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EFFECT OF MASS LOADABILITY, PROTEIN CONCENTRATION AND *n*-ALKYL CHAIN LENGTH ON THE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOUR OF BOVINE SERUM ALBUMIN AND BOVINE FOLLICULAR FLUID INHIBIN

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SUMMARY

The chromatographic behaviour of bovine serum albumin and partially purified preparations of the 58 kilodalton form of bovine follicular fluid inhibin has been investigated using two different *n*-alkylsilica stationary phases. In particular, the effects of mass loadability, protein concentration and loading conditions on the relative retention, peak width and recovery of these proteins have been studied over a dynamic range up to 100 mg protein per g packing material per injection. The influence of variable amounts of more abundant contaminating proteins such as bovine serum albumin in crude inhibin preparations on the chromatographic purification of trace quantities of the 58 kilodalton inhibin protein has also been examined. Based on these observations, recommendations are offered for the selection of protein loading conditions with *n*-alkylsilica stationary phases, particularly for the trace recovery and purification of hydrophobic proteins similar to inhibin where self-aggregation, adsorption to glass or plastic surfaces, and aberrant chromatographic behaviour on size exclusion or adsorptive chromatographic supports may occur.

INTRODUCTION

The influence of solute concentration and mobile phase composition on the mass loadability of polypeptides and proteins separated by reversed-phase high-performance liquid chromatography (RP-HPLC) has not been extensively investigated despite theoretical and practical recognition of the relevance of these experimental variables to preparative separations in the overload condition. More commonly, the separation of polypeptides and small proteins on chemically bonded, microparticulate *n*-alkylsilicas has been studied[1,2] under analytical loading conditions where serendipitously chosen chromatographic conditions, assumed to operate within linear regions of adsorption isotherms, have been used. Extension of the experiences gained

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with analytical separation technologies to semi-preparative purification procedures has usually been approached empirically and with variable success. In typical experiments, stainless-steel columns of standard dimensions (5–25 cm × 0.4–0.8 cm I.D.), packed with reversed-phase support materials of particle diameters nominally within the range 5–10 μm and average pore diameters typically between 10–30 nm have been employed. The retention dependencies which most proteins exhibit with such *n*-alkylsilica supports are usually characterised in terms of bimodal relationships between the logarithmic capacity factor (log k') and the mole fraction of the organic solvent modifier, φ[3,4]. Typically, the slopes of the plots of log k' *vs.* φ are large, *i.e.*, S values greater than 25 and this adsorption behaviour is consequently reflected in the ability of a particular protein solute to be eluted only over a very narrow range of φ values under isocratic conditions.

It was realised early in the application of RP-HPLC procedures to protein purification that the total mass loadability for polypeptides or proteins separated on mesoporous *n*-alkylsilica phases could under appropriately chosen conditions be very high, for example, mass loads > 50 mg per g of the *n*-alkylsilica support, corresponding to 100–200 mg protein for a column of standard analytical dimensions, depending on the molecular weight and hydrophobicity of the protein. This value equates with mass loadability factors of 10^3–10^4 above that normally attempted in typical analytical separations. How such high mass loadability conditions influence the recovery of biosolutes in their biologically relevant states and how various conditions such as the loading solvent, pH or flow-rate affect in the overload situation zone bandwidths, relative retention and resolution of a particular solute from contaminants remain poorly documented. Even less information is available on the influence of contaminant proteins, as their relative abundances are changed, on the resolution and recovery of a specified trace component. The present study addresses several of these questions in an investigation on the mass loadability behaviour of bovine serum albumin (BSA) and a partially purified preparation of the 58 kilodalton form of bovine follicular fluid inhibin, a hydrophobic protein with a well known[5,6] propensity to form aggregates which cause purification difficulties. The choice of BSA as a model protein solute was very well suited in this particular case since BSA is a major contaminant protein present in bovine follicular fluid preparations and binds to reversed-phase supports with similar selectivity to that found with bovine inhibin.

EXPERIMENTAL

Equipment

Chromatographic separations were carried out using a gradient HPLC system comprising two Model M6000A pumps, a M660 solvent programmer, a Model 441 UV absorbance detector (254 nm) and a Model 730 data module, all from Waters Assoc. (Milford, MA, U.S.A.), and a Model 202 fraction collector from Gilson (Villiers Le Bel, France).

Chemicals and reagents

Glacial acetic acid (chromagen free) was obtained from Merck (Darmstadt, F.G.R.), trifluoroacetic acid was from Pierce (Rockford, IL, U.S.A.) and BSA (fraction V, > 96% pure) was purchased from Sigma (St. Louis, MO, U.S.A.). Aceto-

nitrile was obtained from Waters Assoc. whilst water was quartz distilled and deionised (Milli-Q, Millipore, Bedford, MA, U.S.A.). The Ultrapore RPSC C_3 and RPSC C_8 columns (7.5 cm × 0.46 cm I.D.) were generously donated by Dr. Nelson Cooke, Beckman Altex (Berkeley, CA, U.S.A.). These *n*-alkylsilica stationary phases have a mean particle diameter of 5 μm and specific surface area (bare silica) of 95 m^2/g with corresponding average pore size (unbonded, surface average) of 33 nm and *n*-alkyl coverages of 4.5 and 3.3 $\mu mol/m^2$, respectively. Partially fractionated inhibin preparations were obtained following gel permeation chromatography on Sephacryl S200 of bovine follicular fluid under neutral and acidic buffer conditions as detailed elsewhere[5,6]. The lyophilised samples were dissolved in 4 *M* acetic acid and centrifuged prior to injection onto the reversed-phase columns. Bovine serum albumin was radioiodinated by a lactoperoxidase procedure based on the method of Thorell and Johansson[7]. The [^{125}I]BSA was purified on a Ultragel AcA54 (LKB, Bromma, Sweden) column equilibrated in 10 m*M* phosphate buffer, pH 7.2 containing 0.14 *M* sodium chloride, 0.1% thiomersol and 0.2% BSA. The specific activity of [^{125}I]BSA was 1.2 μCi/mg. Prior to RP-HPLC, the [^{125}I]BSA preparation was fractionated on an ODS-silica cartridge (C_{18}-Sep-Pak, Waters Assoc.) with 50% acetonitrile in aqueous 0.1% trifluoroacetic acid (TFA) as elution buffer to remove free ^{125}I. The *in vitro* bioassay for bovine inhibin was based on the dose dependent suppression of the follitropin (FSH) cell content of pituitary cells in culture[8]. Lyophilised chromatographic solvent residues and samples were non-toxic in this assay system.

Chromatographic procedures

Bulk eluents were degassed under vacuum following filtration through Millipore (0.4 μm) membranes as described previously[4]. The linear 30-min gradient, used throughout, was developed from 0.1% TFA in water (solvent A) to 50% acetonitrile in solvent A (solvent B). A flow-rate of 1 ml/min was employed and 500-μl fractions collected. All samples were loaded onto the column via the solvent inlet port of the pump delivering solvent A. Following loading of the sample the fractions corresponding to the breakthrough peak were collected for determination of protein mass, counting of radioactivity or *in vitro* bioassay of inhibin activity. An aliquot was taken from each of the eluted fractions and pooled for assessment of column recoveries of protein mass, radioactivity and/or inhibin activity. The activity retained on the column ("irreversibly bound") was determined from the difference in the sample loaded and recovered in the breakthrough peak and the eluted peaks following a single gradient elution experiment. With the BSA experiments, approximately 65 000 dpm (0.03 μCi) of [^{125}I]BSA was added to each injected BSA sample.

RESULTS AND DISCUSSION

Theoretical considerations

Irrespective of the nature of the adsorption isotherm, as the loading of a sample to a column packed with a microparticulate reversed-phase adsorbent exceeds the saturation limit, then resolution will decrease and approach a minimum value. Resolution, even under these overload conditions, is still determined by the average capacity factors (k') for adjacent components, the average selectivity factors (α) for the particular phase system and the average efficiency (usually expressed as the average

plate number, N) generated by the system such that for two proteins, i and j, the resolution R_{ji} can be given by

$$R_{ji} = \frac{(\alpha - 1)k'}{4(1 + k')} N^{0.5} \quad (1)$$

In order to achieve adequate resolution in preparative separations, large differences in α factors are desirable. Maximum control should thus be exercised over changes in k' and N which may be induced by the overloading conditions. Typically, when column saturation occurs a breakthrough peak is observed, the magnitude of which is determined by the composite interplay of diffusion and adsorption kinetic phenomena. Depending on the flow-rate, and other column residence time effects, the resolution of a column can thus vary significantly as a function of dynamic loadability and solute concentration. Under defined linear elution conditions of fixed flow-rate with a constant value for the mass of solute injected no significant change in column resolution is anticipated for solutes injected, at slightly different concentrations provided the stationary phase performance or the injection volume do not vary in a gross manner throughout the experiments. Since severe dependencies between relative retention and eluent composition are frequently found with proteins chromatographed on *n*-alkylsilicas, dynamic mass loadings up to 0.9 times the statically measured saturation limit in reversed-phase systems has been found feasible[2] without necessarily leading to significant changes in retention. However the apparent reduced plate height ($h = L/Nd_p$; where d_p is the particle diameter), an accurate measure of the overall efficiency of the chromatographic system, is known from theoretical and experimental studies with low molecular weight solutes to be strongly influenced by the injected sample size, the solute concentration and the eluent composition.

The effect of mass loadability on changes in relative retention and peak efficiency for Langmuir type adsorption systems can be given by[9–11]

$$\Delta k = 2Mk_\infty^2/\varepsilon W_C(1 + k_\infty) \quad (2)$$

and

$$\Delta N = N_\infty[1 - \beta M(LF)^2/M_S] \quad (3)$$

where Δk, ΔN are the change in relative retention and plate number, respectively from the most favourable analytical loading situation corresponding to k_∞ and N_∞; M is the weight of solute injected; M_S is the weight of solute to saturate the column; W_C is the weight of the stationary phase in the column; LF is the loading factor which is proportional to $[M_S/M_{max}^{10\%}]^{1/2}$ where $M_{max}^{10\%}$ is the mass load required to cause a 5% increase in the observed standard deviation; L is the column length; and β and ε are solute dependent coefficients. Because of the nature of the inverse proportionality which exists between the average plate number, N, and the peak width, w_v, of an eluted zone, *i.e.* $N = (V_r/w_v)^2$, a mass load which corresponds to a peak width increase of 10% will result at a constant k' value in a 21% decrease in N. If k decreases as well during such a chromatographic experiment then the value of N will be reduced even further.

As part of our studies on the purification of the protein hormone, inhibin, the chromatographic behaviour of crude bovine follicular fluid inhibin was assessed with a variety of *n*-alkylsilicas and different elution conditions. It was evident from these preliminary experiments that complex relationships existed between the mass injected, the injection buffer, the injection volume, the column capacity and the solute recovery. As a consequence of these investigations, additional experiments were designed to evaluate the column loadability of bovine serum albumin, the major protein contaminant in bovine follicular fluid inhibin preparations, and crude bovine inhibin using standard (7.5 × 0.46 cm I.D.) columns packed with Ultrapore RPSC C_3 and C_8 stationary phases. The experimental design involved three investigations in order to characterise these effects more quantitatively. In the first study the effects on retention and column efficiency caused by varying the injected mass of BSA over the range 50 μg to 100 mg loaded per injection on the chromatographic performance of the Ultrapore RPSC C_3 stationary phase was examined. In particular, we were interested to determine the effect of loading BSA at various concentrations in different solvents such as 1, 5, 10 or 15 mg BSA in (a) 4 ml 0.1% TFA, (b) 100 ml 0.1% TFA, (c) 100 ml 1 *M* acetic acid, (d) 4 ml 4 *M* acetic acid and (e) 100 ml 4 *M* acetic acid. The 100 ml loading volume experiments were chosen since they correspond to the typical trace recovery situation which arose as an integral part of the purification studies with inhibin and are representative of the trace recovery of many proteins present in low abundance as partially fractionated samples. In the second study, the effects of varying the mass loading conditions with an inhibin containing bovine follicular fluid preparation on the chromatographic performance of the Ultrapore RPSC C_3 stationary phase was evaluated using the inhibin preparation at various concentrations (1, 5 and 10 mg in 100 ml 4 *M* acetic acid). Finally, a comparison of the performance of two related Ultrapore stationary phases (C_3 and C_8) was carried out using again the inhibin preparation at a concentration of 5 mg/100 ml 4 *M* acetic acid.

Bovine serum albumin loadability study

Since their introduction for the separation and analysis of peptides and proteins in the mid-1970s, reversed-phase silicas, notably the dimethylpropyl, dimethyloctyl and dimethyloctadecyl bonded phases, have been very widely employed for trace enrichment and recovery of biomacromolecules, typically loaded at the 1–100 μg level. Despite this wide usage very little attention has been paid to the influence of the loading solvent or the solute concentration on subsequent resolution. The data obtained in the present study from the bovine serum albumin loadability experiments thus provide useful insights into the influence of these parameters. In particular, we were interested to compare the effect of 0.1% TFA, 1 *M* and 4 *M* acetic acid as loading solvents on the column capacity as the BSA mass injected was increased over the range 10 μg to 15 mg at a constant flow-rate of 1 ml/min.

The results of these experiments summarised in Figs. 1–4 illustrate the effects of changing protein mass and loading solvent on the overall column capacity and performance as assessed from the magnitude of the breakthrough peak, the mass of protein eluted by a single gradient run and the change in the peak width of the eluted zone.

Several observations can be made about the data shown in these figures. First,

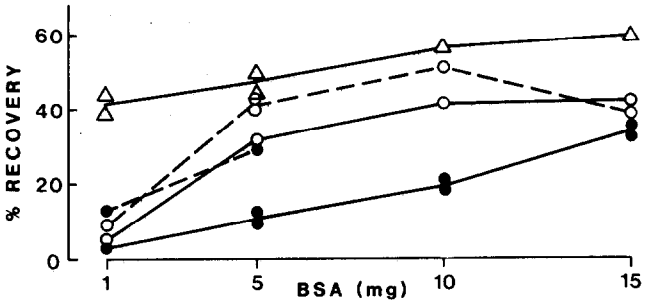

Fig. 1. Plot of percentage of BSA recovered in the breakthrough peak as a function of mass loaded over the range 1–15 mg BSA. The code for the loading condition is: ●—● 0.1% TFA (4 ml), ●---● 4 *M* acetic acid (4 ml), ○—○ 0.1% TFA 100 ml, ○---○ 4 *M* acetic acid (100 ml), △—△ 1 *M* acetic acid (100 ml). The stationary phase was ultrapore RPSC-3, packed into a 7.5 × 0.46 cm I.D. column.

the amount of BSA which appears in the breakthrough peak (Fig. 1) at a fixed flow-rate is dependent on the nature and concentration of the organic acid modifier employed to dissolve the protein sample. Irrespective of the size of the injection volume, the use of 0.1% TFA as the solvent for dissolution of the protein appears to be more effective in minimising the amount of BSA in the breakthrough peak, particularly in the 10–15 mg mass loading range, whilst 1 *M* acetic acid was less effective than either 0.1% TFA or 4 *M* acetic acid at comparable loading volumes. Secondly, as is evident from the 4 ml and the 100 ml loading experiments proportionally less protein was found in the breakthrough peak when more concentrated protein solutions were injected in 0.1% TFA, rather than in 1 *M* or 4 *M* acetic acid. Thirdly, the mass of protein not bound to the reversed-phase support increased progressively over the 1–15 mg mass loaded range irrespective of the loading solvent or volume. Fourthly, the amount of BSA which eluted following a particular 4 ml or 100 ml loading experiment with 0.1% TFA as solvent was essentially constant. As anticipated, the percentage of the loaded BSA which could be retained on the column

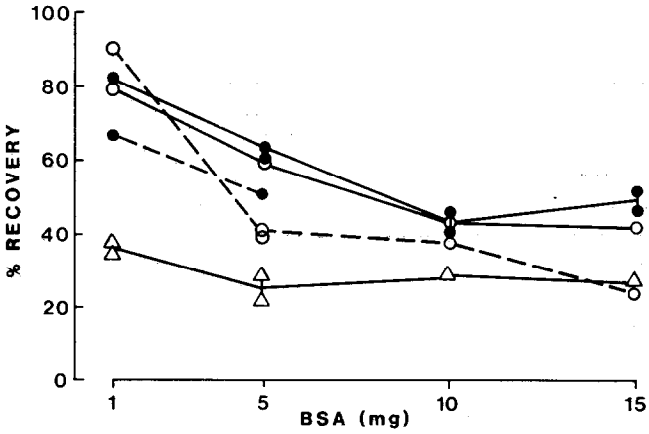

Fig. 2. Plot of the percentage of BSA recovered in the eluted peak as a function of mass loaded over the range 1–15 mg BSA. The code for the loading condition is given in the legend to Fig. 1.

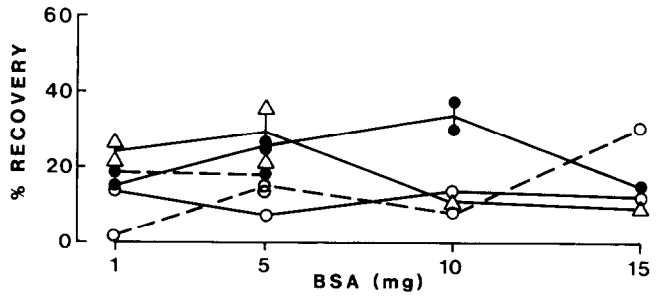

Fig. 3. Plot of the percentage of BSA not recovered in the breakthrough or eluted peak following a single gradient elution as a function of mass loaded over the range 1–15 mg BSA. The code for the loading condition is given in the legend to Fig. 1.

and subsequently eluted under linear gradient conditions was highest at the low mass loadings (Fig. 2). The smaller amount of BSA, which proportionally could be eluted after the 100-ml 1 *M* acetic acid loading experiment, reflects the decreased amount of the protein which could be initially adsorbed. Fifthly, the amount of BSA not eluted by the initial gradient elution run ("irreversibly bound") was dependent on the loading solvent and varied between 5 and 35% for the different loading conditions (Fig. 3). This behaviour underlies the so called "ghost peak phenomena" often seen in the RP-HPLC of polypeptides or proteins when consecutive blank gradients are run immediately following a protein fractionation and further amounts of the proteins can be desorbed. Sixthly, for all loading conditions, the observed retention time for the eluted BSA peak showed a small but significant decrease as the mass load was increased. [t_g = 24.43 ± 0.07 min at a loading of 1 mg BSA to t_g = 24.19 ± 0.01 min ($P < 0.01$) at a loading of 15 mg BSA in 0.1% TFA.] Seventhly, the peak width of the eluted BSA zone responded significantly to the loading condition. For example, the peak width of the eluted BSA zone increased by *ca.* 100% over the mass loading range 1–15 mg BSA when the sample was injected in 0.1% TFA irrespective of whether the injection volume was 4 ml or 100 ml. Finally, major differences in the

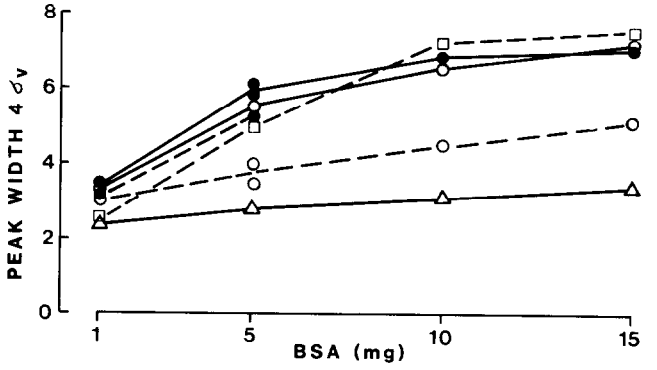

Fig. 4. Plot of the change in peakwidth in volume units of the eluted BSA peak as a function of mass loaded over the range 1–15 mg BSA. The code for the loading condition is given in the legend to Fig. 1.

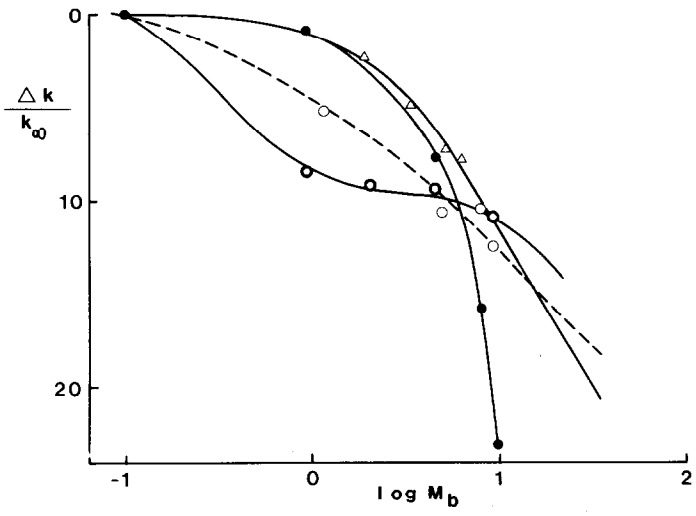

Fig. 5. Plot of the percentage change in relative capacity factor, Δk, (*i.e.* $\Delta k/k_\infty \cdot 100$) as a function of the mass bound for the eluted BSA peak. The code for the loading condition is given in the legend to Fig. 1.

eluted peak width occurred depending on whether the sample was injected in 0.1% TFA, 1 M or 4 M acetic acid.

Collectively the influence of these various loading parameters can be further assessed by replotting the data in terms of incremental changes in relative retention Δk and bandwidth ΔN as functions of the mass of protein adsorbed (Figs. 5 and 6). If it is assumed that the adsorption follows a Langmuir type isotherm, *i.e.* if the change in k' and N can be represented by eqns. 2 and 3, then the mass load which

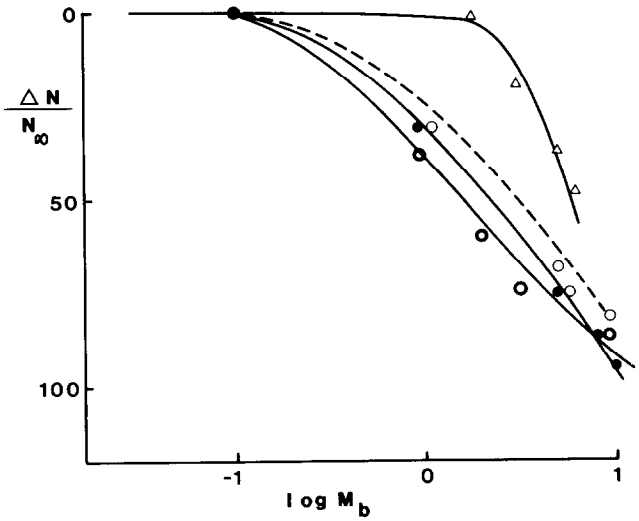

Fig. 6. Plot of the percentage change in theoretical plates, ΔN, (*i.e.* $\Delta N/N_\infty \cdot 100$) as a function of the mass bound for the eluted BSA peak. The code for the loading condition is given in the legend to Fig. 1.

would result in a significant change in relative retention or peak efficiency such as a 10% change can be calculated from eqn. 2. Using the data obtained from the k' *versus* M experiments and eqn. 2, the calculated mass load of BSA of $M \simeq 124$ mg corresponds, with the columns employed in the present study, to a decrease in apparent k' of $k' = 2$ from an initial analytical value of $k' = 22.6$. Experimental values corresponding to this k' change due to mass loadings of *ca.* 140–180 mg were typically observed with BSA and other proteins of similar retention with this reversed-phase packed into columns of the same configuration. This mass loadability value is smaller than the value calculated on the basis that the BSA protein generates a protein monolayer coverage of the stationary phase with free access to all the bonded surface area. The monolayer value for the above column configuration and packing materials for BSA is *ca.* 440 mg assuming that the protein adopts a uniform globular shape with an average hydrodynamic diameter of 7.0 nm[11] and uniformly stacks so that each molecule effectively occupies 49 nm^2 of surface area on the column support material. The four-fold differences between the predicted and observed mass loadability are consistent with our previous demonstration[4] that even with wide pore *n*-alkylsilicas significant percentages of the stationary phase surface area are not accessible to globular protein depending on their size, hydrophobicity, shape and charge distribution. This effect presumably arises as a consequence of the tortuous contours of the mesoporous surface which prevent free access to all intraparticulate regions of the hydrophobic porous environment.

In the present case if the average Stokes diameter for BSA is to be taken to be 7.0 nm with a *ca.* 33 nm pore diameter mesoporous *n*-alkylsilica, the hindrance factor, as derived from the Renkin equation[12], for free access to all pore chambers, is *ca.* 0.36. Since the loadability depends on both the diffusion kinetics and the adsorption kinetics, it follows that the amounts of BSA in the breakthrough peak and the retained peak will vary significantly as the hindrance factor diverges from unity and, in the extreme case of total exclusion from the pores, goes to zero. Over the range of restricted diffusion of 0.1–0.4, solute loadability is expected to show pronounced dependencies on flow-rate and will be typified by a prominent split peak phenomenon (*i.e.* a non-bound zone and a bound or retarded zone) such as that seen in the present study. As is evident from the data, even at 1 ml/min, the magnitude of this split peak effect can be considerable when protein samples are injected as very dilute solutions in large volumes. Although flow-rates smaller than 1 ml/min should reduce the diffusion-limited peak breakthrough phenomenon, from practical considerations such low flow-rates are of marginal value particularly when large volumes (such as 100 ml) of dilute protein samples are to be loaded onto a reversed-phase column. Besides the greatly increased time of loading which would be required at, say, 0.1 ml/min rather than 1.5 ml/min, other effects mediated by dwell phenomena[13] could lead to time-dependent protein unfolding and denaturation.

The plots of $\Delta k/k_\infty$ *vs.* log (mass bound) and $\Delta N/N_\infty$ *vs.* log (mass bound) reveal several important facets of protein adsorption phenomena with *n*-alkylsilicas and the propensity of these biomacromolecules to engage in secondary equilibrium phenomena such as conformational unfolding at hydrocarbonaceous surfaces. As is evident from Figs. 5 and 6 the change in k' for BSA is similar when the sample is injected as a 4-ml 0.1% TFA aliquot or a 100-ml 1 *M* acetic acid aliquot. However, the change in ΔN for BSA is significantly different for these two loading conditions.

Several possibilities must be considered to account for these observations. Firstly, the adsorption of the protein in different loading solvents could involve the participation of several binding sites of different affinities due to the heterogeneity of the stationary phase surface. Secondly, the different solvents may affect the structural hierarchy of the protein in the loading solution so that the protein is in different conformational forms when it binds to the non-polar stationary phase. Thirdly, the different loading conditions may affect the solvated pore structure of the stationary phase to such an extent that the protein accessibility is different under the different loading conditions. The latter two possibilities require additional spectroscopic/circular dichroism measurements or porosimetric/permeability measurements before their relative importance can be ascertained. However, the first possibility can be evaluated directly from the chromatographic data and mass recovery measurements if it is assumed that the "near" equilibrium criteria applies such that the chromatographic data can be evaluated in terms of Scatchard analysis, *i.e.* from the (bound/free *vs.* bound) plots. Results of this analysis are shown in Fig. 7. From these Scatchard plots it is clearly evident that at least two phenomena possibly associated with at least two populations of binding sites are involved in the protein adsorption to the *n*-alkylsilica phase, at least for BSA. One phenomenon attributable to a population, of high affinity but low capacity sites is predominantly associated with the binding of BSA at low mass levels and is particularly noticeable in the more acidic 0.1% TFA conditions whilst a second (and possibly additional) class(es) of phenomenon associated with binding sites of lower affinity but much greater capacity becoming evident at higher mass levels. By loading the protein in 1 *M* acetic acid, or at higher mass loadings with the other loading solvents, the adsorption is dominated by this

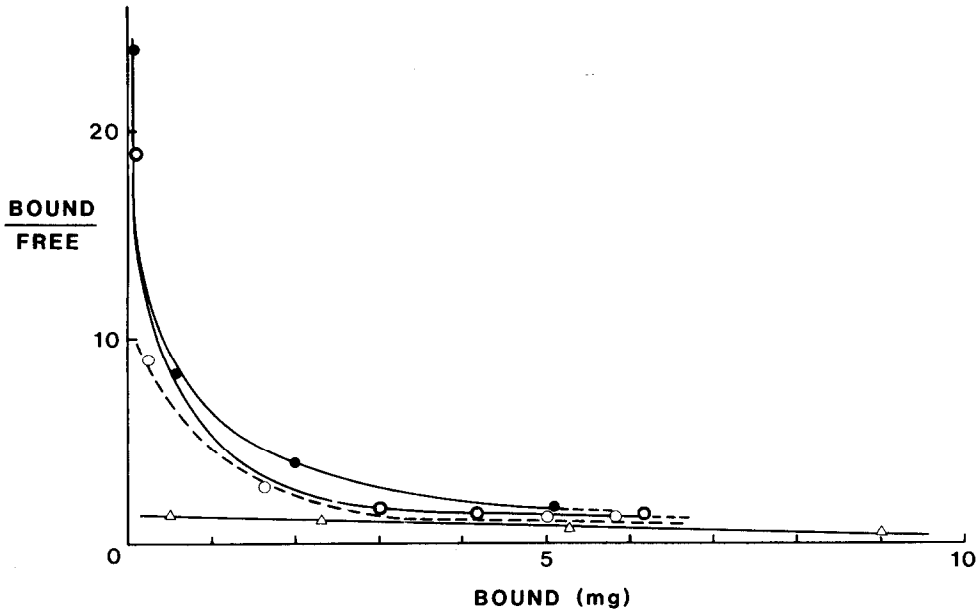

Fig. 7. Plot of mass bound/mass free *vs.* mass bound for the BSA experiments shown in Figs. 1–6. The code for the loading condition is given in the legend to Fig. 1.

second class of sites. Recalculation of the above data to allow derivation of association constants and the relative distribution of the various binding sites will be reported subsequently. However presentation of the adsorption data in terms of Scatchard plots graphically illustrates the basis for the experimental observation that less BSA can be bound to (and subsequently eluted from) the reversed-phase support with the 1 *M* acetic acid condition over the lower mass loading ranges than with the other loading or solvent conditions.

Since the concentration of an eluting band can be approximated by

$$C_x = \frac{1}{\sqrt{2\pi}} M_x \sigma_v^{-1}$$

where M_x is the mass of the solute in the eluted zone of band width $4\sigma_v$, it can be shown that the concentrations of the protein in the eluted zone for the 1 mg load experiment was 0.4 ± 0.1 ($\bar{x}$ ± S.D.) mg BSA/ml irrespective of whether 0.1% TFA, 1 *M* or 4 *M* acetic acid was used as the loading solvent. With the higher 15 mg load experiment the corresponding concentration of the eluted zone was 1.5 ± 0.3 mg BSA/ml again irrespective of the loading solvent used. At a particular mass loading, irrespective of the loading solvent, essentially the same concentration of an eluted zone is achieved with a common 0–50% acetonitrile: 0.1% TFA gradient system. In view of the differences in concentration of the eluted peak, which arise when low or high mass loading conditions are employed, it is likely the limits set by solute solubility in the desorbing solvent composition have not yet been reached. It is also noteworthy that narrower peak widths (and smaller recovered masses) were obtained for the 1 *M* acetic acid loading experiments than with the other loading conditions.

Several mechanistic possibilities can now be considered for these loading effects observed between the 0.1% TFA and the acetic acid conditions. Differences in mass of BSA bound to the column under the various loading conditions must reflect changes in the solute affinity for the support, a decrease in the number and type of available binding sites or both. With large loading volumes such as used in the present study the opportunity arises for re-equilibration of the stationary-phase surface by the loading solvent. This will lead in the case of acetic acid based solutions to a acetic acid-solvated hydrocarbonaceous surface onto which the protein is progressively adsorbed. In this environment the rates of surface mediated phenomena such as protein binding and conformational transitions will be different than those experienced in solely TFA-mediated adsorption and desorption steps. We and others have shown[13,14] that TFA is an effective deformer of protein conformation leading to denaturation in RP-HPLC systems. Since resistance to mass transfer arising from the interplay of heterogeneous binding sites as well as conformational effects are known to directly influence peak shape, an increase in $4\sigma_v$ for the TFA system is thus not unexpected. If the rate constants for these protein binding and relaxation events are smaller in an acetic acid–*n*-alkylsilica environment than in a TFA–*n*-alkylsilica environment then at a particular mass loading condition the change in peak width is expected to be smaller under acetic acid loading conditions. The results shown in Fig. 6 of the plots $\Delta N/N_\infty$ *versus* log (mass) are in accord with this conclusion.

Why then is the breakthrough peak under acetic acid loading conditions larger? Important considerations here are the effective pH of the loading solvent and the

ability of the organic acid to intercalate with the protein. Beside the influence of the stationary-phase re-equilibration effect on the availability of binding sites mentioned previously, the solvation state of the protein and its orientation as it approaches the stationary-phase surface must also be taken into account. Since it is unlikely that significant differences exist in the protein's diffusion coefficient, D_m, due to subtle differences in the viscosity of the acetic acid or TFA-based eluents, then the diffusional kinetics in both eluents will presumably be very similar. As a consequence, the differences seen between the various loading conditions must reflect in the adsorption rate constants due to changes in the availability of adsorption sites at the stationary-phase surface and changes in the surface hydrophobicity of the protein, including the orientation of the protein's hydrophobic domains to the hydrocarbonaceous surface. This conclusion implies that subtle differences in the solute orientation at the instant of binding, and subsequently re-orientation, may arise when proteins are loaded onto a reversed-phase support in a loading buffer different to the eluting buffer. Although such a possibility has yet to be definitively characterised adequate phenomenological precedents exist in other areas of the scientific literature on the high-performance hydrophobic interaction, biospecific affinity and ion-exchange chromatographic behaviour of proteins. Whatever is the origin of these effects they clearly have major ramifications with regard to the choice of eluent composition for

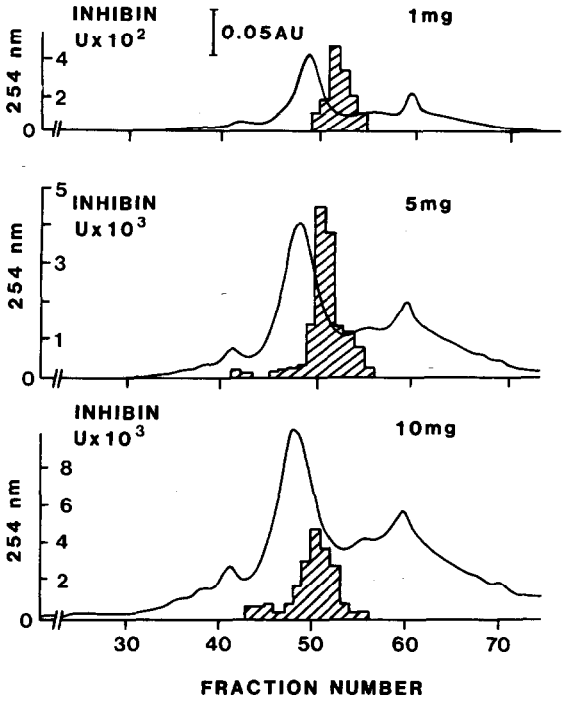

Fig. 8. Absorbance profile for the separation of a bovine inhibin fraction from Sephacryl S200, eluted from a Ultrapore C_3 column at three different loading levels. The chromatogram was developed using a linear gradient of 0.1% TFA to 0.1% TFA in acetonitrile–water (50:50) at a flow-rate of 1 ml/mm over 60 min. Inhibin bioactivity was located using the dispersed pituitary cell culture assay.

preparative loading experiments. The above considerations are based on the assumptions that the elution behaviour of BSA under the different loading conditions is operating within a linear region of an adsorption isotherm. As discussed elsewhere[9,10], when the assumption of linear distribution is abandoned, such that the distribution can no longer be described by a simplified form of the Langmuir equation, *i.e.* by $C_s/C_m = \Phi^{-1}k'$, then a large number of alternative adsorption models must be considered. Evaluation of the above data in terms of such alternative models will be reported in a subsequent paper.

Bovine inhibin loadability study

Ovarian follicular fluid is a rich source of inhibin, a hydrophobic protein which suppresses in a dose dependent manner the FSH content of cultured pituitary cells and FSH secretion *in vivo*. In a recently described procedure[5,6] for the isolation of bovine inhibin, partially fractionated inhibin preparations were chromatographed on Sephadex G100 or G200 in 4 *M* acetic acid with appropriate chromatographic fractions pooled and without concentration immediately chromatographed by RP-HPLC. This stratagem was predicted by the loss of bio-activity of inhibin following lyophilisation probably associated with resolubilisations. Although this direct loading procedure has several advantages, such as minimal handling and the option of trace enrichment, it did require the loading of relatively large volumes of dilute inhibin fractions directly onto the reversed-phase support. Fig. 8 shows the chromatographic profile and zones of inhibin bioactivity following the loading of 10 mg of crude inhibin preparations dissolved in 100 ml 4 *M* acetic acid at a loading flow-rate of 2.0 ml/min, *i.e.* total injection time 50 min. In common with the BSA results, the amount of bioactive inhibin which could be eluted by a single gradient run depended markedly on the initial loading conditions. Table I summarises the results of these experiments. As is evident from these data as the protein content of the load increased up to 10 mg, the relative percentage of inhibin activity which was not adsorbed under the loading conditions increased from *ca.* 4% to *ca.* 50%. With lower protein loads, the percentage of irreversibly bound inhibin was highest. This behaviour has been noted previously with many other proteins which exhibit distribution phenomena typical of the so-called "ghost effect". In common with the BSA

TABLE I

THE EFFECT OF MASS LOADABILITY AND *n*-ALKYL LENGTH ON THE RP-HPLC RECOVERY OF INHIBIN BIOACTIVITY

The samples were loaded in 100 ml 4 *M* acetic acid at a loading flow-rate of 2.0 ml/min and eluted with a 0–50% linear acetonitrile gradient containing 0.1% TFA over 30 min at a flow-rate of 2.0 ml/min.

Column	*Load (mg)*	*Breakthrough peak (%)*	*Eluted (%)*	*Total recovered (%)*
C_3 Commercial	1	4	50	54
	5	15	84	99
	10	51	54	105
C_3 Experimental	5	1	79	80
C_8 Experimental	5	<1	67	67

binding data, these results can be accommodated if two or more classes of stationary phase binding sites of different affinities are involved.

The major protein contaminant in most partially fractionated bovine inhibin preparations is BSA which is generally present in 10–100 fold greater amounts at the penultimate Sephacryl S200–4 *M* acetic acid purification step. This protein constitutes the major UV absorbing component which elutes just prior to the inhibin peak shown in Fig. 8. Although resolution for the earlier eluting components appeared to improve for the C_8 over the C_3 phase, relative selectivity for inhibin was unchanged. At much higher protein loads, or with columns of lower performance, significant decrease in purification factors could be anticipated if the retention of the inhibin protein selectively decreased due to overload effects. Such changes are evident in Fig. 8. Similar results have been noted in the purification of ovine inhibin where in this case the slightly more polar ovine inhibin protein elutes earlier in the chromatographic profile and overlaps with the albumin zone (unpublished results). Improved procedures which allow the removal of albumin from crude inhibin preparations prior to the RP-HPLC step are clearly required but to date methods to achieve this selectively have proved recalcitrant due to the propensity of inhibin to associate with, and hence copurify with, albumin under many conditions.

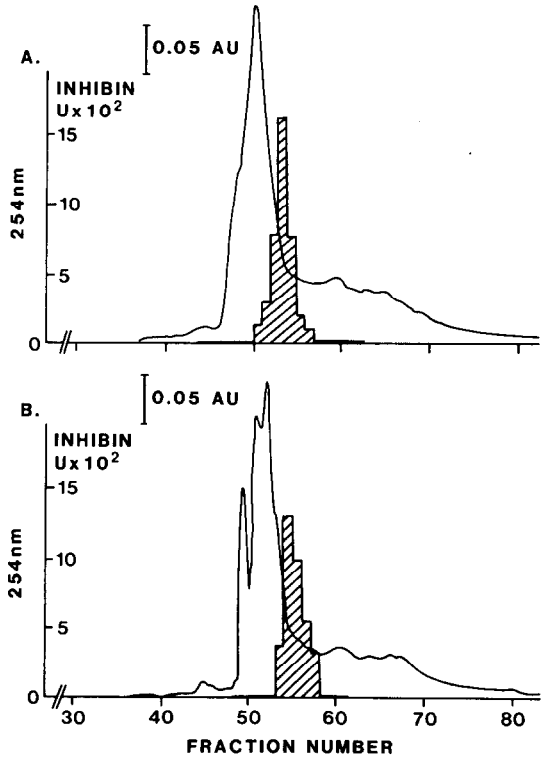

Fig. 9. Absorbance profile and bioactivity profile for crude bovine inhibin preparation eluted from an Ultrapore C_3 (A) and C_8 (B) column under the same gradient conditions at a flow-rate of 1.0 ml/min. In both chromatograms 15 mg of the inhibin preparation was injected and the gradient developed from 0.1% TFA to 0.1% TFA in acetonitrile–water (50:50). Inhibin bioactivity was determined using the disperse pituitary cell culture assay.

Effect of n-alkyl chain length on comparative loadability performance

Comparative elution and bioactivity profiles for the separation of the bovine inhibin preparation on the dimethylpropyl- and the dimethyloctyl-Ultrapore supports with an injected load of 1 mg/100 ml are shown in Fig. 9. Similar data for an Ultrapore RPSC C_3 column after extensive use (> 95 injection cycles) is also shown in Fig. 8. As is evident, little change in selectivity occurs between the propyl- and the octyl-*n*-alkyl phase. Similarly, recovery of inhibin bioactivity (Table I) from both phases was essentially equivalent. Although the peak width of the eluted bioactive zone appears smaller with the C_3 phase, the differences were sufficiently subtle to pre-empt any unequivocal conclusions. Since the C_3 and the C_8 phases represent high coverage ligand densities, prepared from the same pretreated porous silica batch, the conclusion can be drawn that *n*-alkyl chain length *per se* has only a minor influence on biological recovery, at least for this hydrophobic protein. The above results share common features with early results reported from this and other laboratories[4,17–20]. These previous studies demonstrated that when the accessible ligand densities of different porous supports, bonded with *n*-alkyl chains of different chain length, were normalised, essentially identical resolution and recovery could be achieved.

CONCLUSIONS

This study has examined the effect of mass loadability, protein concentration, loading solvent and *n*-alkyl chain length on the reversed-phase high-performance liquid chromatographic behaviour of BSA and bovine inhibin. Based on these investigations several proposals can be made for the scale-up of the purification by RP-HPLC of hydrophobic proteins such as inhibin. Firstly, in order to optimize loading capacity, several loading solvent conditions in addition to the column equilibration solvent, should be explored in preliminary experiments. Where advantages are evident, such as the 1 *M* acetic acid system at the lower mass range in the BSA experiments, this solvent combination should be used, even though it may differ from the elution mobile phase, and could result in a larger breakthrough peak. The breakthrough peak can always be directly recycled or returned to the sample feed reservoir prior to the commencement of elution. Secondly, adsorption phenomena associated with lower affinity binding sites are preferred since intrinsically they can lead to lower residence times on the support and obviate to some extent conformational or other secondary equilibrium effects. Thirdly, a practical compromise between the protein concentration in the feed injection and the protein concentration which can be adsorbed can be reached by changing the loading volume or loading solvent. Where feasible, more concentrated protein solutions should be injected in the smallest volume to minimise peak breakthrough. This may necessitate a partial concentration of very dilute chromatographic fractions, prior to loading. Since the effective column capacity and the theoretical column capacity may differ by factors up to 100-fold or more, the protein throughput will be limited by the maximum concentration possible for the eluted protein to stay in solution. Consequently, the upper limit for loadability and the upper limit for solute throughput should be chosen so that these parameters do not significantly diverge. Since mass loadabilities are predicted to be high with reversed-phase supports, mass loads in excess of 200 mg protein per g support with, for example, crude inhibin fractions may still permit adequate preparative through-

put and recovery as a penultimate purification stage depending on the characteristics and complexity of the protein mixture. This approach would then permit sequential refractionation of bioactive zones on tandem columns with the same high coverage porous *n*-alkylsilica stationary phase. Finally, even in the overload situation advantage should still be taken of the benefits of peak capacity optimisation with the chromatographic system since changes in k' (or the centre of gravity of the concentration profile) are much less responsive to mass changes than changes in bandwidth.

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REFERENCES

1 M. T. W. Hearn, *Adv. Chromatogr.*, 20 (1984) 1.
2 M. T. W. Hearn, in J. C. Jansen (Editor), *High Resolution Protein Purification*, Springer Verlag, Deerfield Beach, FL, 1987, in press.
3 B. Grego and M. T. W. Hearn, *J. Chromatogr.*, 218 (1981) 497.
4 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 282 (1983) 541.
5 D. M. Robertson, L. M. Foulds, L. Leversha, F. J. Morgan, M. T. W. Hearn, H. G. Burger, R. E. H. Wettenhall and D. M. de Kretser, *Biochem. Biophys. Res. Commun.*, 126 (1985) 220.
6 D. M. Robertson, F. L. de Vos, L. M. Foulds, R. I. McLachlan, H. G. Burger, F. J. Morgan, M. T. W. Hearn and D. M. de Kretser, *Mol. Cell. Endocr.*, 44 (1986) 271.
7 J. I. Thorell and B. G. Johansson, *Biochim. Biophys. Acta*, 251 (1971) 363.
8 R. S. Scott, H. G. Burger and H. Quigg, *Endocrinology*, 107 (1980) 1536.
9 M. T. W. Hearn, M. I. Aguilar and A. N. Hodder, unpublished results.
10 H. Poppe and J. C. Kraak, *J. Chromatogr.*, 255 (1983) 395.
11 R. E. Beck and J. Schultz, *Science (Washington, D.C.)*, 170 (1970) 1302.
12 E. M. Renkin, *J. Gen. Physiol.*, 38 (1954) 225.
13 M. T.W. Hearn, A. N. Hodder and M.-I.Aguilar, *J. Chromatogr.*, 327 (1985) 47.
14 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr.*, 317 (1984) 227.
15 H. Engelhardt and H. Muller, *Chromatographia*, 19 (1984) 77.
16 F. V. Warren and B. A. Bidlingmeyer, *J. Liq. Chromatogr.*, 8 (1985) 691.
17 N. H. C. Cooke, B. G. Archer, M. J. O'Hare, E. C. Nice and M. Capp, *J. Chromatogr.*, 255 (1983) 115.
18 J. D. Pearson and F. E. Regnier, *J. Liq. Chromatogr.*, 6 (1983) 497.
19 R. V. Lewis and D. DeWald, *J. Liq. Chromatogr.*, 5 (1982) 1367.
20 L. A. Witting, D. J. Gisch, R. Ludwig and R. Eksteen, *J. Chromatogr.*, 296 (1984) 97.