CHROMATOGRAPHY OF PROTEINS ON CELITE ION-EXCHANGE RESINS

I. PREPARATION OF THE RESINS AND A STUDY OF THE CHROMATOGRAPHIC BEHAVIOUR OF CYTOCHROME *c*

N. K. BOARDMAN*

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge (Great Britain)

INTRODUCTION

Most of the adsorbents used for the chromatography of low molecular weight substances are not suitable for the chromatography of proteins. The main reason for this probably lies in the ease of denaturation of proteins at interfaces, although in some instances adsorbents have proved unsatisfactory for the chromatography of proteins because their capacity to adsorb proteins was too low. Due to their size, proteins are unable to penetrate into the finer pores of an adsorbent. Interaction between protein and resin takes place mainly on the surface of the adsorbent particles with a result that only adsorbents with large surface areas per gram are able to adsorb appreciable quantities of protein.

In the field of ion-exchange chromatography, the most successful adsorbent so far has been a finely divided form (XE-64) of the weakly acidic ion-exchange resin, Amberlite IRC-50. Several basic proteins[1] and the neutral proteins, haemoglobin[2] and myoglobin[3] were successfully subjected to chromatography on this resin. Ion-exchange resins prepared from cross-linked polystyrene have proved, in general, unsuitable for the chromatography of proteins because of their low capacity for protein[4]. In fact, the low capacity of polystyrene resins for protein was used to advantage for the separation of amino acids and peptides from proteins[5,6] and for the desalting of proteins[7]. However, there are reports in the literature of the use of resins derived from polystyrene for the chromatography of proteins. Columns of Dowex 50 were used in the purification of prostatic phosphatase[8] and for the separation of chymotrypsin and chymotrypsinogen[9], and a number of proteins were subjected to chromatography on columns of the anion-exchange resin, Dowex 2, by BOMAN AND WESTLUND[10]. But BOMAN AND WESTLUND[10] conclude from their experiments that the greatest shortcoming of Dowex 2 for the separation of proteins is its low capacity for protein.

* Present address: Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, A.C.T., Australia.

References p. 397.

A number of ion-exchange adsorbents with high capacities for protein have been derived from cellulose[11]. Columns of these cellulose ion-exchange resins were used for the purification of some enzymes and in the fractionation of proteins from serum[12].

In the work reported here, ion-exchange resins with high capacities for protein were prepared by coating particles of a diatomaceous earth with ion-exchange materials. This communication describes preparations of a carboxylic acid ion-exchange resin and a sulphonic acid ion-exchange resin, but the method is also applicable to the preparation of anion-exchange resins. A preliminary account of the preparation of the sulphonic acid resin and its use in the chromatography of insulin were published previously[13]. A composite Celite–sulphonated polystyrene ion-exchange resin, with a low degree of cross-linking was prepared in this laboratory by FEITELSON AND PARTRIDGE[14]. This resin was specially designed for the chromatographic separation of large peptides.

The preparations of a weakly acidic ion-exchange resin and a strongly acidic ion-exchange resin, both with high capacity for protein offered a good opportunity for a detailed study of the behaviour of a particular protein on both types of resin under identical conditions of pH and cation concentration.

The protein chosen for study was cytochrome *c* as it was shown previously[2] that this protein could be successfully subjected to chromatography on columns of Amberlite IRC-50 over ranges of pH and cation concentration.

MATERIALS

Diatomaceous earth. This was Celite 545 obtained from John Manville and Co. Ltd., Artillery Row, London, S.W.1. Fines were removed by suspending the material in water, allowing it to settle for 30 min and removing the supernatant suspension. This process was repeated several times until the supernatant was clear after a settling time of 30 min. The product was dried in an oven at 105°. Celite, treated in this manner was used for the preparation of the carboxylic acid resin, but for the preparation of the sulphonic acid resin the Celite was made water-repellent by treatment with dichloro-dimethyl-silane[15].

Styrene. The commercial material contains a polymerisation inhibitor which was removed by shaking 4 or 5 times with 10% NaOH, followed by washing with water until the aqueous phase was no longer alkaline. The styrene was dried over anhydrous Na_2SO_4.

Divinyl-benzene. The commercial material which contains approximately 50% divinyl-benzene was used. The inhibitor was removed as described for styrene.

Methacrylic acid. This was purified by distillation under reduced pressure.

Cytochrome c. The material prepared from horse heart extracts by the method of KEILIN AND HARTREE[16] was purified by chromatography on columns of Amberlite IRC-50. The oxidised fraction was used in the chromatographic experiments.

References p. 397.

METHODS

Preparation of Celite–carboxylic acid ion-exchange resin

The ion-exchange material was polymethacrylic acid, cross-linked with divinyl-benzene. Particles of Celite were coated with this material by heating a methanolic solution of methacrylic acid and divinyl-benzene in the presence of Celite and a suitable catalyst such as benzoyl peroxide. The method of polymerisation was similar to that used for the formation of vinyl polymers in wool[19,20] and the amount of polymethacrylic acid deposited on the Celite was controlled by the concentration of methacrylic acid in the methanol. The following procedure gave a product which contained 7.5% (w/w) polymethacrylic acid to Celite. The nominal degree of cross-linking was 10%. Benzoyl peroxide (0.1 g) was dissolved in a small quantity of benzene (1–2 ml) and added to methyl alcohol (75 ml). To this solution was added methacrylic acid (6.5 ml) and divinyl-benzene (1.2 ml) and the resulting mixture was stirred into dry Celite (50 g) to give a homogeneous mixture. Polymerisation was carried out in a sealed tube at 80° for 24 h. The product was washed thoroughly with methyl alcohol followed by water and dried at 105°. The total capacity of the resin for Na^+ was determined by reacting samples of resin (0.1 g) with 0.01 *N* NaOH (25 ml) and allowing to stand for several hours with occasional shaking. Aliquots (10.0 ml) of the supernatants were back-titrated with 0.01 *N* HCl using a mixture of methyl red and methylene blue as indicator. The capacity of the above preparation for Na^+ was 0.69 m.equiv./g dry resin, compared with 9.2 for Amberlite IRC-50.

The polymer was found to be uniformly spread over the particles of Celite. A small amount of the new resin was treated with the dye, basic fuchsin, and after thoroughly washing with water the particles were examined under the microscope. Dye was found to be evenly distributed over the particles: untreated Celite showed no uptake of dye. The resin was purified by alternate treatments with 0.1 *N* NaOH and 0.1 *N* HCl and it was finally converted to its sodium form with 0.1 *N* NaOH.

Preparation of Celite–sulphonic acid ion-exchange resin

The ion-exchange material was cross-linked sulphonated polystyrene. Celite was coated with cross-linked polystyrene by a method similar to that described for the carboxylic acid resin. However, in experiments with untreated Celite, polymer was formed preferentially in the methyl alcohol and it was difficult to obtain a stable film of polymer on the Celite particles. This difficulty was overcome by using Celite which had been made water-repellent by treatment with dichloro-dimethyl-silane. As in the preparation of the carboxylic acid resin, the amount of the polymer on the Celite was controlled, to a large extent, by the concentration of monomer in the methyl alcohol. Celite coated with 5% (w/w) cross-linked sulphonated polystyrene was used in the experiments reported in this paper.

Benzoyl peroxide (0.07 g) was dissolved in benzene (5 ml) and then methyl alcohol (10 ml) was added. This solution was added to methyl alcohol (60 ml), containing styrene (6.0 ml) and divinyl-benzene (0.6 ml) and the resulting mixture was

References p. 397.

stirred into 45 g of water-repellent Celite. The mixture was placed in a glass tube, the neck of which was partly drawn out. The tube was immersed in liquid oxygen and evacuated. Nitrogen was admitted and the tube was re-evacuated and nitrogen admitted again. The nitrogen filled tube was sealed off and placed in an oven at 65–70° for 7 days. The product of the polymerisation was washed thoroughly with methyl alcohol, followed by water, and after drying, it was sulphonated at 100° in concentrated sulphuric acid with silver sulphate as catalyst[21]. The sulphonated material was washed with repeated changes of distilled water until free from acid. The capacity of the resin for Na^+ was 0.28 m.equiv./g dry resin; the nominal content of divinyl-benzene was 5% of the weight of styrene. The sulphonic acid resin was purified by alternate treatments with 0.05 *N* NaOH and 0.1 *N* HCl and finally converted to its sodium form with 0.05 *N* NaOH.

Preparation and operation of columns

Purified resin was suspended in the buffer used for developing the chromatogram, allowed to settle for 1 h and the supernatant removed. This procedure was repeated 2 or 3 times until the supernatant was clear after a settling time of 1 h. The procedures employed in packing and operating the columns were the same as described previously[2], except that the columns were packed under a pressure of 5–10 cm Hg. The experiments with cytochrome *c* were carried out in jacketed columns at 25°.

0.3 ml samples containing 1–3 mg of purified cytochrome *c* were chromatographed on individual columns of the Celite–sulphonic acid resin, Celite–carboxylic acid resin and also on untreated Celite. The diameter of the columns was 0.9 cm and the heights of resin varied from 6 to 10 cm. In each experiment, the total volume of effluent, $V + Ve^2$, which passed through the column from the time of application of the sample until the maximum concentration of cytochrome appeared in the effluent was determined and then corrected to a standard resin height of 10 cm. The rate of flow of buffer through the column was 1 ml/h and the effluent was collected in 0.3 ml fractions.

Buffers

The effect of Na^+ concentration on the elution of cytochrome *c* from each of the coated Celite resins and from untreated Celite was studied at a constant pH of 7.0. Buffers contained 50.0 g of $Na_2HPO_4 \cdot 12H_2O$ and 8.6 g of $NaH_2PO_4 \cdot 2H_2O$/litre. NaCl was added to bring the Na^+ concentration to the required level and the pH was adjusted to pH 7.0 with *N* NaOH. The effect of pH on the elution of cytochrome *c* was studied at a constant Na^+ concentration of 1.0 g ions/litre. Between pH 5 and pH 8, citrate buffers were used, from pH 6 to pH 8 phosphate, and from pH 9 to pH 10 borate buffers. In all cases NaCl was added to bring the total concentration of Na^+ to 1.0 g ions/litre.

Analysis of effluent

Cytochrome *c* in the effluent was determined photometrically at 407 mμ in a Hilger "Uvispec" spectrophotometer.

References p. 397.

RESULTS

Figs. 1 and 2 show the effects of pH and sodium ion concentration on the elution of cytochrome *c* from columns of Celite coated with cross-linked polymethacrylic acid (Cel-MX), Celite coated with cross-linked sulphonated polystyrene (Cel-SPX), and untreated Celite. In the experiments where the pH was varied, the Na^+ concentration was 1.0 g ions/litre and in the experiments where the Na^+ concentration was varied, the pH was 7.0.

The behaviour of cytochrome *c* on columns of Cel-MX was very similar to its behaviour on columns of Amberlite IRC-50[2]. In the pH range 5.5–6.5 the adsorption

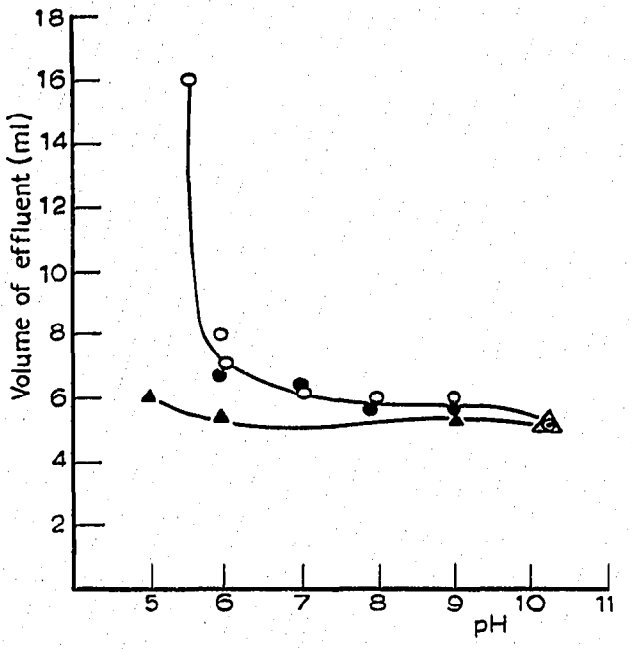

Fig. 1. Effect of pH on the elution of cytochrome *c* from columns of ion-exchange resin 10 × 0.9 cm. –○–○–○– Cel-SPX ion-exchange resin; –●–●–●– Cel-MX ion-exchange resin; –▲–▲–▲– Celite 545. Na^+ concentration 1.0 g ions/litre.

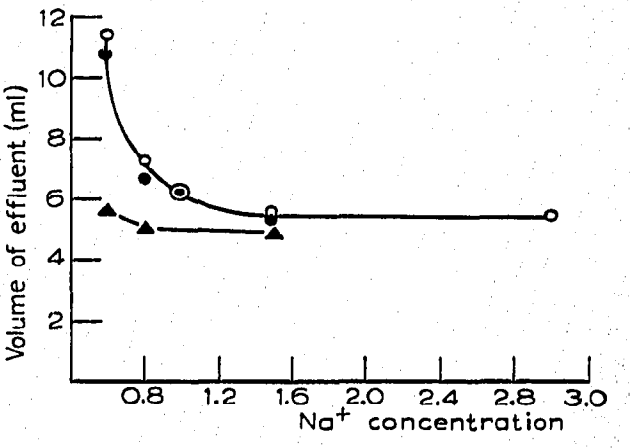

Fig. 2. Effect of sodium ion concentration on the elution of cytochrome *c* at pH 7.0 from columns of ion-exchange resins. –○–○–○– Cel-SPX ion-exchange resin; –●–●–●– Cel-MX ion-exchange resin; –▲–▲–▲– Celite 545.

of cytochrome *c* was sharply dependent on the pH; a large increase in the adsorption occurred at pH values below 6 and under still more acidic conditions the cytochrome band remained stationary at the top of the column. In the pH range 7–9 the adsorption of protein was almost constant and it decreased slightly as the pH approached the isoelectric point of cytochrome *c*. As in the experiments with IRC-50 the adsorption of cytochrome *c* was dependent on the Na^+ concentration of the eluting buffer but the concentration of Na^+ required to elute cytochrome *c* from columns of Cel-MX at pH 7 was considerably higher than that required to elute the protein from columns of IRC-50. For example, with a column of IRC-50 and an eluting buffer of pH 7.0 and Na^+ concentration 0.34 g ions/litre the cytochrome band moved with an $R_F = 0.4$, but for the same rate of movement on columns of Cel-MX, a buffer of a Na^+ concentration 0.7 g ions/litre was required.

References p. 397.

The variations with pH and Na^+ concentration in the rate of movement of cytochrome *c* down columns of the sulphonic acid resin were almost identical with the variations observed on columns of Cel-MX. Similar changes in the adsorption of cytochrome *c* on both resins with Na^+ concentration at pH 7 were more or less expected since both resins are highly charged at this pH, but a surprising feature of the work was the rapid rise in the adsorption of cytochrome *c* on the sulphonic acid resin at pH values below 6.

Reversibility of the adsorption of cytochrome c

Although the R_F values of cytochrome *c* on columns of the carboxylic acid resin and the sulphonic acid resin were nearly identical, the shapes of the elution peaks on the two resins were not the same. Whereas the bands of cytochrome *c* on the carboxylic acid resin were almost symmetrical, the bands on the sulphonic acid resin showed tailing, particularly at R_F values < 0.5. This indicates that there was some irreversible binding of cytochrome *c* on the columns of the sulphonic acid resin. Thus, for the chromatography of a basic protein, such as cytochrome *c*, a carboxylic acid resin appears preferable to a sulphonic acid resin.

Untreated Celite showed a small adsorption for cytochrome *c* at pH 7 and Na^+ concentration 1.0 g ions/litre and this adsorption increased only slightly at low concentrations of Na^+ or at pH values below 6 (Figs. 1 and 2).

Gradient elution analysis

Weakly acidic ion-exchange resins, like IRC-50, with a high capacity for sodium ions, are not particularly suitable for chromatographic experiments involving either stepwise or gradual changes in the pH or sodium ion concentration of the eluting buffer, in the pH range 5–7. These resins are very strong buffers in this pH range and

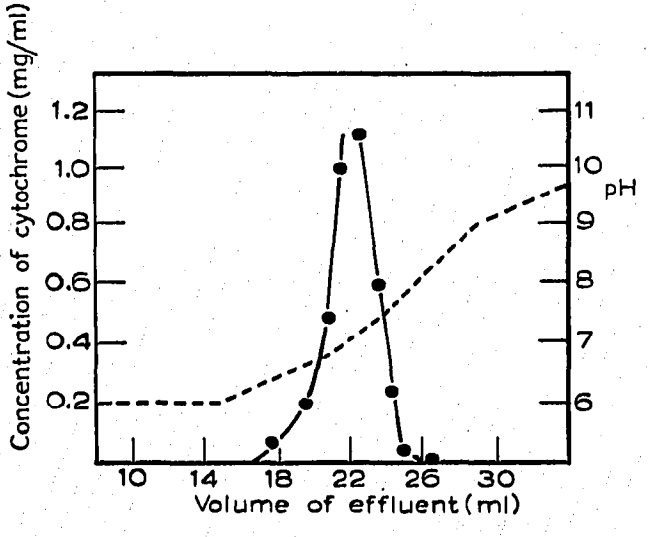

Fig. 3. Chromatogram of cytochrome *c* on a column of Cel-MX ion-exchange resin, 4.2 × 1.8 cm. An eluting buffer of gradually increasing pH was used to elute the cytochrome *c*. Amount on column: 3.8 mg. Dotted line shows the calculated pH values of effluent.

References p. 397.

large volumes of eluting buffer are required to effect changes in the pH of the effluent from columns of such resins. Because of its low buffering capacity per ml of column volume, compared with IRC-50, Cel-MX ion-exchange resin may be used to advantage where it is desired to elute the proteins by a stepwise or gradual increase in the pH or cation concentration of the eluting buffer.

As an example, Fig. 3 shows the chromatogram obtained when purified cytochrome *c* was eluted from a column of Cel-MX by a buffer of gradually increasing pH. It may be noted that the rear of the cytochrome *c* band is sharper than the front, due to the higher pH gradient in the vicinity of the rear boundary. Although the pH of the eluting buffer applied to the column varied linearly with the volume passed through the column over the pH range 6–11, the pH of the emerging buffer increased more steeply with volume as the pH rose above 7, due to the progressively lower buffering capacity of the resin as the resin becomes saturated with sodium ions. It is possible to calculate the pH of the buffer emerging from the column from a knowledge of the titration curves of both resin and buffer and the pH of the buffer applied to the column[4]. The calculated values agree well with those determined experimentally.

Capacity of the Celite ion-exchange resins for protein

In the experiments with cytochrome *c* and insulin, loads of 5–10 mg protein/cm^2 cross-section of column were successfully chromatographed on the Celite ion-exchange resins.

DISCUSSION

Chromatography of cytochrome c

Previously, it was suggested that the large increase in the adsorption of cytochrome *c* on Amberlite IRC-50 at pH values below 6 was due to uncharged carboxyl groups on the resin, since it was observed that the rapid rise in the elution volumes of cytochrome *c* occurred over the same range of pH as the fall in amount of sodium ions retained by the resin from buffers of comparable concentration[2]. A similar hypothesis would explain the observed behaviour of cytochrome *c* on columns of Cel-MX.

However, the experiments with the sulphonic acid resin show that cytochrome *c* is adsorbed strongly at pH values below 6 in the absence of uncharged carboxyl groups on the resin. With the sulphonic acid resin, the high adsorption of cytochrome *c* is not due to a change with pH in the ionization of the groups on the resin. It now seems unlikely, therefore, that uncharged carboxyl groups on the resin are responsible for the increased adsorption of cytochrome *c* on columns of IRC-50 or Cel-MX, although they may contribute to the overall adsorption. The factors mainly responsible for the adsorption of cytochrome *c* either on the sulphonic acid or carboxylic acid resin are most likely changes in the configuration or ionization of the protein molecule.

The rapid rise in the adsorption of cytochrome *c* on both carboxylic acid and sulphonic acid resins at pH values below 6 is probably due to an increase in the number of points of attachment between protein and resin at these pH values. An increase in

References p. 397.

the binding of protein to resin could result from a simple increase in the nett charge on the protein molecule or it could arise from configurational changes in the protein molecule, since rearrangement of the protein molecule may lead to the formation of new points of attachment between protein and resin. It follows from a consideration of mass action that there will be a large increase in the adsorption of cytochrome *c* on lowering the pH from 7 to 5, if the number of points of attachment between protein and resin is increased while the sodium ion concentration in the ambient phase remains constant. For example, an increase in the number of binding points from (say) 5 to 8 could result in a very large increase in elution volume[2].

Chromatography of acidic proteins

Although cytochrome *c* showed similar adsorptions on both the carboxylic acid and sulphonic acid resins, the acidic protein insulin behaved in a different way[22]. Whereas insulin was reversibly adsorbed on Cel-SPX- at pH 3.4 from an acetate buffer of sodium ion concentration 1.0 g ions/litre, with a distribution coefficient of approximately 1, it was irreversibly adsorbed on the carboxylic acid resin, Cel-MX. This would suggest that in the case of insulin uncharged carboxyl groups are playing a prominent role in the adsorption of the protein.

Previous studies on the adsorption of proteins by Amberlite IRC-50[4] indicated that this carboxylic acid resin was not suitable generally for the chromatography of acidic proteins. The proteins studied either were not adsorbed by the resin or they were strongly adsorbed, depending on the pH of the eluting buffer. All the proteins were strongly adsorbed at pH values below 5, where the ionization of the carboxyl groups on the resin was suppressed. It seemed to us that a sulphonic acid resin with a high capacity for protein may prove a suitable adsorbent for the chromatography of acidic proteins, as the sulphonic acid groups are fully ionized within the pH range 1–12.

Experiments with insulin showed that the Celite-SPX ion-exchange resin was a suitable adsorbent for the chromatography of a relatively stable, acidic protein of low molecular weight. However, studies with a number of other proteins such as bovine serum albumin, bovine haemoglobin, lactoglobulin and tobacco mosaic virus showed that the chromatographic behaviour of these proteins on columns of Cel-SPX was sharply dependent on the pH and cation concentration of the eluting buffer in the pH range 5–6. The experiments with tobacco mosaic virus will be reported in a future publication. Thus, the behaviour of these acidic proteins on the sulphonic acid resin closely resembled their behaviour on a carboxylic acid resin such as Amberlite IRC-50. Conditions for the successful chromatography of an acidic protein on Cel-SPX may therefore be fairly sharply defined and the ideal pH and salt concentration should be evaluated for each protein by carrying out test tube experiments[23].

The fact that a protein can be eluted from the resin only over a narrow range of pH and Na^+ concentration and that this range varies from protein to protein makes the method of elution with a buffer of constant composition unsuitable for the separation of all proteins in a mixture. A buffer which elutes one of the proteins in the mixture is unlikely to effect a satisfactory separation of all the proteins in the

References p. 397.

mixture. The elution of all the proteins, individually, will require a series of buffers of increasing eluting power or, alternatively, a buffer of gradually increasing eluting power[24]. Both the stepwise elution and gradient elution techniques were used by TISELIUS, HJERTEN AND LEVIN[25] and by BOMAN AND WESTLUND[10] in their experiments on the separation of proteins on calcium phosphate and ion-exchange resins, respectively. Although elution of proteins by stepwise changes in the buffer or by the gradient method offers many practical advantages over elution with a buffer of constant composition, great care must be exercised in interpreting the results where the former methods are used. This point is discussed in greater detail in the following paper[22].

As postulated for the basic protein cytochrome *c*, the rapid change in the adsorption of the acidic proteins on Cel-SPX with pH of buffer is probably due to an increase in the number of points of attachment between protein and resin. It is postulated that the increased binding of cytochrome *c* to the resins could result either from an increase in the nett charge on the protein or from configurational changes in the protein. With the acidic proteins, the increased binding is probably due to configurational changes in the protein molecules, since the adsorption of the acidic proteins changes so markedly over narrow ranges of pH. It appears that the adsorption of a protein on an ion-exchange resin can be a very sensitive method for distinguishing between protein molecules. Changes in the configuration of a protein may result in dramatic changes in the adsorption of the protein. If a particular configurational change in a protein molecule is sharply dependent on pH, then the adsorption of that protein may be sharply dependent on pH. This situation bears comparison with the acid denaturation of haemoglobin. From studies on the kinetics of denaturation of haemoglobin, STEINHARDT AND ZAISER[26] were led to the view that the combination of hydrogen ions with a small number of "trigger groups" in the protein sufficed to initiate its denaturation as measured by loss of solubility at the isoelectric point and by the all or nothing appearance in each molecule of 36 acid binding groups.

The adsorption and desorption of a protein on a cation-exchange resin may possibly be described by the following scheme.

Adsorption $$P_1 + xH^+ \rightleftharpoons P_1H_x \rightarrow P_2H_x \rightarrow \text{Resin } P_2H_x$$

Desorption $$\text{Resin } P_2H_x - xH^+ \rightarrow \text{Resin } P_2 \rightarrow \text{Resin } P_1 \rightarrow P_1$$

The protein molecule represented by state P_1 is not adsorbed by the resin, but combination with a few protons initiates a change in the configuration of the protein molecule (P_2H_x) and then adsorption of the protein by the ion-exchange resin takes place. The desorption of the protein is brought about by removing protons from the adsorbed protein. This removal of protons enables the protein to assume the configuration which is not adsorbed by the resin. This hypothesis, no doubt, represents an over-simplified picture, as it is possible to visualize a number of ionization states for the protein molecule, resulting in configurational changes in the protein. However, such changes in the configuration of a protein may explain the dramatic changes in the adsorption behaviour of proteins with small changes in pH.

References p. 397.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. S. M. PARTRIDGE for his interest in this work and for helpful suggestions. The author is indebted to the University of Cambridge for an Imperial Chemical Industries Fellowship and to Dr. E. C. BATE-SMITH for the provision of laboratory facilities.

SUMMARY

1. Ion-exchange resins with a high capacity for protein were prepared by coating particles of the diatomaceous earth, Celite 545, with ion-exchange materials. Preparations of a carboxylic acid resin and a sulphonic acid resin are described.

2. A detailed study of the behaviour of cytochrome *c* on both resins was made under varying conditions of pH and cation concentration. The surprising feature of the results was the very high adsorption of cytochrome *c* on the sulphonic acid resin at pH values below 6.

3. A possible hypothesis to explain the high adsorption of proteins on cation-exchange resins in the region of pH 5 is outlined.

REFERENCES

1 S. MOORE AND W. H. STEIN, *Advances in Protein Chem.*, 11 (1956) 191.
2 N. K. BOARDMAN AND S. M. PARTRIDGE, *Biochem. J.*, 59 (1955) 543.
3 N. K. BOARDMAN AND G. S. ADAIR, *Nature*, 177 (1956) 1078.
4 N. K. BOARDMAN, *Dissertation*, Cambridge (1953).
5 A. R. THOMPSON, *Nature*, 169 (1952) 495.
6 S. M. PARTRIDGE, *Nature*, 169 (1952) 496.
7 H. H. TALLAN AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 507.
8 H. G. BOMAN, *Biochim. Biophys. Acta*, 16 (1955) 245.
9 N. SAKOTA, *J. Biochem. (Japan)*, 42 (1955) 649.
10 H. G. BOMAN AND E. WESTLUND, *Arch. Biochem. Biophys.*, 64 (1956) 217.
11 H. A. SOBER AND E. A. PETERSON, *J. Am. Chem. Soc.*, 76 (1954) 1711.
12 H. A. SOBER, F. J. GUTTER, M. M. WYCKOFF AND E. A. PETERSON, *J. Am. Chem. Soc.*, 78 (1956) 756.
13 N. K. BOARDMAN, *Biochim. Biophys. Acta*, 18 (1955) 290.
14 J. FEITELSON AND S. M. PARTRIDGE, *Biochem. J.*, 64 (1956) 607.
15 G. A. HOWARD AND A. J. P. MARTIN, *Biochem. J.*, 46 (1950) 532.
16 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 39 (1945) 289.
17 N. K. BOARDMAN AND S. M. PARTRIDGE, *Nature*, 171 (1953) 208.
18 E. MARGOLIASH, *Nature*, 170 (1952) 1014.
19 M. LIPSON AND J. B. SPEAKMAN, *J. Soc. Dyers Colourists*, 65 (1949) 390.
20 N. K. BOARDMAN AND M. LIPSON, *J. Soc. Dyers Colourists*, 69 (1953) 335.
21 K. W. PEPPER, *J. Appl. Chem. (London)*, 1 (1951) 124.
22 N. K. BOARDMAN, *J. Chromatog.*, 2 (1959) 398.
23 C. H. W. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.
24 R. S. ALM, R. J. P. WILLIAMS AND A. TISELIUS, *Acta Chem. Scand.*, 6 (1952) 826.
25 A. TISELIUS, S. HJERTEN AND O. LEVIN, *Arch. Biochem. Biophys.*, 65 (1956) 132.
26 J. STEINHARDT AND E. M. ZAISER, *J. Am. Chem. Soc.*, 75 (1953) 1599.

Received November 25th, 1958