The control experiment with labeled ligand alone also serves to check on the radiotriguity of the labeled material. Mr. William Jakoby (personal communication) has informed us of a case in which the tritium in a so-called \( \text{H} \)-labeled nucleotide was largely in a form which was rapidly exchangeable with water. (See also Bradbury and Jakoby \textsuperscript{11}.)

The nucleotide nevertheless appeared "radiochemically pure" by paper chromatography. However, in the dialysis apparatus the exchangeable tritium appeared rapidly in the effluent as a large peak of radioactivity which declined rapidly because of the extremely fast dialysis of \( 3\text{H} \) ions out of the upper chamber. Similarly, an appearance of a transient peak of radioactivity in the effluent on addition of \( \text{T} \)-labeled insulin to the upper chamber has been noted and tentatively ascribed to iodine.

Once it is established by control experiments with labeled ligand alone that the system is reasonably free of the above artifacts, the actual binding measurement can be undertaken. For this purpose, enzyme at a molar concentration of binding sites estimated to be equal to or greater than the expected dissociation constant of the enzyme-ligand complex (see Fig. 2), is added to the upper chamber in the selected buffer, with other additions, such as metal ions, metal chelates, sulfhydryl compounds, as required. The buffer flowing through the lower chamber should be supplemented with all dialyzable components other than the ligand to be tested.

The radioactive ligand is then added to the upper chamber at a molar concentration about one-tenth of that of the anticipated enzyme sites, and the collection of fractions of effluent is begun. With a flow rate of 8 ml per minute in our apparatus, we collect 2-ml samples every 15 seconds. Since the counts per minute per milliliter in the effluent reaches a constant value after 1.5 minutes, additions of unlabeled ligand can then be made every 2 minutes, covering the range from 0.2 to 1.4 times the molar concentration of enzyme sites expected, as indicated in Fig. 2. These additions are made in small volumes (5-10 pl) in order to avoid excessive dilution. The final addition to give 30 times the enzyme concentration, or more when possible, should, when corrected for dilution of the enzyme solution and loss of tritium from the upper chamber, yield a value essentially equal to that obtained in a control sample with labeled ligand and no enzyme. This final value, therefore, serves as an internal control, so that ordinarily there is no need to run control samples without enzyme, once the preliminary tests for artifacts have been carried out.

\textsuperscript{8} S. L. Bradbury and W. E. Jakoby, J. Biol. Chem. 246, 609 (1971).

**[18] Chromatography of Proteins on Hydroxyapatite**

By Giorgio Bernardi

The purpose of the present article is to discuss our present knowledge of the mechanism of adsorption of proteins on hydroxyapatite (HA), particularly as far as the interaction of amino acid side groups with adsorption sites on HA crystals is concerned. The main conclusions arrived at in investigations carried out in the author's laboratory\textsuperscript{11} can be summarized as follows. Two different types of adsorbing sites exist on the surface of HA crystals: calcium sites and phosphate sites. The former appear to bind acidic groups, carboxyls, and phosphates; the latter bind basic groups. This picture fits with the known amphoteric character of HA crystals.\textsuperscript{12} Elution is caused by anions (usually phosphates), which compete with the carboxyl or phosphate groups of proteins for the calcium sites of HA; or by cations (Na\textsuperscript{+}, K\textsuperscript{+} or more effectively, Ca\textsuperscript{2+} or Mg\textsuperscript{2+}), which compete with the basic groups of proteins for the phosphate groups of HA.

\textsuperscript{8} Abbreviations: HA: hydroxyapatite; MW: molecular weight; K\textsubscript{p}: potassium phosphate buffer; NaP: sodium phosphate buffer; if pH indication is given for K\textsubscript{p} or NaP, the pH is 6.8.


G. Bernardi, see Vol. 22; p. 223.


From a practical point of view, our investigations have shown that:
(1) elution of proteins from HA columns can be obtained using a number of clients other than the usual phosphate buffer, pH 6.8; this leads to a remarkable increase in the potentialities of the method; (2) the chromatographic behavior of basic, neutral, and acidic proteins on HA columns operated with different elution systems can be predicted to a considerable extent, thus permitting a less empirical approach to separation problems; in turn, the elution mobilities of proteins in different solvent systems can be used to identify the nature of the interacting amino acid side groups.

Other aspects of chromatography of proteins on HA columns have been reviewed in Volume 22 (p. 325).

Chromatography of Proteins

Basic Proteins. The chromatographic behavior of basic proteins is characterized by relatively high elution molarities when the KP elution system is used; as shown in the table, lysyoxine, cytotochrome e, ribonuclease A, and hystaglysin, and splenic acid DNase are all eluted in the 0.12-0.25 M KP range, a lysine-rich histone (30% lys) was at about 0.55 M KP.

NaCl or KC1 molarity gradients can be used to elute basic proteins: the elution molarities, in this case, are equal to about twice the elution mobilities of phosphate buffer, pH 6.8 (see the table). In the case of the lysine-rich histone, gradient elution was not tried, but a 3 M KC1 step (not indicated) removed the protein from the column.

As indicated in the table, all basic proteins are removed from the columns by very low molarities of CaCl2. The proteins having the lowest KP elution molarities, lysyoxine, and ribonuclease A, are eluted by the initial 0.001 M CaCl2 step; those having increasingly higher KP elution molarities are eluted by the CaCl2 molarity gradient below 0.025 M CaCl2. MgCl2 behaved very similarly to CaCl2 as an eluent of basic proteins.

As shown in the table, elution by KP, pH 7.8, takes place at lower molarities than at pH 6.8, the opposite being true for elution at pH 5.8.

The ratio of the eluting phosphate mobilities at pH 5.8 and pH 6.8 was constant for all basic proteins investigated, the ratio of the eluting mobilities at pH 7.8 and pH 6.8 showed some small fluctuations.

Acidic and Neutral Proteins. These proteins show a distinctly different chromatographic behavior on HA columns compared to basic proteins, being eluted at generally lower molarities by the KP system. When using the RCI system, acidic and neutral proteins are either eluted by very high RCI molarities compared to KP or they are not eluted at all.

### Table: Chromatography of Proteins on HA Columns

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lysocline</th>
<th>Cytotochrome e</th>
<th>Ribonuclease A</th>
<th>Hystaglysin</th>
<th>Splenic acid DNase</th>
<th>CaCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocratic point</td>
<td>10.5-11.0</td>
<td>9.8-10.1</td>
<td>9.0-9.2</td>
<td>8.1-8.2</td>
<td>10.2-10.5</td>
<td>10.2</td>
</tr>
<tr>
<td>Eluting gradients</td>
<td>0.12</td>
<td>0.23</td>
<td>0.22</td>
<td>0.16</td>
<td>0.33</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>0.20</td>
<td>0.15</td>
<td>0.32</td>
<td>0.21</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.35</td>
<td>0.26</td>
<td>0.30</td>
<td>0.21</td>
<td>0.21</td>
</tr>
</tbody>
</table>

**Chromatographic experiments were performed on 1 X 18 cm columns of HA prepared as described in Volume 22, p. 325. RCI gradient was in 0.001 M KP.**

In the CaCl2 chromatography, after loading the proteins on HA columns equilibrated with 0.001 M NaCl, columns were washed with 0.001 M NaCl elution was then performed, in addition, with a 0.001 M CaCl2 step, a 0.001 to 0.005 M CaCl2 molarity gradient, and steps of increasing concentrations (up to 3 M) of CaCl2. When even 3 M CaCl2 could not desorb proteins, columns were washed again with 0.001 M NaCl and proteins were eluted with a 3 M NaCl step. For other experimental details see G. Bernardi, M. G. Giro, and C. Ghidini, Biotechnol. Bioproc. Arts, in press.

- 19-25% of the material was not retained by the columns.
- Aromatic peptide is not known. Chromatographic data suggests that isoelectric point is close to identity.
- Boric acid sodium salt was eluted in one main peak followed by a shoulder, corresponding to the dimer. The elution molarity given is that of the main peak.

- 21-25% of the material was not retained by the columns. Two peaks were eluted by the KP molarity gradient at pH 5.8.

- D. B. Mead and R. G. Bresnick, private communication.
all by KCI mantle gradients up to 3 M. None of the neutral and acidic proteins can be eluted by the column by CaCl2 molarity as high as 3 M. After washing the column with 0.01 M NaCl, all these proteins can, however, be totally eluted by a 1 M NaP step. Spleen exonuclease in proteins whose monosaccharide residues are probably closely packed (tryptophan) resembled neutral and acidic proteins in being not eluted by CaCl2.

For acidic and neutral proteins, elution by KP, pH 7.8, or by KP, pH 5.8, takes place at lower and higher molarities, respectively, compared to KP, pH 6.8. These effects are, therefore, qualitatively the same as for basic proteins; in the case of acidic proteins, they are, however, much stronger than in the case of basic proteins.

Phosphoproteins. Phosphoproteins represent a special case in that they have a very high affinity for HA. It has been shown that the two egg-yolk phosphoproteins, α- and β-lipotrin, which are identical in amino acid and lipid composition, are adsorbed in equal amounts to HA columns. They are easily separated on HA columns. When elution was performed with a sodium gradient, instead of the stepwise technique originally used, it could be shown that β-lipotrin, the electrophoretically slow component, was eluted by 0.4 M KP, whereas α-lipotrin, the fast component, was eluted by 0.75 M KP. The third egg-yolk phosphoprotein, phosvitin, a protein which almost 50% of the amino acid residues are phosphoryl groups, was eluted by an exceptionally high phosphate molarity, 1.2 M KP.

The Chromatographic Behavior of Proteins on HA

This may be summarized and interpreted as follows:

1. The finding that KCl elution molarities of all basic proteins tested are systematically twice as large as the corresponding KP elution molarities may be interpreted as indicating that the proteins are largely protein isoelectric points is caused by the cations of the eluent, since the K+ concentration in the KCl system is roughly the same as that in the KP system.

2. The fact that the elution molarities of basic proteins by CaCl2 are 20-200 times lower than those of NaCl or KCl is in keeping with the suggestion made above that, in the chromatography of basic proteins,


1 The elution molarities of K+ in the KCl system is, in fact, higher by about 0.3 M than that of the KP, pH 6.8, system. This different molarity is presumably due to differences in the activity coefficients of the two salts and to the fact that the pK of the KCl solution, at the elution molarity, is lower than 6.8. In the elution KCl system is exactly the same as that in the KP, pH 5.8, system.

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Elution is caused by the cations, not by the anions of the eluent.

The fact that cations having a very strong affinity for phosphate ions like Ca2+ and Mg2+ are endowed with a much greater eluting power than cations having a weak affinity for phosphate, like K+ and Na+, suggests two important points: that the adsorbing sites for basic proteins (trityle) resemble neutral and acidic proteins in being not eluted by CaCl2.

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which are eluted by KCl. The peak in the 0.2-0.8 M range cannot be eluted by CaCl_2, indicating that it is not KCl. It is interesting to ask whether in these cases CaCl_2 leads to the carboxyl groups of these proteins and strengthens the association by forming bridges to phosphate groups on HA.

7. The data of the table show that the elution behavior of proteins by KCl appears to be controlled by a constant factor at pH 5.8 and decreased (also by a constant factor, but with larger fluctuations) at pH 7.8, compared to the usual pH 6.8. Both effects appear to be much greater for acidic than for basic proteins. A satisfactory explanation for this phenomenon is not yet available.

8. The very high elution velocities required by phosphate buffer in comparison with buffer acids may be due, in part at least, to the monoesterified phosphate groups in contrast to the diesterified groups of the acid radicals. Another consideration is that phosphates have units of phosphoric acid, which form areas of very high density of groups able to interact with HA.

Effect of Secondary and Tertiary Structure of Proteins on Their Chromatographic Behavior on HA Columns.

Investigation by Bernardi and Kawasaki showed that proteins are much less retained or not retained at all by HA columns equilibrated with 0.001 M KCl when they are in their denatured state. It appears that the denaturation of the secondary and tertiary structure of proteins by 8 M urea or heat causes a strong reduction in their interaction with HA. This phenomenon can be explained by the fact that the random coil conformation of the denatured protein causes a decrease in the number of amino acid side groups able to interact per unit of protein surface in contact with HA, and therefore a decrease in the elution velocity of denatured proteins. This decrease may be due to the following reasons: (1) acidic or basic groups present at the surface of the native protein will, in part, disappear from the "surface" of the denatured protein, which is known to have a random coil configuration; (2) local clusters (due to the existence of secondary and tertiary structure) of acidic and basic groups will disappear in the denatured state in favor of a more random distribution over the entire protein "surface."

Chromatography of Synthetic Peptides

Acidic Peptides.

These peptides show a strong affinity for HA; poly-L-glutamate and poly-L-aspartate are eluted at about 0.25 M and 0.35 M KCl, respectively. Elution is not due to the ionic strength of the eluting buffer, but to a specific competition by phosphate ions for HA sites binding carboxyl groups, as shown by the following findings: (1) 3 M KCl does not elute poly-L-glutamate; (2) elution with a linear gradient between 0.001 M KCl to 3 M KCl and 0.5 M KCl first at a practically constant ionic strength, KCl forming a peak which is close to twice its mobility; does not change the phosphate eluting solubility of poly-L-glutamate; (3) stereoisomers of poly-L-glutamate with phenylalanine, lysine, and serine, are eluted by KCl, but at a slightly lower ionic strength than with poly-L-glutamate, the phosphate concentration needed for elution decreasing with decreasing glutamate content. (4) A copolymer of L-lysine and L-arginine (1:1 mol ratio) was not retained by a column equilibrated with 1 M KCl.

Neutral Peptides.

The chromatographic behavior of poly-L-lysine, poly-L-glutamic, and poly-L-ornithine is characterized by an even stronger affinity for HA than that of acidic polypeptides. In fact, basic polypeptides are so strongly retained by HA columns that they cannot be eluted by KCl gradients reaching a mobility of 1. An exception to this general rule was found with the low-molecular-weight poly-L-lysine samples (MW = 20000); in this case a large aliquot of the retained material was eluted between 0.1 and 0.2 M KCl as a series of peaks (see Fig. 5 of Bernardi). All basic polypeptides investigated here, however, can be completely eluted by 3 M NaCl or 3 M KCl, or by mobility gradients of these salts. Poly-L-ornithine, in contrast, is eluted at about 1.75 M KCl or NaCl. Both neutral gradients in 0.01 M KCl or 0.01 M NaCl, respectively. Basic polypeptides can also be eluted by rather weak mobilities of CaCl_2. This solvent can also elute material which is not eluted by KCl.

Neural Peptides.

Poly-L-dipeptide, poly-L-serine, poly-L-tyrosine, and poly-L-proline are not retained by HA columns equilibrated with 1 M KCl. The behavior of the latter two polypeptides is in agreement with the fact of interaction of nonaqueous amino acids with HA (see below).

It can be concluded that the chromatographic behavior of synthetic polypeptides fits very well with the general ideas derived from the study of proteins. A complication existing in the case of synthetic polypeptides is due to the fact that their elution mobilities appear to
depend upon their molecular weights, at least when this is below a certain level.

Adsorption of Amino Acids

- Tissiaus et al. found that neutral and dicarboxylic amino acids show very weak or no absorption on HA columns. Basic amino acids were found to have slight affinity, arginine and lysine having an $R_f$ of about 0.4 in 0.1 M NaP, but displaying a considerable tailing. In contrast, Hofmann reported that aspartic acid has by far the lowest $R_f$ of 20 amino acids chromatographed on thin layers of HA. Eilen and Howell found, using light-scattering measurements, that glycine, tyrosine, arginine, and histidine fail to interact with HA at pH 7, whereas lysine, aspartic and glutamic acids, serine, threonine and hydroxyproline do interact.

In order to resolve the apparent conflict of these results, Bernardi et al. investigated the adsorption isotherms of amino acids on HA equilibrated with 1 mM KP, pH 6.8 or 7.8.

At pH 6.8, all polar amino acids tested (arginine, lysine, histidine, aspartic acid, glutamic acid, serine) were adsorbed to a much greater extent. Among nonpolar amino acids, alanine, valine, and phenylalanine were not adsorbed at all; glycine, but not glycyglycine, was adsorbed. If 0.01 M KCl was present in the equilibration buffer, arginine was not adsorbed anymore, whereas aspartic acid was slightly more adsorbed; histidine and serine were not affected in their adsorption properties.

At pH 7.8, arginine and lysine were more strongly adsorbed than at pH 6.8. In contrast, acidic amino acids were less adsorbed, glutamic acid being not adsorbed at all. The adsorption of histidine and serine varied very little at the two different pH values. All other amino acids, including alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan, proline, hydroxyproline, glutamine, asparagine, and glycine, were not adsorbed.

In conclusion: (1) adsorption on HA was only found with polar amino acids; (2) the adsorption of basic amino acids is decreased by 0.01 M KCl, in agreement with the similar effect on basic polypeptides and proteins. Other features, like the adsorption of glycine at pH 6.8 and the increased adsorption of aspartic acid in the presence of 0.01 M KCl, are not easily explainable at the present time. Similarly, the effect

of pH on the adsorption of amino acids does not show any clear correlation with the results obtained with proteins. The finding that histidine and serine are adsorbed raises the question of the possible intervention of hydroxylamine and histidine in the protein-HA interaction. This seems unlikely, however, in view of the fact that poly-L-histidine and poly-L-serine are not retained by HA equilibrated with 0.001 M KP.