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ADSORPTION OF PROTEINS ON SEPHAROSE AFFINITY ADSORBENTS OF VARYING PARTICLE SIZE

B. J. HORSTMANN*, C. N. KENNEY and H. A. CHASE
University of Cambridge, Department of Chemical Engineering, Pembroke Street, Cambridge (U.K.)
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SUMMARY

The binding of bovine serum albumin (BSA) and lysozyme to a series of samples of Cibacron Blue F3G-A Sepharose CL-6B with different narrow-range mean particle diameters has been investigated. Significant variation with particle size has been found for the parameters measured. For both proteins the maximum capacities obtained increased with decreasing particle size. For lysozyme, this percentage increase in capacity is less than the percentage increase in external surface area as the particle size decreases. For BSA, however, the percentage increase in capacity is more than four times the increase in surface area. The dissociation constants and forward rate constants describing the affinity interaction also differ with particle size. Again the effects are more pronounced for the larger protein, BSA, than for lysozyme. The effect of these differing parameters on the performance of fixed beds has been assessed using a computer simulation of the adsorption process.

INTRODUCTION

Although Blue Sepharose (Cibacron Blue F3G-A covalently attached to Sepharose CL-6B), and other Sepharose-based supports are widely used in affinity chromatography[1], little consideration is usually given to the extent to which the available total surface area of the porous support is utilised. Sepharose CL-6B can be used as a gel filtration medium for the fractionation of proteins from molecular weights 10 000 to 4 000 000[2], so its pores must be roughly of the same dimensions as proteins. When the support is derivatised with a relatively small ligand in the formation of affinity adsorbents such as Cibacron Blue, the presence of the ligand should not alter the pore size significantly. However, there is the possibility that once protein molecules become adsorbed to the affinity ligands they will block the passage of other molecules to other adsorption sites, so that the accessible regions of the adsorbent become reduced as adsorption occurs. When an affinity adsorbent is prepared by binding a large affinity ligand such as an antibody to an activated Sepharose, the penetration of the ligand during the initial immobilisation process may be limited in an analogous fashion. If a large protein is to be absorbed subsequently onto the ligand, the problem of pore blocking becomes even more severe. It is likely that for

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most situations the binding will be neither over the total (*i.e.* internal and external) surface area of the porous adsorbent, nor entirely to the outer surface, but some intermediate situation. In order to model the adsorption technique for predictive purposes, such as the scaling up of a process for commercial use, it is necessary to know whether the adsorption approximates to one of these extremes, or if the degree of penetration must be taken into account. A study of the binding of proteins to samples of adsorbent with different particle diameters, involving measurement of the maximum binding capacity of each sample, should, in principle, distinguish between these two extremes, if it is assumed that the particle is made up of a uniform internal structure which is independent of the size of the particle. If the adsorbent is entirely porous to the binding species then the maximum adsorption capacity should be independent of the particle size. If the adsorption occurs exclusively to the external surface of the particles, then the maximum adsorption capacity should be proportional to this external surface area. Sepharose-based adsorbents are not supplied in a range of particle sizes, but a preliminary analysis of the size distribution of a standard batch of Blue Sepharose showed a sufficiently large size range for sieving to be considered as a method of obtaining a range of samples with narrow particle size distributions.

Two proteins with affinity for Blue Sepharose were selected which, on the basis of gel filtration data, would be expected to penetrate the Sepharose beads to different extents. Gel filtration data supplied by the manufacturers of Sepharose shows that the accessible void fraction is 0.75 for lysozyme (mol.wt. 14 000) and 0.55 for bovine serum albumin (BSA, mol.wt. 66 000). As the dye molecule is small, the void fractions for unsieved Blue Sepharose should be similar to these figures.

EXPERIMENTAL

Materials

Cibacron Blue F3G-A Sepharose CL-6B was obtained from Pharmacia, Uppsala, Sweden. BSA and lysozyme were obtained from Sigma (U.K.). Sieves used for preparing samples with narrow particle size distributions were manufactured by Endecotts (test sieves) (U.K.).

Preparation of adsorbent

A quantity of Blue Sepharose was swollen and washed according to the manufacturers' instructions. It was then sieved, using flowing water and 106-, 90-, 75- and 63-μm sieves, giving five fractions. The four largest sieved fractions, together with an unsieved sample were analysed using a Coulter Counter and Channelyzer (Coulter Electronics, Harpenden, U.K.), to give the particle size distribution. The largest of the four fractions was not used in subsequent analysis, due to the observed broad distribution of particle sizes. It was assumed that these narrow-range samples would settle to form a packed bed as if they consisted of identical sized, spherical particles, *i.e.* with an external voidage independent of particle size. Therefore, identical volumes of settled adsorbent were assumed to contain the same amount of gel, independent of the size of the particles in the sample. The external surface area per unit volume for each fraction was calculated from the size distribution data, so that it represented the exact distribution, rather than being based on the mean particle diameter.

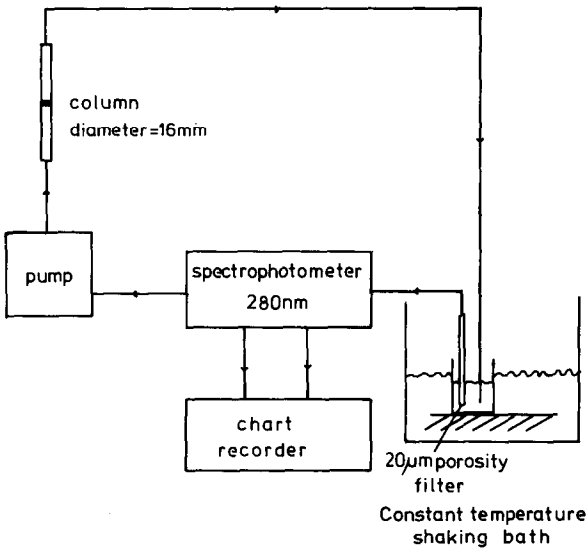

Fig. 1. Experimental apparatus for the measurement of equilibrium adsorption isotherms for the sieved fractions of Blue Sepharose.

Determination of equilibrium adsorption isotherms

The apparatus is shown in Fig. 1. A small amount of the adsorbent (0.4–0.8 ml) was placed in a 1.6-cm diameter column. Buffer (50 m*M* Tris, pH 7.2) was pumped continuously from a reservoir in a constant temperature shaking bath, through a continuous flow spectrophotometer, then through the column, and back to the reservoir. A volume of 0.5 ml of protein solution (10 mg/ml in buffer) was pipetted into the reservoir, and the system was allowed to come to equilibrium. The decrease in the optical density (at 280 nm) of the solution in the reservoir was recorded continuously by a chart recorder, so it was known when equilibrium had been attained. The concentration of protein in solution at this point was noted, and the amount of protein adsorbed to the bed was calculated by difference. Another 0.5 ml of protein was added to the reservoir, and the process was repeated until an equilibrium solution concentration of about 1 mg/ml had been attained. The adsorbent was regenerated by elution with 1 *M* potassium chloride followed by washes with alternate high and low pH, and then re-equilibration with buffer. The whole process was repeated several times for each adsorbent sample. The volume of liquid in the apparatus was determined by pumping the liquid in the system into a measuring cylinder. The spectrophotometer was calibrated before and after each run with solutions of known concentration.

The equilibrium adsorption isotherm was plotted using the amount bound to the adsorbent (calculated by difference) and the solution concentration for each equilibrium point. Reciprocal plots were used to determine both the fit to the Langmuir isotherm equation and the corresponding parameters of maximum binding capacity (q_m) and dissociation constant (K_d).

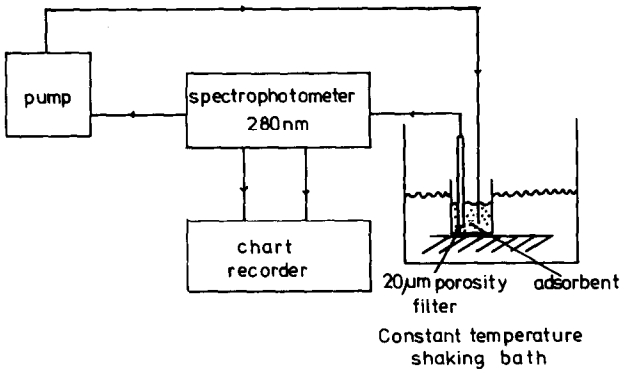

Fig. 2. Experimental apparatus for the study of the kinetics of batch adsorption.

Determination of batch kinetics

Batch adsorption studies were carried out using the apparatus shown in Fig. 2. The protein concentration was measured continuously by pumping the solution through a 20-μm porosity filter, then through a continuous-flow UV spectrophotometer, and then back to the beaker. The volume of the recycle was kept to a minimum, and a fast flowrate was used to ensure a rapid response time. The output of the spectrophotometer was connected to a chart recorder to give a permanent record of the reduction of protein concentration with time. In a typical experiment, at time 0, 1.5 ml of settled adsorbent was added to 20 ml of protein solution at 1 mg/ml in Tris buffer pH 7.2 and the recycling was continued until equilibrium had been achieved.

Determination of the relative dye concentrations on the sieved fractions

Samples (100 μl) of each of the adsorbent fractions were hydrolysed in 1 ml of 6 *M* hydrochloric acid at 37°C overnight. The resulting non-turbid solutions were diluted with 2 ml of water, and the optical densities at 615 nm were determined.

Computer analysis

Batch adsorption studies. The results from batch experiments were analysed to determine values for the forward rate constant, k_1. The details of the adsorption model have been reported previously[3].

The model assumes that the immobilised binding sites (B) on the adsorbent have an affinity for only one species of adsorbate (A) and that the interaction can be described by an equilibrium relationship of the form:

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AB$$

where k_1 and k_2 are the forward and reversed rate constants respectively, and their ratio (k_2/k_1) is equal to the dissociation constant (K_d) that describes the equilibrium relationship. For a batch system, the rate of transfer to the solid phase is given by

$$\frac{dq}{dt} = k_1 c(q_m - q) - k_2 q$$

where c is the concentration of adsorbate in solution, q is the solid phase concentration of the adsorbed material per unit volume of adsorbent, q_m is the maximum capacity of the adsorbent, and t is time. Earlier work used a numerical solution to this equation, but recently an analytical solution has been used. The analytical solution takes the form

$$c = C_0 - \frac{v}{V}\left\{\frac{(b+a)\left[1 - \exp\left(-\frac{2av}{V}k_1 t\right)\right]}{\left(\frac{b+a}{b-a}\right) - \exp\left(-\frac{2av}{V}k_1 t\right)}\right\}$$

where

$$b = \frac{1}{2}\left(\frac{C_0 V}{v} + q_m + \frac{K_d V}{v}\right); \qquad a^2 = b^2 - \frac{C_0 V}{v} q_m$$

and C_0 is the initial concentration of adsorbate in solution. V is the total volume of system and v the volume of adsorbent.

Once the isotherm constants (q_m and K_d) are known, the analytical solution contains only one unknown parameter, k_1, and it is therefore possible to do a least squares fit of the solution to the experimental data to determine a value for k_1.

Prediction of packed-bed performance. In order to predict packed bed performance, a rate model, assuming that the rate of interface mass transfer in a packed column can be described in an identical manner to that for batch systems, was used. Again, this model is described in detail elsewhere[3]. Using equilibrium data, and data from batch experiments, the model can be used to predict the shape of the breakthrough curve that would be obtained in a given operating situation.

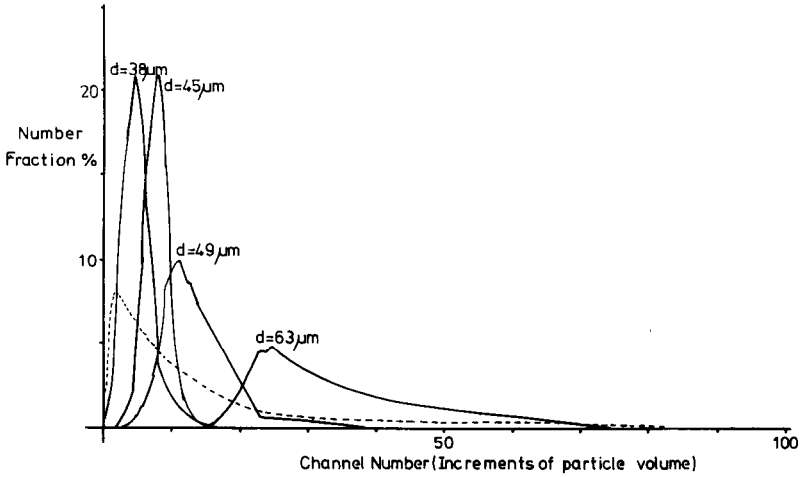

Fig. 3. Size distribution of sieved (—) and unsieved (---) Blue Sepharose. Results from analysis on a Coulter counter. The vertical axis represents a number and not a volume fraction. On a volume basis, the results for the sieved fractions are relatively unchanged, but the distribution for the unsieved fraction is considerably broadened and flattened.

TABLE I

ISOTHERM PARAMETERS FOR THE ADSORPTION OF BSA AND LYSOZYME TO BLUE SEPHAROSE OF DIFFERING PARTICLE SIZE

	Mean diameter (μm)	*Maximum capacity*		*Dissociation constant*		*Coefficient of determination*
		mg/ml	*mol/ml*	*mg/ml*	*M*	
BSA	38	12.0 ± 0.9	$1.8 \cdot 10^{-7}$	0.84 ± 0.08	$1.3 \cdot 10^{-5}$	0.989
	45	7.7 ± 0.4	$1.2 \cdot 10^{-7}$	0.23 ± 0.03	$3.5 \cdot 10^{-6}$	0.988
	49	5.4 ± 0.2	$8.2 \cdot 10^{-8}$	0.13 ± 0.02	$2.0 \cdot 10^{-6}$	0.997
Lysozyme	38	16.6 ± 0.3	$1.19 \cdot 10^{-6}$	0.10 ± 0.010	$7.1 \cdot 10^{-6}$	0.998
	45	15.8 ± 0.1	$1.13 \cdot 10^{-6}$	0.06 ± 0.003	$4.3 \cdot 10^{-6}$	1.000
	49	15.1 ± 0.4	$1.07 \cdot 10^{-6}$	0.04 ± 0.008	$2.9 \cdot 10^{-6}$	0.998

RESULTS AND DISCUSSION

The size distribution for the unsieved Blue Sepharose, as determined using the Coulter counter (Fig. 3), showed that it contained a large number of particles with a diameter of less than 45 μm and very few with a diameter as great as 100 μm, which is rather different to the manufacturers quoted range of 45–165 μm. A similar fact has been noted by other authors from their studies of electron micrographs of Sephadex[4].

All the isotherms measured for the sieved fractions fitted the Langmuir equation well. The determined parameters (q_m and K_d, see Experimental section) and the coefficients of determination (a measure of the fit of the straight line) are shown in Table I. We have shown in previous work that the Langmuir isotherm equation is most appropriate for affinity systems in general[3]. The isotherms for the adsorption of lysozyme (Fig. 4), and BSA (Fig. 5), are distinctly different for the three different particle sizes. The maximum binding capacity increases with decreasing particle size, but not in proportion to the increase in external surface area (Table II). For lysozyme, the capacity increase is less than the increase in surface area. On the assumption of uniform internal structure this would indicate that although the adsorbent particles are not entirely pervious to lysozyme, there is a high degree of penetration. For BSA, however, the increase in capacity was considerably greater than the increase in surface area. This would imply that the internal structure may not be the same for the different sized particles, or more likely, that the dye is not evenly distributed within the particles. The actual amounts of dye bound per unit volume of adsorbent for each of the sieved fractions (as determined by freeing the dye by hydrolysis of the adsor-

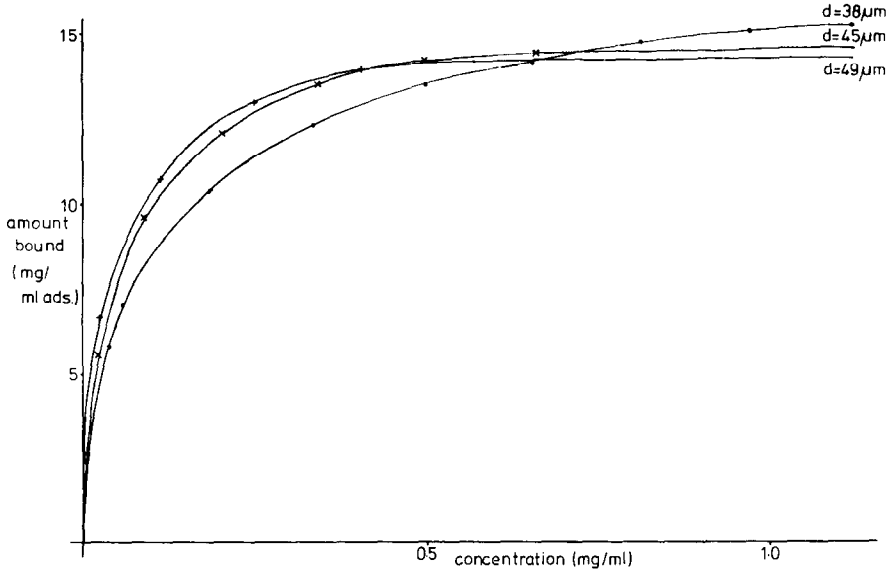

Fig. 4. Equilibrium adsorption isotherms for the adsorption of lysozyme onto the sieved fractions of Blue Sepharose CL-6B at 25°C. d = 38 μm, q_m = 16.6 mg/ml adsorbent, K_d = 0.10; d = 45 μm, q_m = 15.8 mg/ml adsorbent, K_d = 0.06; d = 49 μm, q_m = 15.0 mg/ml adsorbent, K_d = 0.04.

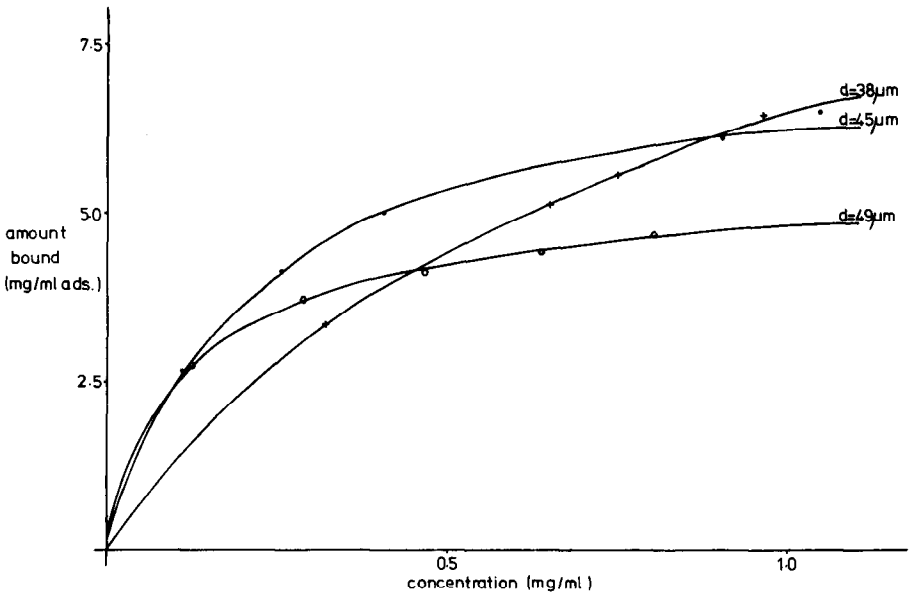

Fig. 5. Equilibrium adsorption isotherms for the adsorption of BSA onto the sieved fractions of Blue Sepharose at 25°C. d = 38 μm, q_m = 12.0 mg/ml adsorbent, K_d = 0.84; d = 45 μm, q_m = 7.7 mg/ml adsorbent, K_d = 0.23; d = 49 μm, q_m = 5.4 mg/ml adsorbent, K_d = 0.13.

bents) were found to be identical. The observed 120% increase in capacity with only a 30% increase in surface area was unexpected. This increase in capacity cannot be explained in terms of a fixed depth penetration independent of the size of the sphere. If this were the case, only a 25% increase in capacity would be predicted. It is possible that for the larger protein, BSA, the restriction to further binding imposed by protein already bound has a more significant effect.

Another unexpected result was that the dissociation constants were found to vary considerably. Again, the variation is greater for BSA than for lysozyme. With

TABLE II

INCREASE IN MAXIMUM CAPACITY COMPARED WITH INCREASE IN EXTERNAL SURFACE AREA WITH DECREASING PARTICLE SIZE

	Mean diameter (μm)	*Maximum capacity (mg/ml)*	*Increase in external surface area (%)*	*Increase in maximum capacity (%)*
BSA	49	5.4	–	–
	45	7.7	13	43
	38	12.0	30	122
Lysozyme	49	15.1	–	–
	45	15.8	13	5
	38	16.6	30	10

TABLE III

FORWARD AND REVERSED RATE CONSTANTS FOR THE ADSORPTION OF BSA AND LYSOZYME TO BLUE SEPHAROSE OF DIFFERING PARTICLE SIZE

r.m.s. = Root mean square.

	Mean diameter (μm)	*Foward rate constant*		*r.m.s. error*	*Reverse rate constant (1/min)*
		ml/mg · min	*1/M · min*		
BSA	38	0.31	20 460	0.002	0.26
	45	0.45	29 000	0.003	0.10
	49	0.52	34 320	0.003	0.07
Lysozyme	38	1.20	16 800	–	0.120
	45	1.32	18 480	–	0.079
	49	1.44	20 160	–	0.058

decreasing particle size and increasing capacity, the dissociation constants become greater, *i.e.* the adsorption appears less favourable.

Values for the overall forward rate constants determined by batch kinetic studies are given in Table III. For both BSA and lysozyme, they decrease with decreasing particle size, but to a lesser extent than would account for the observed increase in the dissociation constant, if the reverse rate constant k_2 were remaining constant. In fact, calculations of k_2 (Table III) show that it is a large increase in k_2 rather than the decrease in k_1 that results in the increase of K_d with decreasing particle size.

Tables I and III give the figures discussed above both on a mass basis, and on a molar basis. The adsorbents all bind a greater mass of lysozyme than BSA. As the proteins are assumed to bind to individual sites on the adsorbent, they will bind in a monolayer fashion, and the actual surface coverage of a given mass of protein will be dependent upon the size of the protein. A comparison of the capacities on a molar basis shows the much greater numbers of lysozyme molecules that can be bound compared to BSA. However, in order to make a comparison of the surface coverage the lysozyme figures should be reduced by a factor of 2.5, as approximately 2.5 times as many lysozyme as BSA molecules can be packed onto a given surface, assuming that both proteins are spherical molecules of the same density. The observation that the surface coverage of lysozyme is much greater than for BSA, suggests that the smaller molecule can penetrate a greater extent of the matrix. The dissociation constants (on a molar basis) are in the same range for the two different proteins. The forward rate constants for lysozyme are approximately three times those for BSA on a weight basis, but on a molar basis, those obtained for BSA are on average approximately 1.5 times greater than those obtained for lysozyme.

The overall effect on adsorption performance of using different particle sizes was analysed. With decreasing particle size, the maximum capacities become more favourable, but conflictingly, the dissociation constants become less favourable. The degree of variation in the dissociation constants is, however, much greater than the degree of variation in the maximum capacities. This effect is particularly pronounced with lysozyme, where a 10% increase in capacity is achieved with a 150% increase in dissociation constant when the particle size is reduced from 49 to 38 μm. The variations of these parameters have effects on fixed bed performance, as both the

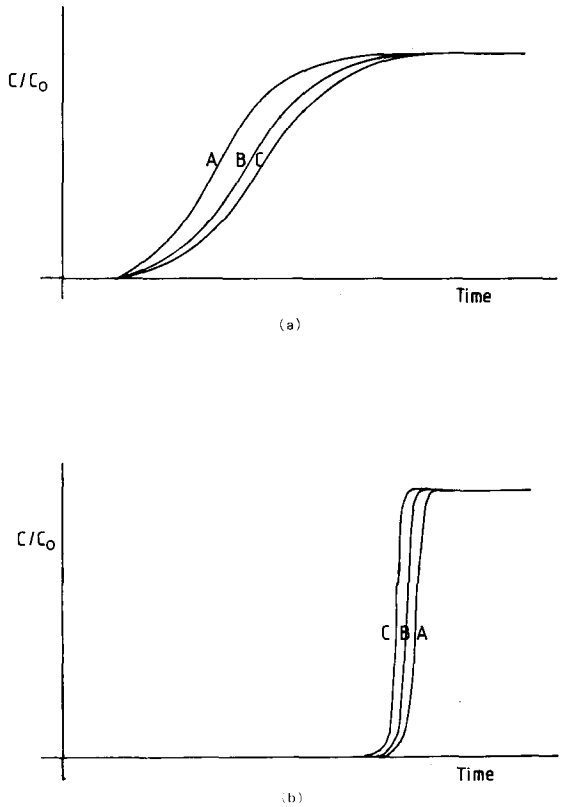

Fig. 6. Breakthrough curves produced by the computer simulation. (a) Inlet concentration = 0.1 mg/ml; (b) inlet concentration = 2.0 mg/ml. A = 38-μm particles; B = 45-μm particles; C = 49-μm particles.

maximum capacity and the dissociation constant influence the shape of the breakthrough curve[3]. Analysis of these effects using the computer based model was carried out by setting the column size, flow-rate and inlet concentration, and then predicting the breakthrough curves for beds consisting of each of the particle sizes, using the appropriate equilibrium and rate data. As expected, it was found that the degree of influence of the unfavourable alteration of K_d depended upon the actual inlet concentration of adsorbate. For lysozyme, at an inlet concentration of 2 mg/ml (approximately 20 times K_d), the adsorbent with the highest maximum capacity gave the best performance (Fig. 6b). However, at an inlet concentration of 0.1 mg/ml (approximately equal to the value of K_d), the order of breakthrough is reversed, with the larger differences in K_d outweighing the differences in the maximum capacity (Fig. 6a). At an inlet concentration around 0.5 mg/ml, the breakthrough curves are almost co-incident. For BSA, a similar effect is seen, but due to the much larger variation in the maximum capacity with varying particle size, the inlet concentration must be reduced to less than the value of the dissociation constant in order to alter the order of the breakthrough curves.

These results clearly show the influence of the dissociation constant in considerations of fixed bed performance. Particular attention must be paid to the dis-

TABLE IV

ISOTHERM PARAMETERS FOR THE ADSORPTION OF BSA AND LYSOZYME ONTO UNSIEVED BLUE SEPHAROSE

	Maximum capacity		*Dissociation constant*	
	mg/ml	*mol/ml*	*mg/ml*	*M*
BSA	13.4	$2.0 \cdot 10^{-7}$	0.36	$5.45 \cdot 10^{-6}$
Lysozyme	17.0	$1.2 \cdot 10^{-6}$	0.07	$4.86 \cdot 10^{-6}$

sociation constant where low inlet concentrations of adsorbate are to be employed.

The isotherm parameters obtained for the unsieved Blue Sepharose are given in Table IV. The values given are per unit volume of settled adsorbent, but in this case the broad particle size distribution will probably allow a tighter packing (with small particles filling the gaps in between the large particles). It is difficult, therefore, to make a true comparison with the sieved fractions, as the amount of gel per unit packed (or settled) volume may well be greater.

The maximum capacity of the unsieved material for both BSA and lysozyme is higher than for any of the sieved fractions measured. This increase may be due to the increased particle packing density, but may also be partly due to the contribution of the fine particles (which have not been studied separately), which would be expected to have an even greater capacity than the 38-μm particles, the smallest sieved fraction studied. The dissociation constant of the unsieved material is in between that of the 38- and 45-μm particles. For most applications, it is the adsorption capacity per unit volume of bed that is of most importance, and compared with the sieved fractions, the unsieved fraction has a higher maximum capacity without an increased dissociation constant.

For the adsorption of large proteins, the large variation of adsorption capacity with particle size has many implications. If Blue Sepharose with a different size distribution were to be used, either because of a different distribution being formed during manufacture, or the size distribution being altered in handling, then different results may be obtained. Small particles in particular can easily be lost in transfer, and whereas for gel filtration their removal is desirable, their presence has been shown to be desirable here.

From these results, it appears that sieving of the adsorbent in order to achieve a constant particle size is not desirable, unless very small particles are used. However, the use of very small particles is often impracticable, due to the resulting high pressure drops and flow-rate limitations.

ACKNOWLEDGEMENT

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