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# PREPARATION AND CHARACTERIZATION OF ADSORBENTS FOR USE IN HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY

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## SUMMARY

Adsorbents suitable for use in high-performance liquid chromatography were produced by covalently immobilizing Procion Blue MX-R to microporous silica. The adsorption capacities of the resulting materials were investigated. It was found that a Langmuir isotherm gave the best fit to data obtained for the adsorption of lactate dehydrogenase, while a Freundlich isotherm best described adsorpion of lysozyme. A 12-$\mu$m diameter silica with 28-nm diameter pores and a bound ligand concentration of 7 $\mu$mol (Procion Blue MX-R)/g (silica) had a capacity of 8.7 mg (lactate dehydrogenase)/g and 40.0 mg (lysozyme)/g. The rate of the overall adsorption process was found to be rapid. A model which assumes instantaneous equilibrium between solid and liquid phases was used to describe chromatography performed on these adsorbents. This gave a good approximation to the breakthrough and washout profiles.

## INTRODUCTION

High-performance liquid affinity chromatography (HPLAC) is a technique that has been developed for the separation and purification of biomolecules. It combines the high specificity of affinity chromatography with the advantages of high-performance liquid chromatography (HPLC). A review of the technique has been presented by Larsson *et al.*[1]. Applications of HPLAC have been presented in papers by Clonis *et al.*[2], who used HPLAC for the process-scale purification of lactate dehydrogenase (LDH) from a crude extract, and by Chase[3], who showed that HPLAC can be used in monitoring various bioprocesses.

Adsorbents for HPLAC have to be rigid, to withstand the high pressure drops associated with a packed bed of small diameter particles without suffering from deformation. They must also present a high surface area for adsorption and have properties which allow suitable affinity ligands to be attached to their surface. Microporous silica has been used extensively as a solid phase as it meets the above criteria. Typical pore sizes range from 5 to 500 nm, and particle diameters from 2 to 20 $\mu$m. Protein diameters range from 2 to 25 nm, and so judicious choice of the silica

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properties ensures that the pore size will not hinder the diffusion of the proteins to the adsorpion sites. In addition, it is possible to modify the surface of the silica so that non-specific protein–adsorbent interactions due to charged groups are eliminated.

Ligands used in affinity chromatography can be chosen to be extremely specific, *e.g.* antibody–antigen systems. Such a choice results in a high degree of purification but suffers the drawback that once a packing material is prepared it is useful for only one type of separation. An alternative is to use "group-specific" ligands that mimic the behaviour of biomolecules. Triazine textile dyes such as Cibacron Blue F3G-A and Procion Blue MX-R have been found to engage in biospecific interactions with certain proteins[1,4]. These dyes can be attached to the surface of microporous silica and the resulting adsorbent used for a range of separations.

In this study Procion Blue MX-R is attached to the surface of microporous silica and the resulting adsorbents are characterized with regard to their capacity to bind lysozyme and lactate dehydrogenase. The effects of bound dye concentration, buffer ionic strength and pH on the equilibrium capacity are studied. The kinetics of this adsorption are investigated and a simple model is proposed to predict the breakthrough and washout profiles produced when lysozyme is applied to a column packed with adsorbent. Breakthrough profiles obtained under varying inlet lysozyme concentrations and varying column flow-rates are compared to predictions made using the simple model.

## THEORY

### *Adsorption isotherms*

An adsorption isotherm is used to characterize the capacity of an affinity adsorbent to bind protein[5]. It provides a relationship between the concentration of the protein in the bulk solution and the amount of protein adsorbed to the solid phase when the two phases are at equilibrium. Two types of adsorption isotherm are typically used in describing affinity systems. These are the:

*Langmuir* which assumes that the molecules are adsorbed at a fixed number of well defined sites each of which can only hold one molecule. These sites are also assumed to be energetically equivalent, and far enough apart on the surface so that there are no interactions between molecules adsorbed to adjacent sites. This results in the following relationship between equilibrium concentrations in the two phases (for definitions of symbols, see Symbols section at the end of the paper):

$$q^* = \frac{c^* q_{\max}}{K_d + c^*} \tag{1}$$

and *Freundlich* which is an empirical isotherm corresponding to the assumption that the enthalpy of adsorption varies exponentially among the available sites.

$$q^* = k(c^*)^n \tag{2}$$

Neither of these isotherms gives any information on the rate at which equilibrium is approached. It is possible to obtain the constants in the above equations from a series of batch experiments in which fixed quantities of adsorbent are allowed to reach equilibrium with varying amounts of protein[5].

*Local equilibrium theory*

It is desirable to be able to model the performance of fixed bed adsorption processes from the point of view of optimizing the process and also for scaling up purposes. The simplest model used to describe fixed bed adsorption assumes there are no mass transfer or kinetic limitations on the process. Thus the solute in the adsorbed and liquid phases reaches equilibrium instantaneously, and ignoring the effects of axial dispersion, the equation of motion for a chromatographic column is[6]

$$U_0 \frac{\partial c}{\partial z} + \varepsilon \frac{\partial c}{\partial t} + \rho_b \frac{\partial q}{\partial t} = 0 \tag{3}$$

This is a first-order partial differential equation and it can be shown[6] that concentration is constant along lines in the $z$–$t$ plane given by

$$\frac{z}{t} = \frac{U_0}{\varepsilon + \rho_b f'(c)} \tag{4}$$

Where $f'(c)$ is $dq^*/dc^*$, the slope of the isotherm at the concentration of interest. This leads to the following results.

*Adsorption.* When a front of protein is applied to the column and the concentration of protein in the buffer leaving the bed is plotted as $c/c_0$ against a dimensionless throughput parameter $\Gamma$ given by

$$\Gamma = \frac{Q\left(t - \dfrac{\varepsilon L}{U_0}\right)}{\rho_b q_0 V} \tag{5}$$

local equilibrium theory predicts a step change in $c/c_0$ from 0 to 1 when $\Gamma = 1$.

*Washing.* When protein-free buffer ($c = 0$) enters a saturated column ($c = c_0$) at time $t = 0$, the shape of the bed exit profile is governed by the shape of the isotherm describing the system. In this study the Freundlich isotherm describes the lysozyme–Procion Blue system used in the chromatographic runs, and for a Freundlich isotherm[7]

$$\frac{c}{c_0} = \frac{1}{c_0} \left[ \frac{\left(\dfrac{U_0 t}{\varepsilon L} - 1\right)\varepsilon}{k n \rho_b} \right]^{\left(\frac{1}{1-n}\right)} \tag{6}$$

EXPERIMENTAL

*Preparation of adsorbents*

The adsorbents used in this work were prepared by attaching Procion Blue MX-R to microporous silica. All silicas used were supplied by Dr. K. Jones (Phase Separations, U.K.) whose assistance is gratefully acknowledged. Three separate batches of adsorbent were prepared. Henceforth these are referred to as adsorbents a, b and c, respectively. The first two of these were prepared by the authors as detailed

below. The silica used in preparing these was spherical, had a nominal pore size of 28 nm and a nominal diameter of 12 $\mu$m. The third was the gift of Y. Clonis and was prepared by a procedure which is detailed elsewhere[2]. This material had a particle diameter of 20 $\mu$m and a nominal pore size of 30 nm.

*Adsorbent a.* In order to silanize the surface of the silica, 10 g of silica was suspended in 100 ml of 0.01 *M* sodium acetate buffer, adjusted to pH 5.5 and containing 5% (v/v) 3-glycidoxypropyltrimethoxysilane (Aldrich, U.K.). This provides a silane in a four-fold excess to the amount stoichiometrically required for complete coverage of the silica surface, as recommended by Dean *et al.*[8]. The buffer was heated to 70°C, and the silane added followed immediately by the silica. The flask was placed in an ultrasonic bath for 3 min to remove air from the pores in the silica, and then placed in an oil bath at 90°C for 4 h. The silica was kept in suspension by stirring with a magnetic bar. The resulting epoxy silica was then washed with 500 ml water on a Buchner funnel through a Whatman filter paper and was then placed in a flask with 450 ml of 1 m*M* hydrochloric acid. The mixture was stirred at 90°C for 1 h to hydrolyse it to the corresponding diol. The diol-silica was then washed with 500 ml water followed by 500 ml acetone and placed in an oven (110°C) for 45 min. Procion Blue MX-R (2 g) was dissolved in 100 ml 0.1 *M* sodium bicarbonate and the diol-silica introduced to the flask. The suspension was sonicated for 2 min, then placed in an oil bath at 38°C for 18 h with an overhead stirrer to provide agitation. The blue silica was washed with water (500 ml) until the blue colour of the unbound dye was washed out, followed by 500 ml acetone prior to being dried in the oven. All steps were carried out at atmospheric pressure.

*Adsorbent b.* A 50-g amount of silica was prepared in this batch. The ratios of reagents (*i.e.* 10 ml/g for silanization and incubation, 0.2 g dye/g silica) were the same as for adsorbent a, except for hydrolysis (adsorbent a 45 ml/g, adsorbent b 20 ml/g). The times and temperatures of all the steps were the same as for adsorbent a. All reactions were carried out in a rotary evaporator flask rotating at 130 rpm and at atmospheric pressure.

*Determination of bound ligand loading*

Following the method of Lowe *et al.*[4] approximately 20-mg samples of adsorbent were weighed into test tubes. The silica was solubilized by adding 5 ml 1 *M* NaOH to each test tube and placing the test tubes in a water bath at 60°C for 30 min. The pH was then adjusted to 6–7 using 1 m*M* HCl and the solution volume made up to between 15 and 25 ml with water. The absorbance was measured at 620 nm against a blank prepared in the same fashion but using an unmodified silica. Assuming the molar extinction coefficient of Procion Blue MX-R is 10.5 ml $mol^{-1}$ $cm^{-1}$ (Clonis *et al.*[2]), the loading of bound ligand ($\mu$mol/g) can be found from

$$\frac{\text{Absorbance} \cdot \text{final volume (ml)}}{10.5 \text{ ml mol}^{-1} \text{ cm}^{-1} \cdot \text{mass of silica (g)}} \tag{7}$$

The molar extinction coefficient of Procion Blue MX-R is taken as 10.5 ml $mol^{-1}$ $cm^{-1}$ (Clonis *et al.*[2]).

*Determination of diol content of diol-bonded silica*

The diol content of the diol-bonded silica was determined following the modified periodate oxidation method outlined by Dean *et al.*[8].

*Adsorbent capacities*

A known mass of adsorbent was placed into buffer containing a known concentration of solute. The change in solute concentration (after allowing 1 h for equilibrium to be attained) can be equated to the amount of solute adsorbed by the solid, and by using varying starting solute concentrations, equilibrium data is obtained. When values of $c^*$ *vs.* $q^*$ have been obtained it is necessary to determine which theoretical isotherm best fits the data. For the Langmuir, a plot of $c^*/q^*$ *vs.* $c^*$ should yield a straight line with a slope of $1/q_{max}$ and with an intercept on the $c^*$-axis at $c^* = -K_d$. For the Freundlich, a plot of ln $q^*$ *vs.* ln $c^*$ should yield a straight line of slope $n$ and intercept ln $k$. Fitting straight lines to the transformed data using the method of least squares, it can be determined which theoretical isotherm gives the best fit to the experimental data.

The capacity of the adsorbent for two proteins was investigated; these were LDH and lysozyme.

*Lactate dehydrogenase.* Data were obtained using adsorbents a and c and both pure and crude extracts of LDH. Pure LDH was purchased from Boehringer (F.R.G.; product No. 127 876) and crude LDH from Sigma (U.S.A.; product No. L-2375). The desired concentration of the stock solution for the experiments was 300–400 units/ml of enzyme activity. (One unit will convert 1 μmol of phospho(enol)pyruvate to pyruvate per minute at pH 7.0 at 25°C.) This was obtained by dialysing 500 μl of pure or 1000 μl of crude enzyme at a ratio of 500:1 in 0.05 *M* pH 7.0 phosphate buffer overnight and making the extract up to a total volume of 2 ml.

Varying dilutions of the stock enzyme solution were added to 1.5 ml Eppendorf tubes containing approximately 0.02 g of adsorbent, keeping the total volume of solution at 400 μl. The samples were placed in a water bath at 20°C for 1 h, being removed at intervals to agitate and re-suspend the silica. For each experiment a control was carried out at half the concentration of the stock solution to check the enzyme suffered no loss of activity during the experiment. The Eppendorf tubes were then centrifuged, the supernatant removed, and the concentration of LDH determined by assaying enzyme activity.

LDH was assayed by following the reaction of pyruvate in the presence of NADH. An assay solution of 0.1 *M* phosphate buffer pH 7.0 containing 0.18 mg/ml NADH (nicotinamide–adenine dinucleotide, reduced; Boehringer) and 0.115 mg/ml sodium pyruvate (BDH, U.K.) and having an absorbance > 1.3 a.u. (path length 1 cm) was prepared. For each assay 50 μl of sample were added to 2.95 ml of assay solution and the absorbance monitored at a wavelength of 320 nm and 25°C on a Pye Unicam (U.K.) PU8160 kinetics spectrophotometer. Samples were diluted as necessary so that the decrease of absorbance with time could be followed accurately. The concentration of LDH in mg/ml is then calculated from:

$$c^* = \frac{\Delta A_{min} \cdot 3 \cdot \text{dilution}}{6.22 \cdot 655} \tag{8}$$

The molar extinction coefficient of NADH is 6220 l $mol^{-1}$ $cm^{-1}$; the specific activity of pure LDH, measured from assays at 25°C, is 655 units/mg, and the adsorbent phase loading is then calculated from:

$$q^* = \frac{(c_{\text{initial}} - c^*) \cdot 0.4}{w} \tag{9}$$

*Lysozyme.* Known amounts (approximately 0.1 g) of adsorbent were weighed into 1.5 ml Eppendorf test tubes. Lysozyme (Sigma) solution in 0.05 *M* pH 7.0 phosphate buffer (except where stated) was prepared as a stock solution at 0.8 mg/ml and diluted to yield solutions at varying concentrations. Of each of these solutions 1 ml was added to an Eppendorf tube containing silica, 1 ml to an Eppendorf tube not containing silica to act as a control and for use in determining the calibration curve to relate optical density to lysozyme concentration. The tubes were placed in a water bath at 20°C for 1 h with agitation at regular intervals. The Eppendorf tubes were then centrifuged and the supernatant removed. These liquid samples were then diluted by factors between 7 and 15 and the absorbance measured at a wavelength of 280 nm using a 1-cm path length. Phosphate buffer was used as a blank and a calibration curve prepared from linear regression of the absorbance/concentration data provided by the control samples. The solid phase concentration was then calculated from:

$$q^* = \frac{(c_{\text{initial}} - c^*) \cdot 1.0}{w} \tag{10}$$

*Adsorption kinetics*

The equilibrium isotherms give no information on the rate of the adsorption reaction. The theoretical model used in this work assumes that equilibrium is instantaneously achieved between the two phases. In order to test whether the adsorption reaction is indeed fast enough for this assumption to be valid, batch tests to ascertain the rate of adsorption were undertaken.

*Lactate dehydrogenase.* A 800-$\mu$l volume of pure LDH was dialysed overnight in pH 7.0 0.05 *M* phosphate buffer and further diluted to a total volume of 5 ml after dialysis. Adsorbent a (0.5 g) was suspended in 15 ml of phosphate buffer and held in suspension by a magnetic stirrer in a 25 ml beaker surrounded by a water jacket at 25°C. At time zero the LDH solution was introduced into the beaker. Samples (50 $\mu$l) were taken at regular intervals, centrifuged and the supernatant removed and assayed for enzyme activity, as described earlier.

*Lysozyme.* A 40-ml volume of 0.05 *M* pH 7.0 phosphate buffer containing 1 mg/ml of lysozyme was agitated using a magnetic stirrer. Adsorbent b (2 g) was suspended in 10 ml of the same buffer and added to the beaker at time zero. Samples were removed using syringes with filters attached so that no centrifuging was necessary. The absorbance was measured at 280 nm and the concentration of lysozyme in the liquid phase determined.

*Chromatographic performance*

Affinity chromatography was studied using a column packed with adsorbent b

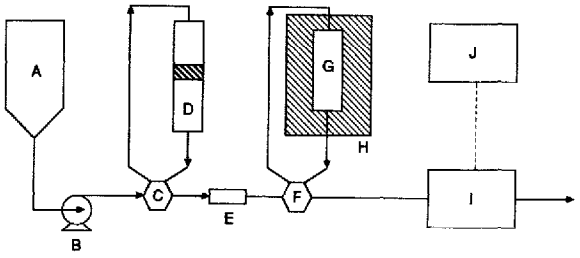


Fig. 1. Chromatography apparatus. A = Buffer reservoir; B = pump; C = valve; D = sample loop; E = guard column; F = valve; G = column; H = water bath; I = spectrophotometer; J = microcomputer. ——— = Fluid flows; – – – – = data flow.

and lysozyme as a solute. Breakthrough profiles were obtained when a front of protein was applied to the column and washout profiles were obtained when protein free buffer was applied to a protein saturated column.

*Apparatus.* A diagram of the apparatus is shown in Fig. 1. Two sample injection valves were used to divert the flow to the sample loop and column as required. It was necessary to use a sample loop to forestall problems associated the passage of protein through the pump and resulting in fouling onto the internal valves. The sample loop was designed and constructed by the authors and consisted of a 1.6 cm I.D. stainless-steel pipe approximately 40 cm long (Fig. 2) with a working volume of 70 ml. An internal piston separates the protein and buffer and has a length of polished steel

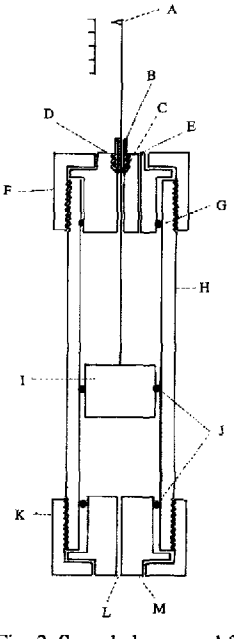


Fig. 2. Sample loop used for applying protein to column. A = Indicator; B = sealing nut; C = "O"-ring seal; D = end piece; E = liquid entry; F = retaining cap; G = "O"-ring seal; H = 16 mm I.D. stainless-steel tube; I = piston; J = "O"-ring seal; K = retaining cap; L = liquid exit; M = end piece.

wire attached which protrudes from the sample loop and acts as an indicator of the piston position. An "O"-ring seal prevents the protein and buffer from mixing. As buffer is pumped behind the piston, it moves down the tube and forces the liquid ahead of it out and through the column.

The column was 0.86 cm in diameter and 5.86 cm long, giving a volume of 3.4 ml. The cell used in the spectrophotometer was a low-volume HPLC type designed to give minimal dispersion. Tubing throughout the system was 0.76 mm I.D. HPLC tubing.

*Packing columns.* In preparation for column packing, adsorbent (3 g) was slurried in 75 ml of methanol ("Hipersolve", BDH, U.K.), sonicated for 5 min to remove air and then poured into a packing bomb. The column (with a frit at one end) was screwed onto the bomb and an upward flow of methanol was maintained through the column at sequential backpressures of 1500, 2500 and 3000 p.s.i. for periods of 5 min each. The column was removed, the cake adhering to the end was broken off and packed down smoothly with a spatula, and a frit screwed on. The mass of silica initially slurried was measured, and the mass of silica not packed into the column at the end of the packing process was also determined. The difference between these two figures gives the mass of silica packed into the column. Thus the bulk density was determined as 0.44 g/ml.

*Column runs.* All buffers used in the column experiments were filtered through a 0.22-$\mu$m filter and then degassed under vacuum. The column was suspended in a water bath at 20°C. All runs were carried out using 0.05 *M* phosphate buffer. At the end of each run the column was eluted with 1 *M* KCl solution to ensure that the protein was completely removed before the next run.

*Column void volume.* The system delay due to the volume of tubing, valves and guard column, *i.e.* the time delay between switching valve 1 to pump through the sample loop and the spectrophotometer registering an increase in concentration, was estimated using runs where the column was bypassed. The additional dead time due to the tubing between the column and valve 2 was added to this delay time.

The column void volume is an important parameter and occurs in eqns. 3–6. It was estimated in this work by three separate methods:

(1) When 1 *M* KCl is applied to a loaded column containing adsorbed protein the affinity interaction is greatly reduced and protein is desorbed from the column. The time lag between salt being applied to the base of the column and protein appearing at the exit is proportional to the void volume of the column, $\varepsilon V/Q$. This gave a mean value of $\varepsilon = 0.59$.

(2) The mass of silica packed into the column was 1.5 g. The density of solid silica is 2.34 g/ml. This gives a value of $\varepsilon = 0.81$.

(3) The actual volume occupied by solid silica was measured by mixing 1 g of silica and 5 ml of methanol in a 10-ml measuring cylinder and noting the increase in volume. This was 0.6 ml/g, giving $\varepsilon = 0.73$.

The average value for $\varepsilon$ was taken to be 0.7. This assumes that lysozyme can enter all the pores of the silica. The nominal pore size is 30 nm and the molecular diameter of lysozyme is 4 nm, so provided the pore size distribution is not too wide this should be the case.

## RESULTS AND DISCUSSION

### *Bound ligand loading*

The results of the analysis of the bound ligand loading are given in Table I. A separate analysis by the same technique on adsorbent a gave 8.1 μmol/g and Clonis *et al.*[2] arrived at 2.9 μmol/g for adsorbent c. Thus these values have errors of ±1 μmol/g. It can be seen that there is a large variation in the final loading of bound ligands on the silica. The reason why adsorbent c has a low bound ligand loading is possibly due to the low dye/silica ratio used in the final derivatization step (0.05 g Procion Blue/g silica, *vs.* 0.2 g Procion Blue/g silica for adsorbents a and b). Adsorbents a and b, however, were produced by the authors in the same laboratory by ostensibly the same process, except for the ratio of 1 m*M* HCl used during the hydrolysis step. The difference in the final surface loading of bound ligands may be due to varying degrees of surface coverage of the silica by diol groups following the hydrolysis step. A diol-bonded silica was produced following an identical procedure to that used in preparing adsorbent b up to the end of the hydrolysis step. This was analysed for diol content and found to have a diol loading of 601 μmol/g. The surface area of the silica was determined using a BET machine (Sorptomatic Series 1800, Carlo Erba) as being 210 $m^2$/g. The theoretical monolayer coverage of diol is[8] 2.3 μmol/$m^2$, and so a monolayer coverage of this material would yield a diol loading of 489 μmol/g. The actual diol loading of 610 μmol/g indicates that there are more than sufficient diol groups present to form a monolayer, and that in places there is a multilayer covering of diol groups. However the aqueous procedures used herein for the derivatization process are known to result in a polymeric diol layer[8] and so it is still possible that significant areas of the silica surface may not be covered with diol groups and so are unable to bind dye molecules in the ligand attachment step. To investigate the effect of the acid/silica ratio during the hydrolysis step further, small amounts of adsorbents were produced in four separate batches, using identical conditions. All reagent quantities and steps were the same as for adsorbents a and b except the hydrolysis ratio, which was 10 ml 1 m*M* HCl/g silica. Bound ligand loading was analysed by the same method. The four samples were found to have a mean ligand loading of 4.3 μmol/g with a standard deviation between samples of 0.6 μmol/g. Thus there is no significant difference between the ligand loading on these samples and adsorbent b. The reason for adsorbent a having a higher bound ligand loading is not understood and needs to be investigated further, so that high bound ligand loadings can be obtained consistently.

TABLE I

BOUND LIGAND LOADING FOR ADSORBENTS a, b AND c

| *Adsorbent* | *Bound ligand loading (μmol/g)* |
|---|---|
| a | 7.0 |
| b | 3.8 |
| c | 1.7 |

*Adsorbent capacities*

*Lactate dehydrogenase*. The data for the adsorption of LDH from a crude extract binding onto adsorbents a and c are shown in Fig. 3. Each experimental point is the average of two enzyme assays for that sample. The error in the assay was found to be less than 25% between two repetitions. The Langmuir isotherm was found to give a better fit than the Freundlich, and to give a good fit to the data. Table II lists the binding parameters for the adsorption of both pure and crude LDH.

The reduced binding capacity for LDH of both adsorbents with crude rather than pure LDH extract is possibly due to the "wide band" specificity of the Procion Blue MX-R as a ligand, *i.e.* many of the available sites could be engaging in affinity reactions with proteins other than LDH which are present in the crude extract.

The data also shows that adsorbent a has a higher maximum binding capacity than adsorbent c. The ratios $q_{max}$ (adsorbent a)/$q_{max}$ (adsorbent c) are 1.7 and 3.2 for pure and crude LDH, respectively. The ratio of bound ligand loading on adsorbent a/adsorbent c is in the region 2.8 to 4.1. Thus it is probable that the higher capacity of adsorbent a is due to the larger number of available sites. The molecular weight of LDH is 140 000 g/mol. Thus the figure of 8.7 mg LDH/g can be equated to 0.062 $\mu$mol LDH/g which corresponds to less than 1% of the total ligand sites being occupied. This could be due to steric hindrance of the LDH molecules in some of the pores or to only a small fraction of the ligands being "active" in terms of binding LDH. The molecular diameter of LDH is[1] 8 nm. Thus if several LDH molecules are bound to ligands at the mouth of the pore, there may be insufficient room for any additional LDH molecules to pass the pore mouth. It should also be bourne in mind that the length of the ligand will further reduce the effective pore diameter. The nature of the affinity interaction may thus be critical in determining whether the LDH can penetrate the pores of the

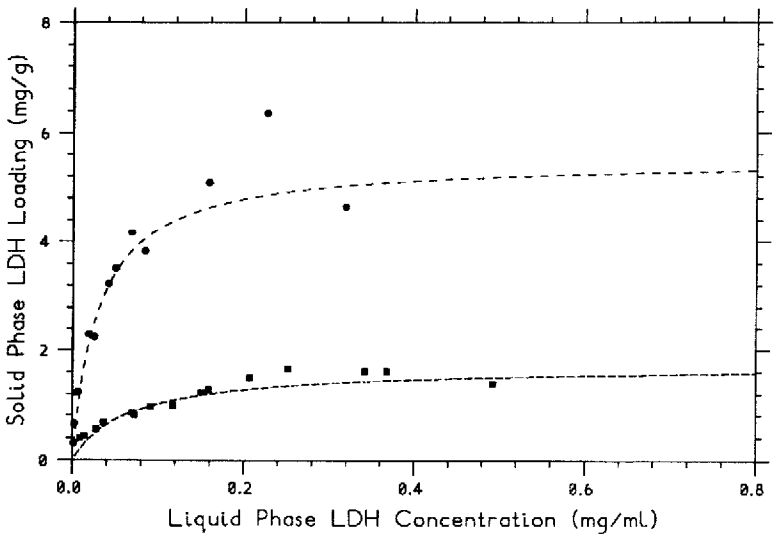


Fig. 3. Equilibrium adsorption of crude LDH onto silicas derivatized with Procion Blue MX-R; 20°C, 0.05 *M* pH 7 phosphate buffer. ● = Adsorbent a (7.0 $\mu$mol ligand/g silica); ■ = adsorbent c (1.7 $\mu$mol ligand/g silica). Langmuir isotherms fitted with the following constants: Adsorbent a (– – – –); $q_{max}$ = 5.50 mg/g, $K_d$ = 0.023 mg/ml; adsorbent c (- - - - - - - - -); $q_{max}$ = 1.70 mg/g, $K_d$ = 0.053 mg/ml.

TABLE II

BINDING PARAMETERS FOR LDH

| | $K_d$ (mg/ml) | $q_{max}$ (mg/g) |
|---|---|---|
| Pure LDH and adsorbent a | 0.031 ± 0.005 | 8.70 ± 0.52 |
| Pure LDH and adsorbent c | 0.107 ± 0.040 | 5.25 ± 0.88 |
| Crude LDH and adsorbent a | 0.023 ± 0.007 | 5.51 ± 0.32 |
| Crude LDH and adsorbent c | 0.053 ± 0.012 | 1.70 ± 0.09 |

adsorbent. If an LDH molecule binds itself irreversibly to a ligand until the conditions are altered to elute the material, there may be no further penetration of the pores after the pore mouth is blocked by the first few molecules. If the LDH–ligand interaction is a reversible process during which the protein is periodically adsorbed/desorbed it is possible that there will be a migration of protein along the length of the pore. The figure of 8.7 mg/g for the maximum capacity of adsorbent a for LDH is in agreement with the data of Larsson *et al.*[1] who measured 6 and 9 mg/g for LDH binding to NAD-derivatized silicas with pore diameters of 10 and 100 nm, respectively. Their results also indicate that steric hindrance may be a problem in LDH adsorption, as 10-nm pore size silica has ostensibly a far greater surface area for adsorption than 100-nm silica, and yet they measured the 100-nm silica as having the larger capacity.

*Lysozyme.* A Freundlich isotherm was found to fit these data more accurately than the Langmuir, and a good fit was obtained with the experimental data. Fig. 4 shows the experimental values with the fitted isotherm drawn in as well. Table III lists

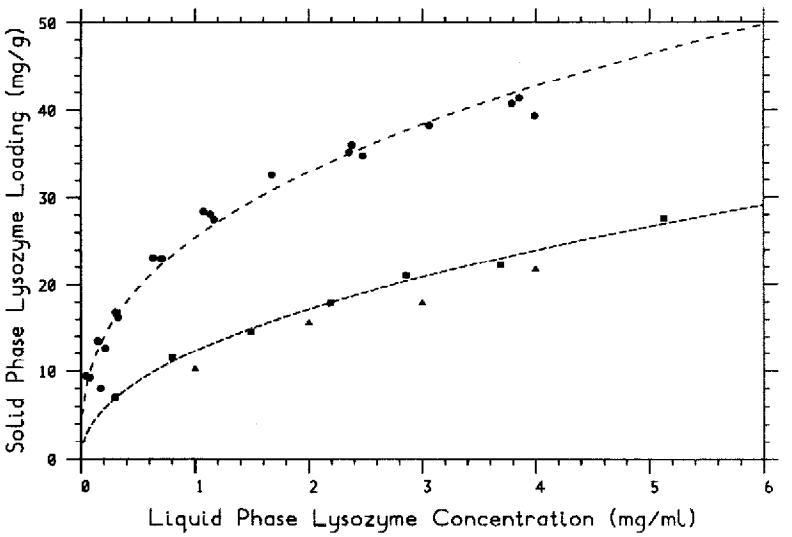


Fig. 4. Equilibrium adsorption of lysozyme onto silicas derivatized with Procion Blue MX-R; 20°C, 0.05 *M* pH 7 phosphate buffer. ● = Adsorbent a (7.0 μmol ligand/g silica); ■ = adsorbent b (3.8 μmol ligand/g silica); ▲ = adsorbent b results from breakthrough profiles. Freundlich isotherms fitted with the following constants: Adsorbent a (-----); $k$ = 25.4 ml/g, $n$ = 0.375; adsorbent b (·····); $k$ = 12.3 ml/g, $n$ = 0.481.

TABLE III

BINDING PARAMETERS FOR LYSOZYME

| *Adsorbent* | $k$ $(mg^{(1-n)}\ ml^n/g)$ | $n$ |
|---|---|---|
| a | 25.4 ± 1.0 | 0.37 ± 0.02 |
| b | 12.3 ± 1.0 | 0.48 ± 0.01 |

the binding parameters for adsorbents a and b. It is unclear why a Freundlich isotherm gives a better fit to this data. A Freundlich isotherm implies no theoretical limit exists for the maximum capacity of the adsorbent for lysozyme. This is obviously unrealistic in practice because the adsorbents used have physical limits as to how much protein can attach itself to its inner pore surface. It is possible that at higher concentrations of lysozyme than used in this work the solid phase loading would have reached a constant value. It is also possible that there is an affinity binding effect taking place between the ligand and the lysozyme, and at the same time there is also some kind of non-specific interaction occuring between the ligand and the lysozyme. The Freundlich isotherm may be the best representation of the superposition of these two effects. Whatever the reason, the utility of the isotherm lies in its use as a tool for quantifying adsorbent capacity. The Freundlich isotherm, while causing theoretical difficulties, serves this purpose well.

The ratio of binding capacity for different adsorbents is a function of liquid phase concentration for the Freundlich isotherm, *e.g.* $q^*$ (adsorbent a)/$q^*$ (adsorbent b) = 2.1 $(c^*)^{-0.11}$. Over the range of concentrations in these experiments this is equal to approximately 1.9. The ratio of bound ligand loadings is approximately 2.0. Thus it seems reasonable to assume for the adsorption of lysozyme the capacity is limited by the number of ligands available at the adsorbent surface. Lysozyme has a molecular weight of 13 930 g/mol[9] and a molecular diameter of 4 nm[10]. This equates to 3 $\mu$mol lysozyme/g adsorbent for adsorbent a, or approximately 40% of the available sites being used, assuming that one ligand binds one lysozyme molecule. If it were assumed that more than one dye molecule is required to bind a lysozyme molecule, or that 40% of the bound ligands are available to adsorb lysozyme, the equivalence in the ratio of capacity to bound ligand loading is explained. Data for the adsorption of lysozyme onto Blue Sepharose[11] show a maximum binding capacity of 16 mg lysozyme/ml settled adsorbent. Adsorbent b was measured to have a packed volume of 2.3 ml/g. Thus 40 mg/g corresponds to 17 mg/ml (packed volume) which is of the same order as the capacity of the Blue Sepharose. These results cannot be directly compared because the immobilized ligand used on Blue Sepharose is Cibacron Blue F3G-A, and the two adsorbents have different surface areas. However, it is interesting to consider the relative capacities of the two adsorbents with view to scale up and process economics.

The ionic strength of the liquid phase significantly effects the capacity of the adsorbent (Fig. 5). The pH of the solution however does not have a significant effect over the range 6.5–7.5, while a liquid phase concentration of 1 *M* KCl was found to reduce the bound protein loading by an order of magnitude. Tests (results not shown) on diol-silica showed that it is effectively "sealed off" and binds no lysozyme, while the unmodified silica was found to bind protein irreversibly. As discussed earlier, the

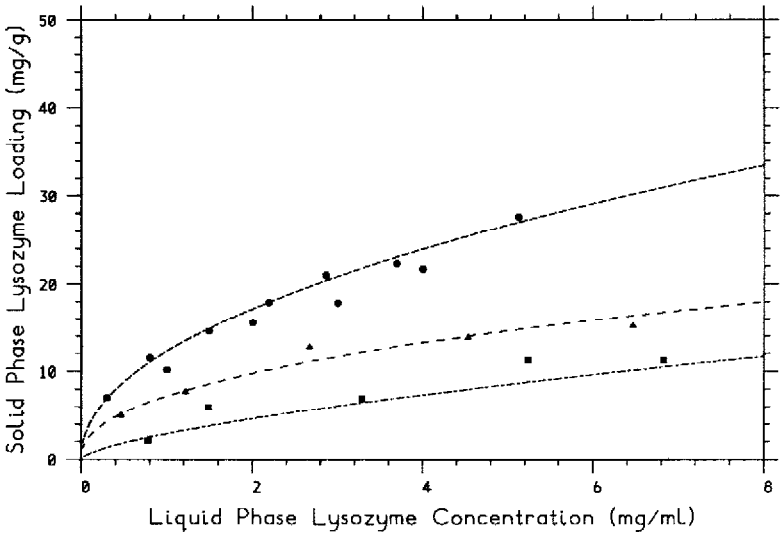


Fig. 5. Effect of buffer ionic strength on equilibrium adsorption of lysozyme to adsorbent b; 20°C. Phosphate buffer pH 7: ■ = 0.2 *M*; ▲ = 0.1 *M*; ● = 0.05 *M*. Freundlich isotherm fitted with the following constants: (-·-·-·-·-) 0.2 *M* buffer; $k = 2.9$, $n = 0.67$; (– – –) 0.1 *M* buffer; $k = 7.2$, $n = 0.44$; (---------) 0.05 *M* buffer; $k = 12.3$, $n = 0.48$.

diol-silica was analysed for diol loading and found to contain diol groups excess to the requirement for a monolayer. The fact that there was no binding of lysozyme to the diol silica indicates that the diol groups present probably constitute a monolayer covering most of the silica surface, as opposed to a patchwork of polymeric diol layers. The approximate coverage of available surface area on the adsorbents can be calculated. For LDH, 0.062 μmol/g corresponds to a projected area of 1.9 $m^2/g$. Similarly for lysozyme 3 mmol/g corresponds to 22 $m^2/g$. The surface area of adsorbent a was 210 $m^2/g$ so that if the molecules are spread out on the surface uniformly the lysozyme molecules may be close enough together to limit the capacity due to steric hindrance, since the surface coverage is 10% of the total available surface. However, the LDH molecules would be some distance apart, as they cover only 0.8% of the total available area. However, as mentioned above, the capacity of the adsorbent for LDH may well be limited by adsorbed molecules at the pore mouth hindering the passage of other molecules past them to the empty sites.

*Adsorption kinetics*

The results of the kinetics experiments on LDH are shown in Fig. 6. Equilibrium was reached in under 2 min. For lysozyme, the rate of adsorption was so rapid as to be almost instantaneous compared to the resolution obtainable by the method described here (15 s), and this data is not shown on the plot. The faster rate of adsorption for lysozyme may be due to the greater diffusivity of lysozyme (the diffusivities of the proteins in water at 20°C are $1.12 \cdot 10^{-7}$ and $0.45 \cdot 10^{-7}$ $cm^2/s$ for lysozyme and LDH, respectively[9]) which allows it to make its way down the pores more quickly. Alternatively, the larger size of the LDH molecule may hinder diffusion due to collisions and interactions with the pore walls and adsorbed LDH molecules.

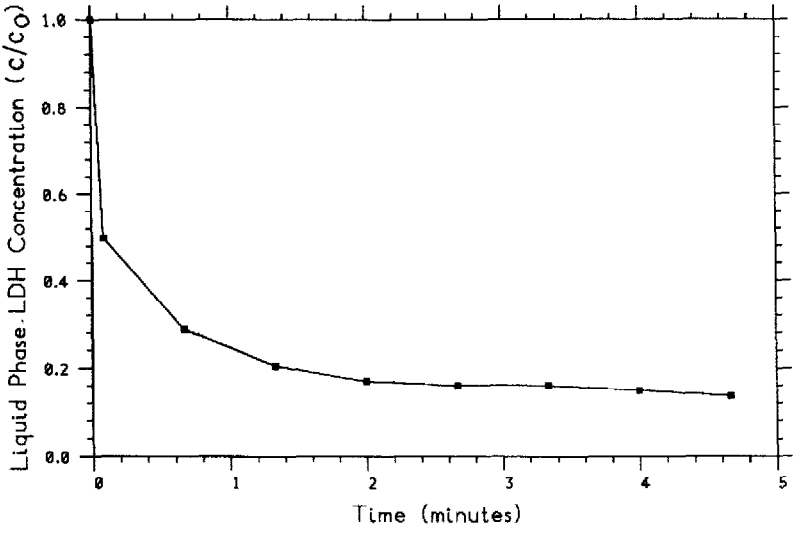


Fig. 6. Rate of adsorption of LDH onto adsorbent a in a stirred beaker; 20°C, 0.05 *M* pH 7 phosphate buffer. ■ = Experimental data.

*Chromatographic performance*

Plots of breakthrough curves for varying inlet concentrations and flow-rates are shown in Figs. 7 and 8. The dimensionless concentration $c/c_0$ is plotted against $\Gamma$, the dimensionless throughput parameter. The area behind the breakthrough curve, from $\Gamma = 0.0$ to the point where $c/c_0 = 1.0$ should be equal to 1.0. Equilibrium theory predicts a step change in $c/c_0$ from 0 to 1 at $\Gamma = 1.0$. The results show that breakthrough generally occurs slightly before this and that the breakthrough profile is not affected very much by either flow-rate or inlet concentration over the range of

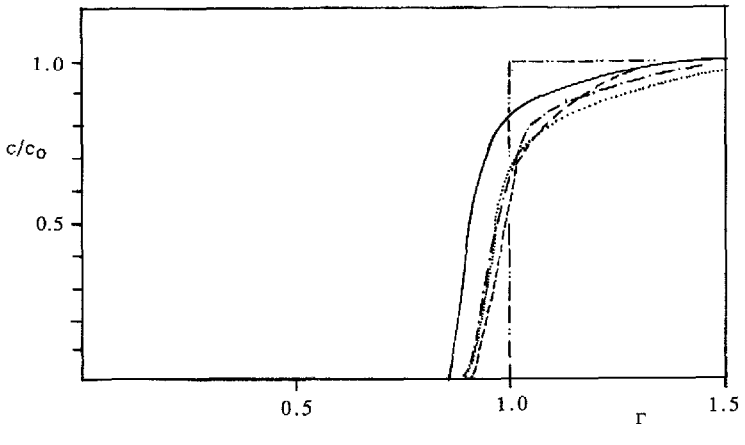


Fig. 7. Breakthrough profiles for lysozyme on adsorbent b at varying inlet concentrations. Superficial velocity = 3.7 cm/min, 20°C, 0.05 *M* pH 7 phosphate buffer. – · – · –, Local equilibrium theory; ———, $c_0$ = 1 mg/ml; · · · · · · · · ·, $c_0$ = 2 mg/ml; – · – · –, $c_0$ = 3 mg/ml; – – – – –, $c_0$ = 4 mg/ml.

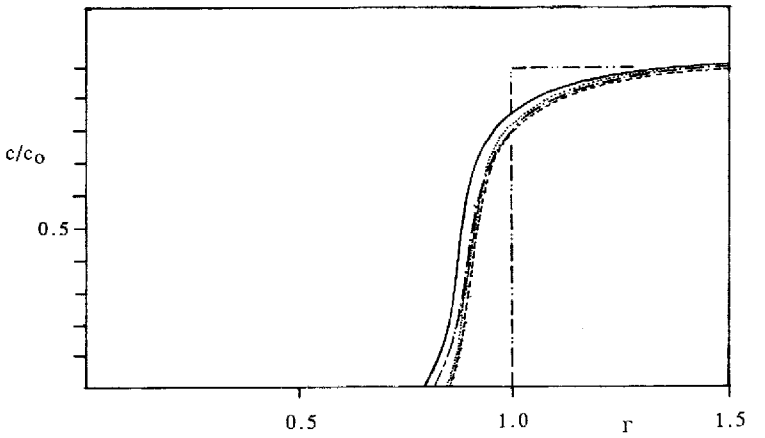


Fig. 8. Breakthrough profiles for lysozyme on adsorbent b at varying superficial velocities. Inlet concentration ($c_0$) = 1 mg/ml, 20°C, 0.05 *M* pH 7 phosphate buffer. – · – · –, Local equilibrium theory; · – · – ·, superficial velocity ($U_0$) = 3.7 cm/min; ———, $U_0$ = 5.2 cm/min; – – – –, $U_0$ = 7.2 cm/min; · · · · · · · · ·, $U_0$ = 8.6 cm/min.

values used in this work. The broadening that is evident is thought to be due primarily to dispersive and mixing effects within the column and pipework.

Using eqn. 6 and the values for the Freundlich isotherm parameters obtained previously for adsorbent b (Table III), theoretical washing curves can be obtained. These are shown superimposed on the experimentally determined curves in Fig. 9 and show a good agreement. The apparent offset in the theoretical and experimental curves may be due to the uncertainty in estimating the system delay time. Thus for both breakthrough and wash situations the local equilibrium model, which makes no allowance for any rate determining steps, can be seen to give a reasonable approximation of the performance of the column. This is due to the rapidity with which equilibrium is approached using the small-diameter HPLAC adsorbents. Obtaining the equilibrium adsorption parameters required to make predictions of column performance using the local equilibrium model requires a minimum of

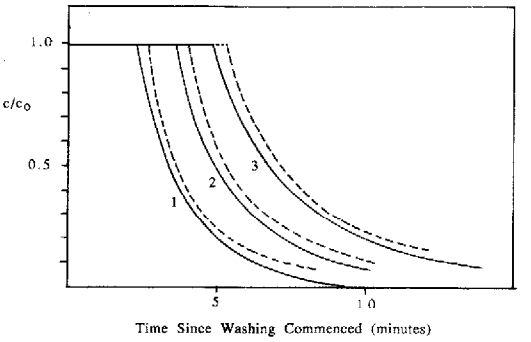


Fig. 9. Washout profiles obtained when protein-free buffer is applied to a protein-saturated column; 20°C, 0.05 *M* pH 7 phosphate buffer. **1**, Superficial velocity ($U_0$) = 5.2 cm/min, inlet concentration ($c_0$) = 1 mg/ml; **2**, $U_0$ = 3.7 cm/min, $c_0$ = 2 mg/ml; **3**, $U_0$ = 3.7 cm/min, $c_0$ = 1 mg/ml. ——— = Experimental; – – – – – = predicted from eqn. 6.

experimental work. This model is thus useful for process evaluation and scale up studies.

The area behind the breakthrough curve can be used to calculate the solid phase concentration that is in equilibrium with the liquid feed concentration. This has been done for the various feed concentrations employed herein and the isotherm data obtained in this fashion are shown plotted with the results from the batch tests discussed previously (Fig. 4). These two methods for obtaining isotherm data agree very well, thus confirming the validity of the method used herein.

## SYMBOLS

| | |
|---|---|
| $\Delta A_{min}$ | rate of change of absorbance (a.u./min) |
| $c$ | liquid phase concentration (mg/g) |
| $c^*$ | equilibrium liquid phase concentration (mg/g) |
| $c_{initial}$ | liquid phase concentration at the start of the isotherm experiment |
| $c_0$ | liquid phase concentration in column feed (mg/g) |
| $K_d$ | Langmuir isotherm dissociation constant (mg/g) |
| $k$ | Freundlich isotherm constant ($mg^{(1-n)}$ $ml^n/g$) |
| $L$ | column length (cm) |
| $n$ | Freundlich isotherm exponent (dimensionless) |
| $q$ | adsorbent phase loading (mg/g) |
| $q^*$ | equilibrium adsorbent phase loading (mg/g) |
| $q_{max}$ | Langmuir isotherm maximum capacity constant (mg/g) |
| $q_0$ | solid phase loading in equilibrium with column feed (mg/g) |
| $Q$ | flow-rate through column ($cm^3$/min) |
| $t$ | time (min) |
| $U_0$ | liquid phase superficial velocity (cm/min) |
| $V$ | volume of column (ml) |
| $w$ | mass of silica used in batch tests (g) |
| $z$ | length variable along column axis (cm) |
| $\Gamma$ | dimensionless throughput parameter |
| $\varepsilon$ | void fraction of column |
| $\rho_b$ | packed density of adsorbent (g/ml) |

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