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CHROMATOGRAPHY OF POLYPEPTIDES AND PROTEINS ON HYDROXYAPATITE COLUMNS: SOME NEW DEVELOPMENTS

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SUMMARY

The chromatographic behavior of polypeptides and proteins and the adsorption of amino acids on hydroxyapatite have been investigated in order to reach a better understanding of the adsorption–elution process and to obtain further information on the amino acid side-groups involved in the protein–hydroxyapatite interaction.

The main findings of the present work are the following: (1) basic proteins can be eluted from hydroxyapatite columns, not only by the usual phosphate buffer (pH 6.8), but also by NaCl or KCl solutions. In contrast, the interaction of neutral and acidic proteins with hydroxyapatite by very low (0.001–0.02 M) concentrations of $CaCl_2$, whereas this solvent does not affect the adsorption of neutral and acidic proteins even at very high (3 M) concentrations.

The above results, as well as other findings concerning the chromatographic behavior of synthetic polypeptides and the adsorption properties of amino acids, very strongly support the idea that crystals have two different adsorption sites on their surface, to be identified with phosphate and calcium, and that these are responsible for the binding of basic and acidic side groups of proteins, respectively.

INTRODUCTION

In order to understand the mechanism of adsorption of proteins on hydroxyapatite columns, it is important to know which amino acid side-groups are involved in the protein–hydroxyapatite interaction and what type of interaction takes place. Chromatography of a number of water-soluble homo- and heteropolypeptides on hydroxyapatite columns led to the conclusion that only those containing carboxyl sidegroups (poly(L-aspartate), poly(L-glutamate) and some of their copolymers) are strongly adsorbed, implying that carboxyl groups are responsible for the interaction of proteins with hydroxyapatite[1].

Further work on synthetic polypeptides[2], while confirming the previous results

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obtained with carboxylic polypeptides, has shown that: (a) basic polypeptides also interact with hydroxyapatite, thus correcting the negative conclusion previously reached[1]; (b) the interaction of basic polypeptides with hydroxyapatite is different from the interaction of acidic polypeptides in that it is strongly affected by salts like NaCl or KCl, which can cause the elution of basic, but not of acidic polypeptides.

These observations prompted further investigations on the chromatographic behavior of proteins on hydroxylapatite.

Two other problems which have been investigated in the present work are: (a) the operation of hydroxylapatite columns using phosphate buffers at two pH values, 5.8, 7.8 different from the usual pH 6.8, and the effect of these different experimental conditions on the elution molarities of several proteins; (b) the adsorption of amino acids on hydroxylapatite.

MATERIALS AND METHODS

Materials

The following polypeptides, purchased from Miles-Yeda, Ltd., (Rehovoth, Israel) were used in the present work: three poly(L-lysine) preparations having molecular weights of 75 000, 7000, and 1500–8000, respectively; poly(L-arginine) (mol. wt 9500), poly(L-ornithine) (mol. wt 15 800); all molecular weights quoted are those given by the manufacturer.

Lysozyme, ribonuclease A, α-chymotrypsin, pepsin, and pancreatic deoxyribonuclease, Code D, were obtained from Worthington (Freehold, N.J.); bovine serum albumin, fraction 5, from Armour (Chicago, Ill.); horse heart cytochrome *c*, type VI, from Sigma (St. Louis, Mo.); horse heart myoglobin from Koch–Light Laboratories (Colnbrook, Bucks, England). Spleen acid deoxyribonuclease B and spleen acid exonuclease were preparations obtained according to Bernardi *et al.*[3], and Bernardi and Bernardi[4], respectively. Snail hepatopancreas acid deoxyribonuclease[5] was prepared according to a procedure to be described elsewhere. A lysine-rich histone fraction F_1 from calf thymus (30% lysine) was the gift of Dr M. Champagne, Strasbourg, France.

Amino acids were obtained from Calbiochem (Los Angeles, Calif.), except for glycylglycine which was purchased from Fluka (Buchs, Switzerland).

Chromatography of proteins and polypeptides on hydroxyapatite columns

Hydroxylapatite was prepared as described elsewhere[2]. As a rule, chromatographic experiments were performed as follows: small amounts of proteins or polypeptides were dissolved in or dialyzed against the equilibrating buffer and loaded on 1 cm × 18–23 cm hydroxyapatite columns. After washing the columns with about 30 ml of equilibrating buffer, elution was performed using one of the following systems: (A) a 0.01 to 0.5 M gradient of potassium phosphate buffer, pH 6.8; (C) a 0.01 to 0.5 M gradient of potassium phosphate buffer, pH 7.8; in the case of pepsin, elution at pH 7.8 was done with a 0.001 to 0.2 M potassium phosphate buffer gradient; (D) a 0.1 to 0.5 M gradient of potassium phosphate buffer, pH 5.8; equilibration of the columns at pH 5.8 was preceded by extensive washing of the columns with 0.5 M potassium phosphate buffer, pH 5.8; in the case of pepsin, elution at pH 5.8 was done with a 0.01 to 0.2 M potassium phosphate buffer gradient;

(E) in this case the procedure used was different: after loading the proteins on hydroxyapatite columns, equilibrated with 0.001 M sodium phosphate buffer, pH 6.8, columns were washed with 0.001 M NaCl; elution was then performed, in succession, with a 0.001 M $CaCl_2$ step, a 0.001–0.05 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 sodium phosphate buffer step. In all cases, flow rate was close to 20 ml/h.

The slopes of the gradients, $grad = \Delta M/V$, where ΔM is the difference in molarity between the starting and the final eluent and V is the total volume of the gradient, were equal to 2.4 M/l in case A and C (except for pepsin in the latter system, where it was 1 M/l), 2.0 M/l in case D, (except for pepsin where it was 1 M/l), 5–7.5 M/l in case B, and 0.25 M/l in case E. Molarity gradient elution was performed using Technicon (Chauncey, N.J.) Varigrads with 100 or 150 g of buffers in the chambers. Elution molarities were checked by using an immersion refractometer (Zeiss; Oberkochen, Germany). The molarity of elution of a protein was taken as the molarity at which the maximum of the peak was eluted.

Concentrations of materials in the chromatographic fractions were determined by measuring their enzymatic activities (deoxyribonucleases and exonuclease) or their ultraviolet absorption at 220 nm (polypeptides), 410 nm (myoglobin), 485 nm (cytochrome *c*) and 280 nm (other proteins).

It should be noted that, in the present work, standard chromatographic conditions were used, the comparison of the chromatographic behaviors of different proteins being performed with identical or very close values of column length, and slope of the molarity gradient. These comparisons are valid because of the very similar dependence of elution molarity of small globular proteins upon these parameters[6]. Loads were in all cases small enough not to affect the elution molarities.

Adsorption of aminoacids on hydroxyapatite crystals

This was investigated using the following procedure. Hydroxyapatite was washed, on a column, with one bed volume of 0.1 M potassium phosphate buffer and 3–5 vol. of 0.001 M potassium phosphate buffer having pH values of 6.8 and 7.8, respectively. This procedure was ineffective for equilibrating hydroxyapatite at pH 5.8, as shown by the fact that the column effluent had a higher pH even after extensive washing. Hydroxyapatite was then extruded from the column, suspended in 0.001 M potassium phosphate buffer of appropriate pH and allowed to sediment overnight in 10-ml cylinders. The volume of settled hydroxyapatite was adjusted to 5 ml by suspending the top layers in potassium phosphate buffer and syphoning off the excess of hydroxylapatite. 5 ml of potassium phosphate buffer were then used to transfer all hydroxylapatite into a 50-ml erlenmeyer flask. 10 ml of amino acid solution of known concentration were then added to the hydroxyapatite suspension, which was very gently shaken for 15 min in a water bath thermostated at 25 °C. Control experiments showed that adsorption equilibrium was reached under these conditions. The hydroxyapatite suspension was then allowed to sediment; the supernatant was withdrawn and its concentration in amino acid was measured using the ninhydrin reaction. In the pH 7.8 buffer, the supernatant was slightly turbid because very fine hydroxyapatite particles did not sediment at this pH; in this case the super-

natant was clarified prior to the ninhydrin reaction by acidification to pH 3.0 with a small volume of 0.02 M HCl.

RESULTS

Chromatography of polypeptides

The chromatographic behavior of poly(L-lysine), poly(L-arginine) and poly(L-ornithine) was characterized by a strong affinity for hydroxyapatite. The basic polypeptides were so strongly retained by hydroxylapatide columns that they could not be eluted by potassium phosphate buffer gradients reaching a molarity of 1. An exception to this general rule was found with the low molecular weight poly(L-lysine) samples; in this case a large aliquot of the retained material was eluted between 0.1 and 0.5 M potassium phosphate buffer as a series of peaks (see Fig. 1a and Fig. 5 of ref. 2).

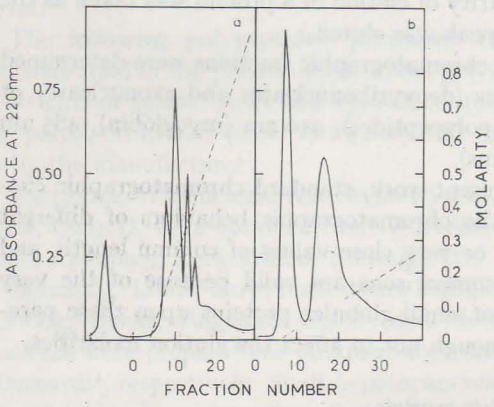

Fig. 1. (a) Chromatography of a low molecular weight poly(L-lysine) sample (Miles-Yeda, code No. 71–120A; mol. wt 1500–8000; 20 mg) dissolved in and dialyzed against 0.001 M potassium phosphate buffer. Load was 39 $A_{220\ nm}$ units. Elution was done with a 0.001–3.0 M potassium phosphate buffer molarity gradient. 3.1-ml fractions were collected during loading and washing, 6.2-ml fractions during gradient elution. 5% of the loaded $A_{220\ nm}$ was not retained by the column, 50% was recovered in the fractions eluted with the gradient. This result is similar to that reported for a different poly(L-lysine) sample (Fig. 5 of ref. 2). (b) Chromatography of the same poly(L-lysine) sample (22 mg) used in the experiment of (a) under different experimental conditions. In this case the sample was dissolved in, but not dialyzed against, 0.001 M sodium phosphate buffer. Load was 41 $A_{220\ nm}$ units. After rinsing the column with 0.001 M NaCl and 0.001 M $CaCl_2$, elution was performed with a 0.001–0.5 M $CaCl_2$ molarity gradient. 3.3-ml fractions were collected. 34% of the loaded $A_{220\ nm}$ was not retained by the column, 62% was recovered in the fractions eluted with the gradient.

All basic polypeptides investigated could, however, be completely desorbed by 3 M NaCl, or 3 M KCl, or by molarity gradients of these salts. Poly(L-ornithine), for instance, was eluted at about 1.75 M by KCl or NaCl molarity gradients in 0.01 M potassium phosphate buffer or 0.01 M sodium phosphate buffer, respectively. Basic polypeptides could also be eluted by rather weak molarities of $CaCl_2$. This solvent could also elute material which was not eluted by potassium phosphate buffer (compare Figs 1a and 1b).

All basic polypeptides used contained a fraction, forming 10 to 30% of the total loaded absorbance at 220 nm (except in the case of the poly(L-arginine) preparation where it formed 60%), which was not retained by hydroxyapatite columns equilibrated with 0.001 M potassium phosphate. It is likely that this non-retained fraction was mainly or exclusively formed by ultraviolet-absorbing impurities, since it was dialyzable to a large extent (Fig. 1) and since ninhydrin-positive material was practically absent from it. The presence of very high levels of these ultraviolet absorbing impurities (in spite of the dialysis step used prior to the chromatography) in the basic polypeptides previously used[1], along with the very low $A_{220\ nm}$ of polypeptides containing no carboxyl groups, was responsible for our former conclusion that basic polypeptides were not retained by hydroxyapatite columns[1].

As far as acidic polypeptides are concerned no effect of KCl had been previously detected on the adsorption of poly(L-glutamic acid)[1]. In the present work, it was verified that native calf thymus DNA and poly(L-glutamic acid) could not be eluted from the columns by either 3 M KCl or 3 M $CaCl_2$.

Chromatography of proteins

The chromatographic behavior of basic proteins is characterized by relatively high elution molarities when using the phosphate, pH 6.8, elution system; as shown in Table I, lysozyme, cytochrome *c*, ribonuclease A, α-chymotrypsin, and spleen acid deoxyribonuclease are all eluted in the 0.12–0.23 M potassium phosphate buffer,

TABLE I

ELUTION MOLARITIES OF SOME PROTEINS FROM HYDROXYAPATITE COLUMNS

Protein	*Isoelectric point*	*Refs*	*Eluting solvents*				
			Potassium phosphate pH 6.8	*Potassium phosphate pH 7.8*	*Potassium phosphate pH 5.8*	*KCl*	*$CaCl_2$*
Lysozyme	10.5–11.0	16	0.12	0.08	0.15	0.25	0.001
Cytochrome *c*	9.8–10.1	17	0.23	0.20	0.30	0.48	0.007
Ribonuclease	9.7	18	0.12	0.09	0.15	0.23	0.001
α-Chymotrypsin*	8.1	19	0.16	0.10	0.20	0.32	0.01
Spleen acid deoxyribonuclease	10.2	20	0.22	0.115	0.32	0.44	0.02
Spleen acid exonuclease**			0.125	0.065	0.195	0.25	> 3.0
Myoglobin	7	17	0.12	0.08	0.17	0.80	> 3.0
Snail acid deoxyribonuclease	5.9	5	0.11	0.04	0.20	0.54	> 3.0
Pancreatic deoxyribonuclease	4.7	21	0.04	0.01	0.12	0.4	> 3.0
Bovine serum albumin***	4.7	22	0.06	0.01	0.17	> 3.0	> 3.0
Pepsin§	1	23	0.03	0.01	{ 0.08 { 0.12	> 3.0	> 3.0

* 19–23% of the material was not retained by the columns.

** No isoelectric point data are available.

*** Bovine serum albumin was eluted in one main peak followed by a shoulder, corresponding to the dimer (Menzel, D. B. and Richards, E. G., personal communication). 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 potassium phosphate molarity gradient at pH 5.8.

pH 6.8, range; the lysine-rich histone was eluted at about 0.55 M potassium phosphate buffer, perhaps the highest elution molarities found so far a non-phosphorylated protein.

NaCl or KCl molarity gradients could be used to elute basic proteins: the elution molarities, in this case, were equal to about twice the eluting molarities of phosphate buffer, pH 6.8 (see Table I). In the case of the lysine-rich histone fraction, gradient elution was not tried, but a 3 M KCl step completely removed the protein from the column.

Fig. 2 displays chromatograms obtained with ribonuclease A using different elution systems. In experiments performed with lysozyme and ribonuclease A, it was found that NaCl has practically the same eluting power as KCl.

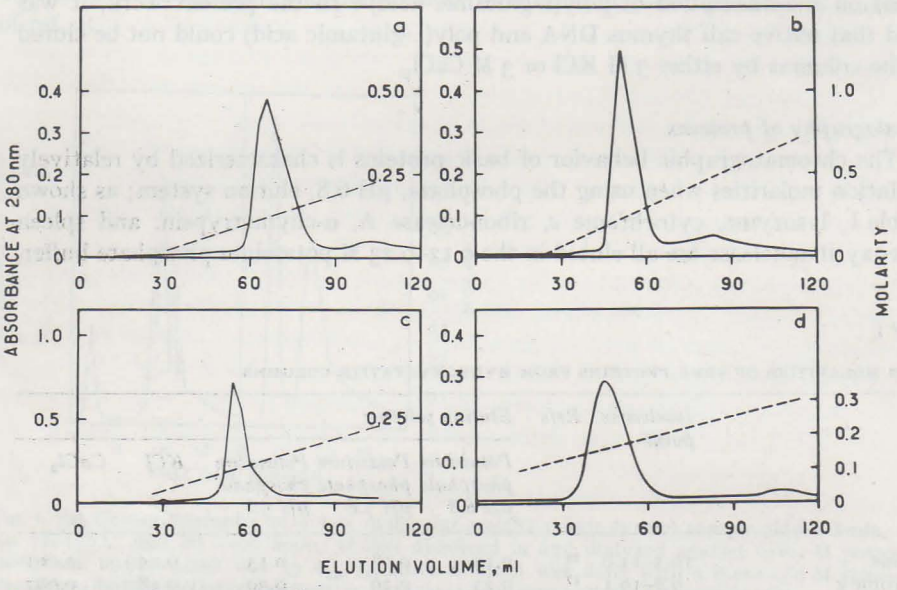

Fig. 2. Chromatography of ribonuclease A. Loads were comprised between 6 and 7 $A_{280\ nm}$ units. Elution was done with (a) potassium phosphate, pH 6.8, (b) KCl in 0.01 M potassium phosphate, pH 6.8, (c) potassium phosphate, pH 7.8 and (d) potassium phosphate, pH 5.8.

As indicated in Table I, all basic proteins were removed from the columns by very low molarities of $CaCl_2$. The proteins having the lowest potassium phosphate buffer elution molarities, lysozyme and ribonuclease A were eluted by the initial 0.001 M $CaCl_2$ step; those having increasingly higher potassium phosphate buffer elution molarities were 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 Table I, elution by potassium phosphate buffer, pH 7.8, took 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.

The data obtained with neutral and acidic proteins are also shown in Table I.

Acidic and neutral proteins showed a distinctly different chromatographic behavior on hydroxyapatite columns compared to basic proteins being eluted at generally lower molarities by the phosphate, pH 6.8, eluting system. When using the KCl eluting system, acidic and neutral proteins were either eluted by very high KCl molarities compared to the potassium phosphate buffer, pH 6.8, eluting molarities, or they were not eluted at all by KCl molarity gradients up to 3 M. None of the neutral and acidic proteins could be removed from the columns by $CaCl_2$ molarities as high as 3 M. After washing the columns with 0.001 M NaCl, all these proteins could, however, be totally eluted by a 1 M sodium phosphate buffer, pH 6.8, step. Spleen exonuclease (a protein whose isoelectric point is not known) resembled neutral and acidic proteins in being not eluted by $CaCl_2$.

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

Figs 3 and 4 show chromatograms obtained with myoglobin and bovine serum albumin, respectively, using different elution systems.

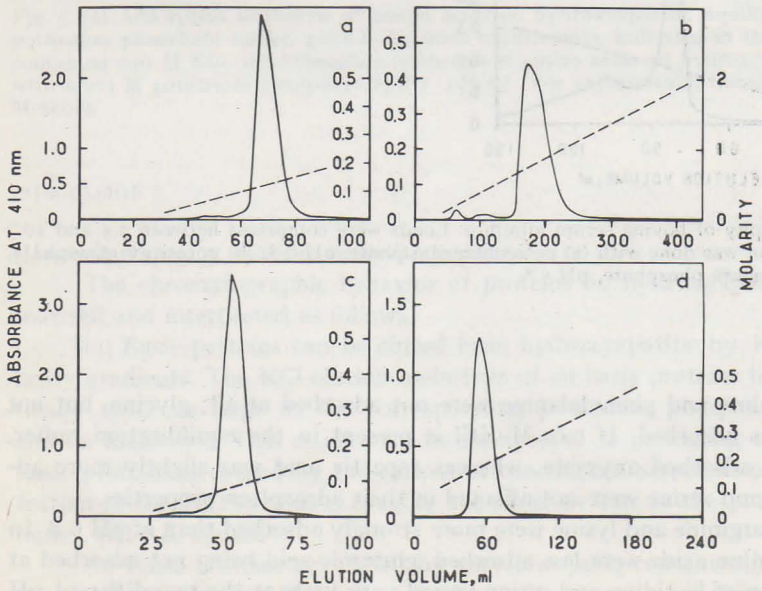

Fig. 3. Chromatography of horse heart myoglobin. Loads were comprised between 22 and 32 $A_{410\ nm}$ units. Elution was done with (a) potassium phosphate, pH 6.8, (b) KCl in 0.01 M potassium phosphate, pH 6.8, (c) potassium phosphate, pH 5.8 and (d) potassium phosphate, pH 7.8.

Adsorption of amino acids

At pH 6.8, all polar amino acids tested (arginine, lysine, histidine, aspartic acid, glutamic acid, serine) were adsorbed to rather similar extent. Among non-polar amino

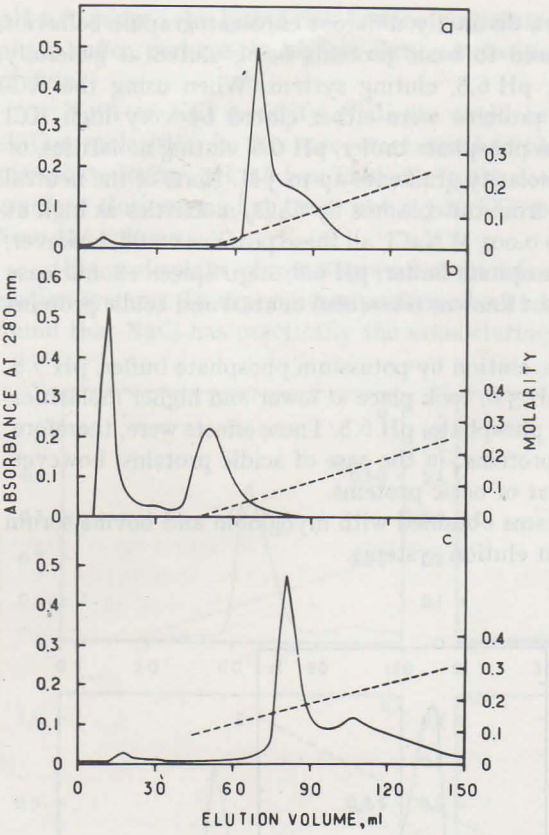

Fig. 4. Chromatography of bovine serum albumin. Loads were comprised between 7.5 and 10.3 $A_{280\ nm}$ units. Elution was done with (a) potassium phosphate, pH 6.8, (b) potassium phosphate, pH 7.8 and (c) potassium phosphate, pH 5.8.

acids, alanine, valine and phenylalanine were not adsorbed at all; glycine, but not glycylglycine, was adsorbed. If 0.01 M KCl is 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.

Fig. 5 shows the adsorption isotherms exhibited by several amino acids at pH 6.8 and 7.8. Except for glutamic acid at pH 6.8, isotherms were linear in the explored range or showed slight downward curvatures.

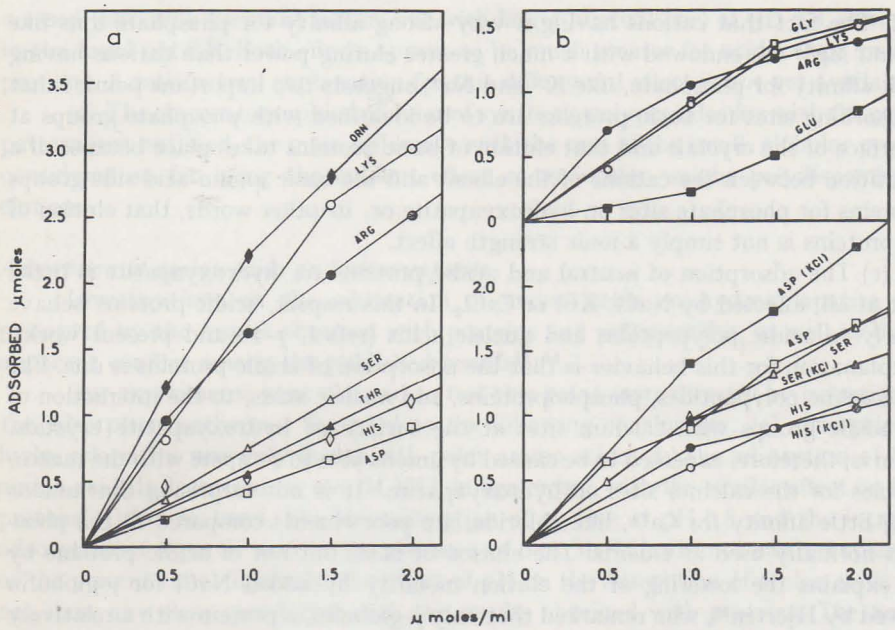

Fig. 5. (a) Adsorption isotherms of amino acids on hydroxyapatite, equilibrated with 0.001 M potassium phosphate buffer, pH 6.8. In some experiments, indicated in the figure, the solvent contained 0.01 M KCl. (b) Adsorption isotherms of amino acids on hydroxyapatite, equilibrated with 0.001 M potassium phosphate buffer, pH 7.8. For experimental details, see Materials and Methods.

DISCUSSION

Chromatography of proteins on hydroxyapatite

The chromatographic behavior of proteins on hydroxyapatite may be summarized and interpreted as follows.

(a) Basic proteins can be eluted from hydroxyapatite by KCl and NaCl molarity gradients. The KCl elution molarities of all basic proteins tested are systematically twice as large as the corresponding potassium phosphate buffer, pH 6.8, elution molarities. This result 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 potassium phosphate buffer, pH 6.8, system*.

(b) Basic proteins are eluted by very low $CaCl_2$ concentrations. The fact that the eluting molarities of basic proteins by $CaCl_2$ are 20 to 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 and not by the anions of the

* The eluting concentration of K^+ in the KCl system is, in fact, higher, by about 30%, than that of potassium phosphate buffer, 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 potassium phosphate buffer, pH 5.8, system).

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 hydroxyapatite or, in other words, that elution of basic proteins is not simply a ionic strength effect.

(c) The adsorption of neutral and acidic proteins on hydroxyapatite is little, or not at all, affected by NaCl, KCl or $CaCl_2$. In this respect, acidic proteins behave similarly to acidic polypeptides and nucleic acids (refs 1, 7–10 and present work). An explanation for this behavior is that the adsorption of acidic proteins is due, like that of acidic polypeptides, phosphoproteins, and nucleic acids, to the interaction of their acidic groups with calcium sites at the surface of hydroxyapatite crystals. Elution is, therefore, expected to be caused by anions able to compete with the macromolecules for the calcium sites on hydroxyapatite. It is not surprising that anions having little affinity for Ca^{2+}, like chloride, are poor eluents compared to the phosphates normally used as eluents. The elution of basic but not of acidic proteins by NaCl explains the lowering of the elution molarity by added NaCl for γ-globulin observed by Hjerten[11], who remarked that only γ-globulin, a protein with a relatively high isoelectric point, showed this effect among serum proteins.

(d) Basic proteins are eluted from hydroxyapatite columns at relatively high molarities of phosphate, pH 6.8. The five proteins listed in Table I 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*. This different behavior may be understood in the following terms: the usual eluents, sodium phosphate buffer or potassium phosphate buffer, pH 6.8, while very effective, because of their phosphate ions, in competing with the carboxyl groups of proteins for the calcium sites on hydroxyapatite, are much less effective in competing with the basic groups of the proteins for the phosphate sites on hydroxyapatite, 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.

(e) An unexpected finding obtained with spleen exonuclease, myoglobin, snail acid deoxyribonuclease and with pancreatic deoxyribonuclease is that these proteins, 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 may be wondered whether in these cases Ca^{2+} binds to carboxyl groups of these proteins and strengthens the adsorption by forming bridges to phosphate groups on hydroxyapatite.

(f) The data of Table I show that the elution molarity of proteins by potassium phosphate buffer, appears to be increased by a constant factor at pH 5.8 and de-

* Phosphoproteins are an exception to this rule, being eluted at very high phosphate molarities (see ref. 23); the reason is that in this case the phosphate groups of the protein are mainly, or only, involved in the interaction with calcium sites on hydroxyapatite.

creased (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 differential effect is not yet available.

(g) The chromatographic behavior of synthetic polypeptides fits with the general pattern just outlined, the main difference with the case of the small, globular proteins considered so far being the evident effect of molecular weight polydispersity on elution.

Adsorption of amino acids on hydroxyapatite

Investigations on the adsorption of amino acids on hydroxylapatite were prompted by the results obtained with proteins and polypeptides, as well as by the apparent conflict among the published results[11–14].

Our experiments were planned to test this point more directly by investigating the adsorption isotherms: these led to the following conclusions: (a) adsorption on hydroxylapatite was only found with polar amino acids; (b) the adsorption of basic amino acids is decreased by 0.01 M KCl, in agreement with the similar effect on basic 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 explanable 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 serine is adsorbed raises the question of the possible intervention of hydroxy-amino acids in the protein–hydroxyapatite interaction. This seems unlikely, however, in view of the fact that we could confirm that poly(L-serine) is not retained by hydroxyapatite[1].

Conclusion

The main finding of the present work is the demonstration of the existence of two different types of adsorbing sites on the surface of hydroxyapatite crystals: calcium sites and phosphate sites. The former appear to be responsible for the binding of acidic groups, carboxyls and phosphates; the latter ones for the binding of basic groups. This picture fits with the known amphotheric character of hydroxyapatite crystals[15]. Elution is caused by anions (usually phosphates) which compete for the calcium sites of hydroxyapatite with the carboxyl or phosphate groups of the macromolecules; or by cations (Na^+, K^+ or , more effectively, Ca^{2+} or Mg^{2+}) which compete for the phosphate groups of hydroxyapatite with the basic groups of proteins.

From a practical point of view, this work shows that: (1) elution of proteins from hydroxyapatite columns can be obtained using a number of eluents other than the usual phosphate buffer, pH 6.8; (2) the chromatographic behavior of basic and acidic proteins on hydroxyapatite columns operated with different elution systems can be predicted to a considerable extent.

Some applications of these findings to problems of protein fractionation will be presented elsewhere in due course.

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