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CHROMATOGRAPHY OF PROTEINS ON CELLULOSE ION EXCHANGERS

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INTRODUCTION

Chromatography of proteins on cellulose ion exchangers was first successfully used in 1956 by Peterson and Sober [1-3] and by Ellis and Simpson [4], and in the following years it became the most widely used procedure in preparative protein chemistry, particularly in the later purification stages. Cellulose ion exchangers have several properties that make them suitable for the chromatography of proteins, the most outstanding being high separating power; many crystalline proteins, and also protein preparations, that appear homogeneous under electrophoresis and ultracentrifuging have been found to be heterogeneous when they are chromatographed on cellulose ion-exchange columns [5-10]. Proetins suffer very little damage on these columns and the yield often reaches 100%. The chemical stability of the columns, their low adsorptive capacity for low-molecular-weight compounds, and their low resistance to liquid flow are also of great importance.

Experimental work on the chromatography of proteins on cellulose ion exchangers has been summarised in several reviews [11-14]. However, no attempt has yet been made to examine the experimental results in a methodical manner with the object of elucidating the mechanism of adsorption and separation. The development of this promising method is greatly hindered by the absence of information of this kind, and the necessity of selecting experimental conditions empirically means that full use cannot be made of all the possibilities of the technique.

In the present paper we do not aim at covering all the literature on the subject, but we have collected and summarised the main results that throw some light on the mechanism of adsorption and separation and which can be used for the setting up and planning of experiments. Not all the theories in the paper have a reliable experimental basis; some are partly hypotheses. However, their discussion is justified by the almost complete absence of special studies on the chromatography of proteins, and we hope it will promote the development of investigations of this kind.

I. CELLULOSE ION EXCHANGERS

1. The Synthesis of Cellulose Ion Exchangers

The fibre in natural cellulose is partly oriented, having a semicrystalline structure, but when cellulose is treated with a concentrated alkali solution, as in mercerisation, the alcoholate (alkali cellulose) is formed:

$$[C_6H_7O_2(OH)_3]_n + n\,NaOH \longrightarrow [C_6H_7O_2(OH)_2ONa]_n + n\,H_2O\,.$$

When the solution is diluted with water, free cellulose is re-formed, but the orientation of the fibres is completely destroyed. This is accompanied by an increase in hydrophilic properties and reactivity. The general method of preparing cellulose ethers, the class of compounds to which the cellulose ion exchangers at present in use belong, consists in the reaction of an alkyl halide with alkali cellulose in the presence of an excess of alkali:

$$[C_6H_7O_2(OH)_2ONa]_n + m\,n\,R\,CH_2Cl + 2n\,NaOH \rightarrow [C_6H_7O_2(O\,CH_2\,R)_m]_n + 3n\,NaCl\,.$$

Here m = 1, 2, or 3, and n depends on the degree of polymerisation of the cellulose, being generally 6000–10 000. If R contains ionising groups, the cellulose ether formed will be an ion exchanger. The number of ether bonds m per polymer unit is determined by the concentration of the alkyl halide in the reaction mixture. The more ionising groups are present, the greater is the adsorptive capacity of the exchanger, but the presence of too many of these groups leads to gelatinisation, or even to dissolution of the cellulose. In the exchangers generally used, the number of ionising groups does not, as a rule, exceed 1–2 mg-equiv. per gramme of adsorbent, *i.e.* about one ionising group per 4–8 glucose residues.

Phosphocellulose is an ester of phosphoric acid and cellulose. Esters of cellulose with organic acids have also been synthesised and used as ion exchangers [15-17]. Diethylaminoethyl cellulose was first synthesised by Hartmann [18] in 1930. More recently, aminoethylcellulose [19], *p*-aminobenzylcellulose [20], triethylaminoethylcellulose [21], ecteola-cellulose, and cation exchangers [21], including carboxymethylcellulose [22], have been prepared. The ion-exchange properties of cellulose exchangers were first pointed out by McIntire and Schenk [17], and the first attempt to employ them for purifying proteins was made by Hoffpauir and Guthrie [23]. Syntheses of the most widely used cellulose ion exchangers are described by Peterson and Sober [2] and by Ellis and Simpson [4], and also in "Biochemical Preparations" [24]. The use of these exchangers in paper form appears to be very promising [25,26]. Recently, diethylaminoethyl Sephadex [27-31] has been synthesised and used in the fractionation of proteins and other compounds; this differs from cellulose ion exchangers in the structure of the insoluble ion, being based on the polysaccharide dextran, in which the chains are cross linked. The adsorbent therefore has a three-dimensional mesh structure.

Attempts have been made to prepare enzyme-specific adsorbents in which the ionising group is an analogue of the substrate of the enzyme to be separated [32]; simple unmodified cellulose acts as a specific adsorbent for enzymes that hydrolyse cellulose [33]. For enzymes that act on ionising substrates, these attempts can hardly be regarded as soundly based, because the non-specific electrostatic interaction between the protein and the exchanger will predominate over the interaction between the exchanger and the active centre of the enzyme.

2. General Properties of Cellulose Ion Exchangers

All cellulose ion exchangers are weak acids or bases. The number of ionising groups and the ionisation constants can be determined by titrating a suspension in a 1 M solution of NaCl with a glass electrode; Fig. 1 shows typical titration curves. The number of ionising groups may also be found from nitrogen, phosphorus, or sulphur determinations.

The presence of like charges on molecules of the ion exchanger leads to mutual repulsion between sections of the chain; this renders the ionising groups more readily accessible even to large exchanging ions.

The rate of exchange on synthetic resins is determined by the time required for ions to diffuse into the adsorbent granules, this being the slowest process. This factor is of particular importance in the adsorption of high-molecular-weight compounds such as proteins[32]; for this reason, in protein chromatography on synthetic resins extremely slow flow rates must be used (~ 2 ml h^{-1} per cm^2 of cross-section). In cellulose ion exchangers, the matrix is not a three-dimensional mesh, as in synthetic ion-exchange resins, but consists of "one-dimensional" fibres, separated from one another in the column by a layer of hydrate or capillary water through which protein molecules pass freely. The internal diffusion factor is therefore eliminated and very high flow rates (up to 50 ml h^{-1} per cm^2 of cross-section) may be used without impairing the results.

Because of the hydrophilic properties of cellulose in aqueous suspensions, these ion exchangers do not form sharp liquid–solid boundaries, unlike mineral adsorbents, or liquid–oil boundaries, and unlike synthetic ion-exchange resins. The adsorbent is, as it were, covered by an envelope of water of hydration. This has two important consequences: first, the sorption of proteins on surfaces is excluded, and second, surface denaturation is eliminated. As has been pointed out, the yield of proteins in cellulose ion-exchange chromatography can reach 100%; when thyrotropine is chromatographed on Amberlite, ~ 50% is lost, but on a column of carboxymethylcellulose the same degree of purification is achieved with 100% yield[35].

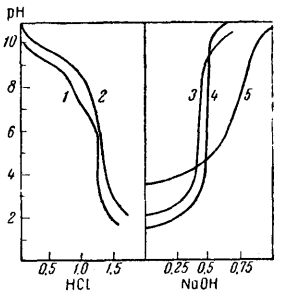


Fig. 1. Titration curves of cellulose ion exchangers:
1) diethylaminoethylcellulose; 2) triethylaminoethylcellulose; 3) sulphomethylcellulose; 4) sulphoethylcellulose; 5) carboxymethylcellulose.

The pK values of cellulose cation exchangers range from 2 to 2.5, and those of anion exchangers from 7 to 9.5. The exchangers are thus weak acids or bases. Their salts should therefore be strongly hydrolysed, especially those with weak acids or bases, for example, amino-acids. This means that low-molecular-weight compounds of this type move along the column at the same rate as the solvent, or at a rate differing only slightly from that of the solvent[36,37]. Proteins are also weak acids or bases, but they are strongly adsorbed, because the multiplicity of charge on the protein ion allows multiple bonds to be formed between the protein and the exchanger.

An increase in the content of ionising groups increases the adsorptive capacity and separating power of the cellulose ion exchanger[3], but the tendency to gelatinisation with increasing capacity leads to increased resistance to liquid flow, which hinders chromatography or make it impossible. Completely soluble polysaccharides with a high content of ionising groups (1–3 per glucose residue) may be used for the fractional precipitation of proteins[38,39].

Particle size has no appreciable effect on adsorptive properties. The adsorbents generally used have a particle size of 200–400 mesh. If the particles are in the form of long fibres (over 1 mm long), uniform packing of the column is impeded, as ion exchangers of this size form lumps; moreover, after the column has been packed and the external pressure removed, the volume of the packed exchanger may increase considerably because of the mechanical elasticity of the fibres. The type of cellulose from which the sorbent has been prepared has some effect on the properties[2].

3. Cellulose Anion Exchangers

Diethylaminoethylcellulose, or DEAE-cellulose, is prepared by the reaction of 2-chlorotriethylamine hydrochloride with cellulose in concentrated alkali solution. In aqueous suspension it forms a hydrate, which dissociates to give a hydroxide ion, the ion which undergoes exchange:

$$\text{Cell.}C_2H_4N(C_2H_5)_2 + H_2O \rightleftharpoons \text{Cell.}C_2H_4\overset{+}{N}H(C_2H_5) + OH^-.$$

DEAE-cellulose is a weakly alkaline anion exchanger (pK' = 9.5) and the most versatile cellulose ion exchanger. It is widely used for the fractionation of proteins; it can also be used for the separation of nucleic acids[40], nucleotides[41–43], viruses[44], peptides[45–48], polysaccharides[49], and other compounds[50,51].

Triethylaminoethylcellulose, or TEAE-cellulose, is obtained by the reaction of DEAE-cellulose with ethyl bromide[21]. The ionising group appears to have the structure of a quaternary ammonium base with hydroxide as the exchangeable ion. It differs little from DEAE-cellulose in properties and range of application[52–54].

Aminoethylcellulose, or AE-cellulose (Cell. .$C_2H_4NH_2$), is a somewhat less alkaline exchanger than DEAE-cellulose (pK' = 9.3).[55,56]

Ecteola-cellulose, or ecteola, is a product of the reaction of epichlorohydrin and triethanolamine with cellulose in concentrated alkali solution. The structure of the ionising group is unknown. It is a less alkaline anion exchanger than DEAE-cellulose (pK' = 7.6). Ecteola is most suitable for fractionating nucleic acids[57–60] and

nucleoproteins[61-63], and it is also used for isolating nucleotides[64,65], heparin and hyaluronic acids[66,67], and viruses[68-70]. It is curious that the chromatographic behaviour of bacteriophage on ecteola columns is determined by the protein and not by the nucleic acid component[71]. Ecteola has been successfully used for purifying the alkaline phosphatase from pig's kidney, but this enzyme is probably not a protein[72].

Paraminobenzylcellulose, or PAB-cellulose (Cell.$CH_2C_6H_4NH_2$), is a still less alkaline anion exchanger than ecteola. It is mainly used in the preparation of protein ion exchangers for the isolation of antibodies and the fractionation of nucleic acids[73-75].

Guanidoethylcellulose, or GE-cellulose (Cell.$.OC_2H_4NHC{=}NHNH_2$), is prepared by the guanidation of aminoethylcellulose. The ionising groups are titrated at pH 8.5–10.[56]

4. Cellulose Cation Exchangers

Carboxylmethylcellulose, or CM-cellulose (Cell.CH_2COOH), is obtained by the reaction of cellulose with monochroroacetic acid in concentrated alkali solution. It is a weak cation exchanger ($pK' = 3.6$), and is used for separating alkaline, neutral and weakly acid proteins[76-82], and also peptides[83-85] and viruses[86].

Phosphocellulose, or P-cellulose, is an ester of phosphoric acid and cellulose [Cell.$PO(OH)_2$]. It is prepared by the reaction of cellulose with phosphorus oxychloride in concentrated alkali solution and is more strongly acid than CM-cellulose. In each phosphoric acid residue two hydroxy-groups are capable of dissociating ($pK_1' = 3$; $pK_2' = 7$). It is used in the chromatography of proteins and some other compounds[54,88].

Sulphoethylcellulose, or SE-cellulose (Cell.$.C_2H_4SO_2OH$) and sulphomethylcellulose (SM-cellulose, Cell.CH_2SO_2OH) are still more acid cation exchangers than phosphocellulose[21]; they are used for fractionating proteins[89-92].

II. NATURE OF THE INTERACTION OF PROTEINS WITH CELLULOSE ION EXCHANGERS

1. Adsorption by an Ionic Mechanism

The adsorption of proteins on cellulose ion exchangers is mainly due to electrostatic forces. This hypothesis is supported by the following facts: (1) the overwhelming majority of proetins that are adsorbed on these exchangers are not adsorbed by untreated cellulose containing no ionising groups; (2) the adsorptive capacity of the exchanger increases with the number of ionising groups; (3) adsorption is rapid, equilibrium being reached in a few minutes[93]; and (4) when proteins are eluted from cellulose cation exchangers by a solution of increasing pH, the components appear in the order of decreasing electrophoretic mobility; when they are eluted from anion exchangers by an eluent of decreasing pH, the fractions appear in the eluate in the order of increasing electrophoretic mobility[3,94,95].

Mitz and Schlueter[96] observed a stoichiometric relationship in the interaction of proteins with cellulose ion exchangers: 1 g of DEAE-cellulose containing 0.8 mg-equiv. per gramme of ionising groups binds 0.8 g of pepsin (equivalent weight 1000); if half the carboxy-groups of pepsin are uncharged, the same amount of adsorbent binds twice as much pepsin; if the number of active adsorbent groups is doubled, the amount of pepsin bound is also doubled.

The adsorption of proteins on cellulose ion exchangers may therefore be represented by the following ion-exchange equations:

$$\text{Cell-a}^+\text{A}^- + \text{P}^- \rightleftharpoons \text{Cell-a}^+\text{P}^- + \text{A}^-, \quad (1)$$

$$\text{Cell-c}^-\text{C}^+ + \text{P}^+ \rightleftharpoons \text{Cell-c}^-\text{P}^+ + \text{C}^+, \quad (2)$$

where Cell-a^+ and Cell-c^- are the insoluble ions of the cellulose anion and cation exchangers respectively, A^- and C^+ are their exchangeable ions, and P^- and P^+ are the anionic and cationic forms of the protein.

In the ion exchange of proteins specific features of the protein molecule must be considered, the main ones being (1) amphoteric character, (2) the multiplicity of charge on the protein ion, and (3) the large dimensions of the protein molecule and the distribution of active ionising groups over its whole surface.

In the adsorption of dipolar ions, in addition to electrostatic attraction that occurs in the adsorption of ions with like charges, electrostatic repulsion also occurs. However, Samsonov and Kuznetsova[97] have shown that the adsorption of dipolar ions on H-exchangers proceeds as follows:

$$\text{RSO}_3^-\text{H}^+ + \text{NH}_3^+\text{R}_1\text{COO}^- \rightleftharpoons \text{RSO}_3^-\text{NH}_3^+\text{R}_1\text{COOH}, \quad (3)$$

that is, the exchangeable hydrogen ion passes to a negatively charged group of the dipolar ion, and the ion is converted to a cation and adsorbed without electrostatic repulsion. This interpretation leads to the conclusion that dipolar ions of the amino-acid type should be adsorbed to a much smaller extent by ion exchangers in the salt form, since the salt is always dissociated, and in this case electrostatic attraction and repulsion will compete. This conclusion has been confirmed experimentally by Samsonov and Kuznetsova. Proteins are also adsorbed better by cellulose from a salt-free medium[93,96], which enables Eqn. (3) to be applied. The further apart the oppositely charged groups, the weaker is the repulsion effect. Thus diglycylglycine and glycylglycine are adsorbed much better than glycine by SBS-2 resin and DEAE-cellulose[36,97]. Although the ionising groups of the polypeptide chain of the protein molecule are generally separated from one another by several amino-acid residues, they may approach when the polypeptide chain curls, as in the formation of secondary, etc. structures.

The following equations represent the adsorption of proteins on cellulose ion exchangers in the hydrogen and hydroxide forms, the special features of the ion exchange of bipolar ions being taken into account:

$$\text{Cell-a}^+\text{OH}^- + {}^-\text{OOCPNH}_3^+ \rightleftharpoons \text{Cell-a}^+{}^-\text{OOCPNH}_2 + \text{H}_2\text{O}, \quad (4)$$

$$\text{Cell-c}^-\text{H}^+ + {}^+\text{H}_3\text{NPCOO}^- \rightleftharpoons \text{Cell-c}^-\text{H}_3^+\text{NPCOOH}, \quad (5)$$

where $^-OOCPNH_3^+$ is the dipolar protein ion. However, these equations cannot be regarded as an accurate description of the process; on the one hand, the number of positively and negatively charged groups on the protein ion may not be the same and, on the other, the spatial arrangement of the charged groups that give rise to repulsion may be such that they remain dissociated.

Another peculiarity of the exchange of dipolar ions is that the same ion may be adsorbed by both cation and anion exchangers; in some separation methods proteins are adsorbed successively by anion and cation exchangers [94,95,98,99]. This property is reflected in Eqns. (4) and (5), in which the protein residues may be identical.

The multiplicity of charge on the protein ion coupled with the large dimensions of the protein molecule lead to another peculiarity in the adsorption of proteins: in adsorption the reacting unit is not the whole molecule but only a limited section of its surface. Since the ionising groups are not distributed uniformly over the molecule, it is clear that under the same conditions, the same protein ion can be held by the same adsorbent with a force that varies according to the orientation of the molecule on the adsorbent surface. This may be one reason why chromatographic zones become diffuse in the elution of proteins from cellulose ion-exchange columns.

Naturally, the properties of the protein molecule as a whole also affect the adsorption properties, the magnitude of the total charge on the molecule being of primary importance. As has been mentioned, when a protein mixture is chromatographed the components are often eluted in the order of increasing (on cellulose cation-exchange columns) or decreasing (on anion-exchange columns) electrophoretic mobility, the pH of the eluent being somewhat lower than the isoelectric point of the protein in elution from anion-exchange columns, and somewhat higher in elution from cation-exchange columns. Thus the protein may remain in the adsorbed state even when the total charge is of the same sign as that of the insoluble cellulose-exchanger ion. On the other hand, if the total charge on the protein molecule is opposite to that of the insoluble cellulose-exchanger ion, this does not mean that the protein will be adsorbed. Thus thetin homocystein methyltransferase, with an isoelectric point of 6.4, is not adsorbed on DEAE-cellulose at pH 6.8. [100] Therefore, although the magnitude of the total charge affects the adsorption properties of proteins, it does not determine them completely.

The presence of a dipole in the protein molecule may also affect the adsorption properties; the adsorption will, of course, increase with the dipole moment. Since cellulose ion exchangers are either weak acids or weak bases, it would seem that their saltlike compounds with proteins, which are also weak acids and bases, should be strongly hydrolysed, that is, adsorption of proteins on cellulose ion exchangers in an aqueous medium should be negligible. The stability of the compounds formed by proteins with cellulose ion exchangers in aqueous suspensions can be explained by the multiplicity of charge on the protein molecule, which makes possible the formation of a large number of salt bonds with the exchanger. Amino-acids and small peptides, which have the same ionising groups as proteins, are either not adsorbed or they form only a weak bond with the ion exchanger. Thus, only the most alkaline amino-acids, histidine, lysine, and arginine, are adsorbed on CM-cellulose, and only aspartic and glutamic acids on DEAE-cellulose [37]. Glycylglycine and diglycylglycine are adsorbed on DEAE-cellulose, but they are very easily eluted, glycylglycine by water and diglycylglycine by a solution of CO_2 at 1 atm. [36] The effect is also observed in the converse experiment; soluble ionising derivatives of polysaccharides form complexes with proteins, but similar derivatives of oligosaccharides (with four carbohydrate residues) are not complexed [101-103]. The effect of the number of charged groups is clearly shown in the chromatography of adenylic acid polymers on cellulose ion exchangers: polymers from the dimer to the hexamer are eluted in the order of increasing chain length, that is, in the order of increasing number of charged groups in the molecule [104]. Statistically, the strength of the multiple bond is explained in the following way: the exchange of one multicharged ion for several singly-charged ions requires the simultaneous approach of these several ions to the bond, which is unlikely; on the other hand, the formation of one salt linkage between a protein and a cellulose ion exchanger promotes the formation of subsequent bonds by binding the protein to the exchanger.

All that has been said above about the relative electrostatic interaction between the protein and the cellulose ion exchanger may be summarised in the following equations. If X is the sum of all the ionising groups of the protein molecule that form charges on it opposite in sign to those on the insoluble exchanger ion, x and x' are ionising groups of the same type as X that do not participate in bond formation with the exchanger for steric reasons (x) or because they are not dissociated under the given conditions (x'), the number of salt linkages will be

$$Z = X - x - x'. \quad (6)$$

We shall term Z, the number of salt bonds formed between the exchanger and the protein molecule, the effective valency of the protein molecule. The main properties of the effective valency are its dependence on pH and on the orientation of the protein molecule on the adsorbent surface.

The total charge E of the protein molecule is

$$E = (X - x') + (Y - y'), \quad (7)$$

where Y is the sum of all the ionising groups of the protein molecule that on ionising acquire charges opposite in sign to the charges of the X groups; y' represents ionised type Y groups. Denoting the dipole moment by D, we can represent the adsorptivity m as a function of the effective valency, the total charge, and the dipole moment:

$$m = f(Z, E, D). \quad (8)$$

We introduce N, the electrostatic charge which would produce the same attractive force between the protein and the exchanger as the combined action of the factors Z, E, and D. We shall henceforth call N the effective charge on the protein molecule:

$$m = f(N); \quad (9)$$

this effective charge may be positive, negative, or zero.

We note further that in accordance with Eqns. (4) and (5) the total charge on the protein ion in the exchanger–ion complex differs from the total charge on the ion in solution. Denoting the charge on the adsorbed ion by E_a, we may write

$$E_a = (X - x') + (Y - y' - y''), \quad (10)$$

where y'' represents type Y groups that are no longer ionised after being adsorbed.

Clearly, the following relationship is always valid:

$$E_a > E. \quad (11)$$

and therefore

$$N_a > N, \qquad (12)$$

where N_a is the effective charge on the adsorbed protein.

In chromatography inequality (11) is reflected in the fact that proteins are eluted from anion-exchange columns at a pH below the isoelectric point, and from cation-exchange columns at a pH above the isoelectric point. However, this does not always hold good, because the effective charge, which determines the adsorption, contains the additional factors Z and D.

It follows from inequality (12) that when $N = 0$ the protein may be adsorbed on both cation and anion exchangers, N_a being positive with the former and negative with the latter. When N is positive the protein is adsorbed only on cation exchangers, and when N is negative only on anion exchangers, N_a having the same sign but a larger absolute value. Under all these conditions the most probable state of the system is that in which the protein is adsorbed on the cellulose ion exchanger, since at the moment of adsorption the interaction between the protein and the exchanger is increased; also for desorption to occur, forces greater than those that led to adsorption must be overcome.

The values of Z, E, and D may change independently from protein to protein; Z and D may depend on the dimensions and shape of the protein molecule (increasing as the dimensions increase or as the shape becomes less spherical), but E does not depend on these properties. These factors have a corresponding effect on the adsorptivity. Thus, cytochrome c, with a molecular weight of 97 000 is more strongly adsorbed on DEAE-cellulose than the rhodospirillum haem protein (RHP), with a molecular weight of 36 000, although both compounds have identical isoelectric points [105]. γ-Globulin (sedimentation constant 6.6 S) is not distinguishable electrophoretically from γ-macroglobulin (sedimentation constant 18 S) but the latter is more strongly adsorbed on DEAE-cellulose [106,107].

The following example is an excellent illustration of how adsorption is affected by the effective valency and the total charge. Nicotinamide-adeninedinucleotide, which has two phosphate residues per molecule, is less strongly adsorbed on DEAE-cellulose than nicotinamide-adeninedinucleotide phosphate, which has three phosphate residues. On the other hand, the oxidised forms of these nucleotides, which have a positively charged quaternary nitrogen in the pyridine ring, are adsorbed less strongly than the reduced forms, which have no positively charged groups in the molecule [108]. In the example given the effects of electrostatic charges on adsorption are all the more convincing because the molecules of the four compounds differ only slightly in structure and dimensions.

2. Other Adsorption Mechanisms

At present the behaviour and interaction of macromolecules in solutions and suspensions is being intensively studied, and problems of the chromatography of proteins on cellulose ion exchangers are a part of this general problem. In the early research work "anomalies" in the thermodynamics of polymer solutions were encountered [109]. The following results are of particular interest here. Several authors noted a positive or very small negative entropy change in the association of protein molecules with one another [110-112] or with carbohydrate polyions [101]. It was difficult to explain the entropy increase by the liberation of water of hydration, because the free energy decreased with association. An analogy can be drawn between these results and the adsorption of proteins on cellulose ion exchangers; in both cases electrostatic interaction occurs between two hydrophilic macroions. This analogy is further justified because in both cases the bond between the ions is extremely sensitive to pH changes; for example, when the pH changes from 8 to 4.4 the constant for the association of soya-bean trypsin inhibitor with trypsin changes by a factor of 100 000; [113] on the other hand, the adsorption properties of proteins on cellulose ion exchangers change so sharply with pH that several workers have referred to the process as adsorption on the "all-or-none" principle.

Proteins are not adsorbed on cellulose containing no ionising groups, but they are precipitated on columns of such cellulose at a lower concentration of ammonium sulphate that that required for precipitation from solution [114,115]. There is therefore some interaction between the protein and the cellulose, and this may increase the strength of the adsorption of proteins on cellulose ion exchangers. This interaction may be due to van der Waals' forces or to the formation of hydrogen bonds. In adsorption on carboxyl ion exchangers, in addition to the usual hydrogen bonds, bonds may also be formed between the protein carboxyls and the cellulose ion exchanger, all the more so because adsorptivity on these exchangers is greater in an acid medium, when dissociation of the carboxyls is largely suppressed. Bonds of this type are formed in protein–protein interaction [116]. The carboxyl–carboxyl hydrogen bond is extremely sensitive to pH changes; even a small change in pH leads to mass destruction of bonds of this type, or to an "ionisation explosion". This could be explained by a sharp change in the adsorption properties of proteins on cellulose ion exchangers as the pH changes. However, the adsorption properties are just as sensitive to pH changes on cellulose ion exchangers containing no carboxyls.

It is thus clear that a rigorous theory of protein chromatography on cellulose ion exchangers is very complex. At present there are no special studies of this kind and we are again compelled to turn to results on the wider problem of polyelectrolyte interaction. General questions of protein interaction (poly-equilibrium theory) have been discussed by Klotz [117].

Harris and Rice proposed a theory for the interaction of flexible polyelectrolytes with charges of the same sign which makes the best allowance for all possible polymer-structure and charge effects on interaction [118,119]. Cellulose ion exchangers are polyelectrolytes of exactly this type. This theory was later extended to polyampholytes [120].

Overbeek and Voorn [121] developed a theory for the interaction that accompanies phase separation. Notwithstanding several simplifying assumptions made by the authors, all the equations proposed are extremely complicated; since the simplifications exclude such factors as hydration, hydrogen bonding, and steric hindrance, these equations cannot be applied to real systems.

The adsorption of proteins on cellulose ion exchangers is characterised by the variety of effective forces and by the complexity of the mechanism. Nevertheless, the predominance of electrostatic forces is clear; the experimental results in the first section show this conclusively. It is therefore advantageous and useful to apply the laws of ion exchange to the interpretation of experimental results

for protein adsorption and chromatography on cellulose ion-exchange columns, even if only qualitative data can be obtained.

III. THE EFFECT OF NEUTRAL SALT AND HYDROGEN ION CONCENTRATION ON THE ADSORPTION OF PROTEINS BY CELLULOSE ION EXCHANGERS

1. Adsorption in a Salt-Free Medium

Proteins are most strongly adsorbed on cellulose ion exchangers from a salt-free medium, or from dilute buffer solutions. It is extremely important to note that the adsorptivity does not depend on the protein concentration; all the protein in solution is adsorbed until the adsorptive capacity is reached[93,96]. A method of concentrating protein solutions may be based on this property. On the other hand, we have here confirmation that the laws of ion exchange apply to the adsorption of proteins on cellulose ion exchangers. The exchange of ions with different charges is represented by Nikol'skii's equation[122]:

$$\frac{m_1^{\frac{1}{z_1}}}{m_2^{\frac{1}{z_2}}} = K\frac{c_1^{\frac{1}{z_1}}}{c_2^{\frac{1}{z_2}}}, \qquad (13)$$

where m_1 and m_2 are the amounts of adsorbed ions with charges z_1 and z_2 respectively and c_1 and c_2 are their concentrations in solution. It can be shown that dilution of the solution always leads to increased adsorption of a multicharged ion. If $c_1/c_2 = P$ (P is a constant), Eqn. (13) takes the form:

$$\frac{m_1^{\frac{1}{z_1}}}{m_2^{\frac{1}{z_2}}} = KP^{\frac{1}{z_1}} c_2^{\frac{1}{z_1}-\frac{1}{z_2}} = A. \qquad (14)$$

If $z_1 > z_2$, then $\lim A_{c_2 \to 0} = \infty$, which can only be satisfied by $m_2 = 0$ and $m_1 = m$ (m is the exchange capacity)[123].

Inequality (12) gives another explanation of the preferred adsorption of proteins from dilute solutions of salts (see p. 481).

2. Effect of Neutral Salts

If

$$c_1 + c_2 = c_0 \qquad (15)$$

and

$$m_1 + m_2 = m, \qquad (16)$$

the dependence of m_1 on c_1 is unambiguously expressed by the equation[123]

$$\frac{m_1^{\frac{1}{z_1}}}{(m-m_1)^{\frac{1}{z_2}}} = K\frac{c_1^{\frac{1}{z_1}}}{(c_0-c_1)^{\frac{1}{z_2}}}. \qquad (17)$$

where m is the adsorptive capacity and c_0 is the concentration of the displacer ion introduced into the system. Parameters of the displaced ion are indicated by the subscript (1) and those of the displacer ion by the subscript (2).

Since the curve of the dependence of m_1 on c_1 has no point of inflection, the curvature of the adsorption isotherm $m_1 = f(c_1)$ will always be either entirely positive (curve convex towards the concentration axis) or entirely negative. Convex isotherms may change to concave with a change in the parameters c_0, m, z_1, and z_2. When m, z_1, and z_2 are constant, this change occurs at a certain critical concentration, $c_0 = c_{cr}$, at which the isotherm is linear. The plot of m_1 as a function of c_0 must therefore be S-shaped, the critical concentration corresponding to a point of inflection on this curve. The plots of the adsorptivity of ribonuclease[124] and other proteins[93] on cellulose ion exchangers at different concentrations of sodium chloride are S-shaped (Fig. 2). This is further confirmation that the laws of ion exchange are applicable to the adsorption of proteins on cellulose ion exchangers.

Since c_{cr} for given values of m, z_1, and z_2 is related to the exchange constant by the expression[125]

$$c_{cr} = mK^{\frac{z_1 z_2}{z_1 - z_2}}, \qquad (18)$$

it is evident that the exchange constant can be determined from the dependence of m_1 on c_0. We have found no experimental confirmation of this in the literature.

Theoretically, the change in adsorptivity from zero to the maximum, which is the adsorptive capacity of the cellulose exchanger, occurs over an infinitely wide range of concentrations of the neutral salt. However, the rate of this change drops sharply as extremal values of the adsorptivity are reached and is large over a certain narrow concentration range. We shall apply the term transition range to the concentration range of neutral salt (or pH values) in which the adsorptivity changes from a value differing little from zero to a value close to the adsorptive capacity. It can easily be shown that this transition range decreases as the effective valency of the displaced ion increases. In fact, from Eqn. (14) we note that if $z_1 \gg z_2$, when c_2 decreases the value of m_1 more rapidly approaches a limit equal to the exchange capacity m. Because of the small width of the transition range in the adsorption of proteins on cellulose ion exchangers, some workers have spoken of the "all-or-none" principle applying to the adsorption[11,126]. The isotherms for adsorption from neutral salt solutions of varying concentration directly disprove this principle: while all

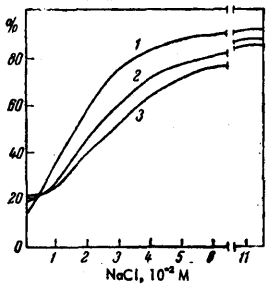


Fig. 2. Dependence of the adsorptivity of ribonuclease on CM-cellulose on pH and salt concentration: 1) pH 8.8; 2) pH 7.5; 3) pH 7.1. Unadsorbed ribonuclease is plotted along the ordinate.

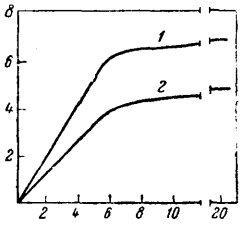


Fig. 3. Adsorption isotherms of asparaginase on DEAE-cellulose: 1) salt-free medium; 2) 0.08 M solution of NaCl. The quantity of asparaginase in the initial solution is plotted along the abscissa and the adsorbed asparaginase is plotted along the ordinate (arbitrary units).

the protein in solution is adsorbed in the absence of salts, irrespective of its concentration, the amount of protein adsorbed from salt solutions depends on its concentration, that is, an equilibrium is established (Fig. 3). In a salt-free medium the equilibrium is displaced towards the adsorbed protein, but when salts are added it is displaced more and more towards the dissolved protein[93]. Statistically, this is explained in the following way: when the concentration of the displacing ion is increased the number of complex collisions required for the displacement of a multicharged protein ion increases.

It should also be noted that in the presence of salts not only the rising but also the horizontal branch of the isotherm is lowered, that is, the adsorptive capacity of the cellulose ion exchanger decreases. This may be one of the causes of diffuse zones when proteins are eluted from these columns.

Salts may affect adsorptivity by another mechanism: salt ions may become bound to the protein, changing its electrostatic charge and therefore the adsorptivity. Changes in the activity of the ions and in the states of ionisation of the exchanger and the protein, due to changes in the ionic strength of the solution, may also be of some importance.

3. Effect of pH

The mechanism of the effect of pH of the medium is essentially different, since a change in pH leads to a change in ionisation of the exchanger and the protein. Since adsorptivity is greater on cellulose cation exchangers in an acid medium and on anion exchangers in an alkaline medium, that is, under conditions such that the ion exchanger is least dissociated and the ionising groups of the protein taking part in adsorption are most dissociated, it is evident that the state of ionisation of the protein, and not that of the exchanger, is important. A change in pH leads to a change in the effective valency, total charge, and dipole moment of the protein molecule, that is, affects all three quantities which determine the effective charge. If the pH changes in such a way as to reduce the effective valency, at a definite pH value ions of the buffer mixture and the protein will begin to exchange as ions that differ little or not at all in the number of charges. It is evident that the protein can then be displaced from the ion exchanger at a low eluent concentration; this has been observed in many studies on the chromatography of proteins on cellulose ion-exchange columns. Here there is also an equilibrium distribution of the protein between the adsorbent and the solution[93]. As the pH changes further, the effective charge may acquire the same sign as the charge on the ion exchanger, which completely excludes the possibility of adsorption.

In this case the very narrow pH range within which adsorptivity changes from zero to the maximum is also noticeable. This narrow range appears to be due to the simultaneous change of all the components of the effective charge, an entirely different reason from that which leads to a narrow pH range in elution by solutions of neutral salts.

IV. MECHANISM OF PROTEIN SEPARATION ON CELLULOSE ION-EXCHANGE COLUMNS

The main process in all chromatographic methods consists in the successive redistribution of the material to be separated between two substances moving relative to each other — between an adsorbent and a solvent, or between two solvents, two phases, etc. If proteins were adsorbed on cellulose ion exchangers according to the "all-or-none" principle, the adsorptivity of the protein should be either zero or equal to the adsorptive capacity of the exchanger, and the rate of movement of the protein along the column should be either zero or equal to the rate of movement of the solvent itself. This means that the protein is not redistributed between the exchanger and the solvent, and there is no true chromatographic process. This view does not explain the extremely high effectiveness of cellulose ion exchangers in fractionating proteins, and especially the tremendous advantages of the column technique over separation under static conditions. Moreover, some experimental results confirm that protein zones can move at rates intermediate between zero and the flow rate of the solvent. Thus, Boman and Kaletta found that when snake venom is chromatographed on DEAE-cellulose columns, an increase in pH or a reduction in eluent concentration leads to lengthening of the chromatogram and to a better separation of components[127]. The "all-or-none" principle excludes the use of elution analysis, but metmyoglobin and carboxymyoglobin can be separated on a DEAE-cellulose column by elution analysis[128].

The equilibrium character of the adsorption of proteins on cellulose ion exchangers over a certain pH range, or range of neutral salt concentration, shows that successive adsorption and desorption occur: the main separation mechanism is a true chromatographic process. It is therefore essential for the separation of proteins that the process be carried out under equilibrium conditions and that the transition ranges should not coincide. In elution with salt solutions the position of the transition range is determined by the effective charge on the protein, and in elution with solutions of varying pH by the pK values of the ionising groups of the protein that form the effective charge. Since the nature of these groups is practically the same in all proteins, selectivity will be low in elution with solutions of varying pH. For this reason, this elution procedure has been less widely used than elution with solutions of neutral salts.

Displacement of protein by protein is another possible separation mechanism, especially in the initial stage of the

chromatography immediately after the protein mixture has been deposited on the column [129-131]. However, great importance can hardly be attached to this mechanism, since at the column outlet protein zones are very often separated from one another by large protein-free zones, and therefore any interaction between the proteins is excluded here.

V. ELUTION OF PROTEINS FROM CELLULOSE ION--EXCHANGE COLUMNS

1. General Principles. Stepwise Elution

There are three main eluting procedures: elution, frontal, and displacement methods. The elution procedure is rarely used in the chromatography of proteins; the separation of myoglobins by Brown [128] on DEAE-cellulose columns is an example. The single-stage elution method is a combination of elution and displacement procedures [131,132]. The frontal method is unsuitable for preparative purposes, but it has been used in the analysis of mixtures of compounds, including protein mixtures [131].

Displacement is the method mainly used in the chromatography of proteins. Displacement is effected either by changing the salt concentration or by changing the pH within limits close to physiological values. The variation of pH with salt concentration can be either stepwise or smooth; if it is smooth, the procedure is termed gradient elution. As has been mentioned, the main condition for the separation of proteins on a column is that the transition ranges of the different proteins in the mixture should not coincide. Let us consider a model experiment.

The protein A is adsorbed on the upper part of a column; curve a represents the dependence of m_1 on c_0 and a' is the transition range (Fig. 4). When the column is washed with eluent of concentration c_1, the zone boundaries do not change. If the concentration is increased to c_2, the protein zone becomes diffuse (because the capacity of the exchanger is reduced) and begins to move along the column at a rate less than the flow rate of the eluent (because of equilibrium distribution of the protein between the adsorbent and the liquid phase). In this movement further spreading occurs, of the front boundary because the concentration of the eluent is below the critical value [125], and of the back boundary because the adsorption isotherms of proteins on cellulose exchangers in the presence of salts are curves convex away from the concentration axis. Fig. 5a shows the shape of the elution

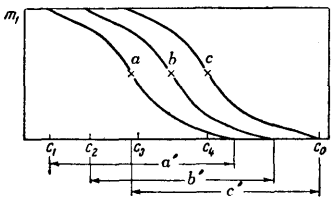


Fig. 4. Transition ranges of proteins. The concentration of the displacer ion is plotted along the abscissa and the quantity of adsorbed protein along the ordinate. Points of inflection on the curves corresponding to critical concentrations are indicated by crosses.

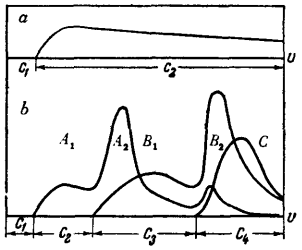


Fig. 5. Elution of a mixture of proteins (explanation in text).

graph or chromatogram. A very large volume of solution of concentration c_2 is required to elute all the protein.

If at the instant when a volume v_1 of eluent of concentration c_2 has passed through the column the concentration is raised to c_3, the remains of the protein form a new zone on the column before the front of the new eluent, and a second peak A_2 of the same protein A is obtained on the chromatogram (Fig. 5b). Since $c_3 > c_{cr}$, the front of the new zone becomes sharper. As before, the back boundary of the new zone spreads.

Let us now consider the elution of a mixture of proteins A, B, and C, for which the dependence of m_1 on c_0 is represented by curves a, b, and c, and the transition ranges are a', b', and c' (Fig. 4). When the column is washed with solution of concentration c_2, only protein A (peak A, Fig. 5b) is eluted; when the concentration is increased to c_3 the remains of protein A (peak A_2) and part of protein B (peak B_1) are eluted. At an eluent concentration c_4 the remains of protein B (peak B_2) are eluted with protein C. Thus, in this model only protein A (peak A_1) is obtained pure.

Fig. 5b shows the results of the chromatographic separation of three thyroglobulin fractions on a column of DEAE--cellulose [133]. With complex mixtures and a lower eluent concentration "step height", the same compound may form not two but several peaks [134,135]. This makes clear the disadvantages of elution in which the conditions are changed in steps. An uncritical approach to this method may have given rise to some unfounded conclusions [136].

Theoretically, even the most complex mixtures of compounds having different transition ranges should be separable by stepwise elution on sufficiently high columns. However, the necessity of using an eluent concentration lower than c_{cr}, with consequent extensive spreading of zones, often means that the method cannot be applied in practice. Nevertheless, stepwise elution often gives good results in the chromatography of simple mixtures of proteins, for example, in final purification stages [137-139]. It is evident that a significant difference in transition ranges is the main requirement for the successful application of the method. Thus, if protein B is excluded from the model system examined above, the remaining proteins A and C can be separated by the stepwise method without significant spreading; protein A is eluted with a solution of concentration c_3 ($c_3 > c^a_{cr}$) and the protein C zone remains unchanged, since its transition range lies at concentrations higher than c_3. Protein C may then be eluted as a compact zone with eluents of concentration $> c_4$ ($c_4 = c^c_{cr}$).

2. Gradient Elution

Gradient elution may be regarded as the automatic selection of the salt or hydrogen-ion concentration at which proteins can be separated chromatographically. This method is particularly suitable when the transition ranges of the proteins to be separated are not known beforehand; and, what is more important, there is an active mechanism compressing the zone boundaries, since the back boundary of the zone moves under conditions of lower capacity.

Elution with a capacity gradient was first used by Harteck and Suhr [140]. Alm, Tiselius, and coworkers [141-143] and others [144,145] set up a capacity gradient by varying the concentrations of the eluent components. Sober and Peterson [146] were the first to use gradient elution in the separation of proteins. Zhukhovitskii and Turkel'taub have developed the theory of this method [147,148].

Physically, the phenomenon of zone compression has the following consequences. Material that accidentally remains behind the zone is under conditions of weaker adsorption (in the chromatography of proteins it is the region where the pH is unfavourable or the salt concentration is high); as a result it begins to move more rapidly. When it overtakes the zone, the material is in a region of strong adsorption and its movement is retarded. At a certain point the whole zone is under conditions of zero adsorptivity (upper limit of transition range), and it begins to move along the column at the same speed as the solvent; the material of the zone is then in the liquid phase. The mechanism of chromatographic separation and active zone compression operates only until the zone reaches its maximum rate of movement.

The chromatographic separation of proteins with gradient elution can be clearly divided into three stages.

1. The adsorption stage. This is sometimes accompanied by separation of components due to protein–protein displacement. In this stage both dilution and concentration of the protein can occur, depending on the concentration of the protein solution and the capacity of the adsorbent.

2. The separation stage. The protein zones move along the column with increasing speed, the limit being the flow rate of the solvent itself. The components are separated by a true chromatographic process. The zones tend to spread because they move for some time at an eluent concentration below the critical, because the effective charge on a given protein is not constant, and because adsorbent capacity decreases. The presence of a capacity gradient moving at a rate faster than that of the zone tends to counteract spreading.

3. The passive transfer stage. The rate of movement of the zones is equal to the flow rate of the solvent and no further separation occurs. Hydrodynamic effects cause the zones to spread.

It follows that for the best separation the relationship between the column height and the capacity gradient should be such that the rate of movement of the zone is a maximum when it leaves the column. The concentration or pH of the solution at which elution then occurs is a characteristic of the given protein (elution concentration c_e or elution pH, pH_e) that can be used for identification. It is evident that c_e or pH_e is the upper limit of the transition range and is close to the minimum salt or hydrogen ion concentration at which the protein is no longer completely adsorbed by the cellulose ion exchanger. For example, Huisman found that when haemoglobins are chromatographed on a CM-cellulose column with different pH gradients, other conditions being the same, they emerge from the column in different volumes of eluate but at the same pH. [94] It is obvious that the exchange capacity of the adsorbent affects the value of c_e. Thus, an increase in the capacity of DEAE-cellulose leads to an increase in the concentration of NaCl at which pepsin and pepsinogen are eluted [149].

If we assume that the adsorption isotherm asymptotically approaches a limit, theoretically an infinitely long column would be required to achieve the maximum rate. However, as experiment shows, a column height of even a few centimetres is sufficient to obtain most of the material in the form of compact zone moving at the same rate as the eluent [150,151]. This is due to the very narrow transition range of proteins.

VI. TECHNIQUE OF PROTEIN CHROMATOGRAPHY ON CELLULOSE ION EXCHANGERS

1. Technique of Gradient Elution

The following is the commonest method of setting up a concentration gradient in a column. Vessel A (Fig. 6*a*), the mixer, contains a solution of concentration c_1; it is equipped with a stirrer and a bottom outlet connected to the column. Vessel B, the supply vessel, contains solution of concentration c_2; solution flowing out of A is replaced by solution from B. The concentration of solution issuing from the apparatus changes from c_1 to c_2 according to the equation [152]

$$c = c_2 + (c_1 - c_2)\, e^{-\frac{v}{q}}, \qquad (19)$$

where c is the concentration when the volume v has run out, q the volume of the mixer, and e the base of natural logarithms. The concentration gradient is represented graphically by a curve asymptotically approaching c_2.

Another elution procedure, which gives a linear variation of concentration, operates in the following way [153]. Two communicating vessels, a mixer A and a supply vessel B, contain solutions of concentration c_1 and c_2 respectively (Fig. 6*b*). The loss of solution from the mixer is made up by solution from the supply vessel, but the volume of solution in the mixer decreases. The variation in concentration of the solution issuing from the mixer is given by the equation [154]

$$c = c_2 - (c_2 - c_1)\left(1 - \frac{v}{V}\right)^{S_2/S_1}, \qquad (20)$$

where S_2 is the cross-sectional area of the supply vessel and S_1 that of the mixer, and V is the total volume of solution in both vessels. When $S_1 = S_2$, the equation is linear:

$$c = c_1 + \frac{v}{V}(c_2 - c_1). \qquad (21)$$

Other methods of setting up a gradient [155-158] include arrangements in which the gradient concentration can be changed at will during the experiment [159,160]. Kočent constructed nomograms for determining the eluent concentration at any time during the experiment with different arrangements for setting up a gradient [161].

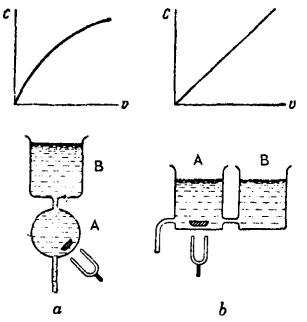


Fig. 6. Diagram of apparatus for setting up (*a*) exponential and (*b*) linear concentration gradients.

Formulae (19)–(21) reflect the variation of the concentration of the solution issuing from the mixer. However, the separation of compounds depends on the actual concentration gradient along the height of the column, and this depends not only on the design of the apparatus for producing the gradient but also on the cross-sectional area of the column and on the packing density. Eqns. (19)–(21) do not therefore represent the complete characteristics of separation conditions in gradient elution. The use of a linear concentration gradient has the advantage that it enables a simple formula to be used for the variation of eluent concentration along the column height, that is, in the region where the actual chromatographic process occurs[162,163]. If h is the height of the column, c the concentration of eluent issuing from the column, and c' the concentration at the upper surface of the column, the gradient G, the variation of concentration per unit height of column, will be given by

$$G = \frac{c' - c}{h} . \qquad (22)$$

From Eqn. (21) it follows that

$$c' = c_1 + \frac{v + v_m}{V}(c_2 - c_1), \qquad (23)$$

where v_m is the volume of mobile liquid in the pores of the column of height h. On substituting the values of c and c' from Eqns. (21) and (23) in Eqn. (22), we obtain

$$G = \frac{v_m}{h} \times \frac{c_2 - c_1}{V} . \qquad (24)$$

Denoting the factor v_m/h, which represents the volume of liquid in the pores of a column 1 cm high, by α, we obtain

$$G = \alpha \frac{c_2 - c_1}{V} . \qquad (25)$$

Thus, for identical separation conditions, values of the gradient G in all experiments must be the same. For example, in two experiments A and B the following conditions must be observed:

$$\alpha_A \frac{c_{2A} - c_{1A}}{V_A} = \alpha_B \frac{c_{2B} - c_{1B}}{V_B} . \qquad (26)$$

The initial concentration in the mixer c_1 is so chosen that the rate of movement of the protein zone is zero at the beginning of the chromatographic process. If in experiments A and B the initial concentrations are the same, Eqn. (26) may be rewritten thus:

$$\alpha_A \frac{c_{2A} - c_1}{V_A} = \alpha_B \frac{c_{2B} - c_1}{V_B} . \qquad (27)$$

In this case it can be shown that in experiments A and B the concentrations of eluent issuing from the column become the same at the point where the following condition obtains:

$$\frac{v_A}{\alpha_A} = \frac{v_B}{\alpha_B} = K_e . \qquad (28)$$

Since the position of the transition range on the concentration scale and the elution concentrations (p. 485) do not depend on the dimensions of the column, values of K_e, the elution constant, will be the same for a given protein on columns of different dimensions under the conditions of Eqn. (27); both the eluent concentration and the elution constant can be used for the identification of proteins.

2. Other Problems in Chromatographic Technique

Choice of adsorbent. The anion exchanger DEAE-cellulose and the cation exchanger CM-cellulose are most frequently used for the chromatography of proteins, and of the two, DEAE-cellulose is much more widely used, probably because the overwhelming majority of proteins are anions at the pH values used in chromatography. This should also determine the choice of adsorbent (anion or cation exchangers) when work is begun on the separation of a protein with unknown properties.

Because of the limited amount of published information on the use of other cellulose ion exchangers, it is not possible to draw any conclusions about their advantages. We merely point out that maltase and saccharase from intestinal juice are separated somewhat better on TEAE-cellulose than on DEAE-cellulose[131]. However, chromatography of thyrotropins gave identical results with these two adsorbents[164]. Identical results were also obtained in the chromatography of phosphoproteins on TEAE-cellulose and ecteola-cellulose[134].

Preparation of columns. In the chromatography of proteins cellulose ion exchangers should be packed uniformly and fairly tightly. The dimensions and shape of the column are determined by the composition and amount of the mixture to be separated and by the elution procedure. In gradient elution the separation may be improved by increasing the height of the column[165] or by reducing the magnitude of the gradient while the height of the column is kept constant[124]. If the conditions of Eqn. (27) are observed, only the productivity of the column, measured by the amount of the mixture separated, depends on the diameter; the productivity, and to a certain limit, the separating power of the column depend on its height. Satisfactory separations of proteins have been reported even on very short columns (~3 cm)[166,167].

Preliminary treatment of cellulose ion-exchange columns. Cellulose ion exchangers adsorb most strongly in a salt-free medium, but when proteins are eluted from unbuffered columns with salt solutions, the pH changes sharply and this may lead to denaturation of the proteins; on cation-exchange columns,

when the medium becomes acid, it may lead to the precipitation of large amounts of protein. To prevent this, the column must be washed before the experiment with a dilute buffer solution (0.001–0.05 M) to equalise the pH of the outflow with that of the initial solution ("column equilibration"). The concentration and pH of the initial buffer are selected so as to make the mobility of the protein zones in the adsorption stage zero; this gives the narrowest zones. In the chromatography of oxidisable proteins antioxidants (mercaptoethanol or cysteine) are added to the buffer solution. However, it must be borne in mind that this can lead to the formation of several zones from a single protein [100,168].

Quantity and concentration of protein chromatographed. The quantity of protein transferred to the column is best expressed in mg per cm^2 of column cross-section; other conditions being equal, the widths of the zones on columns of different diameter will then be the same. Generally 50–150 mg of protein is used per cm^2 of column cross-section. With larger quantities the resulting zones are too broad and the separation stage must then be improved by lengthening the column or by reducing the gradient. Thus, on a 0.9 × 10 cm column of CM-cellulose, 40 mg of ribonuclease can be separated completely, but if 200 mg is used the fractions overlap; with a reduced gradient separation is again achieved [124].

The concentration of protein in the initial mixture is not significant if the adsorption stage is carried out under conditions such that proteins are completely adsorbed.

Repeated chromatography. In the chromatography of proteins on cellulose ion exchangers, sometimes a single protein will form several zones. The formation of multiple zones in stepwise elution has been mentioned above (p. 484). There is evidence that proteins can change reversibly during chromatography and the modifications formed may differ in their mobility on a column [4]. Antioxidants may also react with proteins and change their adsorption properties [100,169]. On the other hand, it is known that some proteins with the same biological activity may differ in physicochemical properties, including adsorptivity on cellulose ion exchangers. In all such cases, repeated chromatography of the separate fractions under the same conditions may give information on the homogeneity or the cause of heterogeneity of a preparation. Final conclusions can only be reached by examining the fractions by different methods.

VII. SOME RESULTS AND POSSIBILITIES

1. Chromatography of Proteins on Cellulose Ion Exchangers as a Criterion of Homogeneity

As already mentioned, many protein preparations that appear to be homogeneous in electrophoresis or ultracentrifuging have been separated into fractions by chromatography on cellulose ion exchangers. Thus, a crystalline preparation of myoglobin was separated by chromatography on CM-cellulose and DEAE-cellulose into three components differing in amino-acid composition, whereas it was homogeneous when ultracentrifuged [6]. A trypsin inhibitor preparation from Lima bean, homogeneous when ultracentrifuged, was separated into four components by electrophoresis and into six by chromatography on DEAE-cellulose; the six components had about the same amino-acid composition but they differed in structure [8]. Trypsin inhibitor from ox blood loses 20–30% of its activity on storage, and electrophoresis and ultracentrifuging indicate that the stored preparation is still homogeneous, but the active and inactive fractions can be separated by gradient elution on a DEAE-cellulose column [9]. Many similar results have been reported [7,10,106,133]. Chromatography on cellulose ion-exchange columns can therefore provide one of the most effective criteria of homogeneity for protein preparations as well as being a good method of identification. This may be explained in the following way. In electrophoresis the principle factor governing the separation of proteins is the total charge, and in ultracentrifuging it is the density of the protein molecule; the mobility of proteins on cellulose ion-exchange columns, however, is governed by several factors, including the total charge, the number of charged groups on the protein molecule and their distribution on its surface, as well as properties such as the dimensions and shape of the molecule, prosthetic groups, etc.

2. Families of Proteins [170]

The problem of families of enzymes, that is the existence in the same organism, or even in the same organ, of more than one protein with the same enzymic activity (isoenzymes [171]), has been discussed for a long time; recently, however, it has attracted particular interest because of new experimental results obtained mainly by cellulose ion-exchange chromatography, but also by electrophoresis on gels [172–178]. Sometimes the number of isoenzymes can be quite large. Thus, Taborsky separated seven isoribonucleases, differing in sedimentation constant and isoionic point, by chromatography on a CM-cellulose column [124]. Press and coworkers found 15 isocathepsins D in ox spleen by chromatography on DEAE- and CM-cellulose columns [179]. Since differences between fractions within a family are often found by other methods [5,180,181], it can be assumed that we are really dealing here with enzyme families. However, another question remains to be answered: do these families arise from modifications in the protein during the separation process, or do they exist in the organism? Obviously, both possibilities have to be considered. It is well known that partial hydrolysis, which sometimes removes over 50% of amino-acid residues, does not lead to a loss of enzymic activity, but much smaller changes are sufficient to alter the physicochemical properties, and therefore the chromatographic mobility. Changes of this kind may be produced in the protein in the separation process by the action of endogenic proteases, or in other ways. The possibility of adsorptivity on cellulose ion exchangers being altered by changes limited to the secondary, tertiary, or quaternary structure must also be considered [182,183]. The reversible interconversion of family components appears to be a phenomenon of this kind [184–187]. The spontaneous synchronous changes in enzymes [188,189] that accompany changes in adsorption properties [190] are an interesting example of reversible changes.

There is evidence that enzyme families exist in the organism. Thus, lactate dehydrogenase, obtained by the same method from different organs was found to be homogeneous in some and heterogeneous in others [191]; crystalline preparations of this enzyme obtained from different organs of the same animal showed greater differences in tryptic hydrolysis than preparations obtained from the same organ of different species [192]. The components of esterase

and of lactate, malate, isocitrate and glycerophosphate dehydrogenases are also not identical in different organs and in different animals [193,194].

3. The Use of Cellulose Ion-Exchange Chromatography of Proteins in Clinical Analysis

Investigation of specific changes in enzymic activity in various pathological conditions is being increasingly used in clinical analysis [195]. Because of the high separating power of cellulose ion-exchange columns and the extreme simplicity of the method, this form of chromatography can be used to detect pathological changes in proteins under clinical conditions. Thus, the chromatograms of serum from patients suffering from leukaemia, cancer, and myeloma differ in many cases from chromatograms of normal serum [196-198]. Using adsorption on DEAE-cellulose, Hess and Walter detected quantitative differences in the serum isolactate dehydrogenases of people suffering from hepatitis, myocardial infarction and haemolytic anaemia [199]. These authors suggest that analysis of serum isolactate dehydrogenases could be used for differential diagnosis [200]. In macroglobulinaemia macroglobulins can be easily separated by chromatography on DEAE-cellulose: CM-cellulose and DEAE-cellulose have been used to separate several pathological haemoglobins [12,94,95,201]. The possibility of separating antibodies [202-204] enables cellulose ion exchangers to be used for studying immunity phenomena [205,206]. Thus cellulose ion-exchange chromatography may prove to be a very useful method in clinical analysis.

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ORGANIC DERIVATIVES OF LAMINAR SILICATES

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1. INTRODUCTION

Organic derivatives of silicates are an extremely interesting group of compounds. Rather more than 20 years ago Berger first reported[1] the preparation of methoxylated montmorillonite; this was the first of a series of intensive investigations of organic derivatives of laminar silicates, and attracted the attention of investigators studying the surface structure of various clays.

An accurate knowledge of the surface structure of clays is necessary for understanding many of their properties, such as their acidity, adsorption and exchange capacity, thixotropy, and so on, which are due to the presence of a negative charge on their particles.

Disperse crystals of clays are known to possess structural irregularities and defects, because of the inclusion of foreign atoms and groups in the lattice, the presence of difficultly removable impurities and of holes, etc. These deviations from the ideal structure in the smallest clay microcrystallites are often used in theories of the negative charge on their surface. The best known hypothesis is that of Marshall and Van der Meulen[2,3], which ascribes the negative charge to the replacement of multiply charged cations in the crystal lattice by less highly charged cations: for instance, Si^{4+} is replaced by Al^{3+} in a tetrahedral layer such as is typical of vermiculite, or Al^{3+} is replaced by Mg^{2+} or Fe^{2+} in an octahedral layer, which often occurs in montmorillonite. The theory of isomorphous substitution, while explaining the existence of wide differences in the chemical composition of similar clays, has its weak points. Calculations show[4] that if their acidity is considered to arise solely as a result of isomorphism, clays should be strong acids in the H-form, but their acidity is known to be constant between pH 3 and pH 5–5.6.

Edelman and Favejee[5] explained the negative charge on clay particles in terms of the acidity due to the presence of acidic Si–OH groups on the surface. Although several years ago the existence of Si–OH groups in laminar silicates was considered to be improbable, more recent studies[6-9], especially of infrared absorption spectra of certain hydrosilicates[10-13], demonstrate their presence in various silicate formations.

Definite proof of their presence is not easy, however. One possibility is the production of organic derivatives of the hydrosilicates by replacing the hydroxy-groups attached to silicon by organic radicals.

The organic derivatives are not merely of interest for investigating the surface structure of laminar silicates, since they have their own practical significance for the sterilisation and stabilisation of soils, the deactivation of poisons, the preparation of hydrophobic silicate materials, and so on. Organic derivatives of laminar silicates will undoubtedly also find practical application as fillers for various types of rubber and as selective adsorbents.

The experience gained in more than a century's study of the chemistry of organosilicon compounds has been applied