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# CHROMATOGRAPHY OF POLYPEPTIDES AND PROTEINS ON HYDROXYAPATITE COLUMNS*

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SUMMARY

Chromatography of a number of water-soluble homo- and heteropolypeptides on hydroxyapatite columns has shown that only those containing carboxyl groups (poly-L-aspartate, poly-L-glutamate and their copolymers) are adsorbed by hydroxyapatite. The presence of urea or salt in the eluting buffer does not modify the elution molarity of poly-L-glutamate.

It is likely that in the case of proteins, too, the chemical groups responsible for the interaction with hydroxyapatite are the carboxyl groups. A special case is that of phosphoproteins where a very strong interaction with hydroxyapatite is due to the presence of the protein-phosphate groups. Denaturation of proteins reduces their affinity for hydroxyapatite probably by reducing the number of carboxyl groups available for the interaction and by destroying the clusters of carboxyl groups present on the surface of native proteins and responsible for the interaction.

## INTRODUCTION

Chromatography of proteins on hydroxyapatite columns is a powerful separation technique developed by TISELIUS, HJERTEN AND LEVIN[1]. The relatively slow acceptance of this technique by protein chemists seems to be due to two main reasons: (a) preparation of hydroxyapatite, according to the procedure of TISELIUS, HJERTEN AND LEVIN[1], or to one of the alternative methods proposed by other authors[2–5], is rather laborious; (b) the extensive investigations by TISELIUS, HJERTEN AND LEVIN[1], HJERTEN[6] and LEVIN[7] have elucidated several aspects of protein chromatography on hydroxyapatite columns, yet nothing is known about the mechanism of interaction of proteins with hydroxyapatite; since no correlation has been found between net charge and/or molecular weight of a protein and its elution molarity from a hydroxyapatite column, chromatography on hydroxyapatite is, so far, an empirical method.

The first difficulty seems to have been overcome with the advent of commercial

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hydroxyapatite preparations (Bio Rad, Richmond, Calif.; Clarkson Chemical Company, Williamsport, Pa.); however no comparative investigations concerning the chromatographic results obtained with the commercial preparations and the preparation of TISELIUS, HJERTEN AND LEVIN[1] have been published yet. As far as as the mechanism of adsorption of proteins on hydroxyapatite columns is concerned, we have attempted in the present work to establish which chemical groups of proteins interact with hydroxyapatite. In order to solve this problem we have studied the chromatographic behavior of several synthetic polypeptides. Our results suggest that carboxyl groups are responsible for the protein–hydroxyapatite interaction. A special case, previously investigated by BERNARDI AND COOK[9], is that of phosphoproteins where the much more important interaction of protein phosphate groups with hydroxyapatite completely offsets that of the carboxyl groups.

Another subject investigated in the present work concerns the influence of secondary and tertiary structures of proteins on their chromatographic behavior on hydroxyapatite columns. This study was suggested by similar investigations carried out in this laboratory on nucleic acids[10–12] and suggests that chromatography on hydroxyapatite columns may be a very useful tool in the study of protein denaturation.

An investigation on the resolving power of hydroxyapatite columns[13], and a theory of the chromatography of macromolecules[14] on hydroxyapatite are being published elsewhere.

## MATERIALS AND METHODS

All polypeptides used in the present work were synthesized in the laboratory of Dr. G. SPACH of this Institute.

Homopolymers: poly-L-glutamate, poly-L-aspartate, poly-L-tyrosine, poly-D,L-histidine, poly-L-lysine; statistical copolymers: copoly-L-glutamate-L-phenylalanine (9:1 and 9.5:0.5 molar ratios); copoly-L-glutamate-L-lysine (9:1), copoly-L-glutamate-L-serine (8.2:1.8; 9:1); copoly-L-benzyl-glutamate-D,L-histidine (1:1). All polypeptides had a molecular weight high enough not to pass through Visking cellulose tubings when dialyzed against 0.01 M phosphate buffer (pH 6.8). This may be taken as an indication that the molecular weights were higher than about 5 000; sedimentation and viscosity measurements performed on several samples indicated molecular weights in the range 5 000–50 000.

Horse heart cytochrome *c* (type III) was purchased from Sigma (St. Louis, Mo.); egg white lysozyme, ribonuclease A and pancreatic deoxyribonuclease were obtained from Worthington (Freehold, N.J.); collagen was prepared according to RUBIN *et al.*[15] (see also KAWASAKI AND BERNARDI[16]).

Hydroxyapatite was prepared according to TISELIUS, HJERTEN AND LEVIN[1]. Elution was carried out using linear molarity gradients of potassium phosphate buffer (pH 6.8). Phosphate molarity of the chromatographic fractions was determined by refractometry. In some cases, indicated in the text, 8 M urea or KCl were added to the potassium phosphate buffer. In the case of collagen, sodium phosphate buffer (pH 6.8) containing 0.15 M NaCl and 1 M urea (a concentration far below that causing denaturation[17]) was used as the eluent. Molarity gradients were obtained, in most cases, with a Varigrad (Technicon, Chauncey, N.J.), the initial buffer being always 0.001 M in phosphate. Elution was under gravity flow (20–60 ml/h). The effect of

flow-rate was not investigated here since it has little effect, at least in the range of flow rates used, on the chromatographic behavior of proteins or polypeptides. 3–5 ml fractions were collected using Gilson (Madison, Wis.) or LKB (Stockholm, Sweden) fraction collectors. Recoveries were close to 100%, except in one case mentioned in the legend of the chromatogram.

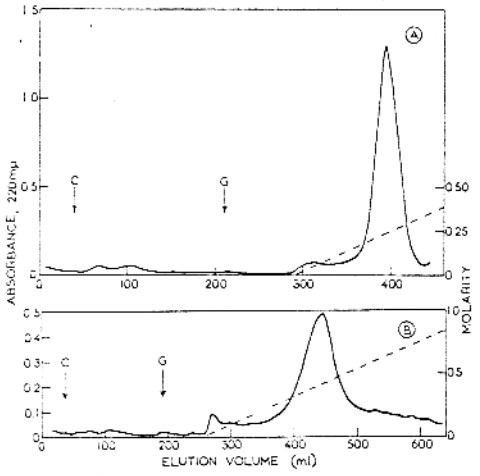
Chromatographic experiments were performed at room temperature, except in the case of native proteins where they were carried out at 4°, unless otherwise stated. In the case of collagen some experiments were carried out at temperatures in the melting range (35–41°). In this case collagen samples were carefully pipetted on the top of a 2–3-cm layer of solvent (the column outlet being closed) and allowed to remain at the equilibration temperature of the column for 40 min before the experiment was started.

Concentrations of materials in the chromatographic fractions were determined by measuring their ultraviolet absorption at 220 mμ (polypeptides), 225 mμ (collagen), 280 mμ (lysozyme, ribonuclease, poly-L-tyrosine), and 415 mμ (cytochrome *c*).

## RESULTS

### *Synthetic polypeptides*

Among the water-soluble synthetic homopolypeptides tested only poly-L-glutamate and poly-L-aspartate were retained by hydroxyapatite columns equilibrated with 0.001 M potassium phosphate buffer. These two polypeptides were eluted at about 0.25 M and 0.35 M potassium phosphate buffer, respectively (Fig. 1 (A and B)). The other synthetic polypeptides were not retained by the columns; Fig. 2 (A and B) shows the chromatograms obtained with poly-L-tyrosine and poly-D,L-histidine, as an example.



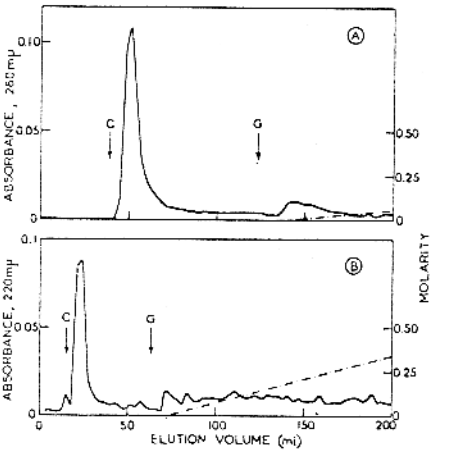
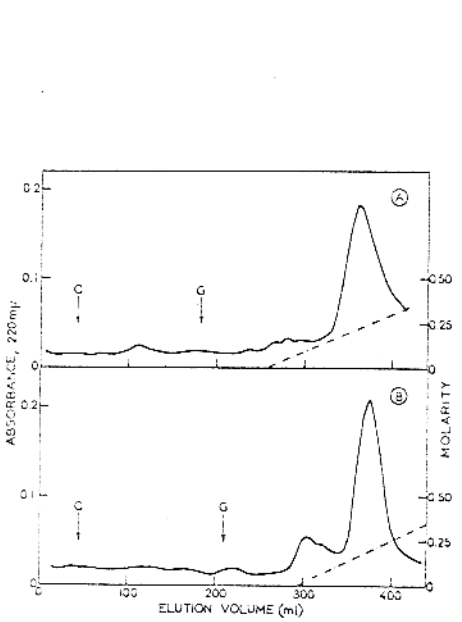




Fig. 1. Chromatography of (A) poly-L-glutamate, (B) poly-L-aspartate. In both cases 10 mg of polypeptide were loaded on 2 cm × 20 cm hydroxyapatite columns. Loading and molarity gradient were started at fractions indicated by arrows C and G, respectively.

Fig. 2. Chromatography of (A) poly-L-tyrosine, (B) poly-L-histidine. In the first case 1.6 $A_{280}$ units were loaded on a 1 cm × 8 cm hydroxyapatite column; in the second case 5 mg were loaded on a 0.8 cm × 10 cm hydroxyapatite column. Other indications as in Fig. 1.



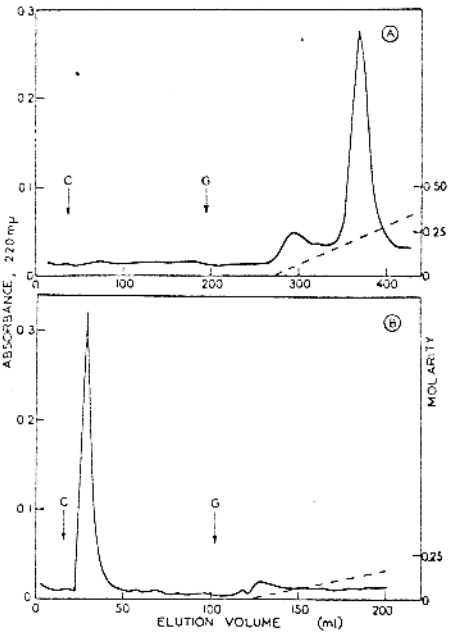




Fig. 3. Chromatography of (A) copoly-L-glutamate-L-phenylalanine (9.5:0.5 molar ratio), (B) copoly-L-glutamate-L-phenylalanine (9:1 molar ratio). Loads were 4 mg and 1.5 mg, respectively, on 2 cm × 20 cm hydroxyapatite columns. Other indications as in Fig. 1. In chromatogram A, the recovery was low for unknown reasons.

Fig. 4. Chromatography of (A) copoly-L-glutamate-L-lysine (9:1 molar ratio); 1.5 mg were loaded on a 2 cm × 20 cm hydroxyapatite column; (B) copoly-L-benzyl glutamate-L-histidine (1:1 molar ratio); 2.8 $A_{220}$ units were loaded on a 1 cm × 7 cm hydroxyapatite column.

Statistical copolymers of poly-L-glutamate were eluted at a lower molarity than poly-L-glutamate, the elution molarity being smaller for copolymers with lower glutamate content. Some typical results obtained with copolymers of L-phenylalanine and L-glutamic acid (having 0.5:9.5 and 1:9 phenylalanine/glutamate molar ratios) and with copolymers of L-lysine and L-glutamic acid (1:9) are shown in Figs. 3 (A and B) and 4 (A), respectively. Similar results were obtained with copolymers of L-serine and L-glutamic acid with different molar ratios. A copolymer of D,L-histidine and benzyl-L-glutamate (1:1 molar ratio) was not retained by the column equilibrated with 0.001 M potassium phosphate buffer (Fig. 4 (B)).

Performing the chromatography of poly-L-glutamate (Fig. 5) and poly-L-aspartate in the presence of 8 M urea caused essentially no change in the elution molarity. Carrying out the elution with a gradient formed by 1 M KCl–0.001 M potassium phosphate buffer and 0.5 M potassium phosphate buffer (therefore at a practically constant ionic strength, since potassium phosphate buffer has a ionic strength which is equal to twice its molarity, when its dissociation is complete) did not change the chromatographic behavior of poly-L-glutamate.

An important observation concerning the chromatography of both poly-L-glutamate and poly-L-aspartate concerns the capacity of hydroxyapatite for these macromolecules. This was found to be at least 5 times lower than that for proteins.

In several experiments described above, an ultraviolet-absorbing peak was

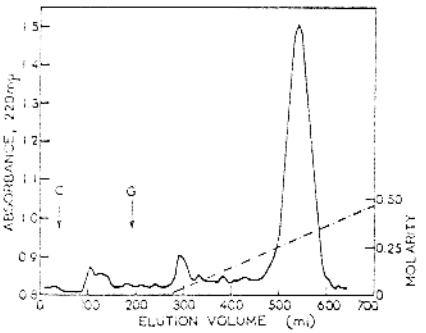



Fig. 5. Chromatography of poly-L-glutamate; 10 mg were loaded on a 2 cm × 20 cm column. The polymer solution and the eluting buffers were 8 M in urea. The molarity gradient obtained in this experiment was not measured because of the presence of urea, but estimated on the basis of experiments done in the absence of urea.

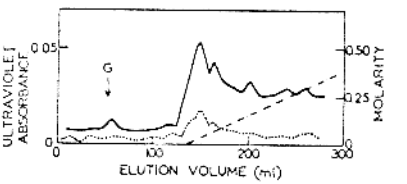



Fig. 6. Solvent (potassium phosphate buffer) run on a 2 cm × 20 cm column. The continuous line and the dotted line indicate absorbancies at 220 mμ and 280 mμ, respectively.

evident at the beginning of the gradient, particularly when the slope of the gradient was high. Blank chromatograms (Fig. 6) showed this to be an artifact probably due to the presence of some ultraviolet-absorbing material in the buffers used.

The results obtained with synthetic polypeptides clearly indicate that carboxylic groups are the only ones, among those investigated, having a strong binding capacity for hydroxyapatite.

*Proteins*

As already mentioned in the INTRODUCTION, nothing is known about the chemical groupings of proteins interacting with hydroxyapatite. On the basis of the results obtained with synthetic polypeptides, it may be thought, however, that here too, carboxyl groups are responsible for the interaction with hydroxyapatite. A direct test of this hypothesis is experimentally difficult, yet not impossible since esterification of the carboxyl groups or deamidation of glutamine and asparagine residues in proteins might help to solve this problem.

The distribution of carboxyl groups at the surface of proteins is not random (in which case a correlation between number or surface density of carboxyl groups and elution molarity might be found), but is unique and genetically determined. It should therefore be expected: (a) that several different modes of interaction of a given protein with hydroxyapatite are possible; (b) that of all possible interactions, the one resulting in the lowest free energy level will be preferred; (c) that this interaction will be associated with a local concentration of carboxyl groups and/or a local distribution of carboxyl groups fitting the distribution of adsorbing sites and will determine the stability of the protein–hydroxyapatite interaction and therefore the elution molarity.

These considerations explain why proteins, otherwise very close in size and net charge, can be separated by hydroxyapatite chromatography. Furthermore they strongly suggest that the tertiary and secondary structure of proteins have a strong influence on their chromatographic behavior. This point, which has already been verified for nucleic acids[10,11], has been tested for proteins also.

The effect of the disruption of tertiary and secondary structures of proteins on their chromatographic behavior on hydroxyapatite columns was investigated by comparing: (a) the elution patterns obtained with native proteins and with the same proteins in 8 M urea, respectively (time effects of urea treatment were not investigated); the experiments performed with poly-L-glutamate in 8 M urea showed that urea does not modify the chromatographic behavior of polymers which already have a random coil conformation like poly-L-glutamate (b) the elution patterns obtained at different temperatures with tropocollagen.

Some of the results obtained are shown in Figs. 7–10. Figs. 7 (A and B) show the chromatographic behavior of lysozyme in the native and the denatured state; in this latter case the protein is no longer retained by the column. The small peak eluted at the beginning of the gradient may be a "false" peak (a chromatographic artifact already investigated by TISELIUS, HJERTEN AND LEVIN[1] and HJERTEN[7] or a partially denatured form of the protein. A similar phenomenon has been shown by ribonuclease

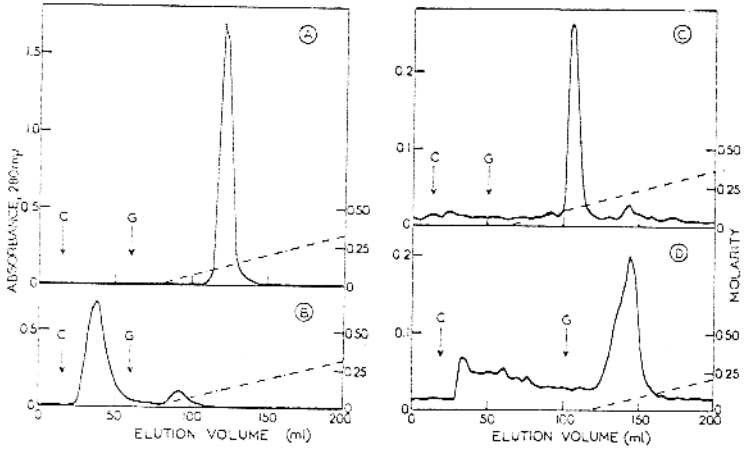



Fig. 7. Chromatography of lysozyme (10 mg) on 1 cm × 10 cm hydroxyapatite columns, run in the absence (A) and in the presence (B) of 8 M urea. Chromatography of ribonuclease (10 mg) on 1 cm × 10 cm hydroxyapatite columns, run in the absence (C) and in the presence (D) of 8 M urea.

(Fig. 7 (C and D)); in this case, however, the elution molarity of the denatured protein is so close to that of the equilibrating buffer (0.001 M potassium phosphate buffer) that the protein is slowly eluted by the washing buffer and a large "false" peak is eluted at the start of the gradient; obviously, the possibility exists here, as in the previous case, that this peak also contains incompletely denatured molecules.

In the case of cytochrome *c* (Fig. 8 (A and B)) the denatured protein is retained by the column, but is eluted at a lower molarity compared with the native protein. In this case, the small trailing peak cannot be a "false" peak, and is likely to be formed by partially denatured molecules. Pancreatic deoxyribonuclease (Fig. 8 (C and D)) was not retained by the column when chromatographed in the denatured state, whereas in the native state it was only eluted by the molarity gradient.

In several cases small amounts of material were not retained when chromatographing crystalline native proteins. As in the case of collagen (see below), the non-retained material may be formed by denatured protein.

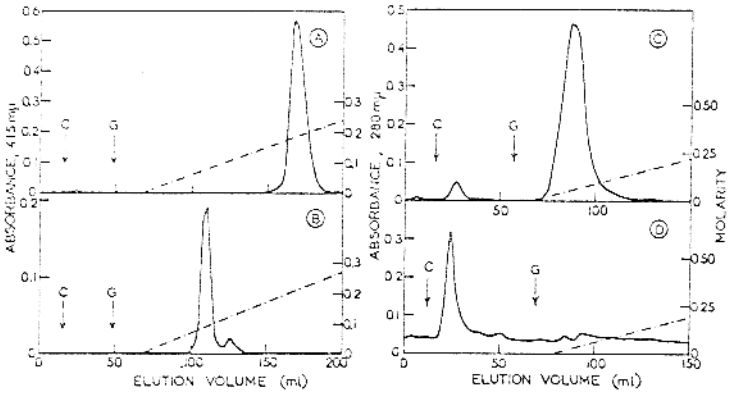



Fig. 8. Chromatography of cytochrome *c* on 1 cm × 6 cm hydroxyapatite columns, run in the absence (A, 6.7 $A_{415}$ units) and in the presence (B, 1.7 $A_{415}$ units) of 8 M urea at room temperature. Chromatography of pancreatic deoxyribonuclease on 1 cm × 10 cm hydroxyapatite columns, run in the absence (C, 10 mg) and in the presence (D, 3 mg) of 8 M urea.

Several of the experiments reported above suggest that partially denatured proteins show a different chromatographic behavior compared to both native and fully denatured molecules. This point has been further investigated using collagen and performing the experiments at different temperatures (Fig. 9 (A and B) and Fig. 10 (A and B)). An experiment run under similar conditions, but at 4°, is presented elsewhere (Fig. 1 of ref. 16); this is not very different from that of Fig. 9 (A). A comparison of the chromatograms run at 4° and 41° indicates that native collagen is eluted at molarities between 0.04 and 0.1 M, whereas thermally denatured collagen is eluted by 0.001 M sodium phosphate buffer. The experiments performed at increasing temperatures indicate that the percentage of non-retained material increases with temperature and the elution molarity of the retained material becoming lower as the

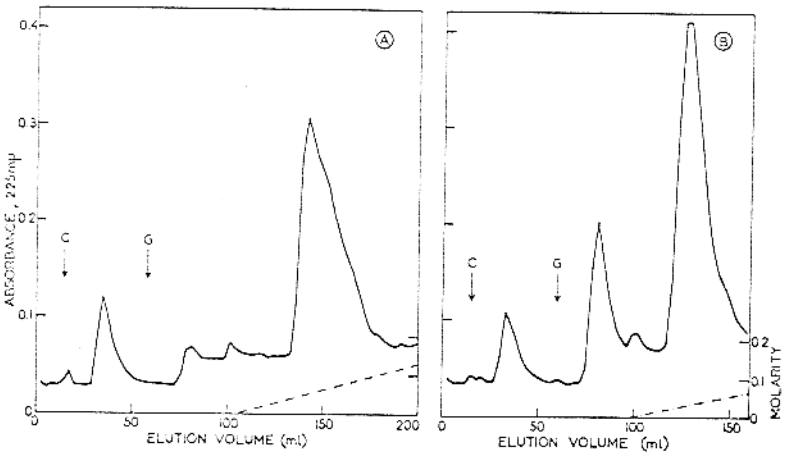



Fig. 9. Chromatography of tropocollagen (2.5 mg) on 1 cm × 20 cm hydroxyapatite columns at 24° (A) and 35° (B).

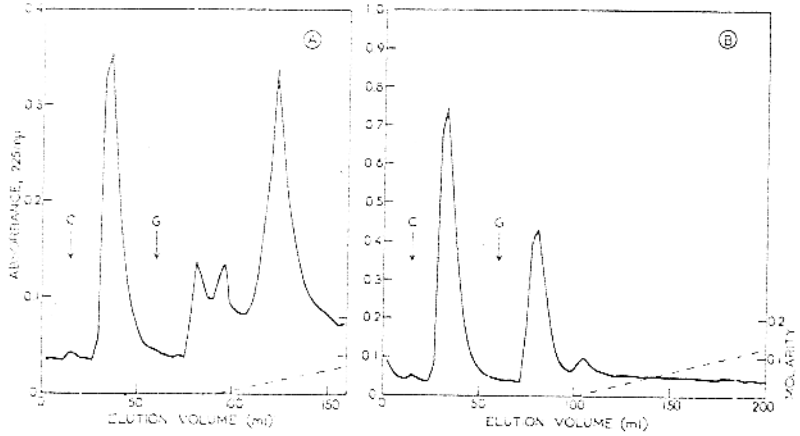



Fig. 10. Chromatography of tropocollagen (2.5 mg) on 1 cm × 20 cm columns at 37° (A) and 41° (B).

temperature increases. Furthermore, two other components are evident in the chromatograms performed at temperatures in the melting range. The first of these components is also eluted by 0.001 M sodium phosphate buffer, but it has a lower $R_F$ value compared to the peak of denatured collagen; the second component is eluted at the beginning of the gradient and may be a "false" peak. The first of these components may correspond to an intermediate state of denaturation of collagen (ENGEL[17]).

*Phosphoproteins*

Phosphoproteins represent a special case among proteins since, in general, they have a much higher affinity for hydroxyapatite than non-phosphorylated proteins. Work by BERNARDI AND COOK[9] has shown that two egg yolk phosphoproteins, $\alpha$- and $\beta$-lipovitellin, which were found to be identical in amino acid and lipid composition, but to differ in their protein phosphorus content, were easily separated on hydroxyapatite columns. When chromatographic experiments were performed with a molarity gradient elution (instead of using the step-wise technique previously employed[9]) it could be shown that $\beta$-lipovitellin, the electrophoretically slow component, was eluted by 0.4 M potassium phosphate buffer, whereas $\alpha$-lipovitellin, the fast component, was eluted by 0.75 M potassium phosphate buffer. The third egg yolk phosphoprotein, phosvitin, a protein in which almost 50% of the amino acids are phosphoryl serines, was eluted by an exceptionnaly high phosphate molarity, 1.2 M potassium phosphate buffer.

## DISCUSSION

The results obtained with synthetic polypeptides show that carboxyl groups are responsible for their interactions with hydroxyapatite, since only polymers containing them were retained by the columns. The different chromatographic behavior is not due to differences in molecular weights since in all cases the number of amino acid residues per polypeptide chains is higher than about 50, therefore, well beyond the range where the chromatographic behavior may depend upon molecular weight.

The statistical copolymers, containing different percentages of glutamic acid residues, studied here confirm the fundamental importance of carboxyl groups in the polypeptide–hydroxyapatite interaction process. In fact, no matter what other amino acid residue was present in the copolymer (histidine, lysine, phenylalanine, serine), its elution molarity was lowered when the percentage of glutamic acid residues in the copolymer was smaller. In agreement with this conclusion, a copolymer of D,L-histidine and benzyl-L-glutamate, in which all carboxyl groups were esterified, was not retained by the column.

The adsorption of polypeptides on hydroxyapatite may be thought to take place because of the interaction between their carboxyl groups and the calcium sites of the hydroxyapatite crystals. Increasing the molarity of the eluting phosphate buffer progressively reduces this interaction to zero, at which point desorption occurs. The decrease in the interaction appears to be due to a specific competition between the phosphate ions of the eluting buffer and the carboxyl groups of polypeptides for the calcium sites of hydroxyapatite, and not simply to an increase in ionic strength. In fact, if elution is performed at a practically constant ionic strength, with a linear gradient between 1 M KCl–0.001 M potassium phosphate buffer and 0.5 M potassium phosphate buffer, poly-L-glutamate is desorbed essentially at the same potassium phospate buffer molarity as in the absence of KCl. The interaction of polycarboxylic acids with hydroxyapatite suggests that carboxyl compounds may be used as eluents. Acetate has already been used successfully in this laboratory to elute weakly adsorbed substances, like oligonucleotides, from hydroxyapatite.

The different elution molarities exhibited by poly-L-glutamate (0.25 M potassium phosphate buffer) and poly-L-aspartate (0.35 M potassium phosphate buffer) cannot be explained with any certainty so far; however, it is possible that, in the random coil conformations possessed by these polypeptides under the experimental conditions used, a larger percentage of carboxyl groups interact with hydroxyapatite in the case of poly-L-aspartate than in the case of poly-L-glutamate.

As far as proteins are concerned, it is very likely, but not yet experimentally proven, that carboxyl groups are also responsible for the interaction with hydroxyapatite.

Our results on the chromatographic behavior of native and denatured proteins show that the latter are much less retained, or not retained at all, by hydroxyapatite columns equilibrated with 0.001 M potassium phosphate buffer. It appears, therefore, that the disruption of the secondary and tertiary structures of proteins causes a reduction in their interactions with hydroxyapatite. This phenomenon could be explained by the fact that the random coil conformation of the denatured protein will cause a decrease in the number of carboxyl groups available for interaction per unit of protein surface in contact with hydroxyapatite and therefore a decrease in the elution molarity of denatured proteins. This decrease may be due to the following reasons: (a) carboxyl groups which were 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 conformation; (b) local concentrations (due to the existence of secondary and tertiary structures) of carboxyl groups able to interact with the adsorbing sites of hydroxyapatite will disappear in the denatured state and the carboxyl groups will become more randomly distributed over all the protein "surface".

Our results show, at the same time, that it is highly improbable that the random coil conformation of denatured proteins is changed, when the protein is adsorbed on hydroxyapatite, into a conformation allowing all or most of the carboxyl groups to interact with hydroxyapatite adsorbing sites.

As far as phosphoproteins are concerned, it is evident that their very strong interaction with hydroxyapatite is due to the presence of protein phosphate groups. The very high eluting molarities shown by phosphoproteins in comparison with nucleic acids may be due to two reasons: (a) phosphoproteins have monoesterified phosphate groups in contrast with nucleic acids which have diesterified groups; therefore one more charge is available per group; (b) phosphoproteins have phosphorylserine runs[19] which form areas of very high density of groups able to interaction with hydroxyapatite.

NOTE ADDED IN PROOF (Received July 6th, 1968)

Poly-L-arginine, poly-L-proline and a low molecular weight (= 1600) poly-L-serine, obtained from Yeda (Rehovoth, Israel) were not retained by hydroxyapatite columns equilibrated with 1 mM potassium phosphate buffer, like all other non-carboxylic polypeptides.

ACKNOWLEDGEMENTS

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