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THE CHROMATOGRAPHY OF BIOPOLYMERS ON CALCIUM PHOSPHATE GELS

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I. INTRODUCTION

An important factor determining the success of a chromatographic separation is the choice of a suitable adsorbents. Progress in this field in recent years has led to the appearance of two main types or groups of adsorbent[1], which are now widely used for the chromatography of the most varied biopolymers.

One of these groups — calcium phosphate sorbents or gels — consists of molecular adsorbents. The other group consists of various ion exchangers, based chiefly on cellulose[2].

Both groups of adsorbent have an extensive, well hydrated surface, but the adsorption of biopolymers on these materials takes place in different ways. The principles of the action of these adsorbents also differ[3] and their chromatographic selectivity with respect to biopolymers is probably not the same[1]. Both groups of adsorbents are now widely used for the chromatography of the most varied biopolymers.

A number of reviews have been published[4-6] on ion exchangers based on cellulose. No reviews have been published on the use of calcium phosphate gels for the chromatography of biopolymers.

The aim in the present review has been to collect and summarise available published data on the chromatography of biopolymers on calcium phosphate sorbents.

II. METHODS OF PREPARATION AND CHARACTERISTICS OF CALCIUM PHOSPHATE SORBENTS

Until recently, only tricalcium phosphate $Ca_3(PO_4)_2$ and calcium hydrogen phosphate or brushite $CaHPO_4.2H_2O$ were generally employed in chromatographic practice. In recent years, a particular form of calcium phosphate — hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ — has begun to be used extensively in the chromatography of proteins. Tricalcium phosphate was at one time used fairly extensively as an adsorbent for the chromatography of proteins. Mention may be made, for example, of the isolation and purification of catalase by the use of tricalcium phosphate[8,9].

Tricalcium phosphate is now seldom used for the chromatography of proteins in column experiments. We shall therefore not deal further with this type of calcium phosphate, except to point out that $Ca_3(PO_4)_2$ usually contains a considerable quantity of hydroxyapatite[10]. This is due to the fact that the primary product formed in the preparation of tricalcium phosphate is pseudoapatite[11], which is subsequently converted into one modification of tricalcium phosphate and partly into hydroxyapatite[12].

A second example of a calcium phosphate gel, which is still used in the chromatography of proteins, in calcium hydrogen phosphate or brushite. The structure of brushite obtained by mixing solutions of $CaCl_2$ and Na_2HPO_4 depends to a marked extent on the pH. Formation of the precipitate usually takes place at pH 6.3–6.5. At the same time, it is known[10] that at pH 6.2 and above, calcium hydrogen phosphate may undergo slow hydrolysis into another form of calcium phosphate with the structure of hydroxyapatite.

Since the chromatography of proteins and other biopolymers is usually carried out in the approximate pH range 6.8–7.2 (i.e. above pH 6.2), the experimenter is in fact concerned with the unstable changing structure of an adsorbent containing a considerable proportion of hydroxyapatite.

In this respect, an undeniable advantage is shown by another calcium phosphate gel, which is now widely used in the column chromatography of biopolymers, namely hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. This material has a high stability over a wide pH range.

The modern chromatography of biopolymers on calcium phosphate columns may be taken as dating from 1956, when Tiselius, after a number of preliminary experiments[13-16], published a method for the preparation of hydroxyapatite suitable for the chromatography of proteins[10]. In this

method, 0.5 M solutions of $CaCl_2$ and Na_2HPO_4 are run at the same rate into a vessel with constant stirring; after decantation, the resulting precipitate of brushite ($CaHPO_4$) is washed with distilled water and boiled with 40% NaOH solution for 1 h. The precipitate is then washed repeatedly with a series of phosphate buffer solutions of different molarities. In this method the operation of boiling the $CaHPO_4$ precipitate with alkali is extremely important, since at this stage the calcium hydrogen phosphate is hydrolysed to a new form of calcium phosphate — hydroxyapatite.

The acidic precipitate of brushite, which usually forms large crystals, is converted, during this operation in alkaline medium, into fine crystals of hydroxyapatite suitable for the chromatography of proteins. X-Ray diffraction study of the resulting adsorbent[10] showed it to be hydroxyapatite, but the molar Ca/P ratio was found to be 1.53 instead of the usual value of 1.67 for hydroxyapatite[10]. This is apparently due to the ability of sodium or phosphate ions to enter the crystal lattice of the adsorbent in different proportions.

Hydroxyapatite prepared by Tiselius' method is now widely used in the chromatography of proteins. The actual preparation of the adsorbent, however, involves a large number of operations and is very time-consuming. To obtain a satisfactory rate of flow it is usually necessary to apply external pressure. A recent paper[17] reports that, to obtain hydroxyapatite by Tiselius' method with a satisfactory rate of flow from the column, it is necessary to prevent the deposition of highly dispersed material after the adsorbent has been washed with water. The need for extremely thorough stirring of the precipitates when the adsorbent is boiled has also been reported[17].

Somewhat later, in 1959, Main and his coworkers proposed a new method for the preparation of hydroxyapatite[18] with properties similar to those of hydroxyapatite prepared by Tiselius' method. In this case the precipitate obtained after mixing solutions of $CaCl_2$ and a sodium phosphate buffer of pH 6.7 is boiled in concentrated NH_4OH solution. The precipitate is converted into hydroxyapatite during this operation. X-Ray diffraction study of the resulting adsorbent[18] showed it to consist of pure hydroxyapatite with Ca/P = 1.53. The hydroxyapatite obtained by this method, however, sorbs proteins comparatively weakly[17]. Main et al.[18] used this adsorbent chiefly for the chromatography of nucleic acids.

Anacker and Stoy[19] recently proposed another method for the preparation of hydroxyapatite suitable for the chromatography of proteins. In this method, 1.5 M K_2HPO_4 and 1.4 M $CaCl_2$ solutions are added with constant stirring to 0.1 M $CaH_4(PO_4)_2$ solution. The precipitate obtained in this way is converted into hydroxyapatite in 0.05 M NaOH at 40° over a period of several days. The resulting precipitate consists of pure hydroxyapatite.

Comparison of the adsorptive properties for proteins exhibited by hydroxyapatites prepared in different ways shows that the adsorbents prepared by the methods of Tiselius[10] and Anacker[19] give similar adsorption curves. Hydroxyapatite prepared by Main's method[18], however, gives a curve which differs sharply from them. The ability of this hydroxyapatite to sorb proteins is much lower.

Thus although hydroxyapatites obtained by different methods have the same chemical composition, their abilities to adsorb proteins are different. There is also a significant difference in the rates of flow of liquid through columns filled with calcium phosphate gels prepared by different methods. For the adsorbent prepared by Tiselius' method[10] the rate of flow through a standard column may reach an average of 20–22 ml h^{-1} with the application of external pressure. For the hydroxyapatite prepared by Main's method[18] the average is given as 25–30 ml h^{-1} without pressure, whereas for columns filled with hydroxyapatite prepared by Anacker's method[19] the rate of flow reaches 200 ml h^{-1} without the application of pressure.

These differences in the adsorptive power of hydroxyapatites prepared by different methods indicate that the magnitude of the adsorption of proteins on calcium phosphate gels depends not only on the chemical nature of the adsorbents (the chemical formula is the same for all three hydroxyapatites) but also on the physical non-uniformity of the adsorbent surface.

In general, the magnitude of the adsorption of a solute is determined both by the adsorption on unit area of the adsorbent–solution interface and also by the specific surface of unit mass of the adsorbent, accessible to the solute molecules[20]. In the comparison of the magnitude of the adsorption on different adsorbents of unknown specific surface, the factor responsible for differences in the adsorption is always unknown. In some cases the difference may be due to a difference in the specific surfaces of the adsorbents[20].

Hydroxyapatites prepared by different methods give precipitates with different degrees of dispersion. These probably have different specific surfaces, and the surface is apparently characterised by different degrees of physical non-uniformity. This is possibly responsible for the difference in the adsorptive power for proteins.

All this points to the need for careful standardisation of methods for the preparation of calcium phosphate sorbents, in order to obtain reproducible results in the chromatography of biopolymers on columns.

III. METHODS OF ELUTION AND CHARACTERISTIC FEATURES OF THE CHROMATOGRAPHY OF BIOPOLYMERS ON CALCIUM PHOSPHATE COLUMNS

1. Methods of Elution

Phosphate buffer solutions of different concentrations are now used almost exclusively as eluants in the chromatography of biopolymers on calcium phosphate. The sorption of proteins on columns is usually carried out at low concentrations of phosphate buffer solution, and elution at higher concentrations of the same buffer, most frequently at the same pH value. Change in pH in chromatography on calcium phosphate sorbents has an extremely small influence on the experimental results compared with change in the buffer concentration[10].

Two main method are now used for the elution of biopolymers from calcium phosphate — gradient elution (gradual increase in the concentration of phosphate buffer added to the solution) and stepwise elution (the increase in the concentration of phosphate in the column is carried out in separate stages).

Under certain conditions, gradient elution usually makes it possible to detect all the components of the mixture being studied. When this method is used, however, the mutual

influence of the proteins[10] often leads to considerable overlap of adjacent fractions on calcium phosphate sorbents, and in a number of cases, even when the solution being studied contains several protein components, only one greatly elongated protein zone is obtained at the column outlet. The homogeneity of a material of this type is determined by a further chromatographic separation.

Usually in such cases it is possible to isolate the individual components of the mixture being studied. One such example is given in a paper by Tiselius[10] on the chromatography of γ-globulin from human serum on hydroxyapatite.

In stepwise elution there is always a danger that the components leaving the column may be of complex composition: proteins of different character may be present in individual fractions. On the other hand, proteins with a broad elution range may give more than one component for a given protein on leaving the column, particularly if the elution is carried out at buffer concentrations differing little from one another. The appearance of these spurious components and the conditions for their formation in the chromatography of proteins on calcium phosphate columns have been discussed in detail[3].

In most cases the chief method used to assess the results obtained in the chromatography of biopolymers on calcium phosphate is further chromatography of the products. Where the biopolymers have in fact been separated into their components, the latter are always eluted from the column in the subsequent chromatographic experiment by the same buffer solution concentrations as in the original chromatographic experiment. At the present time, the chromatography of a test material on calcium phosphate columns is usually carried out by both methods of elution, which to a certain extent supplement one another. In the examination of the material obtained, it is of course desirable to use other methods for the study of protein components in addition to further chromatographic separations.

What is the mechanism of the elution of protein polymers from calcium phosphate columns? Hardly any special study has been made of this problem. Nevertheless, on the basis of work carried out chiefly by Tiselius and his coworkers[10,16], the mechanism can now be assumed to involve replacement of the proteins by phosphate ions. The ability of calcium phosphate to take part in extensive exchange with calcium and phosphate ions from the surrounding medium has been demonstrated directly[21] using the radioactive isotopes ^{32}P and ^{45}Ca. This tendency is particularly marked with phosphate ions. These ions can effectively replace ions from calcium phosphate not only in the hydrate layer but also in the interior of the crystal lattice of the adsorbent[21].

Burley and Cook[22], using a different experimental technique, showed directly that the presence of phosphate groups in proteins essentially determines the behaviour of the latter during chromatography on calcium phosphate columns. Thus egg-yolk α- and β-lipovitellins, which have different phosphate contents, show different behaviour on elution from hydroxyapatite. β-Lipovitellin leaves the column at a phosphate buffer concentration of 0.6 M, and α-lipovitellin only at a concentration of 2.0 M. After treatment of α-lipovitellin with phosphatase (removal of some of the phosphate groups), however, this protein shows behaviour similar to that of β-lipovitellin and is eluted from the column with 0.6 M phosphate buffer. There is need for further studies in this direction in order to elucidate all the details of the mechanism of the elution of proteins from calcium phosphate columns.

2. Characteristic Features of the Chromatography of Biopolymers on Calcium Phosphate Columns

In the chromatography of proteins on calcium phosphate adsorbents, the electrostatic bonds between protein and adsorbent play an important part. Hydrogen bonds and van der Waals forces are also significant[1,7]. Since a large number of bonds are formed between the protein and the adsorbent, their simultaneous dissociation during desorption of the protein is unlikely[1]. Under certain conditions, therefore, there is a danger that reversible partial denaturation of the protein will take place with a possible change in various structures (tertiary and quaternary) in the protein molecule[23].

Moreover, the non-simultaneous dissociation of the large number of bonds of different types between the proteins being chromatographed and the surface of the adsorbent may create a complex picture, whose interpretation is as yet impossible, and may lead to internal interaction between the proteins as they move downwards through the column during the chromatographic process.

The effects of internal interaction and the mutual influence of proteins play an important part in chromatography on calcium phosphate columns[10].

Chromatographic studies are usually carried out with various protein mixtures in order to isolate a definite protein from other proteins present in the mixture. Chromatography is often the final stage in the purification or one of the operations preceding purification. Thus the "ballast" proteins present in the experimental mixture may be present in various proportions relative to the protein to be isolated.

Recent work on the chromatography of proteins on columns has shown that the affinity of a protein for a sorbent often depends on the nature of the other proteins present in the solution[10]. A protein with a high sorptive affinity usually displaces another protein with a lower affinity for the sorbent; mutual replacement of the proteins takes place on the column. The mutual replacement of proteins on calcium phosphate was first utilised by Polis and Shmukler[24] for the purification of milk peroxidase. The following factors play an important part in the mutual replacement of proteins on calcium phosphate columns, the first being the affinity of the proteins for the sorbent. It is important that the difference in the affinities for the sorbent, shown by the displacing protein and the protein being displaced from the column, should be considerable. Satisfactory results can be obtained by the mutual replacement of proteins only if the condition is satisfied. Other important factors are the quantity of replacing protein added to the column, and the concentration of the buffer solution in which the replacing protein is dissolved. All these factors have been examined in detail by Boman[25].

In addition to the replacement of proteins during chromatography on calcium phosphate columns, other effects due to the mutual influence of the proteins may be produced. It has been noted[10] that in many cases a protein (usually strongly sorbed on columns) in the presence of another protein (with a lower sorbability) itself shows a reduced sorbability during chromatography on columns.

The mutual influence of proteins evidently plays an important part in chromatography on calcium phosphate columns and may influence significantly the results of chromatographic experiments. It is thus important to understand and make allowance for all the factors influencing the chromatography of proteins and to extend our knowledge in this field, where, up to the present, a purely empirical approach to the selection of optimum conditions for the chromatography of biopolymers has been adopted.

IV. THE USE OF CALCIUM PHOSPHATE GELS FOR THE CHROMATOGRAPHY OF ENZYME PROTEINS

In recent years, the methods of adsorption chromatography have been widely used in preparative biochemistry, particularly for the purification and isolation of the most varied enzyme proteins. Almost all work on the fractionation of enzymes now involves the use of chromatography. Chromatography is often the chief method leading to successful results in the isolation of enzyme proteins.

It is not our aim to cover completely all the literature on the use of calcium phosphate sorbents for the chromatography of enzymes, and we shall restrict ourselves chiefly to work carried out in the last ten years. It is during this period that particularly extensive use has been made of adsorption chromatography on columns in work on enzyme proteins. Thus French workers[26,27] have recently described the isolation, purification, and study of hog pancreas lipase. This work was carried out with chromatographic columns containing various adsorbents — tricalcium phosphate, brushite, hydroxyapatite, and ion-exchange cellulose. In chromatography on calcium phosphate sorbents, the chromatogram of the eluted enzyme shows one peak, irrespective of the method of elution; several active enzyme fractions were obtained on ion-exchange cellulose. The appearance of a large number of lipase components during chromatography on ion-exchange cellulose was attributed to artefacts[27]. This work is of methodological interest, since different adsorbents were used for the chromatographic study of the same material, and for pancreas lipase calcium phosphate appears to have a definite advantage over ion-exchange cellulose.

Choline esterase from human blood serum has been obtained by chromatography on hydroxyapatite[28]. In this work, the ion-exchange resin Dowex-2 was also used for the chromatographic study of the enzyme, and hydroxyapatite showed considerable advantages over ion-exchange resins. Among the enzymes which split ester linkages, enzymes hydrolysing esters of phosphoric acid have been studied by chromatography on calcium phosphate gels — phosphomonoesterase from human red blood corpuscles[29], and alkaline phosphatase from hog kidneys[30] — the chromatographic separation giving in the latter case several fractions exhibiting enzymatic activity. The question of their nature remains open. Esterase from hog liver has been separated from "ballast" proteins and obtained in the pure state by means of hydroxyapatite columns[31]. The behaviour of the purified liver esterase on hydroxyapatite columns showed that the enzyme can be separated into two or three fractions having similar specific activities[32]. Analysis of these fractions showed that their appearance is due not to differences in their primary structure but apparently to partial association of the molecules of this enzyme[32,33]. This example in particular shows the importance of making an all-round study of the products of the chromatographic separation. Chromatography on calcium phosphate gels has also been used for the study of other enzymes — amylase from wheat flour[34] and phenolase[35], where several fractions exhibiting cresolase activity were obtained. The presence of several active fractions was shown for nicotinamidase[36] by chromatography on hydroxyapatite. In this case the fractions obtained were examined in an ultracentrifuge and also by further chromatographic experiments. It was concluded that the existence of these fractions was due to an artefact produced as a result of autolysis in the early stages of isolation of the enzyme. Among the group of enzymes bringing about transfer reactions — transferases — various dehydrogenases have been subjected to chromatography on calcium phosphate sorbents[37-45]. Among the oxidases, chromatography on calcium phosphate gels has been used successfully for the isolation of monoaminooxidases from cow's blood[46]. In this case, several active enzyme fractions with similar specific activities were obtained. Another enzyme from this group — the aminooxidase from cow's blood — has recently been studied by chromatography on a column of hydroxyapatite[47]. In this case, a combination of column methods with different adsorbents — calcium phosphate and ion-exchange cellulose — was used. The advantages of hydroxyapatite over cellulose for this enzyme were noted.

Among the transaminases, enzymes which bring about the transfer of an amino-group, tyrosine–oxoglutarate–transaminase[48] and alanine–oxoglutamate–transaminase[49] have been examined by chromatography on calcium phosphate gels. In both cases, several active enzyme fractions showing different chromatographic behaviour were detected, but unfortunately they were not investigated further. Other enzymes which have been examined by chromatography on calcium phosphate gels include bacterial polynucleotide phosphorylase[50], trans-ketolase from hog liver[51], transferase from rabbit liver[52], etc. An interesting attempt has been made to use chromatography on a column of hydroxyapatite for the separation of brain enzymes[53]. Some separation of the enzyme peaks was achieved, although a more complete separation of the latter might apparently be achieved by further chromatographic experiments. This work is of interest since it is the first attempt to use chromatography on calcium phosphate for the isolation and fractionation of enzymes from brain tissue.

The above examples show that the chromatography of enzymes on calcium phosphate columns often gives several fractions exhibiting the same enzymatic activity. The same phenomenon is observed in the chromatography of enzymes on ion-exchange cellulose[7]. The question of the chromatographic heterogeneity of enzyme proteins on their elution from columns undoubtedly deserves attention. The results of chromatographic experiments on columns cannot always be interpreted unambiguously, and the reason for the appearance of enzyme fractions with different chromatographic behaviour, but the same activity, is often obscure.

Recent work on the column chromatography of biopolymers has shown that, in addition to the difficulties introduced into the interpretation of the experimental results by the actual elution technique (see Section III for more details), there are a number of other factors, not related to the method of elution, which make difficult an unambiguous explanation of the experimental data. These may be responsible for the appearance of a number of protein fractions with the same (or different) enzymic activity.

We give below examples of such cases and an assessment of the factors responsible for the appearance of a large number of components in protein polymers during chromatography of the latter on calcium phosphate columns. Thus in the chromatography of phycocyanin and phycoerythrin on hydroxyapatite, chromatographically different fractions of these proteins may appear as a result of association–dissociation effects[10].

In the case of ribonuclease from hog pancreas[54], the appearance of chromatographic heterogeneity in the enzyme was due to the removal of polyanions combined with the protein.

Changes produced by various factors in the secondary, tertiary, or quaternary structure of a protein may also be responsible for the appearance of a number of components when the protein is subjected to chromatographic study[23,55].

In other cases, the appearance of a number of components may be due to differences in the age of the material being studied[56]. The nature of the chromatographic curve may sometimes be influenced by spontaneous hydrolysis of the protein material being studied[57]. A difference in the quantity of phosphate groups in the protein can also apparently lead to the appearance of a number of components on elution from calcium phosphate columns[17]. Finally, the existence of individual active forms of the same protein may correspond to its true state. This has recently been shown in work on the chromatography of various proteins on different adsorbents[48,58-60].

Thus the above details show that a variety of factors may be responsible for the appearance of a number of components in the same protein during chromatography on columns. In all such cases, a special study of the protein fractions by different independent methods is desirable in order to evaluate the results obtained. These supplementary data will make it possible in each individual case to establish the reasons for the appearance of a number of components in the proteins being studied. It must apparently be assumed that there are a number of factors leading to the appearance of heterogeneity in protein polymers during column chromatography.

V. THE CHROMATOGRAPHY OF SERUM PROTEINS AND OTHER PROTEINS ON CALCIUM PHOSPHATE

In recent years there has been a considerable increase in the number of papers devoted to the chromatography of plasma and serum proteins on calcium phosphate gels.

The chromatography of the proteins of normal human serum on hydroxyapatite columns was studied in detail by Hjerten[3]. The protein components were found to leave the column in a definite order. It is of interest to compare the behaviour of serum proteins on hydroxyapatite columns with the behaviour of the same proteins during chromatography on ion-exchange cellulose. Work by Sober et al.[61] revealed the distribution of the components of human serum during chromatography on diethylaminoethylcellulose.

Table 1 illustrates the behaviour of serum proteins during chromatography on hydroxyapatite and on diethylaminoethylcellulose columns. The order in which the serum proteins leave the column is different for hydroxyapatite and diethylaminoethylcellulose (Table 1). On elution from hydroxyapatite, α_1-globulin and albumin appear first, and γ- and β-globulins appear last; whereas on diethylaminoethylcellulose columns, γ-globulin and β-globulin appear first, and albumin and α_2-globulin appear last. On elution from columns of ion-exchange cellulose, proteins having higher isoelectric points appear before proteins with lower isoelectric points; whereas on elution from hydroxyapatite, this dependence on the isoelectric points of the proteins is not observed.

TABLE 1.

Adsorbent	Components of serum proteins eluted from column				
	1	2	3	4	5*
Hydroxyapatite	α_1**	Al***	α_2**	γ**	β**
Diethylaminoethylcellulose	γ**	β**	α_1**	Al***	α_2**

* The figures give the order in which the proteins leave the column.
** Globulin.
*** Albumin.

From this it follows that the principles of the separation of proteins on calcium phosphate gels and on ion exchangers based on cellulose are different, that the chromatography of proteins is different for these materials, and that these groups of sorbents cannot replace one another[3].

A number of workers have studied the behaviour of individual serum protein components during chromatography on calcium phosphate columns. The albumins of human serum[3,34], ox serum[10,62,63], and horse serum[17] have been studied by chromatographic methods.

Table 2 summarises data on the chromatography, on hydroxyapatite columns, of serum albumins from different sources. Albumins of different origin, on chromatography on hydroxyapatite, give three components which are always eluted from the columns at definite phosphate buffer concentrations (Table 2). The slight difference in the percentage ratio of components eluted can apparently be attributed to a difference in the purity of the original albumin specimens and to differences in the methods used to prepare the hydroxyapatites.

An interesting feature is that the chromatography of human serum albumin on ion-exchange cellulose[61] also

TABLE 2*.

Albumin from serum	Phosphate buffer used for elution, M			Ref.
	0.07	0.11	0.40	
Ox	50	30	20	10
Ox	50	37	13	62
Ox	67	25	8	63
Horse	67	21	12	17
Human	50	30	20	3
Human	62	28	10	34

* The figures give the percentage of protein eluted from the columns.

gives not less than three different components. The reason for the appearance of chromatographic heterogeneity in serum albumin is not completely clear.

Various workers have studied the behaviour of other serum proteins — α-globulin[3,65], β-globulin[3], and γ-globulin[3,10] — on calcium phosphate columns. In chromatography on hydroxyapatite columns, γ-globulin usually gave three components, whose real existence was confirmed by further chromatographic study[10]. Columns of hydroxyapatite have been used to separate an electrophoretically rapid component of γ-globulin from a component of lower mobility[3]. A scheme has been proposed for the isolation of γ-globulin directly from serum by means of hydroxyapatite columns[3,64].

A number of papers have been devoted to the chromatography of ceruloplasmin on calcium phosphate columns[3,66-68]. This protein has been shown to be chromatographically heterogeneous[68]. The behaviour of fibrinogen from human plasma has been studied on calcium phosphate columns[69,70]. An interesting feature is that the chromatography of fibrinogen was found to be more satisfactory at room temperature than at +4°.[69]

Thus the behaviour of proteins both from whole serum and from its individual components has been studied in some detail on calcium phosphate columns, and a number of components have been shown to be heterogeneous.

Chromatography on calcium phosphate columns has been applied successfully not only to serum proteins but also to other proteins, such as milk albumin[71], egg albumin[10,17], milk proteins[72,73], egg proteins[74], saliva proteins[75], and soya-bean proteins[76,77]. Calcium phosphate gels have also been used for the chromatography of acid mucopolysaccharides (hyaluronic acid and chondroitin sulphuric acid[78]).

It has been found that acidic mucopolysaccharides are sorbed satisfactorily only by calcium phosphate precipitated under definite conditions at pH 9.2, whereas hydroxyapatite prepared by Tiselius' method[10] sorbs these proteins very weakly. This shows again the importance of standardising methods for preparing calcium phosphate sorbents and the possibility of variation in the properties of the latter.

Finally, a number of papers have been devoted to the use of calcium phosphate gels for the chromatography of hormones — insulin[79], lactogenic hormone from sheep hypophysis[80], the luteinising hormone from hypophysis[81], the follicle-stimulating hormone from hog hypophysis[82], and the hormone which stimulates the formation of sheep plasma erythrocytes[83].

These papers show that it is possible to separate and isolate various hormone proteins by means of calcium phosphate columns. In the case of insulin[79] and the luteinising hormone from sheep hypophysis[81], chromatography has shown the existence of several fractions exhibiting hormone activity. Chromatography on calcium phosphate has also been used to isolate and purify certain protein toxins[84,85].

VI. THE CHROMATOGRAPHY OF NUCLEIC ACIDS AND VIRUSES ON CALCIUM PHOSPHATE GELS

Calcium phosphate gels have been used only fairly recently for the chromatography of nucleic acids. One of the first papers in this field is that by Semenza[86], who used hydroxyapatite for the chromatography of DNA from calf thymus. An interesting feature is that DNA is sorbed on hydroxyapatite much more strongly than are ordinary proteins. The chromatography of DNA from calf thymus on calcium phosphate columns has also been carried out by other workers[87].

The DNA eluted from the column appears a single sharp peak[86], in contrast to the results obtained, for example, on ECTEOLA cellulose[88], where considerable heterogeneity of the DNA was observed.

According to modern ideas, DNA from cells have very high molecular weights, and are apparently not divided into subunits, but form continuous polynucleotide chains[89]. In the chromatography of these structures, there is, of course, a real danger of fragmentation and disaggregation of the DNA molecules. It is not impossible that the appreciable heterogeneity of DNA of a given type, observed in chromatography on ECTEOLA cellulose, is due to breakdown of a single DNA molecule[90]. In this respect, calcium phosphate gels apparently have an advantage, since chromatography on the latter usually gives only one peak (or a maximum of two or three) for DNA.[87] Calcium phosphate gels have been used by other workers[18,91] for the chromatography of DNA.

It has recently been shown that chromatography on calcium phosphate columns can be used for the successful separation of mixtures of DNA and RNA.[92,93] A mixture of DNA from calf thymus and RNA from tobacco mosaic virus can be separated[92] in a single operation on a calcium phosphate column.

A good separation of mixtures of DNA and RNA isolated from *Salmonella typhimurium* bacteria has also been achieved[93]. Two peaks are obtained for RNA and one peak for DNA at the column outlet. This work shows the great importance of chromatographic methods using calcium phosphate columns for the separation of complex biological mixtures such as those formed by the nucleic acids. Fairly good separation of the nucleic acids is achieved.

Interesting work has been done on the chromatography of deoxyribonucleotides isolated from the spleen of normal mice and of mice subjected beforehand to radiation[94]: a considerable difference was observed in the elution profiles for the deoxyribonucleoproteins from the irradiated and normal animals. This work indicates new possibilities for the use of calcium phosphate columns and their ability to reflect structural differences between normal and pathological cell deoxyribonucleoproteins. Chromatography on calcium phosphate columns has been carried out not only with DNA but also with RNA from different sources, high-molecular RNA from the cells of Ehrlich's ascites tumour[95], RNA from rabbit liver[96], and soluble RNA from rat liver[97] and from *Escherichia coli* microbial corpuscles[98].

This last paper[98] reported chromatography on hydroxyapatite columns to separate, in one operation, soluble RNA specific to the amino-acids valine and phenylalanine. Development of work in this direction is of considerable interest for the preparation of different types of soluble RNA specific to individual amino-acids.

Noteworthy papers have recently appeared on the chromatography of virus RNA on calcium phosphate gels — RNA obtained from Krebs' ascites tumour cells infected with encephalomyocarditis virus[99], virus RNA isolated

from foot and mouth disease virus[100], and RNA from tobacco mosaic virus[92,101] – and in some of this work[99,100], the RNA was eluted from the column in several peaks. It should be noted, however, that calcium phosphate gels have as yet been comparatively little used for the isolation and characterisation of nucleic acids; nevertheless these sorbents are extremely effective for the chromatographic study of both RNA and DNA obtained from the most varied sources.

The use of adsorption column methods for the chromatography of viruses has been carried out successfully only in recent years. Chromatographic studies on calcium phosphate columns have been carried out for the viruses of influenza[102,103], herpes[104], encephalomyocarditis[105], and encephalitis[106,107], arbor viruses[108], adenoviruses[109], measles virus[110], and also plant viruses[101,111]. The use of column-chromatographic methods in the study of macromolecules such as virus molecules, which have a very high molecular weight and complex structure, presented considerable difficulties, but suitable techniques and adsorbents have been found, which make it possible to isolate virus nucleoproteins from a complex mixture of substances by column chromatography.

Experiments with individual viruses[100] have shown that the medium in which the virus to be tested is cultivated is of importance in chromatography. For example, the chromatographic behaviour of herpes virus depended on whether the virus had been grown in a cell culture or in a chick embryo cavity. Preliminary treatment of the test material with sodium ethylenediaminetetraacetate was also important[100].

Interesting work with arbor viruses[108] has shown that chromatography on calcium phosphate columns may be a convenient and simple method for preparing stable haemagglutinin and complement-fixing antigens, both for the diagnosis of these viruses and for the detailed study of their physicochemical properties.

With adenoviruses, for example, chromatography on calcium phosphate gels can be used for rapid determination of the virus type, using type-specific sera[109], and for the isolation of three different components of adenoviruses, giving the complement-fixing reaction[109]. This last feature has also been shown in the chromatography of adenoviruses on diethylaminoethylcellulose[112].

Finally, mention may be made of the chromatography of coliphages on calcium phosphate columns[113]. An interesting feature is that the ability of calcium phosphate gels to adsorb virus nucleoproteins is much more marked than that for bacteriophages[113].

VII. THE FURTHER DEVELOPMENT AND FUTURE PROSPECTS OF THE CHROMATOGRAPHY OF BIOPOLYMERS ON CALCIUM PHOSPHATE

In recent years a number of papers have pointed out new possibilities for using calcium phosphate gels in the chromatography of biopolymers. It has been shown[10,99,114] that chromatography on calcium phosphate columns can probably differentiate biopolymer molecules according to molecular weight. Protein molecules of small dimensions are eluted from a column by a lower phosphate buffer concentration than are molecules of high molecular weight. Thus chromatography on calcium phosphate columns can be used for the approximate estimation of the molecular dimensions and weight of protein materials to be studied by chromatographic methods.

Interesting papers on the use of calcium phosphate sorbents[10,22] point out that chromatography on calcium phosphate columns can be used successfully to detect and observe the phenomenon of the association–dissociation of protein polymers in cases where these processes take place in a pH range the same as (or adjacent to) that in which the chromatographic process is carried out.

Calcium phosphate sorbents have also been applied in a completely different field[115,116]. It has been shown that chromatography on calcium phosphate can be used to follow the changes in the protein components of blood serum in certain illnesses. Thus the presence, in the patient's blood, of different fractions of the protein ceruloplasmin alters the chromatographic curve[115] and indicates different stages of the pathological process. Chromatography on calcium phosphate columns apparently provides a sufficiently sensitive and flexible method of study and can probably be widely used for the detection of various pathological changes in blood proteins in certain illnesses.

Calcium phosphate gels have found a very interesting application in the study of serological reactions[117,118]. It has been shown possible to study the reaction of an antigen with an antibody directly on the columns. Finally, recent work has revealed a new property of calcium phosphate gels, namely their ability to adsorb certain elements[119] and heavy-metal ions[120] from the surrounding medium.

A recent interesting discovery is related to the behaviour of calcium phosphate gels in the transfer of the calcium phosphate residues of nucleosides to the surface of these gels[121]. This transphosphorylation process may take place over wide ranges of temperature and of pH. It is apparently of definite importance in the chromatography of compounds containing free phosphate groups, which may take part in this reaction.

The above review of the chromatography of biopolymers on calcium phosphate gels shows the various fields of study in which columns of calcium phosphate gels may be used. Further study of the properties of these gels, extension of the range of their application, and detailed investigation of the mechanism of the chromatography of biopolymers on calcium phosphate columns are desirable.

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