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Effect of antigen size on optimal ligand density of immobilized antibodies for a high-performance liquid chromatographic support

JEFFREY B. WHEATLEY
Rainin Instrument Company, 1780 Fourth Street, Berkeley, CA 94710 (USA)

ABSTRACT

The antigen binding capacities for purified polyclonal antibodies immobilized onto a silica-based high-performance liquid chromatographic (HPLC) affinity support are described for three serum proteins over a range of antibody ligand densities. The rate of decline in the specific activity of the immobilized antibodies with respect to increasing ligand density was found to increase with the molecular weights of the antigens. The antibodies used were purified from whole antiserum using high-performance affinity chromatography and were examined using HPLC on an SCX stationary phase. Conditions are also described for efficient coupling of the ligand to the support.

INTRODUCTION

The binding capacity of an immunoaffinity support is related to the amount of antibody immobilized per gram or milliliter of support. Increasing the specific surface area of the support (area per gram) while maintaining a constant antibody ligand density can result in an increased antigen binding capacity. However, for highly porous particles an increase in surface area is often accompanied by smaller pore diameter. As the pore diameter decreases below certain limits, access to the surface becomes restricted for large molecules. Consequently, maximizing the capacity of a support through an increase in specific surface area must be balanced against the selection of a pore size large enough to permit both sufficient immobilization of antibody and reception of the antigen [1,2]. For a particular immunoaffinity support of defined particle dimensions, optimization of the binding capacity depends considerably on access of the antigen to the binding regions of the immobilized antibodies. Access to the binding sites is influenced by such factors as the molecular orientation of the immobilized antibody molecules and the antibody ligand density in relation to antigen size and pore diameter [3,4]. Also of fundamental importance are the association constants intrinsic to the native antibodies and conditions encountered by the antibodies that can lead to denaturation and loss of binding integrity. These factors arise during the initial purification of the antibody and in the procedures under which the antibody is immobilized onto the support. Denaturation can also result from conditions under which the affinity column is used and stored over time.

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It has been shown that the specific activity of immobilized antibodies, expressed as the mole ratio of bound antigen to theoretical antibody binding sites, decreases with increasing ligand density. Eveleigh and Levy [4] found that the mole to mole ratio of bound albumin to antibody decreased with increases in the ligand density of polyclonal anti-human albumin antibody immobilized on cyanogen bromide-activated Sepharose 4B. In a study using a monoclonal antibody for lysozyme, Hearn and Davies [3] reported finding no difference in the specific activities for two antibody ligand densities on cyanogen bromide-activated Sepharose 4B. However, they did observe significant differences for the same antigen–antibody pair on two other porous polymeric supports.

In this study, the effects of increasing ligand density and antigen size on binding capacity were examined for three serum proteins: human IgG immunoglobulin, albumin and α_1-acid glycoprotein. The binding capacities were measured for polyclonal antibodies immobilized onto Hydropore-EP, a silica-based HPLC affinity support. Conditions are also described for the efficient immobilization of proteins and for the examination of purified antibodies by HPLC on an SCX stationary phase.

EXPERIMENTAL

Materials

Proteins and antisera were obtained from Sigma (St. Louis, MO, USA) and serine methyl ester from Aldrich (Milwaukee, WI, USA). Hydropore-EP (12 μm, 300 Å) and Hydropore-5-SCX (5 μm, 300 Å) are products of Rainin Instrument Co. (Woburn, MA, USA).

Apparatus

Chromatographic and affinity purifications were done using Rainin Rabbit HPLC pumps and Knaur Model 71 and 87 UV detectors from Rainin Instrument Co. Control of pumps, HPLC methods, data acquisition and treatment were accomplished with the Macintosh-based Dynamax HPLC Method Manager from Rainin Instrument C. Spectrophotometric measurements were made on a Hitachi (San Jose, CA, USA) Model U-2000 scanning spectrophotometer.

Preparation of antigen columns

The antibodies used were purified from whole goat antiserum using high-performance affinity chromatography. Antigen columns were prepared for this purpose by immobilizing the protein, either human IgG immunoglobulin, albumin or α_1-acid glycoprotein, onto the Hydropore-EP affinity support packed into a 50 × 4.6 mm I.D. stainless-steel column. This was achieved by recirculating 5–10 mg of protein dissolved at about 1 mg/ml in an ammonium sulfate solution buffered in 0.020 *M* potassium phosphate (pH 7.0). The solutions were recirculated through the columns at 0.2 ml/min for 16–20 h. Coupling of human IgG and albumin was carried out in 0.75 *M* ammonium sulfate whereas α_1-acid glycoprotein was coupled in 2.0 *M* ammonium sulfate.

Hydropore-EP is derived from porous, spherical silica possessing an average particle diameter of 12 μm and an average pore diameter of 300 Å. The surface of the silica is chemically modified to produce a covalently bound hydrophilic monolayer

possessing epoxide functional groups. The epoxide reacts with nucleophilic groups found in proteins, resulting in covalent immobilization of the protein onto the surface of the support.

Purification of antibodies

The antiserum, diluted 50% in loading buffer, was applied at 0.5 ml/min to the antigen column, which had been pre-equilibrated in 0.15 *M* sodium chloride in 0.010 *M* potassium phosphate (pH 7.0) (loading buffer). The loaded column was washed with loading buffer at 2.0 ml/min until the detector output (at 280 nm) returned to a steady baseline near zero absorbance. The column was then washed at 2.0 ml/min with about 20 column volumes of 0.020 *M* potassium phosphate (pH 7.0). This step, which was performed in order to remove most of the salt, was required for examination of the product by cation-exchange HPLC. After the desalting step, the bound antibodies were eluted with 0.050 *M* potassium phosphate (pH 2.5) at 0.5 ml/min. The product was collected in three fractions, with the middle, concentrated fraction consisting of eluate collected at an absorbance > 1.0 at 280 nm. The pH of the eluate fractions was measured and a sample of the middle fraction was injected onto the cation-exchange column. The pH of the eluate fractions was then adjusted to 6.9–7.2 with 5% sodium hydroxide.

After storage for at least several hours the eluate was centrifuged to remove the small amount of precipitate that invariably appeared. A sample of the middle fraction was then injected onto the antigen column and the concentrations of the eluate fractions were determined from the absorbance at 280 or 230 nm. The middle fraction, typically containing 85% of the eluate protein, was used for binding capacity determinations in all three antigen–antibody systems. The pH of this fraction prior to neutralization ranged from 5.3–5.7 and the protein concentration was between 5 and 10 mg/ml. In Fig. 1 a chromatogram is shown for the injection of goat anti-human

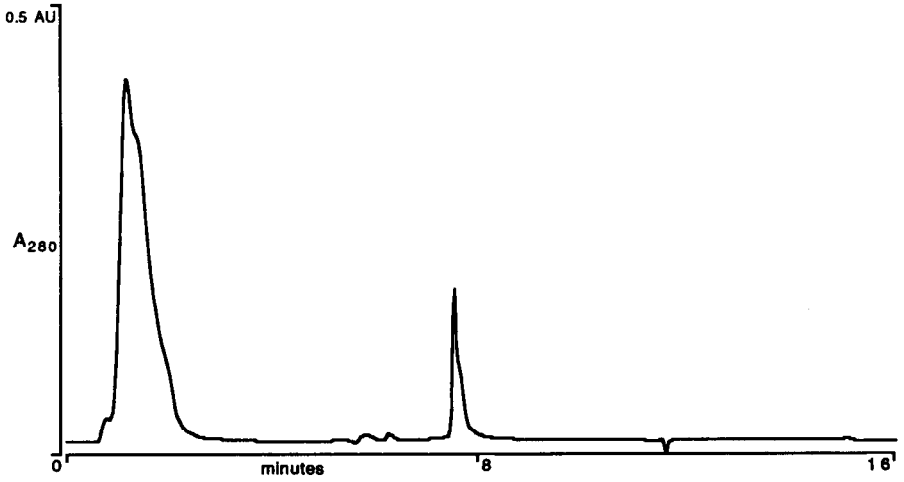

Fig. 1. Goat anti-human albumin antiserum on albumin antigen column. Albumin-specific antibodies are found in the retained band. Column: human albumin immobilized on Rainin Hydropore-EP, 50 × 4.6 mm I.D. Conditions as described in text.

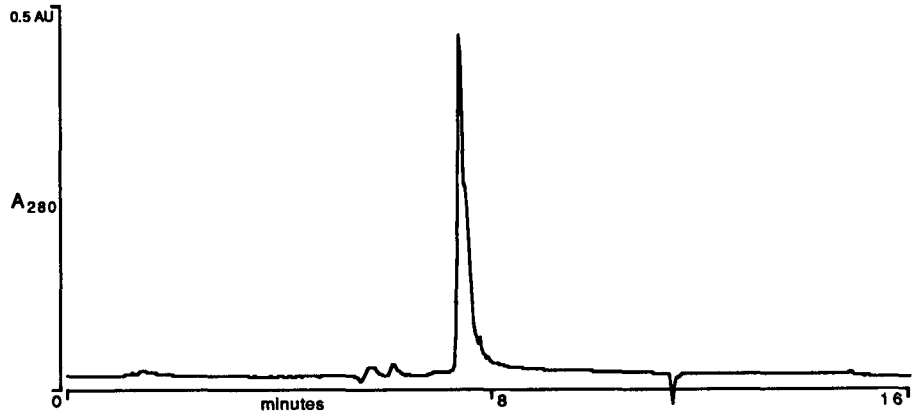

Fig. 2. Purified goat anti-human albumin antibodies injected onto albumin antigen column. Conditions as in Fig. 1.

albumin onto the albumin antigen column. The small retained peak corresponds to antibodies specific for albumin. In Fig. 2 a chromatogram is shown for the injection of the middle fraction of eluate onto the same column, indicating the near absence of the unretained components found in the chromatogram for the whole antiserum.

Measurement of binding capacities and calculations

Purified antibodies were coupled to Hydropore-EP in 0.75 *M* ammonium sulfate buffered in 0.020 *M* potassium phosphate, pH (7.0) according to the following procedure. The antibodies (250–2800 μg) were diluted in 0.020 *M* potassium phosphate; 3.0 *M* ammonium sulfate in 0.020 *M* potassium phosphate was then added to give a concentration of 0.75 *M*. A weighed amount of Hydropore-EP was added to the solution and the mixture was mixed overnight by orbital rotation at room temperature. Most mixtures contained 25 mg of support in a volume of 400 μl. For some samples possessing higher levels of immobilized antibody, the coupling mixture contained either 10 or 25 mg of support in volumes up to 2.5 ml.

The derivatized support was thoroughly washed in phosphate-buffered saline (PBS) (pH 7.0) and mixed overnight in 0.20 *M* serine methyl ester (pH 8.5) to deactivate any unreacted epoxide groups. After thoroughly washing with PBS (pH 7.0), the support was then mixed with an excess of the appropriate antigen for 2 h in PBS (pH 7.0), washed thoroughly with PBS and eluted with 0.10 *M* glycine (pH 2.5). In separate experiments, highly derivatized support was washed in 0.10 *M* sodium acetate containing 0.40 *M* sodium chloride (pH 4.8). Examination of this wash and the serine methyl ester solution following deactivation revealed the presence of only insignificant amounts of IgG.

The amount of antibody bound to the support in the coupling step was determined from the difference between the amount of antibody initially added and that recovered in the post-coupling wash. This and the antigen in the eluate were calculated from their absorbance at either 280 or 230 nm. Absorptivities for 280 nm were taken from the literature, and those for 230 nm were calculated from A_{230}/A_{280} ratios. Molar amounts for antigens and antibodies were based on reported molecular weight values.

The fraction of the theoretical capacity was calculated according to the equation

$$S.A. = \text{mol Ag}/2(\text{mol Ab})$$

where *S.A.* is the specific activity and Ag and Ab refer to antigen bound and antibody immobilized, respectively. If each antibody molecule is assumed to possess two binding sites, a specific activity of 1.0 would indicate that all binding sites are occupied. The purified antibodies used in this study were assumed to be exclusively IgG for the purposes of the study. In point of fact they may have contained small amounts of other immunoglobulin classes.

RESULTS

Immobilization of proteins onto the support

In Fig. 3, the plot of percentage protein coupling *versus* ammonium sulfate concentration determined under static conditions for goat IgG and human α_1-acid glycoprotein shows that the percentage coupling increases with increasing salt concentration. Virtually complete immobilization was achieved for both proteins, occurring at 0.8 *M* for goat IgG and at 2.0 *M* for α_1-acid glycoprotein. In the preparation of the human IgG and α_1-acid glycoprotein antigen columns, which was carried out by recirculating the protein in salt concentrations of 0.75 and 2.0 *M*, respectively, >95% immobilization of protein occurred. The coupling efficiency for the albumin immobilization was found to be 42% at 0.75 *M* ammonium sulfate. Based on the trend seen in Fig. 3, the efficiency of albumin immobilization would probably have benefitted from the use of a higher concentration.

Immobilization of purified goat anti-human serum protein antibodies in 0.75–0.85 *M* ammonium sulfate routinely resulted in coupling efficiencies >90% for antibody ligand densities up to *ca.* 50–60 mg/g support. At higher densities the coupling efficiency decreased. For the most highly derivatized support (114 mg/g), 71% coupling was found (data not shown).

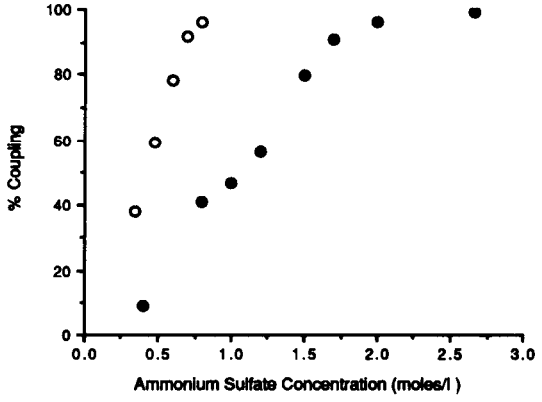

Fig. 3. Percentage protein coupling to Hydropore-EP *vs.* ammonium sulfate concentration. ○ = Goat IgG; ● = human α_1-acid glycoprotein.

Examination of purified antibody on the SCX stationary phase

In Fig. 4 a chromatogram is shown for the injection of whole goat anti-human albumin antiserum onto the sulfopropyl cation-exchange column, using conditions under which IgG is retained and most proteins show early elution. Fig. 5 shows a chromatogram for the injection of goat IgG and in Fig. 6 a chromatogram is shown for the injection of eluate obtained from the purification of anti-albumin antibodies from the antiserum. The chromatogram for the purified eluate indicates a near absence of the early eluting bands seen in the whole antiserum. Although the possibility cannot be excluded that some serum components could be co-eluting in the retained (antibody) band, the absence of early eluting bands and the minimal presence of unretained components in the chromatogram for the injection of the purified eluate onto the antigen column (Fig. 3) suggest that the eluate consists of antigen-specific antibodies. The chromatograms included here are typical of those obtained for other purifications.

Antibody binding capacities and specific activity

The binding capacities for albumin and α_1-acid glycoprotein, expressed as milligrams of antigen bound per milligram of immobilized antibody per gram of support, are shown in Fig. 7 and those for IgG in Fig. 8. The antigen binding capacity is linear for all three proteins at low ligand densities. However, as the ligand density increases, the increases in capacity fall off for albumin and IgG. The capacity for α_1-acid glycoprotein appears to be linear through the highest ligand density examined in this study. The increases in capacity for albumin fall off gradually, and the IgG binding capacity appears to change suddenly, from a linear increase to an actual decline in capacity.

Fig. 9 shows the antigen binding capacities for the three proteins, expressed as micromoles of antigen bound per micromole of immobilized antibody per gram of

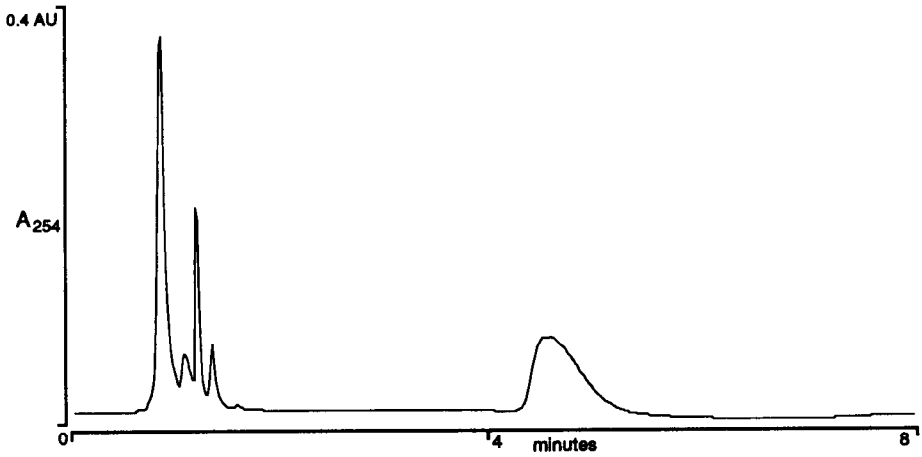

Fig. 4. Goat anti-human albumin antiserum on SCX. The retained peak corresponds to goat IgG as seen in Fig. 5. Column: Hydropore-5-SCX, 100 × 4.6 mm I.D. Conditions: 0–3 min, 0% B, 1 ml/min; 3–6 min, 0–100% B, 2 ml/min; A = 0.02 *M* potassium phosphate (pH 5.9) and B = 1.0 *M* sodium chloride in A; detection at 254 nm, 0.4 a.u.f.s.

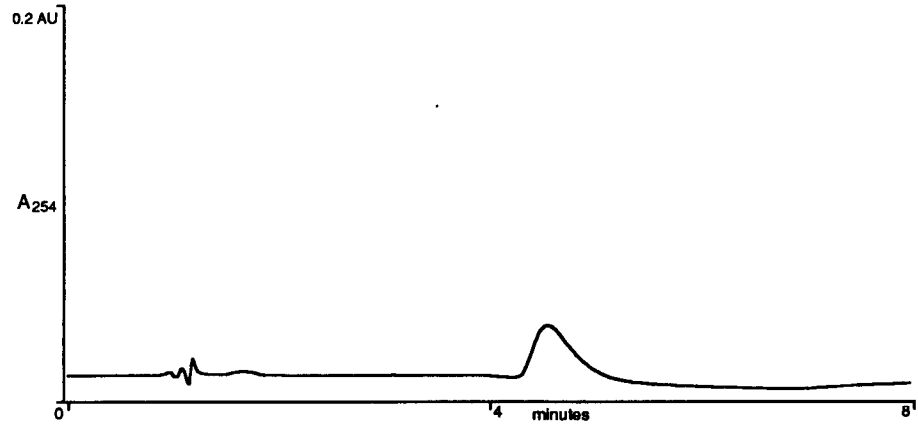

Fig. 5. Goat IgG on SCX. Column and conditions as in Fig. 4.

support, and provides a comparison of antigen binding efficiencies with increasing antibody ligand density. The three antigens show comparable binding efficiencies at low ligand densities. The efficiencies appear to remain fairly linear for both albumin and α_1-acid glycoprotein throughout the range of ligand densities examined. IgG reflects the trend seen in the previous figures, with the efficiency falling off relatively early and suddenly.

Fig. 10 is derived from the data in Fig. 9 and provides a more sensitive indication of individual and comparative efficiencies. In this graph binding of antigen is expressed as a fraction of the theoretical maximum, where 1.0 would indicate that all antibody binding sites are occupied. The efficiency of α_1-acid glycoprotein appears to be steady at *ca.* 27% of the theoretical value; for albumin it decreases from about

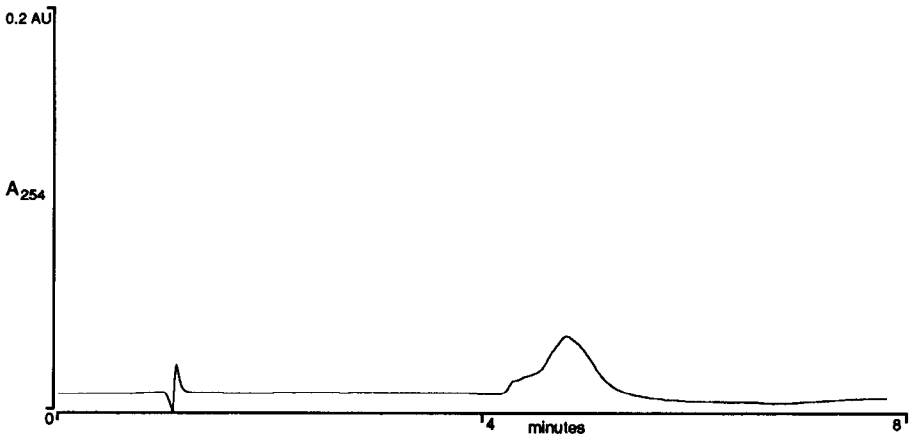

Fig. 6. Purified anti-human albumin antibodies on SCX. The retained band corresponds to goat IgG as seen in Fig. 5. The refractive index peak seen at the void volume is due to differences in the salt content between the sample and mobile phase. Column and conditions as in Fig. 4.

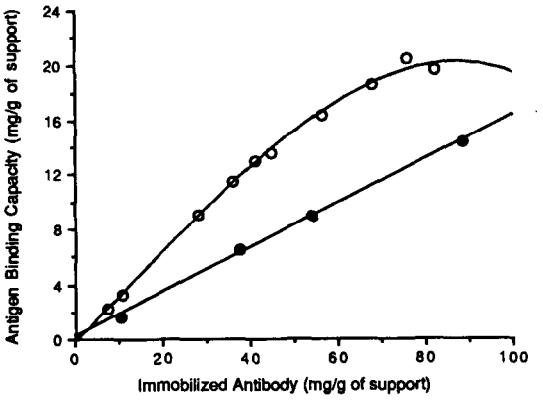

Fig. 7. Binding capacity of albumin and α_1-acid glycoprotein *vs.* immobilized antibody density in mg per gram of support. ○ = Human albumin; ● = human α_1-acid glycoprotein.

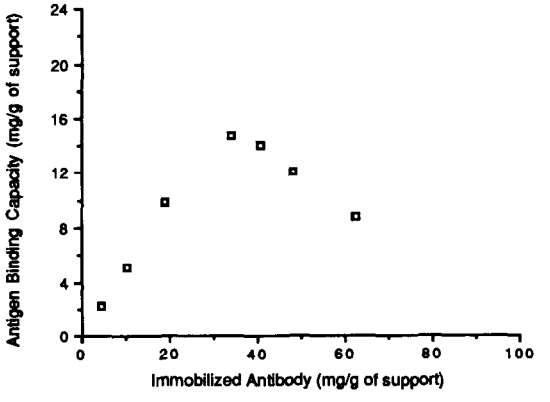

Fig. 8. Binding capacity of human IgG *vs.* immobilized antibody density in mg per gram of support.

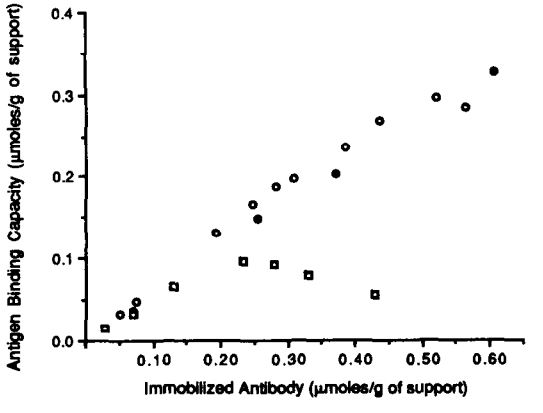

Fig. 9. Binding capacity *vs.* immobilized antibody density in μmol per gram of support. ○ = Human albumin; □ = human IgG; ● = human α_1-acid glycoprotein.

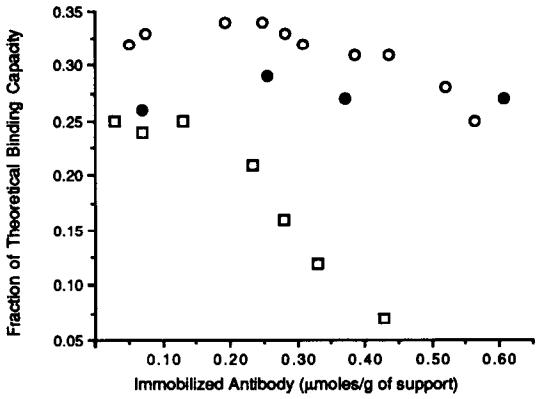

Fig. 10. Fraction of theoretical binding capacity *vs.* antibody ligand density. ○ = Human albumin; □ = human IgG; ● = human α_1-acid glycoprotein.

33% to 25% and for IgG it falls from 25% to only 7% at high ligand densities. Overall, albumin appears to experience the highest efficiency, but it can be seen to decrease, beginning about half-way along the ligand density range. Binding of α_1-acid glycoprotein shows a lower efficiency than that of albumin, but it remains steady throughout the range. The IgG binding efficiency appears to be no greater than that of α_1-acid glycoprotein at any ligand density, and rapidly falls to low levels.

DISCUSSION

It was found that the efficiency with which antibodies, and proteins in general, couple with the epoxide groups of Hydropore-EP is favored at relatively high salt concentrations. This has been observed both with ammonium sulfate and potassium phosphate and probably results from an improved proximity of the reactive groups due to salt-induced association between the protein and support surface. This effect is similar to that seen for hydrophobic interaction chromatography (HIC) [5–8]. It has been shown [9] that for injections of human albumin and human γ-globulin onto a polyether stationary phase under HIC conditions γ-globulin is strongly retained whereas albumin exhibits early elution. This indicates that γ-globulin is more susceptible to salt-induced surface associations, which is consistent with the coupling results obtained here for human IgG and albumin, in which immobilization of IgG was nearly complete at 0.75 *M* whereas only 42% of albumin was immobilized. The salt concentration required for efficient immobilization of albumin would probably be similar to that needed for α_1-acid glycoprotein, which required about 1.9 *M* ammonium sulfate for 95% coupling (Fig. 3). Although it has been reported that epoxide-type affinity supports exhibit low reactivity at neutral pH [10], the measurements described in this study and performed in other contexts indicate that the utilization of a sufficiently high salt concentration will result in highly efficient immobilization of proteins to this support.

The increased coupling efficiency found at high salt concentrations could result from several factors. Salt-induced partitioning of the protein along the surface of the support would lead to localized concentration of the protein near epoxide groups,

possibly resulting in an increased reaction rate between nucleophilic residues and the epoxide. A downward shift in the pK of nucleophilic side-chains, resulting from a salt-induced hydrophobic association between the protein and support surface, would also increase the reaction rate by increasing the concentration of the unprotonated, reactive form of the nucleophile. Shifts in dissociation constants, thought to result from effects of the local environment of the side-chains, have been reported to occur in some proteins [11,12]. In one study a pK of 5.9 was assigned to a lysine residue in acetoacetate decarboxylase [11], representing a downward shift of more than four units from the value usually associated with the ε-amino group. Finally, proteins localized on the surface could provide a proton source for general acid catalysis of the epoxide.

The gradual decrease in specific activity observed for binding of albumin (mol.wt. 69 000) and the absence of any change for α_1-acid glycoprotein (mol.wt. 44 000) can be compared with the stronger decline seen for IgG (mol.wt. 153 000). The relative rates of decline in specific activity with molecular weight suggest that the approach to ligand binding sites is more restricted for larger antigens. This is further supported by the observation that the specific activity first begins to decline at a lower ligand density for IgG than for albumin. These results suggest that as the molecular weight (size) of the antigen is increased, the ligand density at which the initial drop in specific activity is observed should decrease. An increase in the pore size would be expected to shift this threshold to a higher ligand density. However, as increasing pore size is accompanied by reductions in specific surface area, the absolute capacity could be expected to fall. This kind of trade-off was discussed by Narayanan *et al.* [1] for the immobilization of proteins on silica-based supports of increasing pore diameter. It would be interesting to measure the threshold and rate of decline in specific activity for the binding of a wider (molecular weight) range of antigens in order to examine the correlation of these factors. Although it is tempting to contrast the binding efficiencies for IgG and albumin at the lower ligand densities (25% *versus* 33%, respectively), the fact that the antibodies for the two systems were derived from different sources precludes comparisons, as has been pointed out by Eveleigh and Levy [4].

The most dramatic effect of antibody ligand density on capacity was seen for IgG, which showed not only early changes in linearity but also significant declines in absolute capacity for the higher ligand densities. The capacity per gram of support dropped from a maximum of about 15 mg for 34 mg of immobilized antibody to 9 mg for 62 mg of ligand, a decline of 40%. Similar observations have been reported by other investigators for an immobilized monoclonal antibody to tissue plasminogen activator [13]. In this study, actual declines in capacity were not observed for α_1-acid glycoprotein. At the highest ligand density examined for albumin a slight drop in capacity was seen, but more data are required for confirmation. The question arises of whether decreases would occur for albumin and α_1-acid glycoprotein capacities at ligand densities beyond those examined in this study and how steeply such declines might be in comparison with that seen for IgG. Although the maximum ligand density for IgG on Hydropore-EP was not measured, the moderate decline in coupling efficiency observed at the highest density examined (71% for 116 mg/g) suggests that a limit is being approached.

In conclusion, these findings suggest that in optimizing conditions for immu-

noaffinity applications, the size of the antigen should be considered in selecting the ligand density. The protocol used for the preparation of an immunoaffinity support for one kind of application may not be appropriate for another. Whereas in some instances ligand densities resulting in less than maximum binding efficiency might be acceptable in order to increase absolute capacities, in no event should the ligand densities exceed values that result in a decrease in capacity.

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