ upon both L and grad follows an almost parallel pattern for rigid macromolecules having different m_{elu}, as far as x' is the same. But we can also expect that there may be a slight fluctuation in the conformation which will influence σ, but not m_{elu}. As far as this fluctuation is about the same for molecules belonging to the same size class, or small enough, the first expectation is valid.

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