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Adsorption behaviour of albumin and conalbumin on TSK-DEAE 5PW anion exchanger

JUN-XIONG HUANG[a], JENNIFER SCHUDEL and GEORGES GUIOCHON*
**Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600 and Division of Analytical Chemistry, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120 (U.S.A.)*
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SUMMARY

The adsorption behavior of chicken albumin and conalbumin on TSK-DEAE 5PW ion exchanger was studied by determining the equilibrium isotherms of these compounds at different temperatures, with different mobile phase ionic strengths and pH, using frontal analysis. The last two parameters have a considerable influence on the isotherms, including the saturation capacity, whereas temperature has a much less important influence. In most instances, and especially with chicken albumin, these isotherms are poorly approximated by a Langmuir isotherm equation, but rather correspond to high-affinity isotherm types. The Scatchard plots exhibit a downward convexity, indicating non-cooperative adsorption on two kinds of sites. The adsorption process tends to be partially irreversible towards dilution in the mobile phase.

INTRODUCTION

The investigation of protein adsorption at liquid–solid interfaces is of great importance for theoretical and practical reasons. The surface area occupied by a protein molecule on the surface of a known adsorbent, the adsorption energy at low surface coverage, the importance of lateral molecular interactions in the sorbed layer, the competition between different protein molecules for adsorption and the displacement of protein molecules by "strong" solvents all contribute to shed light on the ternary structure, its fluctuations and the surface energy of the proteins studied[1]. From a more practical standpoint, an accurate knowledge of the adsorption isotherms of a number of pure proteins and of their competitive isotherms is required for a better understanding of the chromatographic behavior of these compounds, especially at high concentrations, under the experimental conditions favored in the preparative applications of liquid chromatography.

Preparative liquid chromatography has become the essential tool used in

[a] Present address: Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China.

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biochemical laboratories and in the pharmaceutical industry for the extraction, separation and purification of proteins. The literature concerning these applications of chromatography has experienced a very rapid growth in recent years[2–4]. The optimization of the experimental conditions for preparative separations of proteins is still carried out empirically, however, because of a lack of the information required to apply the results of the theory of non-linear chromatography[5,6]. The most pressing need, at present, is for data on the competitive adsorption behavior of proteins on the most conventional stationary phases.

Among the packing materials that are currently used for the chromatographic separation and analysis of peptides and proteins, TSK gels have become very popular[7,8]. However, to our knowledge no systematic investigation of the adsorption behavior of proteins on these semi-rigid, porous polymer beads has yet been published.

This paper reports measurements of the adsorption isotherms of two common, basic proteins, chicken albumin and conalbumin, carried out by frontal analysis on TSK-DEAE 5PW, a weak anion exchanger, under various experimental conditions, and on the effect of the mobile phase flow velocity, temperature, pH and ionic strength of the mobile phase on these isotherms. A miniaturized equipment, similar to that described previously[9], was used for these determinations because of the special requirements of protein adsorption isotherm determinations.

In a further paper, the use of these isotherm data for the prediction of the profiles of the high-concentration bands obtained on analytical-scale columns will be discussed[10].

THEORETICAL

The experimental results derived from frontal analysis are amounts, q, adsorbed at equilibrium at various concentrations, C, of the protein in the mobile phase. These data can be plotted either as classical isotherms, *i.e.*, plots of q *versus* C, or as Scatchard plots of q/C *versus* q. If the equilibrium isotherm is well accounted for by a Langmuir expression:

$$q = \frac{aC}{1 + bC} \tag{1}$$

the Scatchard plot is a straight line of slope $-b$ and ordinate intercept a. This straight line intersects the abscissa at $q = a/b$, which corresponds to the column saturation capacity (and C infinite). The determination of the best estimates for a and b is thus done by fitting a linear relationship to the data on a Scatchard plot[1]. In most instances, however, the data points are not aligned and the equilibrium isotherm is not accounted for by a Langmuir equation.

The simplest model that can account for the type of data obtained in this work assumes that there are two types of non-cooperative independent adsorption sites[1]. In such a case, the amount adsorbed is the sum of the amounts adsorbed on each of the two types of sites:

$$q = \frac{a_1 C}{1 + b_1 C} + \frac{a_2 C}{1 + b_2 C} \tag{2}$$

In this case, the Scatchard plot is curved, with a downward convexity. The coefficients of the isotherm can be derived from the characteristics of the plot.

It should be emphasized here that the theoretical conditions for the validity of eqn. 1 are very retrictive (*e.g.*, ideal solution and adsorbed layer, no adsorbate–adsorbate interactions). We should be more surprised to see that eqn. 1 accounts well for many sets of adsorption data than to observe failures. There are many sources of failure of this equation. A heterogeneous surface covered with two different types of sites, a model leading to the bi-Langmuir isotherm (eqn. 2), is not the only possible reason. Obviously, a heterogeneous surface is covered by patches which exhibit a certain distribution of surface energy. The resulting isotherm is very complex and cannot be expressed simply. The relative success of eqn. 2 reflects more our ability to fit experimental data to a four-parameter equation of this type than its soundness as a model.

The molecular interactions of a charged protein with an ion exchanger exhibit many possibilities of complex retention mechanisms involving combinations of ionic interactions between the protein (which may carry several charges) with one or several ionic groups bonded to the surface of the ion exchanger, and/or hydrophobic interactions between the neutral patches on the protein surface and the organic groups at the surface of the exchanger. The ion-exchange mechanism alone becomes complex when the eluite carries several distant charges. Regnier and co-workers[11–13] have derived relationships valid at low sample concentrations. Eble *et al.*[14] have shown that, at high concentrations, the equilibrium isotherm resembles a two-site distribution. Finally, at high concentrations, protein–protein interactions may further complicate the phenomenon.

Therefore, the successful use of either eqn. 1 or 2 to account for experimental data does not mean that the retention mechanism is simple or that it proceeds after one or two well defined and identifiable steps.

EXPERIMENTAL

Materials

TSK-DEAE 5PW samples (average particle size *ca.* 10 μm) were a gift from Y. Kato (Toyo Soda, Tonda, Japan). Chicken albumin (denoted throughout the paper as albumin) and conalbumin were purchased from Sigma (St. Louis, MO, U.S.A.). All solvents were of high-performance liquid chromatographic (HPLC) grade from American Scientific Products (McGaw Park, IL, U.S.A.). Reagents were of analytical-reagent grade from Mallinckrodt (Paris, KY, U.S.A.). All these chemicals were used as received, without further purification. The purity of the proteins used was not tested. However, no spurious peak or step appeared during any experiment which could have indicated the presence of impurities.

Columns

Microbore columns (50 mm × 1 mm I.D.) were slurry packed with a suspension of TSK-DEAE 5PW in water under a pressure lower than 800 p.s.i. Under higher pressures the polymer bead particles are destroyed and the efficiency of the column obtained and its permeability may become very low. The columns must be also operated under low or moderate pressures. According to the manufacturer's

recommendations for the use of TSK gel columns, the maximum mobile phase flow-rate for a 7.5 mm I.D. column should be 1.20 ml/min, which corresponds to a flow-rate of 21 μl/min through a 1 mm I.D. column, as used in this work. Probably because of the narrower diameter, higher velocities could be used without damage, and columns were operated at flow-rates between 20 and 50 μl/min. Above this flow-rate, the packing material may be crushed and the column performance severely affected.

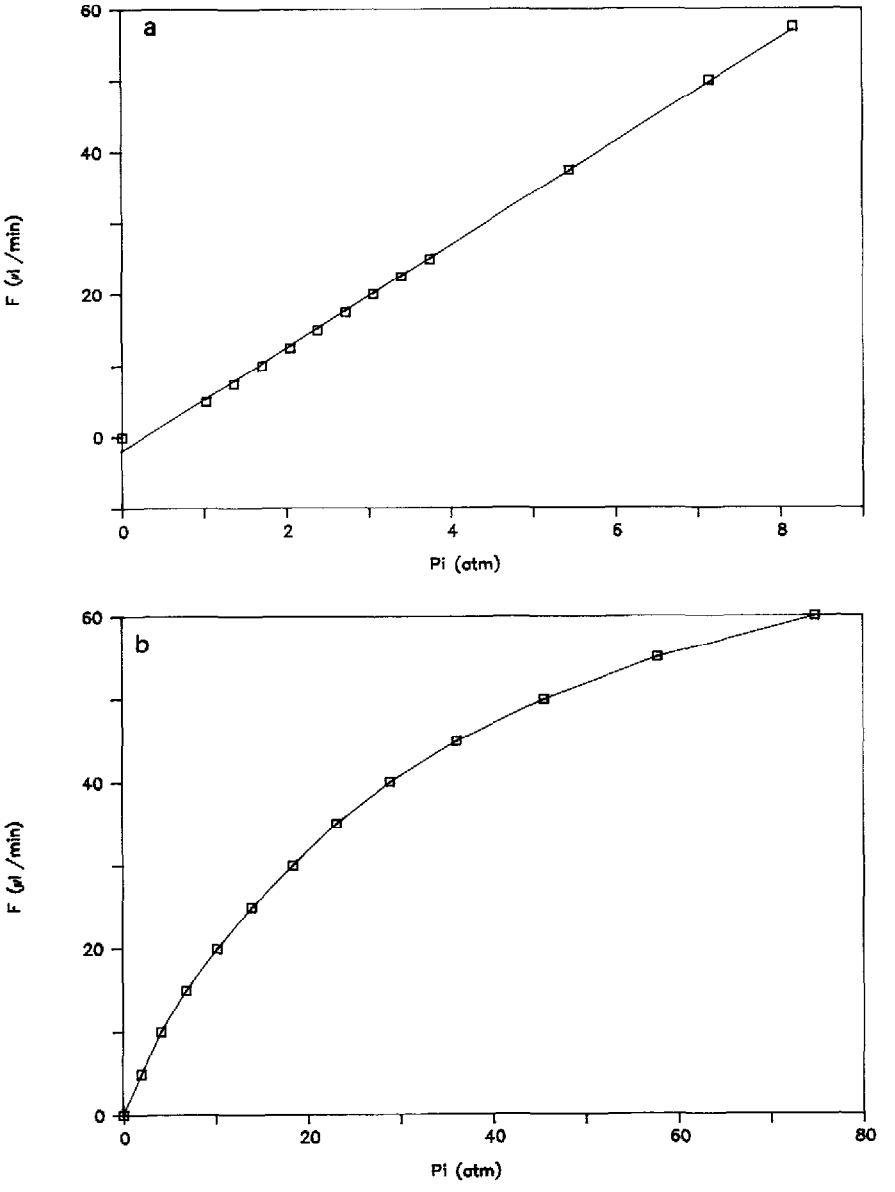

Fig. 1. (a) Plot of mobile phase flow-rate (F) *versus* column inlet pressure (P_i). (b) The same, over a wider pressure range for a poorly packed column.

Fig. 1a shows a plot of flow-rate *versus* inlet pressure for one of our typical columns. The relationship is linear in the pressure range used. Fig. 1b shows that, over a wider pressure range, the flow-rate of a poorly packed column does not increase linearly with increasing pressure, but more slowly. Provided that the pressure remains moderate, the phenomenon is reversible.

Equipment

The isotherm measurements were carried out with a miniaturized liquid chromatograph of conventional design, similar to that described previously[9]. A flow cell of very small volume was used with a Kratos (Ramsey, NJ, U.S.A.) 757 Spectroflow UV detector. All tubings and connections were kept as small as possible.

Procedures

Isotherm measurements were conducted using the classical frontal analysis method[15]. The measurement procedure was identical with that used previously[16]. The aqueous buffer solutions used contained Tris–acetate, adjusted with acetic acid to the required pH. Solutions were stored in a refrigerator and used after filtration on a 0.2-μm nylon 66 membrane filter from Gelman (Ann Arbor, MT, U.S.A.).

When the determination of an isotherm has been carried out under a certain set of experimental conditions, the column is thoroughly washed with a concentrated solution (8 *M*) of urea before any further experiments are carried out. This permits the complete elimination of all traces of strongly adsorbed protein and supplies a clean adsorbent surface for further investigations. Hence all the curves described in this paper are comparable. The need to apply this cleaning procedure carefully demonstrates, however, that a certain, albeit small, amount of protein is quasi-irreversibly adsorbed by the surface, thus pointing to a probable adsorption hysteresis[17].

Calculations

After having determined which Scatchard plots were curved, the values of the coefficients of the bi-Langmuir equations were calculated for those instances. The two asymptotes of these hyperbolic plots were linearly regressed to give the best initial guesses for the four coefficients, a_1, a_2, b_1 and b_2. Those initial values were entered into a simple simplex optimization program, together with the experimental isotherm data, in order to find the best fit. The program iterates until the sum-of-squares error between the experimental and calculated isotherms is minimized.

RESULTS AND DISCUSSION

In all the isotherm determinations, the breakthrough fronts recorded were very steep, showing that the corresponding isotherms are convex upward, *i.e.*, the amount of the studied protein sorbed by the gel at equilibrium increases less rapidly than its concentration in the mobile phase. As the isotherms were determined by frontal analysis, they are adsorption isotherms; no attempt was made to investigate the desorption isotherms, the desorption kinetics or the degree of hysteresis involved in the equilibrium studied[17]. The necessity to clean the column with urea solution after each determination in order to obtain consistent results indicates that such a phenomenon takes place to some extent.

Adsorption isotherms of albumin and conalbumin

Fig. 2a shows the adsorption isotherm determined for albumin and conalbumin with a 50 m*M* Tris–acetate buffer solution at pH 8.6. Both isotherms have a convex upward shape, with a steep initial slope. This isotherm shape is characteristic of macromolecular adsorption. The isotherm for conalbumin is reasonably well fitted by the simple Langmuir isotherm equation, as illustrated by the Scatchard plot[18] shown in Fig. 2b. In contrast, the adsorption isotherm of albumin is poorly fitted by a Langmuir equation. First, the initial slope is extremely steep, much steeper than with conalbumin. At the lowest mobile phase concentration at which a measurement could be carried out, the adsorbed amount is nearly 80% of the column saturation capacity, instead of 25% for conalbumin (Fig. 2a). This steep initial rise is followed by a rapid saturation. A constant amount is adsorbed at mobile phase concentrations above 4.0 mg/ml. The Scatchard plot, shown in Fig. 2c, exhibits the characteristic hyperbolic shape. Such an adsorption behavior cannot be accounted for by a simple mechanism.

Influence of the mobile phase flow-rate on the isotherm

By definition of an equilibrium property, the equilibrium isotherm should be independent of the mobile phase flow-rate during the measurements. Because of the very low molecular diffusion coefficients of proteins in aqueous solutions, however, and because of the possible steric hindrance to their radial mass transfer by diffusion across the porous particles of stationary phase, it is necessary to determine the range of flow-rates within which equilibrium determinations can safely be made.

The influence of the mobile phase flow-rate on the equilibrium isotherm was studied by carrying out isotherm determinations with the same mobile phase under increasing flow-rates. Fig. 3 shows the adsorption isotherm of albumin determined at flow-rates of 25, 50 and 75 μl/min. The data are reproducible, even at the highest

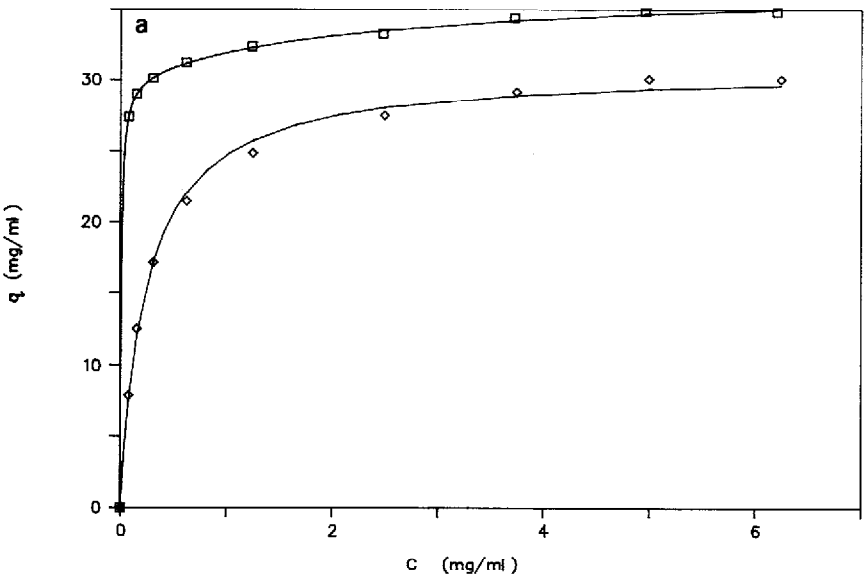

Fig. 2.

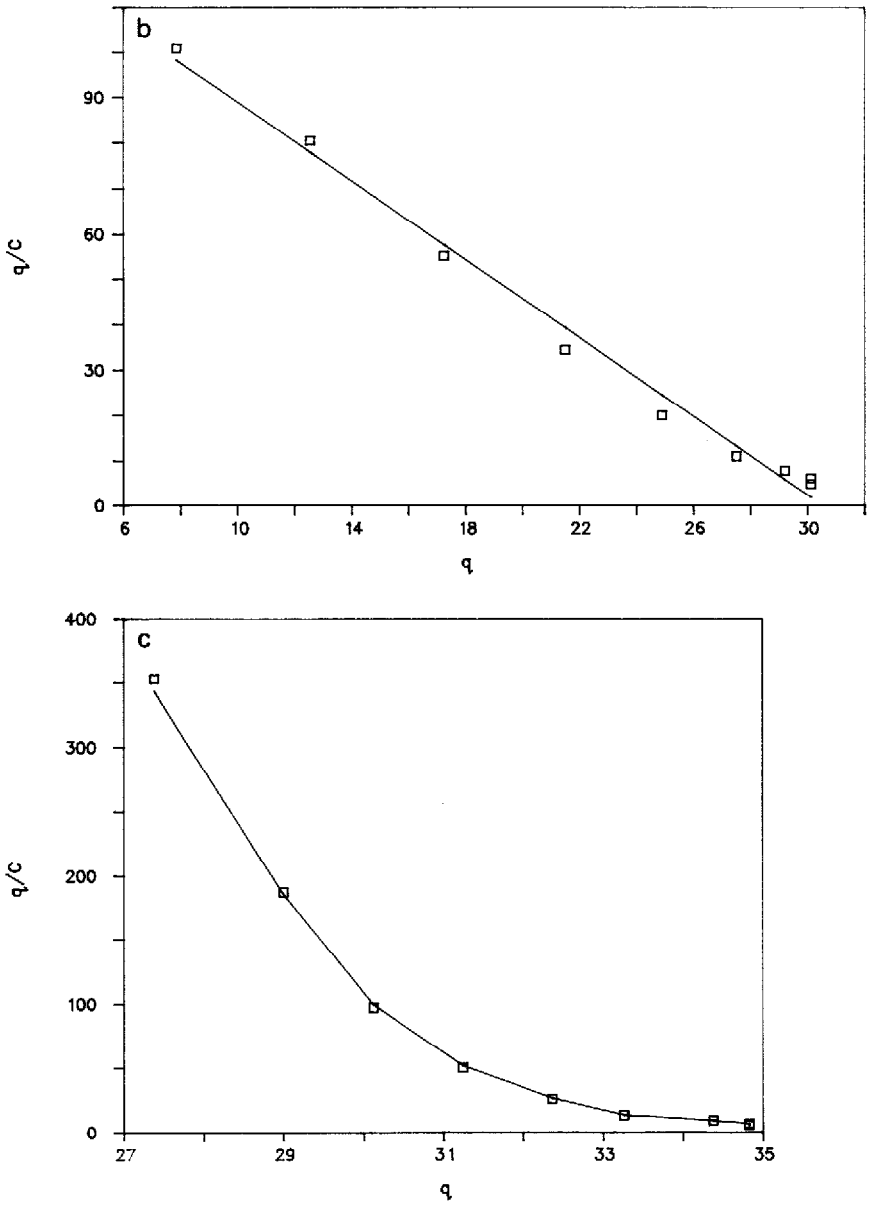

Fig. 2. Experimental data on the distribution equilibrium of chicken albumin and conalbumin between TSK-DEAE 5PW gel and the mobile phase [aqueous solution of Tris–acetate buffer (50 m*M*) at pH 8.6]. (a) Adsorption isotherms of chicken albumin (□) and conalbumin (◇). Plot of concentration in the stationary phase (q) *versus* concentration in the mobile phase at equilibrium (C). Experimental points and best curve obtained by a least-squares fitting of these data points to the Langmuir equation (conalbumin, *cf.*, Fig. 2b) or eqn. 2 (albumin, *cf.*, Fig. 2c). (b) Scatchard plot for the conalbumin data, showing a linear dependence of q/C on q. (c) Scatchard plot for the albumin data, showing a strongly curved plot, with downward convexity (non-cooperative binding).

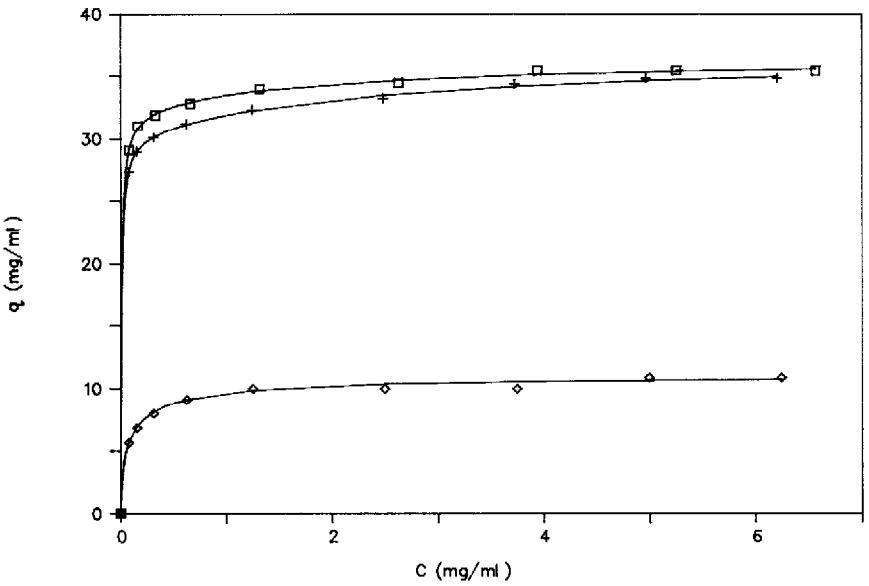

Fig. 3. Influence of mobile phase flow-rate on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot. Flow-rate: □ = 25; + = 50; ◇ = 75 μl/min.

flow-rate used. There is no real significant difference between the results obtained at 25 and 50 μl/min or at lower flow-rates. It is clear, however, that isotherm determinations carried out at flow-rates above 50 μl/min are meaningless, as the system is not equilibrated. The amount of albumin adsorbed decreases dramatically when the flow-rate increases above 50 μl/min. This latter flow-rate corresponds to a linear velocity of 0.13 cm/s and a reduced velocity for albumin of the order of 100–150. Although a 0.13 cm/s velocity may seem unexceptional for conventional HPLC experiments, the reduced velocity is fairly high for this kind of determination, whether or not the packing material suffers from excessive crushing. This illustrates that HPLC of proteins should always be carried out at velocities much lower than that of conventional organics[19].

All further measurements were carried out at a flow-rate of 50 μl/min.

Scatchard plots and representation of the isotherms

The Scatchard plots for Fig. 2 demonstrate that under the corresponding experimental conditions conalbumin exhibits non-cooperative binding to the weak ion exchanger used as the stationary phase, whereas chicken albumin exhibits either negatively cooperative binding or, more probably, adsorption on two different types of interaction sites[17]. Accordingly, Scatchard plots were made for each set of adsorption data collected. In all instances where the plots were not linear, they exhibited the same curvature, with a downward convexity. An iteration program[20] permits the determination of the best values of the slopes and ordinates of the two asymptotes of these plots (Fig. 2c).

TABLE I

ADSORPTION ISOTHERMS OF ALBUMIN

A_1/B_1 and A_2/B_2 are the column saturation capacities for the two kinds of sites.

Variable	*Value*	A_1	B_1	A_1/B_1	A_2	B_2	A_2/B_2	$\Sigma A/B$
Temperature (°C)	15	3248	94.9	36.1	4.14	0.854	4.8	40.9
	25	3472	115	30.2	2.85	0.429	6.6	36.8
	35	5156	215	23.9	7.83	1.168	6.7	30.6
Flow-rate (l/min)	25	3568	111	32.2	2.64	0.609	4.3	36.5
	50	3472	115	30.2	2.85	0.429	6.6	36.8
	75	290	50.00	5.8	16.30	3.19	5.1	10.9
Sodium acetate (m*M*)	0	3472	115	30.2	2.85	0.429	6.6	36.8
	25	331	18.8	17.6	4.101	0.517	7.9	25.5
	50	119	18.0	6.6	6.71	0.868	7.7	14.3
pH	7.6	9.6	9.7	1.0	2.53	0.208	12.1	13.1
	8.6	331	18.8	17.6	4.10	0.517	7.9	25.5
	9.6	105	21.2	5.0	2.14	0.333	6.4	11.4

The data are reported in Tables I (albumin) and II (conalbumin). The values obtained for the coefficients a_1, a_2, b_1 and b_2 are not very accurate, because of the experimental errors and of the compensation between the two terms of eqn. 2. The reproducibility for the column saturation capacity of the first type of sites is not much better than *ca.* 10% (see the data for 25 and 50 μl/min). The reproducibility for the column saturation capacity of the second type of sites is poor, of the order of 40%, mostly because adsorption data cannot be measured accurately at low concentrations. For conalbumin, most isotherms were well accounted for by a simple Langmuir model and in these instances only one set of coefficients is reported. In two instances (*e.g.* pH = 7.6), however, corresponding to very low column saturation capacities, the values of the coefficients calculated by the program had no physical meaning. They are not reported, but the estimated column saturation capacity is given in the last column of the Table II.

In Figs. 4–8, the solid lines represent the isotherms calculated from the coefficients in Tables I and II.

TABLE II

ADSORPTION ISOTHERMS OF CONALBUMIN

Symbols as in Table I.

Variable	*Value*	A_1	B_1	A_1/B_1	A_2	B_2	A_2/B_2	$\Sigma A/B$
Sodium acetate (m*M*)	0	125	4.05	30.8				30.8
	25	36.7	2.77	13.2				13.2
	50	2.0	0.42	4.8				4.8
pH	7.6	–	–	–	–	–	–	2.5
	8.6	36.7	2.77	13.2				13.2
	9.6	353	34.0	10.4	4.93	0.77	6.4	16.8

Influence of the ionic strength of the solution

Figs. 4 and 5 show the equilibrium isotherms of conalbumin and albumin, respectively, obtained with mobile phases of increasing ionic strength. Both the initial slope and the column saturation capacity of these basic proteins decrease dramatically with increasing ionic strength of the bulk solution. The range of sodium acetate concentrations investigated was 0–50 m*M*. In this range, the driving force for protein adsorption on the TSK-DEAE 5PW gel remains the electrostatic interactions between the ionized protein molecules and the charge carriers of the weak anion-exchange resin.

The influence of the mobile phase ionic strength on the equilibrium isotherm of proteins is explained by the increasing competition for adsorption between the negative ions in the mobile phase and the negative protein ions. The hydrophobic interactions of the proteins with the organic groups at the surface of the resin still have a negligible influence. It is most probable, however, that at higher ionic strength an opposite trend would take place and adsorption of the proteins would begin to increase with increasing ionic strength, as reported previously[9].

The equilibrium isotherms of conalbumin at all ionic strengths can be well accounted for by the Langmuir equation, as we have already reported. The same is not true for albumin. The initial slope of the isotherm is too steep and the saturation takes place too fast. The Scatchard plots show the same curvature as the plot in Fig. 2c. The isotherm is well accounted for by the sum of two Langmuir isotherms, corresponding to non-cooperative adsorption on two different kinds of sites. It is remarkable (see Table I) that the saturation capacity for the first sites decreases steadily with increasing

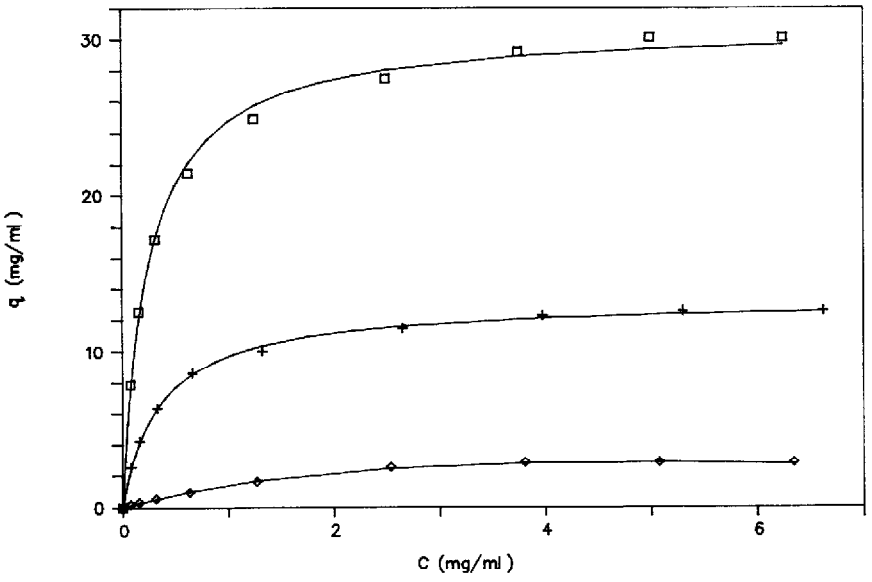

Fig. 4. Influence of mobile phase ionic strength on the equilibrium isotherm of conalbumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot (linear except for 50 m*M* sodium acetate). Additional concentration of sodium acetate buffer: □ = 0; + = 25; ◇ = 50 m*M*.

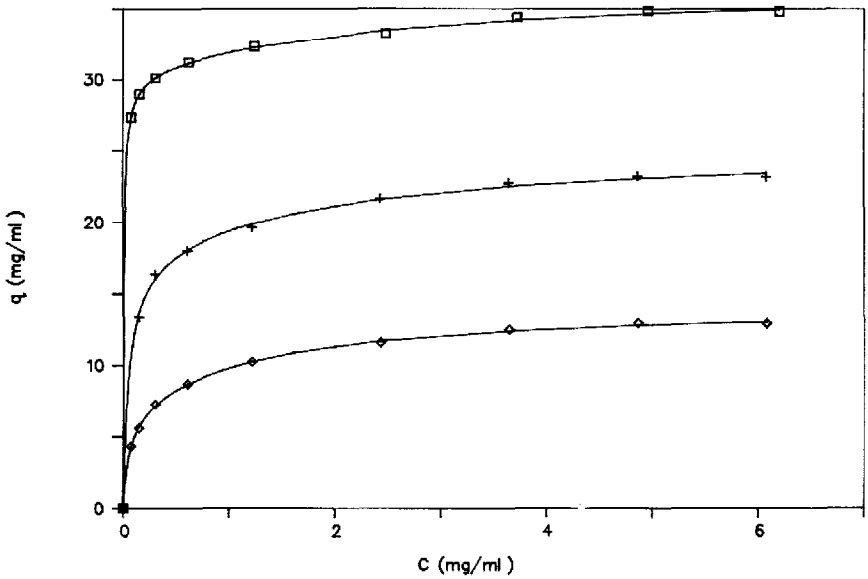

Fig. 5. Influence of mobile phase ionic strength on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot. Additional concentration of sodium acetate buffer: □ = 0; + = 25; ◇ = 50 m*M*.

ionic strength, whereas that of the second sites remain constant, within experimental error. Probably the former sites could be responsible for ionic interactions, whereas the latter would be involved in the hydrophobic interactions.

Influence of the pH of the mobile phase

Figs. 6 and 7 show the equilibrium isotherms of conalbumin and albumin, respectively, at increasing pH of the mobile phase. The ionic strength of the mobile phase was higher in these experiments than in those on the influence of flow-rate and temperature, as 25 m*M* sodium acetate was added to the mobile phase. This explains the difference between the isotherms of the two proteins obtained at pH 8.6 in Figs. 2, 6 and 7.

The amount of conalbumin adsorbed at equilibrium between the two phases increases with increasing pH, from 7.6 to 9.6 (see Fig. 6). The increase in amount sorbed is larger when the pH increases from 7.6 to 8.6 than when it increases from 8.6 to 9.6. The data at pH 7.6 were difficult to measure for conalbumin, partly because the column saturation capacity is small at that pH. The best isotherm obtained by curve fitting has no physical meaning and the coefficients are not reported. The superimposition of several phenomena is probably responsible for the pH dependence of the adsorption behavior of proteins.

The most important of these phenomena are the variation of the relative concentration of the ionized species with increasing pH and the increasing competition for adsorption between the negative protein ions and other negative ions, such as OH^- or AcO^-, whose concentration increases rapidly with increasing solution pH. The first phenomenon dominates in the pH range 7.6–8.6, whereas the concentration of the

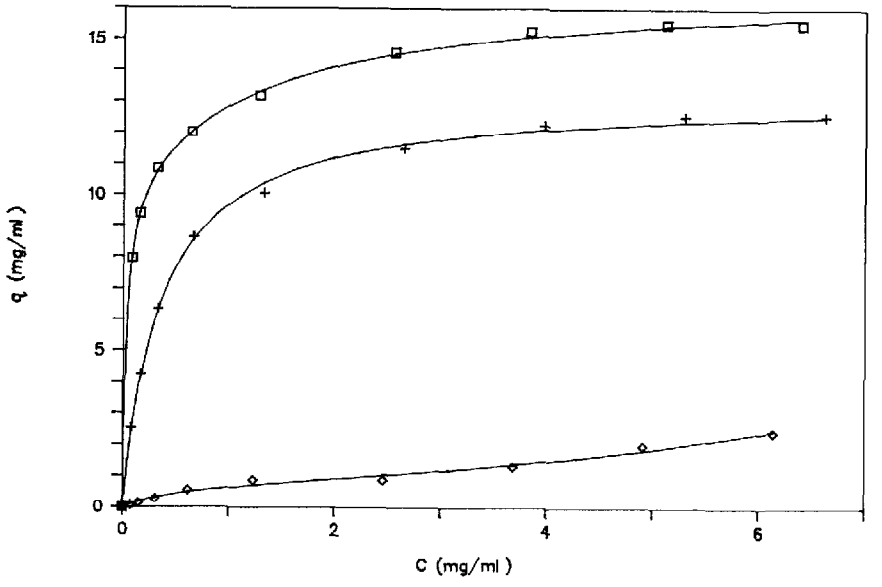

Fig. 6. Influence of mobile phase pH on the equilibrium isotherm of conalbumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot (except pH 8.6, Langmuir isotherm). Mobile phase pH: □ = 9.6; + = 8.6; ◇ = 7.6.

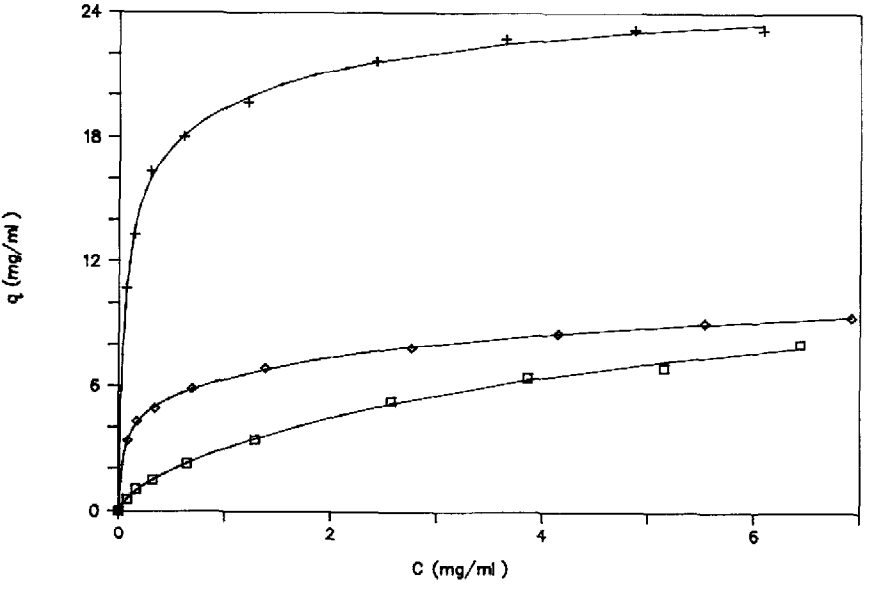

Fig. 7. Influence of mobile phase pH on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2, except the mobile phase also contains 25 m*M* sodium acetate. Solid lines calculated using the best coefficients derived from the Scatchard plot. Mobile phase pH: □ = 7.6; + = 8.6; ◇ = 9.6.

OH^- ions remains low. When the pH is increased from 8.6 to 9.6, however, the concentration of protein ions increases more slowly, and these ions are in increasing competition with the negative ions of the solution, so the amount of protein adsorbed increases only slowly, as observed.

With albumin, the competition between the protein ions and the other negative ions in the solution is strong enough at high pH that the amount of protein adsorbed is actually smaller than at pH 8.6. This decrease is essentially due to the lower column saturation capacity for the sites of the first type, which decreases 3-fold when the pH is raised from 8.6 to 9.6. The column saturation capacity of the sites of the second type decreases sharply when the pH is raised from 7.6 to 8.6, but then remains nearly constant.

Influence of the column temperature

Fig. 8 shows the equilibrium adsorption isotherm determined for albumin at 15, 25 and 35°C. The amount of protein adsorbed at saturation decreases slightly with increasing temperature, by *ca.* 1.5%/°C. The origin slope of the isotherm increases slightly with increasing temperature (see Table I).

The decrease in the column saturation capacity with increasing temperature is essentially due to the decrease in the capacity of the sites of the first type. The effect of a temperature change on the sites of the second type appears to be minimal, within the range of experimental error.

The effect of a change in the column temperature on the amount of protein adsorbed at equilibrium, from a solution at a constant concentration, is much less important than the influence of changes in the mobile phase composition.

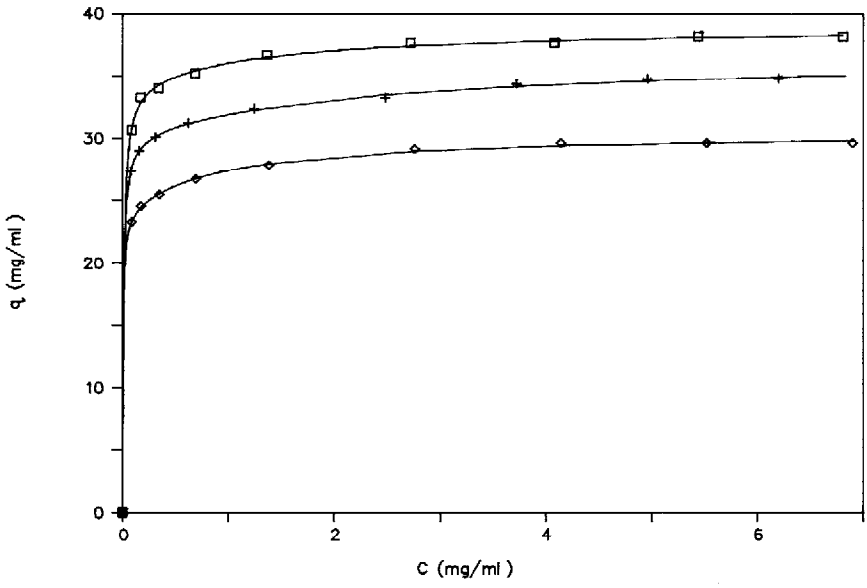

Fig. 8. Influence of the column temperature on the equilibrium isotherm of chicken albumin. Experimental conditions as in Fig. 2. Solid lines calculated using the best coefficients derived from the Scatchard plot. Column temperature: □ = 15; + = 25; ◇ = 35°C.

Adsorption capacity of TSK-DEAE gels

The adsorption capacity of the TSK gel investigated depends heavily on the experimental conditions. At most, it is of the order of 35–38 mg/ml for both proteins. This value is smaller than that obtained in a similar study performed on ion-exchange packing materials based on silica[9]. The difference is mostly explained by the larger average pore size of the polymeric packing particles and its smaller specific surface area.

In most instances, the column saturation capacity for the first type of sites is about 20% of the total column saturation capacity. The sites which are responsible for the highest fraction of the column saturation capacity are also those for which the Gibbs free energy of association is the largest. Adsorption on these sites is also the most sensitive to changes in the electrolytic properties of the mobile phase. The lack of precision prevents a more detailed discussion. The data, however, demonstrate that in most instances the surface of the stationary phase is not homogeneous, which, considering the organic structure of these ion exchangers, is not surprising.

The adsorption data presented here have been used for an investigation of the elution profiles of high concentration zones of albumin and conalbumin on TSK-DEAE 5PW gels under isocratic conditions. A comparison between experimental results and the prediction of the semi-equilibrium model[21] confirmed the slow kinetics of mass transfer observed in this work[10].

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