obic buffer by controlling column flow with *k* and *l* as described above. The stopper with tubing *n* was placed on the column as *j* was closed. The buffer flow rate was established and the gas pressure was adjusted so that each drop of buffer caused a gas bubble to rise from the Mariotte tube. When the system was completely assembled, stopcock *k* had to be opened to provide pressure equalization between *B* and the collecting tube if the column was to flow. When a gradient elution was used, stopcocks *g* and *i* were opened and the magnetic stirrer was started.

Fractions of the desired volume were collected and dispensed (*m*) into preflushed serum bottles while O_2-free gas was directed into the bottle. A serum bottle stopper sealed the bottle and gas was flushed through the head space and vented by hypodermic needles (*D*).

[29] Chromatography of Proteins on Hydroxyapatite

By GIORGIO BERNARDI

Chromatography of proteins on hydroxyapatite[1] (HA[2]) columns is a separation technique developed in Tiselius' laboratory.[3-7] The use of HA as prepared by Tiselius *et al.*[4-6] has superseded that of other calcium phosphates previously employed in protein purification.[8]

The relatively slow acceptance of chromatography on HA columns by protein chemists seems to be due largely to three main factors: the rather laborious preparation procedure; the unknown mechanism of interaction of proteins with HA; the introduction of cellulose ion-exchangers

[1] Hydroxyapatite, not hydroxylapatite, is the name recommended by Wyckoff[34] since "hydroxyl" implies the derivatives being named after the subtituted ion, a usage which is not observed in the corresponding fluorine and chlorine derivatives (e.g., fluorapatite, chlorapatite, not fluoridapatite, chloridapatite).

[2] Abbreviations: HA, hydroxyapatite; NAP, KP, equimolar mixtures of NaH_2PO_4, Na_2HPO_4, and KH_2PO_4, K_2HPO_4, respectively. The pH is close to 6.8, and the ionic strength is equal to about twice the molarity. The abbreviation PB (phosphate buffer) used by some authors does not indicate the cation. Because the eluting power of phosphates is different for different salts, the abbreviation PB is discouraged.

[3] S. M. Swingle and A. Tiselius, *Biochem. J.* **48**, 171 (1951).

[4] A. Tiselius, *Ark. Kemi* 7, 445 (1954).

[5] A. Tiselius, S. Hjertén, and O. Levin, *Arch. Biochem. Biophys.* **65**, 132 (1956).

[6] S. Hjertén, *Biochim. Biophys. Acta* 31, 216 (1956).

[7] O. Levin, this series, 5, 27.

[8] C. A. Zittle, *Advan. Enzymol.* 14, 319 (1953).

by Peterson and Sober in 1956[9] and of Sephadex by Porath and Flodin in 1959.[10]

The first difficulty seems to have been overcome with the advent of commercially available HA preparations (see following section). As far as the second point is concerned, recent work has led to a better understanding of the interaction between proteins and HA,[11,12] the influence of the secondary and tertiary structure of proteins on their chromatographic behavior,[11] the parameters which determine the resolving power of the columns,[13,14] and the theoretical basis of chromatography of macromolecules endowed with a rigid structure on HA columns.[15,16] These investigations have contributed to establishing HA chromatography as a major protein separation technique. Since the basic mechanism of fractionation on HA is quite different from those underlying ion exchange and gel filtration, the three methods usefully complement each other.

The present article will deal only with some basic problems in protein chromatography on HA columns, without attempting to cover the theoretical aspects of this technique or its applications to specific proteins. It may be worthwhile to recall that HA chromatography has become in recent years a standard technique also in the field of nucleic acids.[17-21]

Methods

Preparation of Hydroxyapatite

Preparation Procedure of Tiselius et al.[5]

The following is a description of this procedure as used in the author's laboratory.

Materials. The following analytical grade reagents (Merck, Darmstadt, Germany) are used routinely: $CaCl_2 \cdot 2H_2O$, $Na_2HPO_4 \cdot 2H_2O$, $NaH_2PO_4 \cdot H_2O$, $K_2HPO_4 \cdot 3H_2O$, KH_2PO_4.

[9] E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.* **78**, 751 (1956).
[10] J. Porath and P. Flodin, *Nature (London)* **183**, 1657 (1959).
[11] G. Bernardi and T. Kawasaki, *Biochim. Biophys. Acta* **160**, 301 (1968).
[12] G. Bernardi, *Biochim. Biophys. Acta*, submitted for publication.
[13] T. Kawasaki and G. Bernardi, *Biopolymers* **9**, 257 (1970).
[14] T. Kawasaki and G. Bernardi, *Biopolymers* **9**, 269 (1970).
[15] T. Kawasaki, *Biopolymers* **9**, 277 (1970).
[16] T. Kawasaki, *Biopolymers* **9**, 291 (1970).
[17] G. Bernardi, *Nature (London)* **206**, 779 (1965).
[18] G. Bernardi, *Biochim. Biophys. Acta* **174**, 423 (1969).
[19] G. Bernardi, *Biochim. Biophys. Acta* **174**, 435 (1969).
[20] G. Bernardi, *Biochim. Biophys. Acta* **174**, 449 (1969).
[21] G. Bernardi, this series, Vol. 21 [3].

Preparation of Brushite, $CaHPO_4 \cdot 2H_2O$. Two liters each of 0.5 *M* $CaCl_2$ and 0.5 *M* Na_2HPO_4 are fed at a flow rate of 250 ml/hour, using a multichannel peristaltic pump, into a 5-liter beaker containing 200 ml of 1*M* NaCl. The addition is done while stirring just enough to avoid sedimentation of the brushite precipitate. After addition, brushite is allowed to settle. The supernatant liquid is decanted, and the precipitate is washed with two 4-liter volumes of distilled water.

Conversion of Brushite into Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. Brushite is suspended in 4 liters of distilled water and stirred. 100 ml of 40% (w/w) NaOH is added. The mixture is heated to boiling in 40–50 minutes, and then boiled for 1 hour with simultaneous, gentle stirring. The precipitate is allowed to settle completely, and the supernatant fluid is siphoned off. The precipitate is washed with 4 liters of water and the supernatant fluid is siphoned off when a 2 cm-layer of precipitate is formed on the bottom of the beaker. This is the only time during the procedure when a complete settling of the precipitate is not allowed in order to eliminate the "fines." The precipitate is then washed once more, allowing a complete settling. At this point, the precipitates from two preparations are pooled and suspended in 4 liters of 10 m*M* sodium phosphate buffer, pH 6.8 (NaP[2]), and just brought to boiling; frank boiling at this point is to be avoided. After suspension in 4 liters of 10 m*M* NaP, the precipitate is boiled for 5 minutes. This operation is repeated twice more using 10 m*M* NaP and, again, using 10 m*M* NaP; in both cases boiling is carried out for 15 minutes. A yield of 400–500 ml of packed precipitate is obtained from two pooled preparations.

Storage of Hydroxyapatite. The final precipitate, composed of bladelike crystals, may be stored in 1 m*M* NaP for several months at 4° without any change in its chromatographic behavior. The addition of chloroform as a preservative is not necessary. During resuspension of HA crystals, strong agitation should be avoided, since this fractures the crystals and their aggregates, thereby rendering them unsuitable for column chromatography.

Alternative Preparation Procedures

Other methods for preparing hydroxyapatite have been described by Main, Wilkins, and Cole,[22] Anacker and Stoy,[23] Jenkins,[24] and Siegelman *et al.*[25,26] The results reported with these preparations are limited so that it is difficult to judge their relative merits.

[22] R. K. Main, M. J. Wilkins, and L. Cole, *J. Amer. Chem. Soc.* **81**, 6490 (1959).
[23] W. F. Anacker and V. Stoy, *Biochem. Z.* **33**, 141 (1958).
[24] W. T. Jenkins, *Biochem. Prep.* **9**, 83 (1962).
[25] H. W. Siegelman, G. A. Wieczorek, and B. C. Turner, *Anal. Biochem.* **13**, 402 (1965).
[26] H. W. Siegelman and E. F. Firer, *Biochemistry* **3**, 418 (1964).

Commercial Hydroxyapatite Preparations

A preparation obtained according to the procedure of Tiselius *et al.*[5] is sold by Bio-Rad laboratories (Richmond, California), either as a suspension in 1 mM NaP or as a dry powder. Another preparation is sold by Clarkson Chemical Co. (Williamsport, Pennsylvania). Commercial HA preparations met with criticisms from several laboratories when they were first made available. Comments on the preparations sold during the past two years have been generally favorable.

Experimental Techniques with Columns

For general instructions on column chromatography the reader is referred elsewhere.[27,28] Some features that are more specific to HA columns are briefly noted here.

Packing of the Columns. This is done by adding a suspension of HA crystals in Na or K phosphate buffers, pH 6.8, (NaP or KP[2]), to columns partially filled with the same buffer; the column outlet is progressively opened only after a 1-cm layer of HA has settled. Further additions of the HA suspensions are made to fill the column. The filling operation may be facilitated by the extension of the column with a glass tube of the same diameter. Alternatively, columns may be prepared by adding the HA suspension to a funnel mounted on the top of the column, the whole system being full of starting solvent; the HA suspension in the funnel is maintained under gentle stirring during the procedure. This procedure, suggested for Sephadex,[29] allows homogeneous packing.

Adsorption and Elution. As a rule, the sample is loaded in the solvent with which the column was previously equilibrated; generally this is a low-molarity NaP or KP solution.

NaP or KP of increasing concentration are generally used to elute proteins. NaP cannot be used at 4° at molarities higher than 0.5 M, because of the limited solubility of Na_2HPO_4. Columns are normally operated under a slight pressure (30–50 cm of water). If controlled by a pump, the flow rate should not be kept higher than that of a column flowing under a slight hydrostatic pressure. The phosphate concentration in the column effluent is usually checked by refractive index measurement, phosphorus analysis, or conductimetry.

Column Regeneration. If elution of adsorbed material is complete, the column may be reequilibrated with the starting buffer and used again

[27] This volume, [26] and [27].

[28] L. Fisher, *in* "Laboratory Techniques in Biochemistry and Molecular Biology" (T. S. Work and E. Work, eds.), Vol. I, p. 151. North Holland Publ., Amsterdam, 1969.

[29] P. J. Flodin, *J. Chromatogr.* 5, 103 (1961).

although, preferably, after removal of the top layer. The same column can be re-used 3 or 4 times.

Recovery of Irreversibly Adsorbed Materials. The HA bed may be extruded from the column and treated in one of the following ways: (a) placed in dialysis bags and dissolved by dialysis against 1 M EDTA, pH 8.0; (b) eluted with 0.1 M NaOH; (c) dissolved in 1 N HCl.

The Adsorption-Elution Process

A systematic exploration of the parameters involved in the chromatography of proteins on HA columns has just begun.[13, 14] Therefore, it may be useful to review briefly the basic features of the adsorption-elution process and to present the limited information available so far.

Adsorption

Adsorption may be done in batch or on a column. Five sets of parameters should be considered: (a) the HA bed, (b) the material to be absorbed, (c) the solvent, (d) the temperature at which adsorption takes place, and (e) the time of contact of the protein solution with HA, respectively.

HA Bed. The total volume of packed HA crystals, V_t (total volume), is equal to the sum of three terms—the volume of the "dry crystals," V_c (crystal volume), the volume of the solvent bound to the HA crystals and inaccessible to the material to be adsorbed, V_i (inner volume), and the volume of the solvent between the HA crystals and accessible to the material to be adsorbed V_0 (outer volume):

$$V_t = V_c + V_i + V_0 \qquad (1)$$

(i) The total volume of the packed HA bed, V_t, can be determined by measuring its dimensions. (ii) The outer volume, V_0, can be determined by measuring the elution volume of a nonadsorbed substance, such as methyl orange, eosin, fuchsin, or methyl red,[7] i.e., the volume of the solvent which leaves an HA column between loading and appearance of the dyestuffs in the effluent. (iii) The inner volume, V_i, can be calculated from the difference, $(V_0 + V_i) - V_0$, the term $(V_0 + V_i)$ being determined by measuring the loss in weight, at 110°, of a known amount of packed HA crystals. (iv) The crystal volume, V_c, may be calculated from the difference, $V_t - (V_0 + V_i)$.

HA preparations, obtained as described in the preparation procedure of Tiselius, packed under stirring and equilibrated with 1 mM KP exhibit linear flow rate *vs.* pressure drop diagrams. A pressure drop (hydrostatic pressure divided by the length of the column) of 10 results in a flow rate of ~ 100 ml/cm²/hour. For these preparations, $V_0 = 0.82$, $V_i = 0.10$, and $V_c = 0.08$ ml/ml HA bed. The density of packed HA

crystals (wet) is equal to 1.17 g/ml. The value found for V_0 is quite reproducible for preparations obtained according to the method described above and is definitely higher than that (0.60–0.75) reported by Levin.[7] Obviously, HA preparations obtained by different procedures, or preparations in which crystals were fractured, will have different properties. Since HA crystals are in the form of lamellae, it is likely that mechanical breakdown does not cause a very large increase in the surface available for adsorption.

The Material To Be Adsorbed. Two parameters are of interest: (1) The amount of material to be adsorbed (this should be established from the known capacity of HA), (2) the concentration of the protein in the solution to be adsorbed. The coexistence of different materials to be adsorbed need also be considered, since their presence will lead to competition for the adsorbing sites and cause displacement effects.

The Solvent. The concentration of eluting ions at the adsorption step is obviously a critical parameter in determining the capacity of HA for a given material to be adsorbed. The presence in the solvent of substances having a stronger affinity for calcium than phosphate, e.g., EDTA and citrate, can decrease the capacity of HA to zero.

Temperature. Temperature will affect the adsorption phenomenon itself (the adsorption isotherm), the ionization of phosphate ions, and the secondary structure of the proteins to be adsorbed. The effect of temperature on adsorption and on phosphate ionization is not important, yet deserves to be investigated in detail. The effect on the protein structure may cause serious changes in their affinity for HA; denatured proteins in 8 M urea have a lower affinity for HA than native proteins.[11]

Time of Contact between Proteins and HA Necessary to Reach Adsorption Equilibrium. If adsorption is done on a column rather than in batch, one should consider the flow rate while loading the protein solution.

Elution

This is generally performed by increasing the concentration of eluting ions, usually phosphate, either stepwise or continuously. Stepwise elution may be used in both batch and column operation; elution with a concentration gradient can be used only with columns. In both cases, the flow rate of the eluent should be maintained within certain limits to avoid a deformation of the chromatographic peaks.

Stepwise Elution. This procedure is very useful when separating two or more adsorbed substances which have known and different elution molarities. Its two main disadvantages, when used with columns, are the following: (i) tailing of the peaks: substances with strongly curved adsorption isotherms and therefore extended elution ranges cannot be eluted by a solvent of constant composition without tailing, unless elution is so strong that the R_f is close to 1.0[5]; (ii) "false peaks": single substances with strongly curved isotherms, may give rise to several peaks, each new concentration step releasing an additional amount of substance.[5]

Gradient Elution. Two parameters are very important in determining resolving power: (i) the length of the column, L; (ii) the slope of the gradient of the column, *grad.* In the usual case of linear concentration gradients, *grad* may be calculated from Eq. 2

$$grad = \frac{\Delta M}{V} \cdot \frac{S}{V_0 / V_t} \qquad (2)$$

where ΔM is the difference in the molarity of phosphate between the initial and the final buffer; V, the total volume of the buffer; S, the cross-sectional area of the column; and V_0 and V_t, the outer and the total volume of the column as already defined. If S and V are expressed in cm^2 and cm^3, respectively, *grad* represents the increase in phosphate molarity per centimeter of column.

When elution is carried out with a linear phosphate gradient, the chromatographic behavior of a protein is characterized by two parameters[13]: (i) the elution molarity, m_{elu}, defined as the phosphate molarity at which the center of the protein peak is eluted; the center of gravity of the peak is given by Eq. 3,

$$\bar{V} = \int Vf \cdot dV \; / \int f \cdot dV \qquad (3)$$

where f is the distribution function of the peak, and V the volume of the solvent; (ii) the width of the peak. This can be calculated as its standard deviation and should be normalized by dividing it by S (Eq. 4).

$$\sigma = [\int (V-\bar{V})^2 f \, dV \; / \int f \, dV]^{1/2} \cdot \frac{1}{S} \qquad (4)$$

Both chromatographic parameters, m_{elu} and σ, depend upon several factors including column length, slope of the gradient and the presence of other chromatographic components. These relationships have been studied in some detail[13, 14] for five proteins endowed with rigid structure and of different sizes: cytochrome c, lysozyme, β-lactoglobulin A, calf skin tropocollagen, and T2 phage.

The main conclusion of this work may be summarized as follows:

In the case of small protein molecules (cytochrome c, lysozyme, β-lactoglobulin A) m_{elu} markedly increased with increasing column length and slope of the gradient (Fig. 1 a–c; Fig. 2 a). In the case of tropocollagen, m_{elu} increases with increasing column length, but is not dependent upon the slope of the gradient (Fig. 1 d; Fig. 2 b). T2 phage particles show an m_{elu} which is independent of both column length and slope of the gradient (Fig. 1 e). The behavior of m_{elu} appears therefore to be different for proteins having different molecular weights.

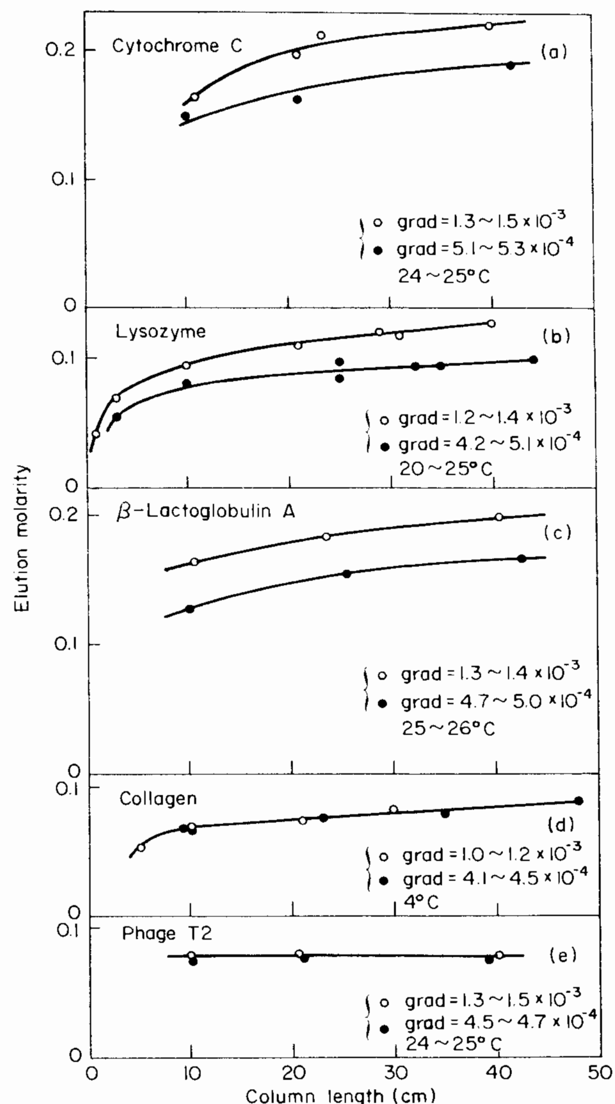

FIG. 1. Elution molarity of (a) cytochrome *c*, (b) lysozyme, (c) β-lactoglobulin A, (d) collagen, and (e) T2 phage, as a function of column length and slope of the gradient Load was 2 mg of protein, except in the case of T2 phage, where 1 A_{260} unit was used. Elution was carried out at room temperature with a linear molarity gradient of potassium phosphate, except in the case of collagen, where elution was done at 4° with sodium phosphate containing 0.15 *M* NaCl and 1 *M* urea. Columns of 1 cm or 0.5 cm diameter were used with flow rates of 30–60 ml/hour or 7–14 ml/hour, respectively. Yields were close to 100% in all cases. The slope of the gradient, the load and the width of the peak have been normalized for a column diameter of 1 cm. [T. Kawasaki and G. Bernardi, *Biopolymers*, 9, 257 (1970)].

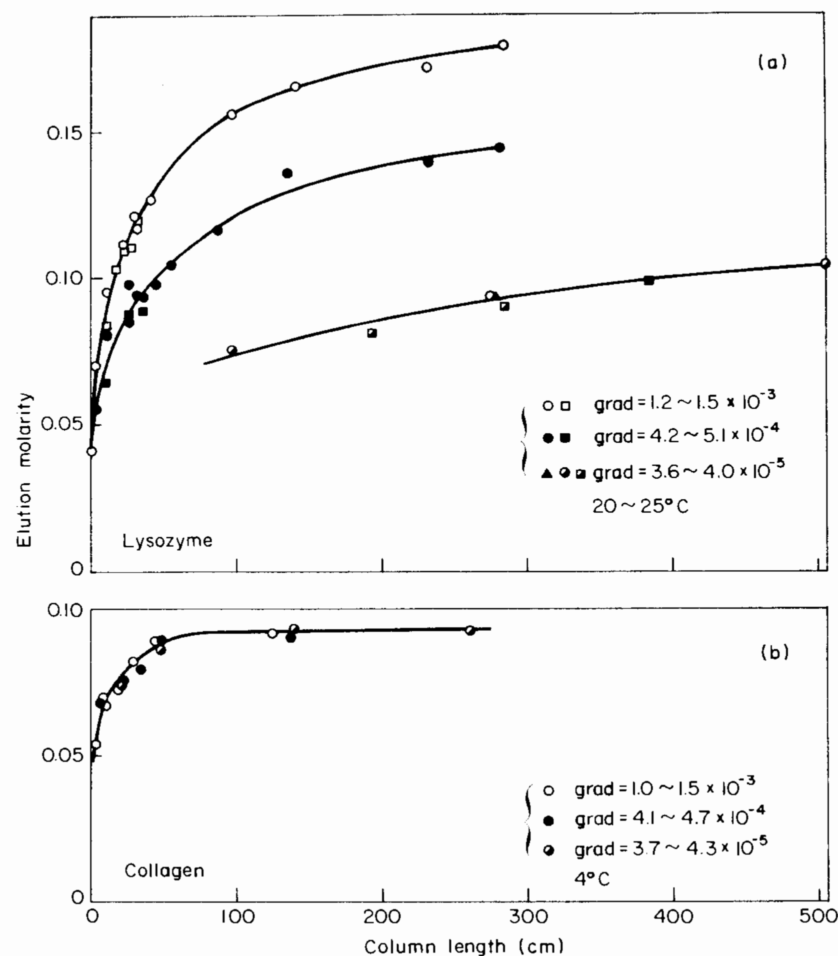

FIG. 2. Elution molarity of lysozyme and collagen as a function of column length and of the slope of the gradient over a wider range of experimental conditions. Loads were (▲) 0.6 mg, (○, ●, ◕) 2 mg (□, ■, ◩), and 10 mg for lysozyme; the load was 2 mg for collagen. Certain values for collagen (◕) were obtained by extrapolation to zero load. In the case of columns longer than 150 cm, several columns connected by capillary tubing were used. Other parameters of the procedure are the same as for Fig. 1. [T. Kasawaki and G. Bernardi, *Biopolymers*, 9, 257 (1970)].

The width of the peak, σ, increases while m_{elu} decreases with increasing load, m_{elu} showing only slight dependence upon load. If two proteins are cochromatographed, the m_{elu} of the lower-eluting one is decreased, whereas that of the highei-eluting one remains the same; this displacement effect decreases with decreasing load (Fig. 3). How-

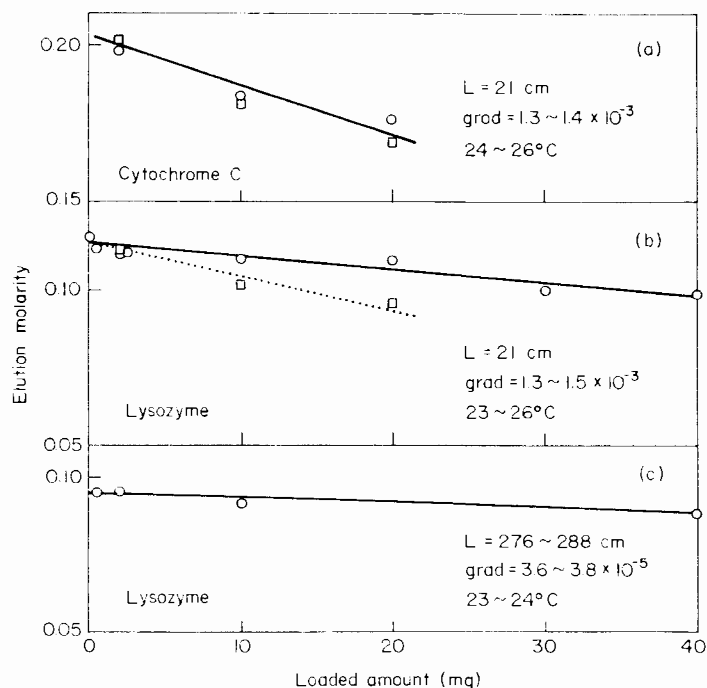

FIG. 3. Elution molarity as a function of load: (a) cytochrome *c*, in the absence (○) and in the presence (□) of the same amount of lysozyme; (b) lysozyme, in the absence (○) and in the presence (□) of the same amount of cytochrome *c*; (c) lysozyme alone, under experimental conditions different from those used in (a) and (b). Other parameters of the procedure are the same as in Fig. 1. [T. Kawasaki and G. Bernardi, *Biopolymers*, 9, 257 (1970)].

ever, the improved separations that one would expect as a consequence of increased load are offset by the concomitant increase in σ (Fig. 4). Within experimental error, σ is not affected by the presence of another chromatographic component (Fig. 4). If the load is small enough, both m_{elu} and σ become essentially independent of both load and presence of other chromatographic components (Fig. 4). Similar observations have been made with collagen.

A lower slope of the gradient leads to a better resolution for the separation of proteins of similar size. In the case of the separation of small proteins among themselves, the best column length increases with the decrease of the slope of the gradient. If proteins are larger, the column length may be shorter and good resolution can still be obtained. In fact, in the case of collagen, a column length of about 80 cm is sufficient, whereas 10 cm is adequate for phage T2.

In the separation of small (10^4 daltons) and large (>10^5 daltons) proteins, the slope of the gradient must be low in order to obtain a good

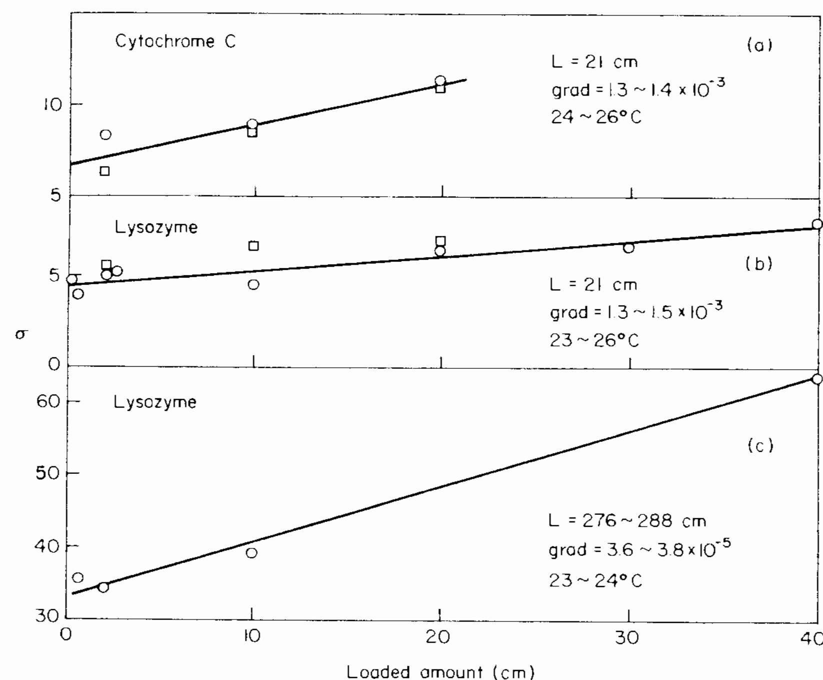

FIG. 4. Standard deviation of the peaks obtained in the experiments of Fig. 3. [T. Kawasaki and G. Bernardi, *Biopolymers*, 9, 257 (1970)].

resolution if the former have a lower elution molarity. No general conclusion can be reached if the latter have a lower elution molarity.

Mechanism of Adsorption and Elution

In order to understand the mechanism of adsorption and elution of proteins, it is important to know which chemical groups are involved in the protein–HA interaction. As far as proteins are concerned, both negative groups, carboxyl and phosphate, and positive groups can interact with HA. The binding sites for the negatively charged groups of proteins (as well as of nucleic acids) are, in all likelihood, the calcium ions at the surface of the HA crystals. The importance of calcium ions in the adsorption process was first recognized by Tiselius *et al.*,[5] who noticed that treatment of HA with compounds having a very strong affinity for calcium, e.g., citrate, decreases its adsorption capacity for proteins. Dyes forming sparingly soluble salts with calcium ions, such as the crystal violet and alizarin dyes, have a strong affinity for HA. In contrast with the negative groups, binding sites for the positive groups of proteins are not known. These may be the same calcium ions or, more likely, negatively charged groups. In this connection it is important to stress that HA crystals are amphoteric and that isoelectric points of

different HA preparations have been found to range from 6.5 to 10.2.[30] HA prepared according to Tiselius *et al.*[5] has a net positive charge in 1 mM KP[20] and is, therefore, a basic HA. A very important difference in the interaction of positive and negative groups of proteins with HA is that the adsorption of positive groups is strongly reduced by salts such as KCl or NaCl whereas that of negative groups is practically not changed.

Chromatography of Amino Acids

Tiselius *et al.*[5] reported that neutral and dicarboxylic amino acids show very weak or no adsorption on the columns. Basic amino acids do have slight affinity; arginine and lysine have an R_f of about 0.4 in mM NaP but display considerable tailing. In contrast, Hofman[31] reported that aspartic acid had by far the lowest R_f of 20 amino acids chromatographed in thin layers of HA.

Chromatography of Synthetic Polypeptides[11,12]

Chromatography of Polypeptides Containing Carboxylic Groups. Poly-L-glutamate and poly-L-aspartate show a rather conspicuous affinity for HA, being eluted at about 0.25 M and 0.35 M KP, respectively. Several observations deserve to be mentioned here in connection with the chromatographic behavior of polypeptides containing carboxylic groups. (1) Statistical copolymers of poly-L-glutamate with phenylalanine, lysine and serine, are eluted at a slightly lower molarity than poly-L-glutamate, the concentration necessary for elution decreasing with decreasing glutamate content. (2) If carboxyl groups are esterified, the polymer is not retained by a column equilibrated with 1 mM KP, as shown by a copolymer of DL-histidine and benzyl-L-glutamate (1:1 molar ratio). (3) Chromatography of poly-L-glutamate and poly-L-aspartate in the presence of 8 M urea caused no change in the eluting molarity. This result, at variance with what is found in the case of proteins endowed with a rigid structure (in which case denaturation causes a drastic drop in the elution molarity of proteins[11]), is not surprising since poly-L-glutamate already is in a random coil configuration at neutral pH. (4) Carrying out the elution with a linear gradient in which the limiting buffers were formed by 1 M KCl–0.001 M KP and 0.5 M KP, respectively (therefore at a practically constant ionic strength, since KP has a ionic strength which is equal to twice its molarity when its dissociation is complete) did not change the eluting molarity of poly-L-glutamate. More recent experiments[12] in the presence of 3 M KCl, also did not alter significantly the phosphate eluting molarity of poly-L-glutamate. These results are identical to those obtained with native DNA, where the phosphate eluting molarity is practically unaffected by the presence of KCl.

[30] S. Mattson, E. Kontler-Andersson, R. B. Miller, and K. Vantras. *Kgl. Lantbruks-Hoegsk. Ann.* **18**, 493 (1951), quoted by S. Larsen, *Nature (London)* **212**, 212 (1966).

[31] A. F. Hofman, *Biochim. Biophys. Acta* **60**, 458 (1962).

Chromatography of Basic Polypeptides. The chromatographic patterns of poly-L-lysine (MW = 75,000) poly-L-arginine (MW = 9500), and poly-L-ornithine (MW = 15,800) are characterized by their strong affinity for HA, except for a fraction not retained by columns equilibrated with 1 mM KP.[32] In fact, these basic polypeptides were so strongly retained that they could not be eluted with gradients reaching a concentration of 1 M KP, and only very poorly with even higher concentrations (see legend of Fig. 5). An exception to this rule is found with a low-molecular-weight poly-L-lysine (MW = 7,000), in which case a large aliquot of the retained material is eluted with the 1 mM–1 M KP gradient as a series of sharp peaks (Fig. 5A); this pattern is not modified by the presence of 7 M urea in the eluting buffer (Fig. 5B), a result to be expected in view of the random-coiled configuration of poly-L-lysine at neutral pH (see above).

The adsorption of the basic polypeptides by HA is strikingly different from that just described for the acid polypeptides, in that adsorbed basic polypeptides can be easily and completely eluted by 3 M NaCl or 3 M KCl.

Chromatography of Neutral Polypeptides. Poly-L-tyrosine, poly-L-proline, and a poly-L-serine of very low molecular weight (MW = 1600) were not retained by HA columns equilibrated with 1 mM KP. Poly-L-histidine also showed this behavior.

Chromatography of Proteins

Only two particular cases will be considered here.

Basic Proteins. The chromatographic behavior of highly basic proteins is similar to that of basic polypeptides. Phosphate concentrations required for elution are high, and NaCl or KCl have strong effects on adsorption.[12] For instance, a lysine-rich histone fraction in which 30% of the amino acid residues consist of lysine is eluted at a high phosphate molarity, 0.55 M NaP. However, if the histone solution is loaded in 0.01 M KP–3 M KCl, the protein is not retained. Another basic protein, lysozyme, normally eluted by about 0.12 M KP, is not retained by HA columns equilibrated with 0.01 M KP–2 M KCl. In fact, it is possible to elute lysozyme with a KCl gradient at a concentration of about 0.25 M. The lowering of the elution molarity by added NaCl had been ob-

[32] This fraction, variable in amount in different polypeptide preparations, consists of ultraviolet-absorbing impurities.

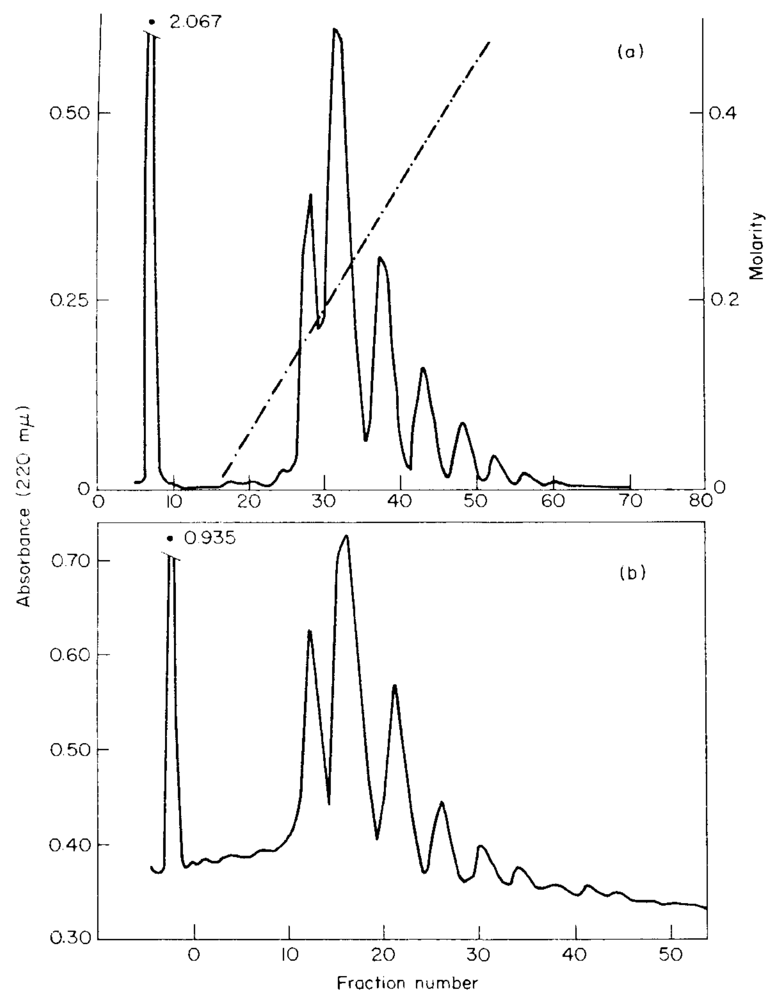

FIG. 5. (a) Chromatography of poly-L-lysine (Yeda-Miles, Rehovoth, Israel; MW = 7000). In 4 ml of 0.01 *M* KP, 59 A_{220} units were loaded onto a 1 × 22 cm column. The unretained sharp peak represents 23% of the load. A 0.01 *M* to 1 *M* KP gradient eluted a series of peaks representing 47% of the load. A similar run with poly-L-lysine (MW = 75,000) showed a first sharp peak representing 20% of the load; a 0.01 to 1 *M* KP gradient did not elute any material; direct elution with 2 *M* KP yielded only 11% of the load. (b) Chromatography of poly-L-lysine (Yeda-Miles; MW = 7000). In 4 ml of 0.01 *M* KP–7 M urea, 57.6 A_{200} units were loaded on to a 1 × 23.5 cm column. The unretained sharp peak represents 10% of the load; a 0.01 *M* to 1 *M* KP gradient in 7 *M* urea eluted a series of peaks representing 56% of the load. [G. Bernardi, unpublished results].

served previously for γ-globulin by Hjertén,[6] who remarked that only γ-globulin, a protein with a relatively high isoelectric point, showed

this effect among the serum proteins. Tiselius *et al.*[5] observed that protamine is adsorbed more strongly at high pH than at neutrality, contrary to the behavior of proteins with acid or neutral isoelectric points, and that both protamine and lysozyme are more easily eluted by cacodylate than by phosphate buffer.

Phosphoproteins. Phosphoproteins represent another special case in that they also have a very high affinity for HA. It has been shown[33] that two egg-yolk phosphoproteins, α- and β-lipovitellin, identical in amino acid and lipid composition but different in their phosphorus content, could be separated easily on HA. When elution was performed with a molarity gradient, instead of the stepwise technique originally used, it could be shown[11] that β-lipovitellin, the electrophoretically slow component, was eluted by 0.4 *M* KP, whereas α-lipovitellin, the fast component, was eluted by 0.75 *M* KP. The third egg-yolk phosphoprotein, phosvitin, a protein in which almost 50% of the amino acid residues are phosphoryl serines, was eluted by an exceptionally high phosphate molarity, 1.2 *M* KP.

The very high eluting molarities required by phosphoproteins in comparision with nucleic acids may be due, in part, to the monoesterified phosphate groups in contrast to the diesterified groups of nucleic acid. Another consideration is that phosphoproteins have runs of phosphorylserine which form areas of a very high density of groups able to interact with HA.

[33] G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta* 44, 96 (1960).
[34] R. W. G. Wyckoff, "Crystal Structures," Wiley (Interscience), New York, 1951.

[30] Preparation of Calcium Phosphate Gel Deposited on Cellulose

By MASAHIKO KOIKE and MINORU HAMADA

Calcium phosphate gel[1] is a common reagent for the purification of enzymes by batchwise procedures. However, it is impossible to use the gel in columns because of poor flow characteristics unless mixed with a suitable filter aid, such as Super-Cel[2,3] or cellulose.[4] Some of the

[1] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. Ser.* B **124**, 297 (1938). See also S. P. Colowick, this series, Vol. 1, [11].
[2] S. M. Swingle and A. Tiselius, *Biochem. J.* **48**, 171 (1951).
[3] A. Tiselius, S. Hjertén, and Ö. Levin, *Arch. Biochem. Biophys.* **65**, 132 (1956).
[4] V. Massey, *Biochim. Biophys. Acta*, **37**, 310 (1960). See also this series, Vol. 9, [52].