Advances in Separation Processes

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Advances in Separation Processes

A two-day symposium organised by the Institution of Chemical Engineers (South Wales Branch) and held at University College Swansea, 3–4 April 1990.

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Preface

The current resurgence of interest in Separation Processes arises from the need to meet tighter market specifications, often starting with a lower grade of raw material as some of the better reserves become depleted. In many cases, the more firmly established separation processes are unsuitable for the isolation of the product in the required degree of purity. This applies particularly in the separation of stereoisomers where differences in physical and chemical properties are often unsufficient for them to form the basis of an economic process. Furthermore, with increasing costs of fuel, there is an incentive to develop energy efficient processes.

The South Wales Branch of the Institution has organised this meeting in co-operation with the Science and Engineering Research Council (SERC) who have set up a special initiative in Separation Processes. This is sponsored by both their Process Engineering Committee and their Biotechnology Directorate, as difficult separations are a feature of both the chemical and biochemical industries.

The programme includes papers in a number of the priority areas of the SERC, including membrane technology, solvent extraction and highly selective adsorption. The poster sessions, which will include presentations from many of the academic workers who are receiving support from the SERC, provide a good opportunity for industrialists to gain an overview of the work of the academic community. This may act as a stimulus to future co-operative programmes of work.

J. F. Richardson

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I.CHEM.E. SYMPOSIUM SERIES NO. 118

ADSORPTION ISOTHERMS OF ALBUMIN ON A CROSS-LINKED CELLULOSE CHROMATOGRAPHIC ION-EXCHANGER

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Cross-linked, regenerated cellulose provides an improved adsorbent matrix for the chromatographic separation of proteins. Adsorption equilibria have been studied for bovine serum albumin on a Vistec granular, cross-linked DEAE-cellulose ion-exchanger. Isotherm points were determined by batch equilibrium. The data are best fitted by a Langmuir isotherm under all except extreme conditions. The adsorption is controlled more strongly by salt concentration than protein concentration so that not only dialysis, but even dilution, increases the protein uptake. The optimum pH was about 7.5. At this pH a salt concentration of about 15 kg/m^3(0.25M) is required to desorb albumin completely. The adsorption capacity is independent of particle size below 250 μm and is shown to reside almost entirely in the internal pores of the cellulose granules. In comparison, synthetic ion-exchange resins have a much lower capacity for protein but much of the capacity may still be located in the pores.

INTRODUCTION

Separation costs account for 50-80% of the production costs of new biotechnology products. Separation protocols increasingly depend on chromatographic separation stages for concentration, recovery and especially fractionation. In order to develop a chromatographic process it is necessary to be able to predict the performance as a function of the operating conditions. This requires fundamental data on the equilibria and kinetics of the chromatographic adsorption and desorption process. Few studies of this nature (recently reviewed (1,2)) have yet been conducted for protein separations. The need for such a study is evident in the increasing commercial interest in the isolation of pure proteins, particularly the fractionation of human blood proteins and the recovery of high-value proteins from cheese whey and animal blood for use in veterinary medicine, in foodstuffs, and as substitutes for human plasma fractions. The adsorption of the protein bovine serum albumin on a

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chromatographic ion-exchange adsorbent provides a model example of systems of commercial interest.

The synthetic resin ion-exchangers widely used for small inorganic ions are unsatisfactory for protein isolation for several reasons. The small pore size is claimed to restrict adsorption of macromolecules to the external surface, giving low capacity; the hydrophobic matrix can denature labile biomolecules; and the high charge density, which gives very high capacity for small ions, tends to bind proteins too strongly for easy desorption. These resins are better used for removal of biological contaminants from water supplies (3,4) than for protein recovery or fractionation. The disadvantages of these resins for proteins are avoided in ion exchangers based on hydrophilic, polysaccharide matrices such as fibrous cellulose (5), which were specifically developed for separating proteins. Unfortunately, the compressibility and irregular shape of fibrous cellulose gives slow, inefficient separations. Attempts to overcome this problem have led to the development of more rigid polysaccharide matrices such as cross-linked agarose (6) and, more recently, granular or beaded cellulose made by reconstitution of natural cellulose pulp (7-10). Controlled cross-linking of cellulose during the manufacturing process further improves the mechanical rigidity and hence flow properties, and also reduces the unwanted tendency of the material to swell or contract when the pH or ionic strength are changed.

We have studied the granular cross-linked diethyl-amino-ethyl-(DEAE-) cellulose manufactured by the Vistec process (7). The work had two aims.

1) To investigate the properties of the regenerated DEAE-cellulose as an ion-exchange medium for proteins.

2) To study the adsorption equilibria of albumin on this medium and examine the effect of the major operating parameters.

An investigation of the adsorption kinetics will be published separately. Knowledge of the equilibria and kinetics taken together provides a basis for developing the chromatographic production of albumin from animal blood (11).

Grant (12) has suggested that the ion-exchange groups in a regenerated cellulose ion exchanger may not be uniformly distributed. Using staining techniques he has shown that larger particles have a decreased density of ion-exchange groups towards their centres, giving lower capacity in a fixed bed of larger particles than in a bed of smaller particles. In Grant's process for manufacturing regenerated cellulose the ion-exchange groups are attached after regeneration and particle sizing. The reduced core density of ion-exchange groups probably reflects diffusional transport resistance during their attachment. By contrast, in the Vistec process (7), ion-exchange groups are added to the cellulose pulp before regeneration. Whether this improves the distribution of ion-exchange groups is not known, but any deficiency in the core will reduce adsorption capacity. An additional influence on adsorption capacity is the degree of cross-linking applied to the cellulose chains. To maintain reasonable flow properties, smaller particles are manufactured with greater cross-linking which tends to reduce capacity. It is clearly important to examine the effect of particle size on adsorption capacity. The other operating conditions studied were ionic concentration, pH and initial protein concentration.

EXPERIMENTAL

Three grades of Vistec DEAE-cellulose ion-exchange adsorbent media (Koch Light Ltd) were used. They have different particle sizes measured under the microscope (Table 1), and different degrees of cross-linking. The adsorbents were pretreated as described previously (11). The model protein was Fraction V Bovine Serum Albumin powder (Sigma London Chemicals Ltd, Poole, Dorset).

Procedures to regenerate the medium fouled with protein (ref.12 and Koch Light literature) were found to be only partly reproducible and effective. Virgin adsorbents were used throughout and gave closely reproducible results. It is important in protein adsorption studies to distinguish between regenerated and virgin adsorbents.

Isotherms were determined as follows. The pH of the pretreated medium was adjusted by addition of HCl or NaOH, as required, and the ionic strength was reduced to the order of 20×10^{-6} Siemens by repeated washing with distilled water. Excess distilled water was decanted off to give a dry-weight-to-slurry-ratio of 1:15. A weighed amount of slurry (approximately 15g) was poured into each of a series of 50 ml test tubes. Protein solutions were made up containing albumin at the desired concentration in Tris buffer (0.01 M trishydroxymethyl methylamine and HCl to pH 7.5) and salt in a concentration calculated to give the desired initial ionic concentration on mixing with slurry. 10 ml of protein solution was added to each slurry tube and the contents mixed. The tubes were equilibrated overnight at 20°C. Samples of the liquid phases were taken and analysed on a Pye SP6 spectrophotometer at 280 nm for protein concentration. The conductivity and pH of the liquid phases were also measured. The solid phase concentration was calculated by using the difference in liquid concentrations at the start and the end of the experiment, the dry weight of the medium in the tube, and the liquid volume in the tube.

RESULTS AND DISCUSSION

Equilibrium Isotherms

The isotherm is a plot of the concentration of protein adsorbed on the ion exchanger against the residual liquid concentration at equilibrium, at constant temperature. Figure 1 shows isotherms for the adsorption of bovine serum albumin on Vistec D2 medium. The capacities are broadly comparable with values obtained by Graham and Fook for "Protion", a DEAE ion exchanger made from a cellulose which is regenerated but not said to be cross-linked (13).

The three models most commonly used to describe adsorption isotherms are those of Langmuir (14), Freundlich (15), and Brunauer, Emmett and Teller (BET) (16). Previous applications of model isotherms to protein adsorption have recently been surveyed by Gosling (1,17). In the present case, Freundlich's isotherm, which is most useful for dilute solutions over small concentration ranges, did not fit the points in Fig.1 well. The BET equation gave the right shape of curve only for the special case of adsorption in a single uni-molecular layer; in this case it is identical with the Langmuir equation, which gave a good fit, as observed in a few other protein-adsorbent systems (2).

Langmuir's model, originally derived for gases, requires that

(a) adsorption is limited to a unimolecular layer,
(b) adsorbate molecules are not free to move on the surface and,
(c) the enthalpy of adsorption is the same for all molecules.

This model is expected to be well suited to proteins adsorbing on an ion-exchange surface. Assumptions (a) and (b) are likely to be valid since each protein molecule would be attached to specific surface sites and would not desorb until all bonds are broken simultaneously (10). Moreover, if the ion-exchange forces are dominant over other forces of attraction, adsorption in multiomolecular layers is insignificant in comparison with the first layer and the enthalpy of adsorption is invariant. A similar situation is found in the adsorption of bovine serum albumin on resinous sorbents, for which Foster (19) has concluded that ionic attraction is the dominant force in the adsorption. He found that the isotherms could be correlated by both Freundlich and Langmuir equations, although Langmuir gave better correlation coefficients.

The Langmuir (18) equation

$$q = \frac{K_m q_m c^*}{1 + K_m c^*} \qquad (1)$$

relates c*, the residual concentration of protein in the liquid phase at equilibrium, to q, the concentration in the solid phase at equilibrium. It involves two constants, K_m, the ratio of the adsorption and desorption rate constants, and q_m, the maximum adsorption capacity of the adsorbent, i.e. the solid concentration for a complete monolayer. It is common practice to obtain the constants from the slope and intercept of a linearised form of the equation. This method is not entirely satisfactory since it weights the lower, less accurate, values of q(c*) unduly and gives constants whose values are highly sensitive to experimental error. We therefore used instead a non-linear least squares fit of equation (1) to the data. Details are given by Leaver (20). Values of q_m and K_m so derived are shown for interest in Table 2 though the data are too few to justify systematic analysis of the values. The main point of these results is the good fit of the corresponding Langmuir curves in Figs 1, 2, 3, 7 and 8. Thus, Langmuir's isotherm correctly predicts the shape of the adsorption isotherm for modelling purposes.

Effect of Ionic Concentration

Fig 1 shows four isotherm curves for a range of initial ionic (sodium chloride) concentration, I, from 5 to 12.5 kg/m^3, at pH 7.5. Clearly, the concentration of salts in the system has a profound effect upon the distribution of protein between the two phases, confirming the results of Peterson (18). Both the adsorption capacity and the initial slope of the curve rise rapidly with decreasing ionic strength.

The plots may be used to illustrate the effect of prior dilution or dialysis upon the protein adsorbed since each curve was obtained using the same set of initial protein concentrations of 4.8, 9.5, 14.3, 19.2 and 24.0 kg/m^3, which are in the ratios 1:2:3:4:5. (As expected from the mass balance, points of the same initial protein concentration lie on a straight line in Fig.1).

The effect of dilution is illustrated by considering the fourth point c* = 14.7 kg/m^3 on the isotherm for I = 12.5 kg/m^3 (0.21M). If the initial liquid were diluted by a factor of two, and half of the new liquid volume were used, then the new data point would correspond to the second point on an isotherm I = 6.25 kg/m^3. Interpolation between the second points on the isotherms at I = 5 and I = 7.5 kg/m^3 shows that q would be about 1.93 kg/kg, which compares with the undiluted value of 0.88 kg/kg. Thus a two-fold dilution more than doubles the amount of protein adsorbed in this case. The reason is that the dilution of protein is more than compensated by the

dilution of salts competing with the protein for the ion-exchange sites.

The effect of dialysing the protein solution to reduce the salt content is even more marked, as can be seen simply by comparing corresponding data points on two curves. Halving I from 12.5 to 6.25 kg/m^3 at a constant initial protein concentration of 19.2 kg/m^3 involves moving from the fourth data-point on the curve I = 12.5 kg/m^3 to the fourth data-point on an interpolated curve I = 6.25 kg/m^3. Thus, q increases by a factor 3.5/0.88 = 4.0 for a two-fold reduction in salt concentration by dialysis. Dialysis is nearly twice as effective as dilution in raising the adsorbed protein concentration. These findings are consistent with the chromatographic results (11).

Effect of the pH of the Medium

The effect of pretreating the ion-exchange medium until the washings were of specified pH values is illustrated in Figs. 2 and 3. Plots were obtained for the D2 medium for all the combinations of ionic strength and pH listed in Table 2. Data for the two highest ionic strengths at the two pH extremes could not be satisfactorily fitted by the Langmuir equation and gave the lowest uptakes of protein.

The plots imply that the optimum pH for maximum adsorption is about 7.5. To check this, the adsorption at a fixed initial protein concentration of 20 kg/m^3 and fixed ionic concentration of 5 kg/m^3 was measured for media pretreated to various pH values. The result is Fig.4 which shows the expected optimum. The divergence between the initial and final pH is due to the protein solution being prepared in a pH 7.5 buffer.

A possible reason for an optimum pH of about 7.5 is as follows. Bovine serum albumin has an isoelectric point of pH5, whereas DEAE-cellulose, being an anion exchanger of moderately weak basicity, probably requires a higher pH, somewhat above 7.5, for half-neutralisation by chloride counter-ion. As the pH increases above 5, the net negative charge on the albumin molecule increases, raising the electrostatic attraction between protein and anion exchanger. As the pH increases above 7.5 there is little further change in the albumin charge but the lower hydrogen ion concentration releases more Cl^- counter-ions to neutralise, and so inactivate, the DEAE groups on the anion exchanger. Thus, the optimum pH is about 7.5. It is possible, however, that other factors contribute to the adsorption behaviour, such as other charged groups on the medium added by side reactions during manufacture. Weak anion exchangers display unpredictable titration curves which indicate the presence of such groups (21).

Effect of Low Ionic Concentrations

Fig.5 shows the isotherms for equilibrium ionic concentrations below 5 kg/m^3. They are characterised by a maximum occurring at a sharp discontinuity. This behaviour may be due to morphological changes in the protein structure. At high protein concentrations the solution requires ions to keep protein in its native state. It is likely that a low ratios of ionic concentration to protein concentration the protein is agglomerated, which may reduce its adsorption or reduce its UV transmittance. This explanation is consistent with the finding that, at the discontinuity, the ratios of initial protein concentration to ionic concentration were essentially equal, at 19 and 21, respectively, for the isotherms at I = 2 and I = 1 (not shown) kg/m^3.

In contrast to the behaviour at higher ionic concentrations, the isotherms for I = 0, 1, 2 and 5 kg/m^3 were not clearly separated, within experimental error, at protein concentrations below the discontinuity. To maximise

protein adsorption, therefore, it is not beneficial to dialyse or dilute the solution beyond a certain salt concentration. This concentration should be maintained at a minimum of one-twentieth of the initial protein concentration to avoid the postulated agglomeration with loss of adsorption.

Desorption

Fig 6 shows the concentrations of sodium chloride required to desorb the protein, i.e. reduce the solid concentration from its equilibrium adsorption value to zero. The upper line represents a series of tubes each containing 10 ml solution with similar initial concentrations of protein (45 g/l), sodium chloride (5 g/l) and D2 medium (dry weight 0.72 g). Two hours were allowed for the albumin to distribute between the liquid and solid phases. Samples of the liquid phases were taken to calculate the solid phase concentration.

The lower curve represents the new solid phase concentration 67 h after the further addition of 5 ml of sodium chloride solution of a concentration calculated to increase or decrease the ionic concentration from its initial value of 5 kg/m^3 to final values indicated by the abscissae of the lower set of points. The difference between the two curves represents the desorption of protein from the medium by increasing the salt concentration and re-equilibrating.

The two negative values of solid concentration for the lower curve incorporate the experimental error when subtracting two liquid phase concentrations to calculate the solid concentration; the mean value for the seven points is zero. Clearly, ionic concentrations of 15 kg/m^3 and above are sufficient to desorb albumin, given time for equilibrium to be reached. This value is consistent with Fig 3 and with the concentration step needed to elute adsorbed albumin during a parallel study of the chromatographic separation of albumin from other blood proteins (11). However, concentrations less than this are still sufficient to elute some albumin (Fig 3). Therefore, to minimise the loss of albumin during the chromatographic elution of non-albumin proteins, a salt concentration of only 5.3 kg/m^3 was used (11).

Comparison of Ion Exchanger Grades and Location of Adsorption Capacity

All the experimental results described so far are for the D2 grade of Vistec ion exchanger. In the Vistec media a controlled degree of cross-linking is incorporated to reduce the compressibility and restrict the amount of swelling or contraction that occurs on change of pH or ionic strength (12). Two other grades of this material are available, D1 and D3. The particle size increases (Table 1) and the degree of cross-linking decreases in the series D1, D2, D3.

Figure 7 shows isotherms for the three media grades under constant conditions of pH and ionic concentration. The fitted Langmuir parameters are listed in Table 2. The difference between the D1 and D2 media is small. The D3 medium clearly has inferior adsorption properties compared with the other two media. This is despite allowing several days for the protein to penetrate the structure of the particles. It was also found that most of the adsorption was complete in the first 15 hours; allowing extra time for equilibration did not significantly affect the results.

Figure 8 shows the effect of ionic concentration upon the adsorption isotherm for the D3 medium. Comparing this with Figure 1 for the D2 medium shows the adsorption by D3 to be about half that of the D2 medium at both values of ionic concentration.

The adsorbed protein concentrations observed in Fig 7 cannot be accounted for on the basis of monolayer adsorption solely at the external surface of the cellulose particle. Calculation (Appendix) shows that external adsorption can contribute only of the order of 10^{-3} of the total adsorption observed. The total adsorption in Fig 7 agrees well with the estimated monolayer adsorption of 0.16-0.31 kg/kg inside the pores of the particles (Appendix). Almost all the adsorption is thus located inside the pores.

Three factors are likely to affect the adsorption capacity of the different grades of ion exchanger. First, for reasons explained in the Introduction, larger particles may have a decreased density of ion exchange groups towards their centres. Secondly, protein molecules entering a pore will tend to adsorb at the entrance initially, partially blocking small pores. This effect, together with constrictions along the pores, will be more significant with larger particles where the pores are longer and more tortuous than in smaller particles. Thirdly, cross-linking, which is greater for the smaller grades of particle, reduces the adsorption capacity. The first two effects act in concert, but oppose the third. On this view the adsorption data in Fig 7 imply that the changes in pore blocking and ion-exchange group distribution from the D1 to the D2 media cancel the changes due to cross-linking to yield similar adsorption capacities. With the larger D3 particles the benefit of less cross-linking is not sufficient to counteract the effect of pore blocking and possibly less uniform ion-exchange group distribution, so that lower adsorption capacities are recorded. Longer diffusion times are also expected, lengthening the cycle time required in a chromatographic process. These defects of the D3 particles outweigh their advantage of a lower pressure drop.

The D1 and D2 media have similar capacities. Of the two, the D2 medium offers the lower pressure drop and is cheaper. For chromatographic separation, a final choice between the two grades depends on their adsorption kinetics.

Comparison with Non-Cellulose Ion Exchangers

Calculations of adsorption capacity, using equations (2) and (3) in the Appendix, are helpful in identifying the locations of the adsorption capacity in different types of ion exchanger. The small pore size of ion-exchange resins used for inorganics is usually held to make them unsuitable for proteins. Thus Foster (3,4), reporting that the adsorption of bovine serum albumin on Duolite A7 and other ion exchange resins increased with decreasing particle size, concluded that the internal surface played a relatively minor role in adsorption. The relationship between adsorption capacity and external surface area, however, was not linear.

Typical adsorption values quoted were 0.05-0.12 kg/kg. Particle sizes to obtain these figures based on external surface only, using equation 2 (Appendix), would be 0.4-0.9 μm. Although particle sizes were not quoted, this is at least two orders of magnitude less than the commercially available material. Foster reports times of 200h to reach equilibrium with albumin. This seems to be too long to be accounted for solely by a stagnant Nernst diffusion barrier on the particle exterior. It is very likely, therefore, that there is some diffusion of albumin into the pores.

Other resins tested by Foster (3,4) gave lower adsorption values. Rohm and Haas XAD8 resin gave the lowest capacity of 5×10^{-3} kg/kg, implying a much larger particle size of 9 μm based on external adsorption only. This is still, however, much smaller than the particle sizes of the commercial resin.

It is clear that there is great variation in the behaviour of ion exchange materials towards proteins. Some ion-exchange resins exhibit largely external

adsorption, whereas others may adsorb in internal pores as well. Vistec regenerated cellulose, however, has a much higher adsorption capacity, of about 0.5 kg/kg, almost all of which is located inside the porous structure of the granules.

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APPENDIX External and Pore Adsorption Capacity

Under the microscope the particles of Vistec ion exchanger were seen to have irregular, but generally equiaxed, shapes resembling pieces of coal, with a few agglomerates containing 3 or more particles. For modelling purposes, it is assumed that monolayer adsorption occurs on particles which are uniform and spherical, and that the adsorbed protein molecules are contiguous in a 4-coordinated configuration.

The adsorption on the external spherical surface area can be shown (22) to be given by

$$q' = \frac{6M\,(1-\varepsilon)}{N\,S\,G\,d_p} \qquad (2)$$

where q' = solid phase concentration (kg/kg), ε = column voidage (-), M = molecular weight of the protein (kg/mol), N = Avogadro number (molecules/mol), S = area of surface occupied by a protein molecule (m^2), G = pseudo-density of medium (kg dry weight/m^3 of swollen bulk volume), d = particle diameter of medium. The following values were used: M = 69 kg/mol (23), ε = 0.4 (based on tracer studies of non-adsorbed protein), G = 154 kg/m^3 (measured for D2 medium). S is calculated from the molecular dimensions of an albumin molecule, which is an ellipsoid of diameter 3.8 nm and length 15 nm (23). The rectangle enclosing the projected ellipse with major and minor axes of these dimensions has an area S = 5.7 x 10^{-17} m^2. Particle diameters (Table 1) range from 150 to 580 μm. Substituting these values into equation (2) yields values of q' from 3.1 x 10^{-4} to 8.1 x 10^{-5} kg/kg.

Adsorption on the surfaces of cylindrical pores of uniform width and depth within the particles is given (22) by

$$q = \frac{4\,M\,\phi}{N\,S\,G\,d} \qquad (3)$$

where ϕ = the porosity of the packed bed (fraction of the bed volume present as intra-particle pores), and d_p = an average pore diameter. A porosity value of 0.3 for porous particles is typical. The average pore size for Vistec media has been estimated to be 50 - 100 nm (24). Substituting these values into equation (3) gives values of q of 0.16 - 0.31 kg/kg. These values agree well with the experimentally measured adsorption in Fig 7.