The control experiment with labeled ligand alone also serves to check on the radiopurity of the labeled material. Dr. William Jakoby (personal communication) has informed us of a case in which the tritium in a so-called ^{3}H-labeled nucleotide was largely in a form which was rapidly exchangeable with water. (See also Bradbury and Jakoby.[12]) 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}H ions out of the upper chamber. Similarly, an appearance of a transient peak of radioactivity in the effluent on addition of ^{125}I-labeled insulin to the upper chamber has been noted and tentatively ascribed to iodide.[11]

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 chelators, 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 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 μl) in order to avoid excessive dilution. The final addition to give 30 times the enzyme concentration, or more when feasible, should, when corrected for dilution of the enzyme solution and loss of isotope 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.

[12] S. L. Bradbury and W. B. Jakoby, *J. Biol. Chem.* **246**, 6929 (1971).

The actual concentrations of enzyme and ligand used in a particular case will depend on the dissociation constant of the system. The radioactivity added to the upper chamber has to be high, of the order of 10^6 to 10^7 cpm, because of the high dilution factor (ca. 10^3 to 10^4) during flow dialysis. Nevertheless, the high specific activities of labeled compounds make it feasible to attain the required number of counts per minute at very low molarities. Thus, with ^{32}P-labeled ATP (4–10 Ci/mmole), dissociation constants as low as 10^{-7} M have been readily measured with this method.[4,5] With [^{14}C]glucose (0.15 Ci/mmole), a K_{diss} around 10^{-5} M is easily measurable.[1,2] In cases where dissociation constants are too low for measurement by this method, the procedure would nevertheless be useful as a means of detection of ligand-binding proteins with such characteristics.

[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[1]), 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[2–4] 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 amphotheric character of HA crystals.[5] 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^+, K^+ or, more effectively, Ca^{2+} or Mg^{2+}), which compete with the basic groups of proteins for the phosphate groups of HA.

[1] Abbreviations: HA, hydroxyapatite; MW, molecular weight; KP, potassium phosphate buffer; NaP, sodium phosphate buffer; if no pH indication is given for KP or NaP, the pH is 6.8.

[2] G. Bernardi and T. Kawasaki, *Biochim. Biophys. Acta* **160**, 301 (1968).

[3] G. Bernardi, see Vol. 22, p. 325.

[4] G. Bernardi, M. G. Giro, and C. Gaillard, *Biochim. Biophys. Acta* **278**, 409 (1972).

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

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 eluents 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 molarities 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, lysozyme, cytochrome *c*, ribonuclease A, α-chymotrypsin, and spleen acid DNase are all eluted in the 0.12–0.23 *M* KP range; a lysine-rich histone (30% lysine) was eluted at about 0.55 *M* KP.

NaCl or KCl molarity gradients can be used to elute basic proteins: the elution molarities, in this case, are equal to about twice the eluting molarities 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* KCl step completely removed the protein from the column.

As indicated in the table, all basic proteins are removed from the columns by very low molarities of $CaCl_2$. The proteins having the lowest KP elution molarities, lysozyme, and ribonuclease A, are eluted by the initial 0.001 *M* $CaCl_2$ step; those having increasingly higher KP elution molarities are eluted by the $CaCl_2$ molarity gradient below 0.025 *M* $CaCl_2$. $MgCl_2$ behaved very similarly to $CaCl_2$ 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 molarities at pH 5.8 and pH 6.8 was constant for all basic proteins investigated; the ratio of the eluting molarities 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 KCl system, acidic and neutral proteins are either eluted by very high KCl molarities compared to KP or they are not eluted at

ELUTION MOLARITIES OF SOME PROTEINS FROM HA COLUMNS[a]

Protein	Isoelectric point	Eluting solvents: KP 6.8	KP 7.8	KP 5.8	KCl	$CaCl_2$
Lysozyme	10.5–11.0[f]	0.12	0.08	0.15	0.25	0.001
Cytochrome *c*	9.8–10.1[g]	0.23	0.20	0.30	0.48	0.007
RNase A	9.7[h]	0.12	0.09	0.15	0.23	0.001
α-Chymotrypsin[b]	8.1[i]	0.16	0.10	0.20	0.32	0.01
Spleen acid DNase	10.2[j]	0.22	0.115	0.32	0.44	0.02
Spleen acid exonuclease[c]	—	0.125	0.065	0.195	0.25	>3.0
Myoglobin	7[g]	0.12	0.08	0.17	0.80	>3.0
Snail acid DNase	5.9[k]	0.11	0.04	0.20	0.54	>3.0
Pancreatic DNase	4.7[l]	0.04	0.01	0.12	0.4	>3.0
Bovine serum albumin[d]	4.7[m]	0.06	0.01	0.17	>3.0	>3.0
Pepsin[e]	1[n]	0.03	0.01	0.08 0.12	>3.00	>3.0

[a] Chromatographic experiments were performed on 1 × 18–23 cm columns of HA prepared as described in Volume 22, p. 325. KCl gradients were in 0.01 *M* KP. In the $CaCl_2$ chromatograms, after loading the proteins on HA columns equilibrated with 0.001 *M* NaP, columns were washed with 0.001 *M* NaCl; elution was then performed, in succession, with a 0.001 *M* $CaCl_2$ step, a 0.001 to 0.05 *M* $CaCl_2$ molarity gradient, and steps of increasing concentrations (up to 3 *M*) of $CaCl_2$. When even 3 *M* $CaCl_2$ could not desorb proteins, columns were washed again with 0.001 *M* NaCl and proteins were eluted with a 1 *M* NaP step. For other experimental details see G. Bernardi, M. G. Giro, and C. Gaillard, *Biochim. Biophys. Acta*, in press.

[b] 19–23% of the material was not retained by the columns.

[c] Isoelectric point is not known. Chromatographic data suggest that isoelectric point is close to neutrality.

[d] Bovine serum albumin was eluted in one main peak followed by a shoulder, corresponding to the dimer.[e] The elution molarity given is that of the main peak.

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

[f] P. Jollès, *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 4, p. 431. Academic Press, New York, 1960.

[g] E. G. Young, *in* "Comprehensive Biochemistry" (M. Florkin and E. H. Stotz, eds.), Vol. 7, p. 25. Elsevier, New York, 1963.

[h] L. B. Barnett and H. B. Bull, *Arch. Biochem. Biophys.* **89,** 167 (1960).

[i] E. A. Anderson and R. A. Alberty, *J. Phys. Colloid Chem.* **52,** 1345 (1948).

[j] G. Bernardi, E. Appella, and R. Zito, *Biochemistry* **3,** 1419 (1965).

[k] J. Laval, Thesis, University of Paris, 1970.

[l] U. Lindberg, *Biochemistry* **6,** 355 (1967).

[m] G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.* **60,** 1633 (1956).

[n] G. E. Perlmann, *Advan. Protein Chem.* **10,** 23 (1955).

[o] D. B. Menzel and E. G. Richards, private communication.

all by KCl molarity gradients up to 3 *M*. None of the neutral and acidic proteins can be removed by the columns by $CaCl_2$ molarities as high as 3 *M*. After washing the columns with 0.001 *M* NaCl, all these proteins can, however, be totally eluted by a 1 *M* NaP step. Spleen exonuclease (a protein whose unknown isoelectric point is probably close to neutrality) resembled neutral and acidic proteins in being not eluted by $CaCl_2$.

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[6] that the two egg-yolk phosphoproteins, α- and β-lipovitellin, which are identical in amino acid and lipid composition, but different in their protein phosphorus contents can be easily separated on HA columns. When elution was performed with a molarity gradient, instead of the stepwise, technique originally used, it could be shown[2] 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 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 elution of basic proteins is caused by the cations of the eluents, since the eluting K^+ concentration in the KCl system is roughly the same as that in the KP system.[7]

2. The fact that the eluting molarities of basic proteins by $CaCl_2$ 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, 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 Ca^{2+} and Mg^{2+} 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 are to be identified with phosphate groups at the surface of the crystals and that elution of basic proteins takes place because of a competition between the cations of the eluent and the basic amino acid side groups of proteins for phosphate sites on HA, or, in other words, that elution of basic proteins is not simply a ionic strength effect.

3. Acidic and neutral proteins behave similarly to acidic polypeptides and nucleic acids[2,4,8,9] in that their adsorption on HA is little, or not at all, affected by NaCl, KCl, or $CaCl_2$. An explanation for this behavior is that the adsorption of acidic and neutral proteins, like that of acidic polypeptides, phosphoproteins, and nucleic acids, is due only, or partly, to the interaction of their acidic groups with calcium sites at the surface of HA crystals. Elution is, therefore, expected to be caused by anions able to compete with the macromolecules for the calcium sites on HA. It is not surprising that anions having little affinity for calcium, like chloride, are poor eluents compared to the phosphates normally used as eluents.

4. Basic proteins are eluted from HA columns at relatively high molarities of phosphate, pH 6.8. The five proteins listed in the table are all eluted in the 0.12–0.23 *M* phosphate range; the lysine-rich histone is eluted at an even higher molarity, 0.55 *M*. In contrast, neutral and acidic proteins seem to be eluted at low phosphate molarities, in the 0.03–0.12 *M* range (phosphoproteins are an exception to this rule, see point 7, below). This different behavior may be understood in the following terms: the usual eluents, NaP or KP, pH 6.8, while very effective, because of their phosphate ions, in competing with the carboxyl groups of proteins for the calcium sites on HA, are much less effective in competing with the basic groups of the proteins for the phosphate sites on HA because of the low affinity of Na^+ or K^+ for such groups. Therefore, the usual phosphate buffers are good eluents for acidic proteins, as well as for acidic polypeptides, phosphoproteins, and nucleic acids and poor eluents for basic proteins. Conversely, $CaCl_2$ is an excellent eluent for basic proteins and a very poor one for acidic proteins.

5. An unexpected finding obtained with spleen exonuclease, myoglobin, snail acid DNase, and pancreatic DNase is that these proteins,

[6] G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta* **44**, 96 (1960).

[7] The eluting concentration of K^+ in the KCl system is, in fact, higher by about 30% than that of the KP, pH 6.8, system. This difference may, however, be due to a difference in the activity coefficients of the two salts and/or to the fact that the pH of the KCl solution, at the eluting molarity, is lower than 6.8 (the eluting K^+ concentration in the KCl system is practically the same as that in the KP, pH 5.8, system).

[8] G. Bernardi, *Biochim. Biophys. Acta* **174**, 423, 435, 449 (1969).

[9] G. Bernardi, see Vol. XXI, p. 95.

which are eluted by KCl molarities in the 0.2–0.8 *M* range cannot be eluted by $CaCl_2$ molarities as high as 3 *M*. It is interesting to ask whether in these cases Ca^{2+} binds to the carboxyl groups of these proteins and strengthens the adsorption by forming bridges to phosphate groups on HA.

6. The data of the table show that the elution molarity of proteins by KP, appears to be increased 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.

7. The very high eluting molarities required by phosphoproteins in comparison with nucleic acids may be due, in part at least, to the monoesterified phosphate groups in contrast to the diesterified groups of nucleic acids. Another consideration is that phosphoproteins have runs of phosphorylserines, 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.

Investigations by Bernardi and Kawasaki[2] showed that proteins are much less retained or not retained at all by HA columns equilibrated with 0.001 *M* KP when they are in their denatured state. It appears that the disruption of the secondary and tertiary structures 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 configuration 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 molarity 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 structures) 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 Polypeptides

Acidic Polypeptides. These polypeptides show a strong affinity for HA; poly-L-glutamate and poly-L-aspartate are eluted at about 0.25 *M* and 0.35 *M* KP, 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* KP–1 *M* KCl and 0.5 *M* KP (therefore at a practically constant ionic strength, KP having a ionic strength which is close to twice its molarity) does not change the phosphate eluting molarity of poly-L-glutamate; (3) statistical copolymers of poly-L-glutamate with phenylalanine, lysine, and serine, are eluted by KP at a slightly lower molarity than poly-L-glutamate, the phosphate concentration needed for elution decreasing with decreasing glutamate content. (4) A copolymer of DL-histidine and benzyl-L-glutamate (1:1 molar ratio) was not retained by a column equilibrated with 1 m*M* KP.

Chromatography of poly-L-glutamate and poly-L-aspartate in the presence of 8 *M* urea caused no change in the phosphate eluting molarity. This result, at variance with what is found in the case of proteins endowed with secondary and tertiary structure (in which case denaturation causes a drastic drop in the elution molarity) is not surprising since both carboxylic polymers already are in a random coil configuration at neutral pH.

Basic Polypeptides. The chromatographic behavior of poly-L-lysine, poly-L-arginine, 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 KP gradients reaching a molarity of 1. An exception to this general rule was found with the low-molecular-weight poly-L-lysine samples (MW = 7000); in this case a large aliquot of the retained material was eluted between 0.1 and 0.5 *M* KP as a series of peaks (see Fig. 5 of Bernardi[3]).

All basic polypeptides investigated can, however, be completely desorbed by 3 *M* NaCl, or 3 *M* KCl, or by molarity gradients of these salts. Poly-L-ornithine, for instance, is eluted at about 1.75 *M* by KCl or NaCl molarity gradients in 0.01 *M* KP or 0.01 *M* NaP, respectively. Basic polypeptides can also be eluted by rather weak molarities of $CaCl_2$. This solvent can also elute material which is not eluted by KP.

Neutral Polypeptides. Poly-L-histidine, poly-L-serine, poly-L-tyrosine and poly-L-proline are not retained by HA columns equilibrated with 1 m*M* KP. The behavior of the latter two polypeptides is in agreement with the lack of interaction of nonpolar 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 molarities appear to

depend upon their molecular weights, at least when this is below a certain level.

Adsorption of Amino Acids

Tiselius *et al.*[10] 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 0.001 *M* NAP,[1] but displaying a considerable tailing. In contrast, Hofman[11] reported that aspartic acid has by far the lowest R_f of 20 amino acids chromatographed on thin layers of HA. Elden and Howell[12] 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.*[4] investigated the adsorption isotherms of amino acids on HA equilibrated with 1 m*M* 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 rather similar extent. Among nonpolar amino acids, alanine, valine, and phenylalanine were not adsorbed at all; glycine, but not glycylglycine, 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

[10] A. Tiselius, S. Hjertén, and O. Levin, *Arch. Biochim. Biophys.* **65**, 132 (1956).
[11] A. F. Hofman, *Biochim. Biophys. Acta* **60**, 458 (1962).
[12] H. Elden and D. S. Howell, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **19**, 142 (1960).

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 hydroxyamino acids 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.