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Effects of stationary phase ligand density in high-performance liquid chromatography of proteins

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Effects of stationary phase ligand density in high-performance liquid chromatography of proteins

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Effects of stationary phase ligand density in high-

performance liquid chromatography of proteins

by

Danlin Wu

A Dissertation Submitted to the
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GENERAL INTRODUCTION

High-Performance Liquid Chromatography of Proteins

The availability of pure substances for special enzymatic
and therapeutic applications or for determining the structure
and function of the enormous array of compounds encountered in
biological systems has always been a problem in clinical and
biochemical sciences as well as other related fields. The
fact that a cell may contain several thousand proteins,
hundreds of RNAs, and multiple DNA and polysaccharide compo-
nents makes the isolation of any single macromolecular species
a challenging task. For this reason, the advancement of
modern life science and the development of macromolecular
separations have been closely related. Contributions to our
present understanding and application of biological systems,
derived from use of the analytical ultracentrifuge, electro-
phoresis, and column chromatography are examples of the impact
of separation science on life science. Unfortunately, many of
the analytical techniques upon which life scientists have been
dependent for the past two decades are labor-intensive, slow,
and of low resolving power.

Liquid chromatography has become a major separation tech-
nique in life sciences since it was first introduced 75 years

ago (1). It has been known for four decades (2) that reducing
the particle size of chromatographic packing materials will
result in sharpening of eluted peaks and an increase in the
separation efficiency. However, implementation of this know-
ledge took many years because (i) beds of very small particles
require a high inlet pressure to achieve reasonable flow
rates, (ii) precision flow high-pressure pumping systems were
not available, (iii) the gel-type packing materials used in
the separation of biopolymers could not tolerate high flow
rates, and (iv) the technology for packing microparticulate
beds had not been developed. By the late 1960s, most of these
problems had been solved for the separation of small mole-
cules, and the name high-performance liquid chromatography
(HPLC) was coined to describe rapid separations in micropar-
ticulate beds. Unfortunately, the high-performance packing
materials of this era were not suitable for separation of
biopolymers because of their relatively small pore sizes and
nonspecific adsorption of biopolymers. Another 10 years
elapsed before the emergence of commercial HPLC packings
specifically designed for the separation of biopolymers.

HPLC columns are characterized by small particle size
packing materials with large pore sizes, high mechanical
strength, and even distributions of both the particle and pore
sizes as well as by high precision pumps and sophisticated

detection systems, which together have greatly enhanced the separation power of the system and reduced the operation time. A typical separation of several days by conventional liquid chromatography takes only 20 minutes with HPLC. The exponential growth of literature on the HPLC separation of proteins indicates that the technique has become vital for the life sciences.

Samples of biological origin may contain 2000 or more constituents. Unfortunately, HPLC technology has not advanced to the point that it is realistic to think of resolving all these components in a single column. Purification of biological macromolecules is still a multiple-step or multiple-column process. Often different chromatographic modes must be used to separate the different protein components of a single sample, due to the diversity of the structural differences of protein molecules.

Resolution of components in a chromatographic system is dependent on their differential distribution between a solid stationary phase and a liquid mobile phase. Components of greater concentration in the mobile phase will elute from the column first, followed by those of lower concentration in the mobile phase. Differences in peak maxima between eluted components are a function of the relative differences in their distribution between the two phases. Since proteins vary in

size and shape, solubility, ionic characteristics, hydrophobi-
city, and affinity for other molecules, discrimination between
any one or combination of these properties may serve as the
basis for differential distribution within a separation
system. Ion exchange chromatography (IEC), reversed phase
chromatography (RPC), affinity chromatography (AC), hydropho-
bic interaction chromatography (HIC), and size exclusion
chromatography (SEC) are fractionation techniques designed to
exploit these chemical or physical differences in proteins.

Ion Exchange Chromatography

In ion exchange chromatography, the degree of solute
retention is governed by the strength of electrostatic inter-
action between the solute and the column. The force of
attraction will depend on (i) the number of charges, which is
related to the number of accessible amino acid residues on the
protein surface that may interact simultaneously with the
packing material, (ii) the charge density of the packing
material, (iii) the ionic strength of the medium, and (iv) the
type of ions in the medium. The chromatographer usually has
three mobile phase variables to use in controlling retention
and selectivity in IEC: (i) the ionic strength, (ii) the type
of displacing ion or ions, and (iii) the pH. The charge on

both proteins and supports is often pH dependent. The struc-
tural complexity of proteins makes their IEC retention very
complicated. Because of their amphoteric nature, proteins
have been chromatographed on both cation and anion exchange
columns. The ion exchange study in this work, however, was
devoted only to cation exchange chromatography, because the
retention mechanisms in principle are the same for both cation
and anion exchange processes.

The most general technique for controlling retention
through manipulation of the mobile phase in IEC is by changing
the ionic strength of the displacing ions. The very narrow
ionic strength range over which most proteins elute and the
broad difference in their ionic characteristics make gradient
elution the most useful technique for resolving protein mix-
tures (3). Columns are loaded at 0.01 M to 0.02 M salt
concentrations and gradient-eluted up to concentrations of 0.5
or 1.0 M salt. The nature of the displacing ions can also
strongly influence IEC retention. The relative displacing
power of various ions generally follows the Hoffmaeister
series (3), but there are numerous exceptions. At present,
there is no predictable pattern to these exceptions.

The importance of pH in retention and resolution of
proteins is determined by their charge characteristics. The
amphoteric nature of proteins reveals that under acidic

conditions, amino groups in proteins will be totally protonated while the ionization of carboxyl groups is repressed. As the solution pH is increased, a point will be reached where the ratio of anionic to cationic groups is unity and the protein is at its isoelectric point (pI). Further increase in pH will cause the protein to acquire anionic character through ionization of carboxyl groups.

In addition to the optimization of mobile phase conditions, much work has been done in the development of ideal stationary phases. Emphasis has been on the synthetic chemistry of stationary phase preparation, the choice of the kind of bonded phase, and the packing material, etc. The stationary phases that have been used in IEC of proteins include: (i) strong anion exchange groups consisting of aliphatic quaternary amines, (ii) weak anion exchange groups consisting of primary, secondary, or tertiary amines, (iii) strong cation exchangers using sulfonic acid groups, and (iv) weak cation exchangers using carboxyl groups. The ion exchange work presented in this study was based on the carboxyl ion exchanger. A good review article summarizing various ways of preparing stationary phases on different supports can be found in ref. 4.

Ion exchange chromatography is uniquely suitable for the fractionation of biologically active macromolecules.

Proteins can tolerate high salt concentrations up to 1 M without any significant conformational changes. Therefore, their biological activities are usually retained after chromatographic separations. Also, mass recoveries are reasonably high, commonly exceeding 90 percent.

Because of the conformational stability of proteins and the exclusively electrostatic interactions between solutes and stationary phase under the IEC separation conditions, the retention of proteins in IEC is relatively simple vs. that of other chromatographic modes such as RPC, where proteins would be denatured and lose their native structures, or HIC, where retention is further complicated by the fact that it is related to both ionic and hydrophobic interactions. This makes IEC a uniquely suitable model system for studying retention mechanisms in protein chromatography. There have been many studies performed regarding the protein retention mechanisms. For example, the retention model proposed by Kopaciewicz et al. has successfully interpreted protein retention as a function of Z number, defined as the number of binding sites on protein surface interacting with the stationary phase (5). By determining the Z numbers for proteins under various conditions they revealed that the electrostatic interaction between the protein and the stationary phase was primarily governed by the distribution of charged groups on

the protein surface, rather than by the pI, as suggested by
the traditional "net charge" concept. Thus, the role that the
protein three dimensional structure plays in chromatographic
retention has been recognized, allowing a better understanding
of retention mechanisms.

Reversed-Phase Chromatography

In reversed-phase chromatography, the separation is based
on the hydrophobic interactions between hydrophobic residues
on the protein and alkyl residues on the support. The
dominant factor controlling protein retention by hydrophobic
interaction is the distribution of hydrophobic residues and
the number of residues that might interact with a surface,
which in turn is determined by the primary, secondary,
tertiary, and quaternary structure of the protein.

Elution of proteins from reversed phase columns requires
an organic solvent and often an acid. The organic solvent is
needed to break down the intense hydrophobic interactions in
the column and effect elution (6). The acid in mobile phase
may contribute to retention by (i) influencing the ionic state
of the solute, (ii) controlling the ionization of surface
silanols on the support, and (iii) forming ion pairs between
cationic solutes and the acid.

Use of acids in the mobile phase has been found to increase both mass recovery and resolution of many proteins. Trifluoroacetic acid (TFA) is the most popular one because it is an excellent solubilizing agent and allows detection of peptides below 230 nm (7, 8). The most popular mobile phase is a gradient ranging from 0.1 percent TFA to 0.1 percent TFA in propanol or acetonitrile.

The most suitable reversed phase columns to date have been organosilane-derivatized porous silica matrices. When an alkoxy- or chlorosilane containing an organic substituent is reacted with a silica surface, the organosilane is attached by siloxane bonding (9). This process causes the surface to acquire some of the properties of the organic moiety. Ethyl-, n-propyl-, n-butyl-, n-octyl-, n-octadecyl-, cyanopropyl-, alkylphenyl-, and diphenyl- silanes applied in a monolayer or polymeric film have all been used successfully in the separation of proteins.

In contrast to ion exchange chromatography, in which the retention of proteins is relatively simple and the protein conformation is usually retained, reversed-phase chromatography has very complicated retention mechanisms because (i) hydrophobic interaction figures prominently in tertiary structure, (ii) proteins have a tendency to internalize hydrophobic residues within their three-dimensional structure,

(iii) pairing agents are often required in the elution, (iv) the mobile phases used for elution at least partially disrupt the three-dimensional structure of the protein, and (v) the proteins are frequently denatured while adsorbed on the surface. It is not surprising that there has been no well accepted theory concerning the retention mechanism for proteins on reversed-phase columns; an exact description of reversed-phase chromatography of proteins is many years away.

RPC currently is the most popular separation technique for protein digests and peptides of various origins.

Affinity Chromatography

Affinity chromatography is based on the exceptional ability of biologically active macromolecules to bind specifically and reversibly to complementary molecules, generally called the affinity ligands. The specificity is achieved through favorable interactions between a ligand and the specific macromolecule to which it binds. The specific interactions which stabilize the ligand-macromolecule complex include (i) close packing of atoms, (ii) a maximum of hydrogen bonds, (iii) complementary pairing of charges, and (iv) buried hydrophobic groups.

If a ligand is immobilized to a solid support and a solution containing the biologically complementary molecule in active form is passed through a column of this ligand, then all the compounds which have no affinity for the ligand will pass through unretarded; while those showing an affinity for the ligand will adsorb onto the column. These can be released later from the complex by using a solution of the soluble ligand or by changing the mobile phase composition. Dissociation of the complex can often be achieved by changing the pH, ionic strength, or temperature.

Through the use of high-speed pumping systems and porous silica supports with covalently bonded ligands, affinity chromatography separations have been achieved in a few minutes or less (10). Such fast separation makes the technique particularly useful for repetitive types of separations such as industrial and laboratory purifications and clinical analyses. Many other applications have also been developed in various fields (11).

Affinity ligands are usually attached to supports by covalent bond formation or immobilization, which is usually a two step process. In the activation step, the support is treated with a reactive compound in aqueous or organic solution to produce an activated matrix. After removal of excess activating agent, the coupling step between activated

matrix and ligand is performed in aqueous solution. Ideally, a dense, stable coverage of the support with fully active ligands is desired. However, many of the ligands are large, fragile molecules whose three-dimensional structure and orientation on the support are important for their activity. Hence, optimum immobilization conditions often must be found empirically.

There are several factors one must consider in choosing immobilization conditions. One is the functional group on the ligand, which is determined by the coupling chemistry. Most immobilization methods use free amino groups for coupling. Second, a spacer arm is often needed so that the coupling sites on the ligand can be reached. Third, the reactivity of the functional groups on the ligand and the hydrolytic reactivity of active groups on the support may be affected by pH. In addition, the ligand could be irreversibly harmed by extremes of pH. Also, a high surface concentration of spacer arms on the support could lead to the immobilization of the ligand via multipoint attachment, which may cause loss of biochemical activity of the ligand as a result of distortion of the ligand, steric hindrance of its binding sites, or other undesirable effects (11). The optimum level of spacer arm concentration in terms of both specific activity and amount of ligands attached mainly depends on the nature of the support,

the coupling chemistry, and the type of ligand to be coupled. This is determined purely on an empirical basis.

The immobilization method is virtually universal for different applications regardless of the nature of the field to which the method is applied. Examples can be found in enzyme engineering where the immobilized enzymes function as catalyst, and in organic synthesis where an enzyme dis-criminates between enantiomers (12). The general strategy in the preparation of affinity chromatographic supports should be applicable to other immobilization practices.

Packing Material

Because its mechanical strength makes it suitable for high speed and high pressure operation, silica has become the most common support material used to prepare phases for protein HPLC. Nonspecific adsorption of proteins by bare silica due to the anionic silanol groups, which was a severe limitation at the time when it was first developed in 1970s (13), has been taken care of by coating the surface with a hydrophilic layer (14). The major drawback of the silica support is the dissolution of the material, especially at extreme pH values (>8, <2). Even well-bonded silicas release small quantities of silicic acid into the medium;

fortunately, silanol anions generally are regarded as nontoxic and inert toward proteins. The cost of silica and polymer supports is about the same for small scale separations, because the unit cost of polymer supports is greater but they are more durable. For large quantity separation of proteins, silica tends to be substantially cheaper. While traditional low performance liquid chromatography is characterized by polymer supports, modern HPLC achieves its high speed by using silica supports. Silica gel was used throughout this work.

Stationary Phase

The eminence of the stationary phase in HPLC can be highlighted by examining the structural features of proteins. Standard works in protein chemistry establish exhaustively that the distribution of amino acids at the surface of a protein is heterogeneous, that it is determined by the primary, secondary, and tertiary structure, and that it is influenced by environmental conditions (15, 16). Hydrophobic residues, for example, have a tendency to turn inward in aqueous systems and outward in organic solvents. The relevance of this to chromatography is that one area at the protein surface may have a higher density of those residues

participating in binding than another area; that is, a specific orientation of macromolecules on an adsorptive surface is to be expected. For example, a series of mutant lysozymes from different species has been used to show that in hydrophobic interaction chromatography only the face of the molecule opposite to the catalytic cleft is involved in chromatographic retention (17). Changes as small as the ionization of a single histidine can be detected easily when they are in this chromatographic contact region. In contrast, amino acid substitutions outside of this chromatographic contact region have no influence on retention at all. There are also cases in which basic amino acids can be added to the external surface of a protein and have no effect on retention in cation exchange chromatography, although additions in other areas increase retention (18). These examples demonstrate that there are specific contact regions or orientations in the different chromatographic modes. The phenomenon is certainly not unique in nature. If true, then the interactions in the contact area between the column and protein are the dominant factors controlling adsorption. Therefore, the characteristics of the stationary phase becomes very important in terms of the binding affinity, retention mechanisms, selectivity, etc., since the properties of protein surface are more or less fixed. The stationary phase determines which spot on the

protein surface binds and in what way. It should not be surprising that changes in stationary phase variables such as ligand density could lead to drastic changes in protein retention, because the protein structure is very delicate and sensitive to the stationary phase.

In fact, the stationary phase plays an even more important role than discussed above. It affects not only retention of solutes but also resolution, capacity, mass recovery, etc. (4, 19). There is no doubt that the stationary phase is the governing factor in HPLC design although the mobile phase is also manipulated to enhance the performance.

With the exception of size exclusion chromatography (SEC), the stationary phase in HPLC is usually a thin layer bonded to the surface of porous particles. Because true partition chromatography is not used for the separation of proteins, retention in all modes of chromatography other than SEC is considered an adsorption process. With adsorption processes, the surface configuration together with the chemical nature of the surface play a prominent role. It is well recognized that the ability to separate proteins with good resolution depends on the design of the stationary phase. More important is the fact that retention of proteins is not a simple extension or summation of the retention properties of the individual amino acids that compose the polypeptide. Any

change in the property of the bonded phase affects protein
retention in a very complex manner. Improving the performance
of HPLC by the optimization of the stationary phase is still
largely done on a "trial and error" basis. Basic aspects
concerning the design of stationary phases include the nature
of the bonded phase or the kind of ligand bonded, the ligand
distribution, the ligand density, etc. The first two factors
have been extensively studied by several research groups. For
example, Lochmuller et al. have used the luminescence of
pyrene silane bonded to silica gel to study the ligand organi-
zation and distribution (20). Regnier and co-workers have
examined many bonded phases in terms of their hydrophobici-
ties, charge characteristics, chain lengths, etc. (21, 22).
However, there have been very few published reports regarding
the stationary phase ligand density. This is primarily due to
technical difficulties of preparing low ligand density
supports without exposing the silanol group that is responsi-
ble for the nonspecific adsorption of proteins, as well as to
the traditional position that high ligand density is always
superior in terms of resolution power.

Ligand Density

There has been growing experimental evidence that macromolecules behave remarkably different from small solutes in the chromatographic process. For example, if isocratic elution, which is routinely used for small molecules, is used to elute macromolecules, extraordinarily broad peaks will be obtained (23). In addition, the elution salt used was found to affect the bandbroadening and selectivity of protein separations, contrary to what has been observed for small molecules (24). Furthermore, mini columns have been demonstrated to be nearly as efficient as large columns for protein separations in several chromatographic modes (25-27), while for small molecules the separation power is proportional to column length (28). Finally, the retention time of proteins, unlike that of small molecules, has been found to be independent of alkyl chain length in reversed-phase chromatography (22). All of this indicates that macromolecules may follow different adsorption-desorption mechanisms than those experienced by small molecules.

The fact that protein retention is so much different from that of small molecules makes many of the chromatographic theories derived from small solutes invalid or indefinite as they are applied to protein chromatography. The fundamental

difference between small solutes and macromolecules is the
valency by which the analytes bind to a support. While small
molecules are generally monovalently bound to a support
regardless of ligand density, proteins can bind to the
support through multiple-sites, depending on the ligand
density. Most of the high-performance IEC and RPC columns in
use today have surface ligand concentrations of 2-4 umol/m^2.
This corresponds to single ligand groups that occupy 50-100 Å
and that are 7-10 Å apart on the surface. Because adsorbed
proteins can occupy thousands of Å^2 of the surface, there is
ample opportunity of multiple-site adsorption. In the case of
bovine serum albumin, it has been shown that the Z number (or
apparent number of interaction sites) is greater than 200
(29). This shows the multiple-site adsorption character and
gives an idea of the contact surface area.

Because of the multiple-site nature of protein adsorp-
tion, it was anticipated that proteins would respond to ligand
density changes in a way different from that seen with small
molecules in the following ways: (i)the multiple-site adsorp-
tion would be expected to make the protein retention depen-
dence of ligand density different from that of small mole-
cules. The proteins should be much more sensitive to ligand
density changes due to changes in the Z number, (ii) multi-
ple-site adsorption would be expected to promote conforma-

tional change of proteins leading to denaturation (30), which is also ligand density dependent, (iii) the large amount of displacing agents bound to the heterogeneous protein surface would further complicate the retention mechanisms (31), (iv) the kinetic constants of proteins would be expected to be affected by ligand density as a result of the changing in binding mechanisms, (v) resolution has been shown to be affected by ligand density (19) in such a way that a selectivity change is anticipated, (vi) mass recoveries are usually found to be lower with proteins (32) due to the multiple-site adsorption.

In order to control and manipulate protein separations, there is a need to establish a theoretical description of protein chromatography since most chromatographic theories pertinent to small molecules are not directly applicable to proteins. Knowledge of ligand density effects on protein retention is essential to the understanding and advancement of protein chromatography. This knowledge was anticipated to be obtainable from a well designed study using variable ligand density approach, in which such retention variables as k' (capacity factor), the Z number, etc., were examined as a function of ligand density.

In addition, detailed information concerning the retention mechanism for proteins drawn from modifying the sta-

tionary phase, such as by altering ligand density, can reveal the manner by which protein molecules interact with the stationary phase. This should aid in understanding the interactions between proteins and other biological molecules as well, since such interactions can be very similar to retention on a chromatographic column. For example, cytochrome C, a strongly cationic protein, has been found to be sensitive to ionic strength in its reaction with cytochrome oxidase (33-36). But whether this is caused by the simultaneous diminishing of binding strength of all sites or by the decrease of number of binding sites remained unknown due to the limitation of the system. It was anticipated that chromatographic studies would uncover whether the decrease in interaction strength was accompanied by a change in the number of sites. This can be done by measuring the Z number at various ionic strength. Such information would be valuable not only for the cytochrome C - cytochrome oxidase interaction but also for other similar systems.

The precision of Z number measurements can also be enhanced by using chromatographic measurements. While the Z number can be determined down to the second decimal place by chromatography, the values reported from peptide mapping (37) are only estimated to the nearest integer.

Published reports involving ligand density studies have been largely found in the preparation of immobilized proteins for affinity chromatography. The specific activity of many of these immobilized macromolecules was found to be related to the surface concentration of spacer arms (38-43). Also, Anderson et al. examined several retention models using concanavalin A as a probe molecule at various ligand densities (44). Retention was found to change from a monovalent interaction to a divalent interaction as the ligand density increased. In addition to affinity chromatography, Kopaciewicz et al. studied protein binding, retention, and resolution as related to ligand density in IEC (19). Vanecek and Regnier measured protein ion exchange capacity as a function of ligand density (45).

The purpose of this study was to develop a silica support of variable ligand density, followed by a careful examination of ligand density effects. Such a support should be easily adaptable to various separation modes so that the ligand density effects can be investigated for several different chromatographic modes. The scope of this work covered both theoretical aspects, such as selectivity as related to protein binding sites and practical aspects such as mass recovery, denaturation, resolution, and specific activity, etc., based on the variation of ligand density.

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SECTION I.   PREPARATION OF STATIONARY
PHASES OF VARIABLE LIGAND DENSITY

INTRODUCTION

Studies of the chromatographic behavior of biomacromolecules on reversed-phase (1-5), hydrophobic interaction (6-9), ion-exchange (5, 10-13), and affinity (14) columns have shown that multipoint adsorption greatly affects the retention and kinetic behavior of these molecules. It is also known that the specific activity of immobilized biomacromolecules is a function of the number of bonds per macromolecule (15-20). A simple method for varying the surface densities of the functional groups responsible for retention or immobilization is needed to study these phenomena to optimize chromatographic performance. A macromolecule may cover an area of approximately 10^4 Å^2 on the surface of a support. When compared to the approximately 40 Å^2 area covered by an alkyl chain in a commercial reversed-phase column, it is apparent that to avoid multipoint interaction one might want to prepare adsorbents with functional group surface densities of as low as 1% of the maximum value. Kopaciewicz et al. synthesized polyethyleneimine-based anion exchangers covering a 3 fold range of ligand density (13). They observed weaker retention of proteins on the low-coverage supports and higher recoveries of some hard-to-elute proteins. However, selectivity did not appear to change significantly with ligand density.

In affinity chromatography, ligand density is often con-
trolled by using limiting amounts of the activating agent.
However, many such reagents, e.g., 1,1'-carbonyldiimidazole,
are simply too reactive, and thus reproducibility and uniform
distribution of active groups may be difficult to obtain (21).

In this study, cyclic anhydrides were utilized for the
modification of hydroxyl-containing supports. Since cyclic
anhydrides are less reactive than acetic anhydride (22) and
require approximately 1 h to completely react with simple
alcohols (23), it was anticipated that ligand density could be
altered by controlling reaction time, temperature, and anhy-
dride concentration. Succinic anhydride has been previously
used in affinity chromatography for the modification of amine-
containing polyacrylamide (24) and agarose (25) supports in
aqueous solution primarily for the purposes of introducing a
spacer arm and/or a carboxyl functionality. Diglycolic
anhydride has been used for the preparation of carboxymethyl
cation exchangers from polyethyleneimine-silica supports (26).
No attempt was made in any of these studies to vary the ligand
density (24-26).

EXPERIMENTAL

Reagents

Tetrahydrofuran (THF) was distilled from sodium metal before use. Ethanol was distilled from calcium oxide. Ferric perchlorate, nonyellow, was obtained from GFS Chemicals (Columbus, OH). 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC), glucosamine hydrochloride, lysozyme (egg), cytochrome c (equine), hemoglobin (bovine), and ribonuclease A (bovine) were from Sigma (St. Louis, MO). N-acetyl-D-glucosamine, ethyl caproate, diglycolic acid, succinic anhydride (SA), and diglycolic anhydride (DGA) were from Aldrich (Milwaukee, WI). Hypersil 100 and 300, 5-um particle diameter, Nucleosil 300, 5-um, and LiChrospher SI300 and SI4000, 10-um, were from Alltech (Deerfield, IL). Dimethyl diglycolate was prepared from diglycolic acid and diazomethane using a Diazald kit (Aldrich).

Diol-Bonded Silica

All of the silicas were silanized by use of an aqueous procedure in which the conditions were adjusted according to

the surface area of the support (27). The diol content was determined by periodate titration (27).

Spacer Arm Attachment

One gram of diol-bonded silica was refluxed in 10-30 mL of 0.05 or 0.5 M anhydride in dioxane or distilled THF. The reaction was performed on a shaking table (Eberbach, Ann Arbor, MI) to keep the silica suspended. The volume of solution was large enough so that the anhydride was present in at least 4-fold excess relative to the hydroxyl content of the support. After reaction, the silica was washed twice with warm solvent and twice with room-temperature solvent on a fine-porosity fritted glass filter, then dried in a vacuum oven overnight at room temperature.

Ferric Hydroxamate Ester Assay

A previously described procedure (28) was used except for the following modifications: ethyl caproate and dimethyl diglycolate were used as standards for the succinic anhydride and diglycolic anhydride modified silicas, respectively. Samples containing 0.2-1 μmol of ester in 0.5 mL of distilled ethanol were treated with 0.25 mL of alkaline hydroxylamine

reagent and sonicated for 5 min. After 1 h of reaction at
room temperature, 4.0 mL of ferric reagent was added followed
by 2 min of sonication and an additional 5-min reaction at
room temperature. The silica was removed by centrifugation
prior to the final absorbance measurement at 530 nm.

Glucosamine Immobilization

Glucosamine was coupled to the carboxyl groups of the
spacer arm using a direct carbodiimide coupling procedure
(25). To 20-40 mg of the silica sample in a test tube were
added 0.5 mL each of 0.2 M glucosamine hydrochloride, pH 5.0,
and 0.2 M EDC, pH 5.0. The mixture was degassed, flushed with
N_2, stopped, and shaken for 2 h at room temperature. The
product was filtered and washed twice with deionized water and
twice with methanol, then dried overnight in a vacuum oven at
room temperature.

Ferricyanide Assay

Silica samples containing 30-250 nmol of glucosamine were
assayed by the alkaline ferricyanide method (29) using N-
acetyl-D-glucosamine as standard. Silica was removed by
centrifugation prior to the absorbance measurement.

Ion-Exchange Capacity

Cation-exchange capacities were determined for supports packed into 10 cm x 4.1 mm columns. Fifty milliliters of 0.1 M sodium phosphate buffer, pH 6.7, was pumped through the column at 3 mL/min to ensure that the ion-exchange sites were in the sodium form. After the column was washed with 100 mL of deionized water, 180 mL of 2 M lithium chloride was applied, followed by washing with 200 mL of water, and finally by 54-105 mL of 0.05-0.5 M potassium chloride (depending on the actual ion-exchange capacity according to eq 5-5 of ref. 30). After dilution of the potassium ion concentration to 0.04 M, the lithium was determined by atomic absorption spectroscopy.

Chromatography

Cation-exchange chromatography was performed at room temperature using a flow rate of 1 mL/min and linear gradients from 0.01 M sodium phosphate, pH 6.0, to the same buffer containing 0.2 M Na_2SO_4. Ten microliters of 5 mg/mL protein solutions were injected. Detection was by absorbance at 280 nm. Two columns, 10 cm x 4.1 mm, containing DGA-modified Nucleosil 300 diol were tested. The "high" coverage support was

prepared by refluxing 1.0 g of diol-bonded silica in 20 mL of 0.5 M DGA/dioxane for 12 h, while the "low" coverage support was prepared by using 40 mL of 0.05 M DGA/THF.

RESULTS AND DISCUSSION

The general approach taken here for the control of ligand density was to prepare a "layered" adsorbent (Figure 1). The first layer was a neutral, nonadsorptive diol-bonded phase attached to silica. The second layer was a "spacer arm" with terminal COOH groups of variable surface density obtained by reacting the diol-bonded silica with a cyclic anhydride under controlled conditions. By use of well-known carbodiimide coupling methods (25, 31-34), a third layer containing the actual chromatographic adsorbent---alkyl chains, ion-exchange groups, immobilized proteins, etc.---may be attached.

Assay of Spacer Arm Surface Concentrations

The major difficulty encountered in this work was in finding methods for the assay of low concentrations of spacer arm (10 μmol/g) in small amounts of silica (1-10 mg). One requirement of a spectrophotometric assay was that the colored product be released into solution rather than remain bound to the silica surface. The ferric hydroxamate method (28) for the determination of ester groups (hereafter called the ester assay) and a second method (hereafter called the glucosamine assay) in which glucosamine was first coupled to the spacer

Figure 1. Diagram of a layered adsorbent of controlled ligand density showing the surface of the silica support (a), the diol-bonded phase (b), the spacer arm layer of controlled density (c), and the adsorbent layer (d)

(d)

(c)

```
      R                                                                R
      |                                                                |
      C=O                                                              C=O
      |                                                                |
      CH₂                                                              CH₂
      |                                                                |
      O                                                                O
      |                                                                |
      CH₂                                                              CH₂
      |                                                                |
      C=O                                                              C=O
```

(b)

```
  OH      O       OH      OH      OH      OH      OH      O
  |       |       |       |       |       |       |       |
  CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂
  |       |       |       |       |       |       |       |
  CHOH    CHOH    CHOH    CHOH    CHOH    CHOH    CHOH    CHOH
  |       |       |       |       |       |       |       |
  CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂
  |       |       |       |       |       |       |       |
  O       O       O       O       O       O       O       O
  |       |       |       |       |       |       |       |
  CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂
  |       |       |       |       |       |       |       |
  CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂
  |       |       |       |       |       |       |       |
  CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂     CH₂
  |       |       |       |       |       |       |       |
 -Si-    -Si-    -Si-    -Si-    -Si-    -Si-    -Si-    -Si-
```

(a)

```
  O       O       O       O       O       O       O       O
  |       |       |       |       |       |       |       |
 -Si-    -Si-    -Si-    -Si-    -Si-    -Si-    -Si-    -Si-
  |       |       |       |       |       |       |       |
```

arm using a water-soluble carbodiimide, then assayed by the
ferricyanide oxidation method (29), were utilized.

Ester Assay Results

Figure 2 shows the spacer arm concentrations as a
function of time under several sets of conditions. As one
would expect, the reaction was faster in dioxane (boiling
point 101°C) than THF (boiling point 66°C). Of the two
anhydrides, DGA was more reactive than SA. More spacer arm
could be attached to Hypersil 100 diol than to the lower
surface area Hypersil 300 diol. The initial diol concentra-
tions of these two supports were 630 μmol/g and 190 μmol/g,
respectively. The maximum ester content was 1.4-1.8-fold
larger, suggesting that the anhydride was reacting with both
hydroxyl groups of the diol. Thus, with this method it was
possible to prepare supports with a very high density of
carboxyl groups.

In the ester assay, blank values obtained by refluxing
diol-silica without anhydride present were subtracted. These
blank values (Table I) were constant with time but varied with
the solvent and silica used. The blanks were significantly
higher if the silica was stirred with a magnetic stirring bar
instead of being shaken, so stirring should be avoided.

Figure 2. Ester content as a function of time for Hypersil
           100 diol reacted with 0.5 M DGA/dioxane (○) and
           0.5 M DGA/THF (□) and for Hypersil 300 diol
           reacted with 0.5 M DGA/dioxane (●), 0.5 M DGA/THF
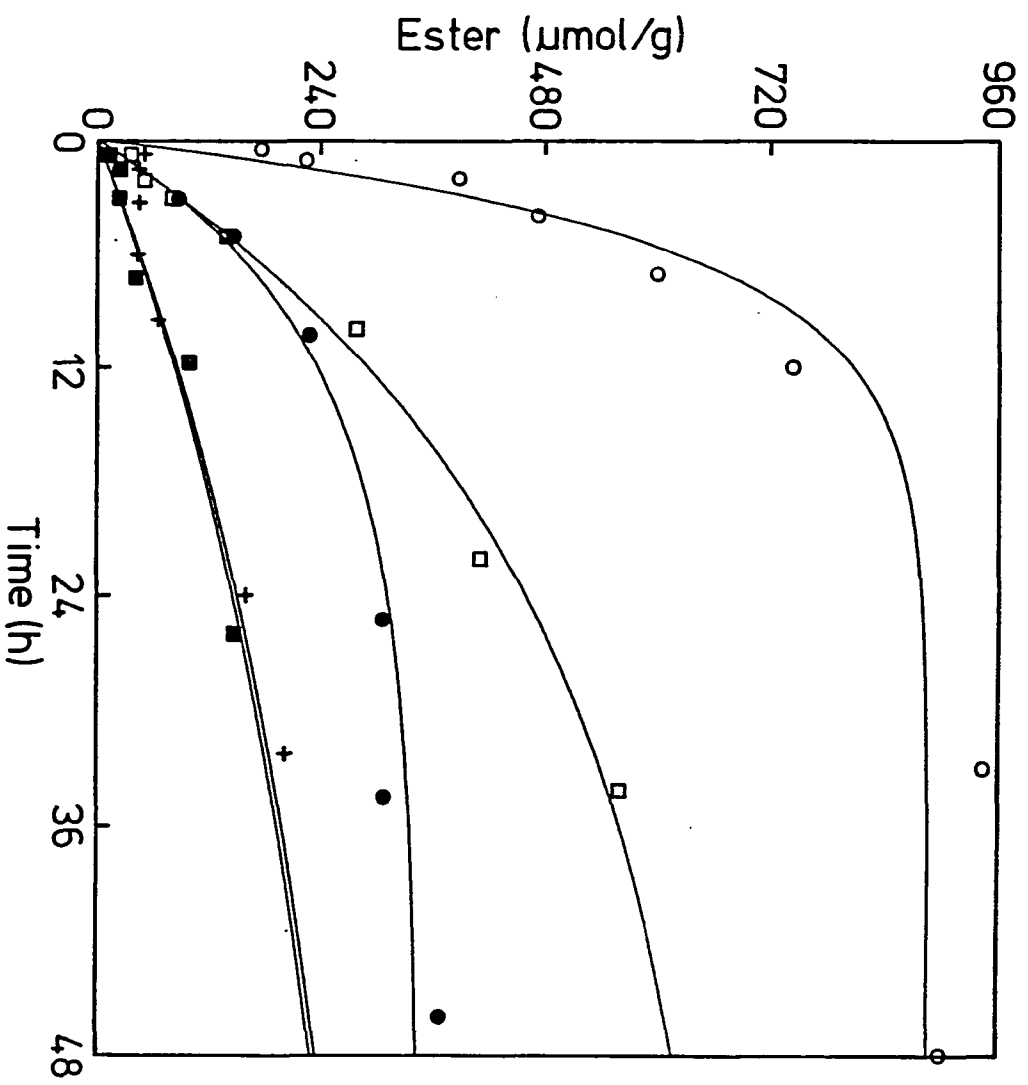   (■) and 0.5 M SA/dioxane (+)

Table I. Precision and Blank Values for the Assay Methods

Assay	Support	Solvent	Blank $\pm\sigma$ (µmol/g)	σ of samples (µmol/g)[a]
Ester	Hypersil 100	THF	26 $\pm$ 12	$\pm$ 18
Ester	Hypersil 300	THF	9 $\pm$ 12	$\pm$ 14
Ester	Hypersil 300	Dioxane	19 $\pm$ 14	$\pm$ 29
Glucosamine	All	Both	2.0 $\pm$ 1.8	$\pm$ 2.7

[a]Including error of blank subtraction.

The free carboxyl end of the spacer arm could potentially form a second ester bond with the support, thus producing a material with a high ester content but a low free carboxyl content. To examine this possibility, the free carboxyl groups were further derivatized with diazomethane to produce the methyl esters. The data in Table II show a doubling of the ester content (within the error of the assay), thus indicating that most of the spacer arms were attached to the support at only one end. The glucosamine assay data below provided further support of this conclusion.

Glucosamine Assay Results

This method could be used to assay low-coverage supports because of its low background. For example, by altering reaction time, solvent (i.e., temperature), and anhydride concentration, it was possible to prepare supports ranging from a few percent to 100% of the maximum spacer arm coverage (Figure 3). Since the reactions occurred on a time scale of hours, the spacer arms were probably randomly located on the support surface. However, further studies are needed to prove that the spacer arms were not clustered on the surface, as has been observed in the preparation of low-coverage supports by silanization (35).

Table II. Effect of Diazomethane Treatment

| Time (hr) | Ester Content (μmoles/g)[a] | | Ratio |
	Initial	Diazomethane-Treated	
1	120	240	2.0
2	210	320	1.5
8	360	1200	3.3
14	370	1080	2.9
24	560	1160	2.1
		Average	2.4 ± 0.7

[a]LiChrospher SI300 diol modified using 0.5M SA/dioxane.

Figure 3. Ester content determined by the glucosamine assay
          as a function of time for Hypersil 300 diol reacted
          with 0.5 M DGA/dioxane (●), 0.5 M DGA/THF (■),
          0.05 M DGA/dioxane (◆) and 0.05 M DGA/THF (▲)
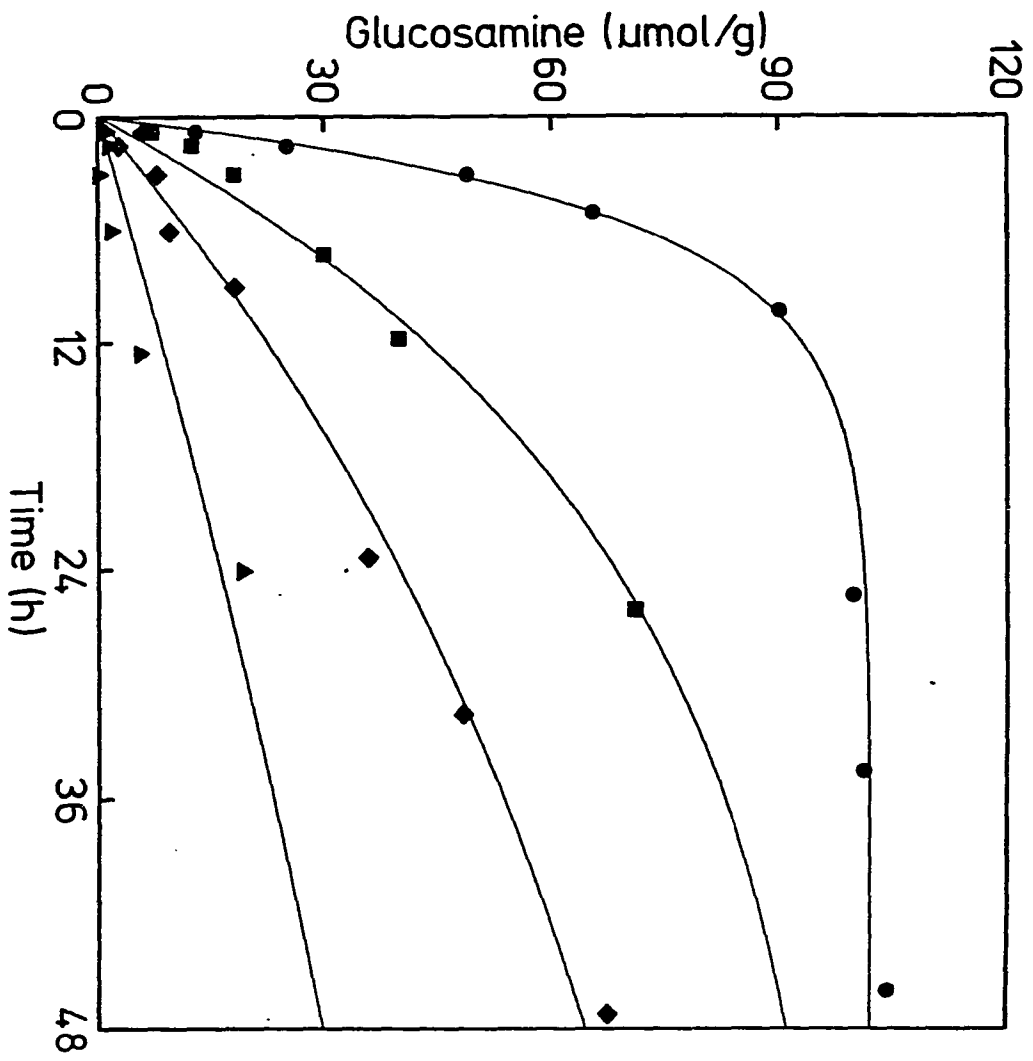
A comparison of the data in Figures 2 and 3 shows that the maximum glucosamine coverage was approximately one-third of the maximum value from the ester assay. This discrepancy apparently was due to a poor yield of the carbodiimide coupling reaction. Thus, the glucosamine assay should only be used for purposes of comparison with ester assay.

Rate Constant Data

To fully utilize the data obtained, the kinetics of the spacer arm incorporation were examined. The reactions were expected to be pseudo zero order with respect to anhydride concentration since a large excess was used. Therefore, the data were fit to a first-order rate expression.

$$A_t = A_\infty (1 - \exp(-kt))$$

Where A_t and A_∞ are the spacer arm concentrations ($\mu mol/g$) at time t and at the completion of the reaction, respectively, and k is the apparent rate constant. The results are listed in Table III, and the goodness of fit can also be judged from Figures 2 and 3.

From the glucosamine assay data of Table III, it can be seen by comparing 0.5 M and 0.05 M DGA data that the reaction is first order with respect anhydride concentration.

Table III. Kinetic Data

Assay	Support	OH Content (μmol/g)	Conditions	A_∞ (μmol/g)	k (hr^{-1})
Ester	Hypersil 100	1260	0.5 M DGA/Dioxane	890	0.20
Ester	Hypersil 100	1260	0.5 M DGA/THF	700	0.045
Ester	Hypersil 300	380	0.5 M DGA/Dioxane	350	0.096
Ester	Hypersil 300	380	0.5 M DGA/THF	350[a]	0.023
Ester	Hypersil 300	380	0.5 M SA/Dioxane	350[a]	0.024
Glucosamine	Hypersil 100	1260	0.5 M DGA/THF	150	0.14
Glucosamine	Hypersil 300	380	0.5 M DGA/Dioxane	100	0.21
Glucosamine	Hypersil 300	380	0.05 M DGA/Dioxane	100[a]	0.021
Glucosamine	Hypersil 300	380	0.5 M DGA/THF	100[a]	0.047
Glucosamine	Hypersil 300	380	0.05 M DGA/THF	100[a]	0.007
Glucosamine	Hypersil 300	380	0.05 M SA/Dioxane	100[a]	0.036
Glucosamine	Lichrospher SI4000	55	0.05 M DGA/Dioxane	20	0.08

[a] A_∞ was fixed at the value from the 0.5 M DGA/Dioxane reaction.

The rate constants determined under a fixed set of conditions were somewhat insensitive to the type of support used. Reaction rates of Hypersil 100 and LiChrosphere SI 4000 were both somewhat faster than Hypersil 300, but considering the much smaller diol content of the LiChrospher SI4000 and the higher diol content of the Hypersil 100, the rates changed relatively little. This is convenient in that the data in Table III may prove to be useful for predicting the surface coverage of other supports under the same conditions.

There is a discrepancy between the rate constants determined by the two assay methods (Table III). This might simply reflect the error in performing the fits to the data, but could also be the result of convoluting the spacer arm reaction with the glucosamine coupling reaction. The latter is more likely, since, as pointed out earlier, the glucosamine coupling reaction was performed under conditions where the yield was only about one-third. Thus the rate constants determined from the ester assay are probably more accurate.

Ion-Exchange Capacities

To confirm the results of the ester assay, large quantities of several supports of variable ligand density were prepared and packed into chromatographic columns. The lithium

ion-exchange capacities were measured and are listed in Table IV. The agreement between calculated and measured values was generally good.

Integrity of Diol Phase

Since bonded phases in HPLC are not usually heated for extended periods of time in organic solvents, it was necessary to ensure that this treatment did not damage the diol phase. This was examined in two ways. First, the diol content of Nucleosil 300 was determined before and after refluxing for 16 h in dioxane. The results were as follows: initial diol content, 251 ± 5 μmol/g; after refluxing in dioxane, 251 ± 3 μmol/g. Thus, there was no apparent loss of the diol groups.

In a second set of experiments, several diol-bonded Hypersil 300 samples were refluxed in dioxane or THF for times raging from 45 min to 46 h. Glucosamine was then coupled to these samples to see if any carboxyl groups had been introduced by oxidation. In each case the amount of glucosamine attached was negligible (0.8 μmol/g average). Therefore, from these two experiments it did not appear that heating in dioxane or THF significantly damaged the diol phase.

Table IV. Comparison of Theoretical and Measured Ligand
          Densities

Solvent	Reaction Time[a](h)	Ligand Density (μmol/g)		
		Theory[b]	Ester Assay	Ion-Exchange Capacity
THF	3.4	20	27	26
dioxane	2.2	50	56	61
dioxane	4.1	83	88	97
dioxane	13.2	183	220	197
dioxane	60.0	313	390	236

[a]The anhydride concentration was 0.5 M DGA in all cases.
[b]Calculated from eq. 1 assuming A_{∞} = diol concentration
and k = 0.02 h^{-1} for THF and k = 0.1 h^{-1} for dioxane. The
support was Nucleosil 300.

Integrity of the Ester Bonds

It is well-known that ester hydrolysis is catalyzed by acidic or basic conditions (36). Since basic conditions also damage silica supports, it is primarily the acidic conditions that are of concern here. For example, pH 2-3 is often used to elute solutes from affinity chromatography columns (37). To examine this, a 0.01 M chloroacetic acid buffer, pH 2.5, was pumped at room temperature through a DGA-modified diol-bonded silica column for 10 days at 0.1 mL/min. Ester and ion-exchange capacity assays indicated a 10-15% loss of the carboxyl groups. In comparison to a study performed using methylacetate in an aqueous solution of the same pH, the ester groups on the silica support appeared to hydrolyze less than one-tenth as rapidly. This relatively slow loss of ester groups should not present problems unless the columns are continually operated at low pH.

Chromatographic Studies

The retention of several proteins on low-coverage (20 μmol/g) and high-coverage (210 μmol/g) supports was examined. These DGA-modified supports were expected to behave in a manner similar to the widely used carboxymethyl cation-

exchange resins. Commercial carboxymethyl resins are typically prepared by using chloroacetic acid under conditions too alkaline for silica supports (38, 39).

A gradient elution chromatogram of four proteins on the high-coverage support is shown in Figure 4a. Retention was quite strong with the cytochrome C eluting after the end of the gradient. Under the same conditions, decreased retention and resolution were observed on the low-coverage column (Figure 4b). By use of a shallower gradient, retention and resolution were increased (Figure 4c). In addition, a significant change in selectivity was observed. The elution orders of the ribonuclease A-hemoglobin and lysozyme-cytochrome C pairs were observed. The cause of the elution order reversal will be presented in the next section.

Figure 4. Cation-exchange chromatography of ribonuclease A
(R), hemoglobin (H), lysozyme (L) and cytochrome C
.(C) on high-coverage (a) and low-coverage (b,c)
columns with gradient times of 10 min (a,b) and 28
min (c). The proteins were applied in a ratio of
3:2:1:1, respectively
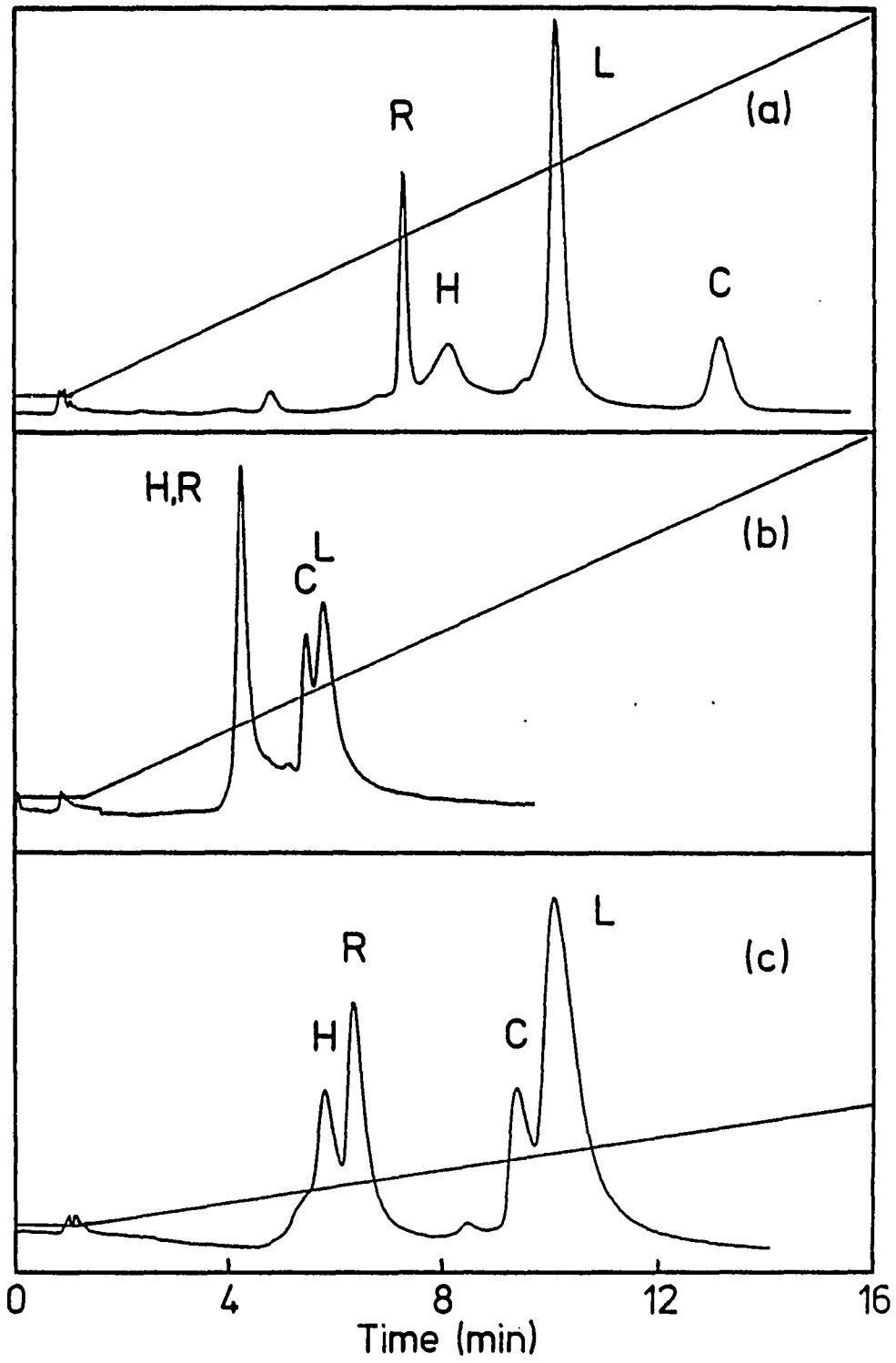
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SECTION II. EFFECTS OF STATIONARY PHASE LIGAND DENSITY
ON HIGH-PERFORMANCE ION EXCHANGE CHROMATOGRAPHY OF PROTEINS

INTRODUCTION

Ion exchange chromatography is a powerful separation tool for the isolation of proteins. This method has seen rapid growth over the past decade (1, 2) and numerous packing materials have been developed to optimize the chromatographic performance (3-9). The main advantage is that it gives a high recovery for proteins in terms of both mass and biological activity. Although the methodology has been frequently applied and the experimental conditions (i.e., mobile phase velocity, pore diameter, column length, salt composition, pH, temperature, loading capacity, etc.) have been thoroughly investigated (10-14), little is understood about the underlying retention mechanism and the factors which contribute to the selectivity.

In a recently proposed retention model for ion exchange chromatography of proteins, Kopaciewicz et al. (15) suggested that as a protein comes into contact with the chromatographic stationary phase, only a fraction of the protein surface covers the binding area and that the retention is exponentially related to the Z number (i.e., the number of binding sites on a protein surface interacting with the stationary phase). Since the number and distribution of charged groups on the surface of a macromolecule are fixed as well as its

chromatographic binding domain, it follows that the Z number and retention should be mainly dependent on the stationary phase ligand density. Consequently, the ligand density should have a profound effect on the binding mechanism and chromatographic behavior of macromolecules. Knowledge of ligand density effects on protein retention would aid in understanding the retention mechanisms of macromolecules, which is essential to achieving good chromatographic results. Unfortunately, there have been very few published reports describing the effect of ligand density on the performance of ion exchange chromatography of proteins. This has primarily been due to the unavailability of supports covering a sufficiently large range of ligand density.

In our laboratory, we have recently developed a technique (16) for the preparation of silica-based supports of variable ligand density. By using this technique, surface ligand densities ranging from a few percent to complete monolayer coverage have been obtained. Significant alteration in selectivity with changes in ligand density have been observed on these supports for cation exchange chromatography of proteins.

In this study, a series of supports of different ligand densities were prepared using the same technique. The ligand density quantitation and ligand distribution were evaluated chromatographically by measuring the k' of benzylamine as a

function of ligand density and the ion exchange capacity for lysozyme. The Z number was then measured for lysozyme and cytochrome C at different ligand densities in an attempt to relate it to the degree of protein retention. Band-broadening was also examined for the different support materials. The resolution and the phosphate-cytochrome C interactions were also investigated. Since the binding of basic proteins to the support and to enzymes have similar characteristics (17), the knowledge of protein-resin interactions may lead to a better understanding of the mechanisms involved in protein-enzyme interactions.

EXPERIMENTAL

Reagents

The cytochrome C (equine) and lysozyme (egg) were from Sigma (St. Louis, MO). The diglycolic anhydride (DGA), benzylamine, and diglycolic acid were from Aldrich (Milwaukee, WI). The Nucleosil 300-5 was from Alltech (Deerfield, IL). The tetrahydrofuran (THF), ethanol and diglycolic acid were treated as described previously (16).

Instrumentation

A model 344 gradient liquid chromatograph (Beckman, Berkeley, CA) was used. Absorbance was monitored at either 280 nm (proteins and acetone) or 262 nm (benzylamine) by a V^4 variable-wavelength absorbance detector (ISCO, Lincon, NE). Data were collected and processed on an Apple IIe computer via an ADALAB interface board (Interactive Microware, State College, PA).

Methods

All of the carboxylate cation exchange supports were prepared according to a previously published procedure (16). The ligand density was quantitated by the ferric hydroxamate ester assay (16). The support was suspended in 0.5 M sodium sulfate and packed into 100 x 4.1 mm I.D. columns at 5000 p.s.i. using the upward-flow method (18).

Chromatography

The weak mobile phase (A) was 0.01 M sodium phosphate (pH 6.0) and the strong mobile phase (B) was 0.01 M sodium phosphate, 0.2 M sodium sulfate (pH 6.0). The sodium concentration was adjusted by premixing A and B in the desired ratio. Chromatography was performed isocratically at room temperature using a flow rate of 1 ml/min. Ten microliters of either a 5 mg/ml protein solution or 10^{-3} M benzylamine solution were injected. The void volume was determined by injecting 10 microliters of 1% acetone. The experimental error of the Z number measurement was estimated to be $\pm$ 5% to $\pm$ 10% depending on the magnitude of the Z number.

The protein adsorption capacities were determined by continuously applying 0.5 mg/ml lysozyme to 6.2 x 2.1 mm I.D.

columns.   The breakthrough curves were then corrected for the void volume.

The statistical moments were found by using the modified B/A (0.5) method (19).

RESULTS AND DISCUSSION

Elution Salt and Its Activity

When proteins are eluted from an ion exchange column, the displacing power of a given salt may be different for different proteins (20). This adds one more dimension of selectivity to ion exchange chromatography and may be used to optimize the separation in addition to such parameters as temperature, stationary phase properties, etc. However, in this investigation emphasis was put on the effect of ligand density on protein retention. Thus, it was desired to avoid any factors that would differentially influence retention of different proteins other than ligand density. This requires that an elution salt be used which has the same displacing power for different proteins.

It was found that the displacing power of sodium sulfate for cytochrome C and lysozyme was virtually the same on a strong cation exchange column (20). Under the pH conditions used here, the ionization of carboxylate is believed to be complete (21), making the weak cation exchanger very similar to the strong cation exchanger from the electrostatic point-of-view. Thus, the sodium sulfate was chosen as the elution salt to simplify the retention mechanism.

For plots of log k' vs. log (l/Na activity) the activity
coefficient was not constant over the concentration range
studied. This was especially true at high salt concentra-
tions (e.g. when B% > 50%), where a two-fold change in B% led
to a 15% change in the activity coefficient (22). To avoid
error due to this, the activity of the sodium salt was used in
all calculations.

Ligand Distribution and Quantitation

In a previous report (16), the ion exchange capacities of
various ligand density packings were measured using atomic
absorption spectroscopy to check the reliability of the ester
assay. To further confirm the precision of the ester assay, a
plot of k' vs. ligand density was made using benzylamine as
the probe molecule, which eluted isocratically at pH 6.0. The
results are given in Figure 1. Apparently a linear relation-
ship was obtained between the k' of benzylamine and ligand
density in all cases except for the highest ligand density
data, indicating that the results of ester assay were general-
ly good.

The binding capacity of lysozyme on supports with various
ligand densities was measured using breakthrough curves. These
were also performed on blank columns which contained either no

Figure 1. Ligand density dependence of k' of benzylamine obtained with phosphate
buffer of pH 6.0
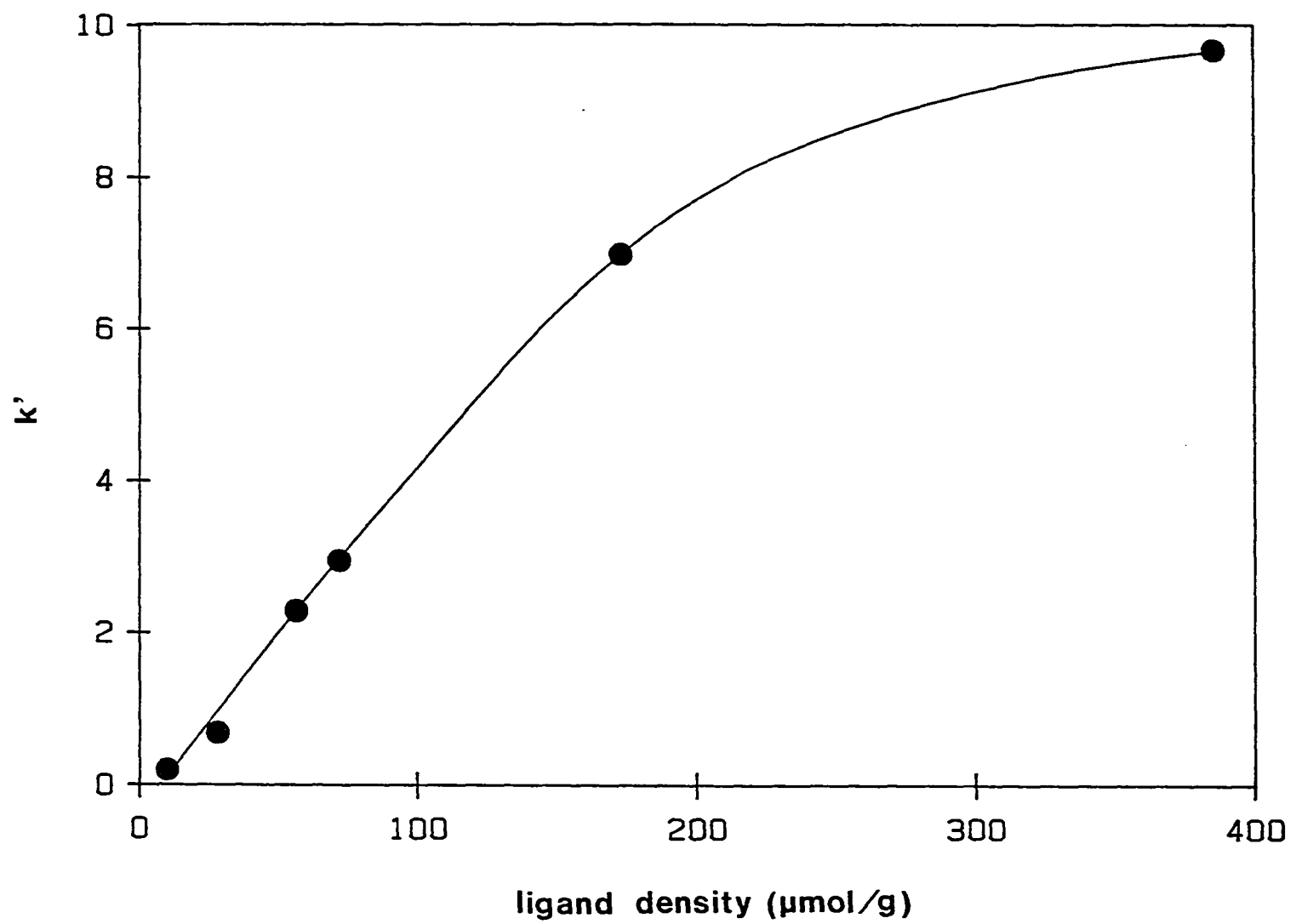
packing material or diol-bonded silica. These were done to
check for the possible presence of nonspecific adsorption.
Both blank columns showed essentially the same ion exchange
capacity, which was negligible vs. even the lowest ligand
density column. This demonstrates that nonspecific adsorption
was insignificant. This is probably due to the fact that the
diol layer is hydrophilic and covers all or most of the sila-
nol sites. For all other columns, lysozyme capacity data were
taken after previously saturating the column several times to
remove any irreversible adsorption sites. Figure 2 presents
the change in protein capacity with ligand density. Lysozyme
capacities remained fairly stable as the ligand density in-
creased from 72 μmol/g up to 494 μmol/g. Over the entire
range tested, the lysozyme capacity increased by 5-fold while
the ligand density increased by 17.5-fold. It appears that
the ligand distribution on these supports was reasonably uni-
form even on the low coverage column. However, further
experiments are needed to look more closely at the ligand
distribution.

Z Number and Stationary Phase Heterogeneity

Figure 3 shows the relationship obtained between the Z
number and the ligand density. The bottom two curves were

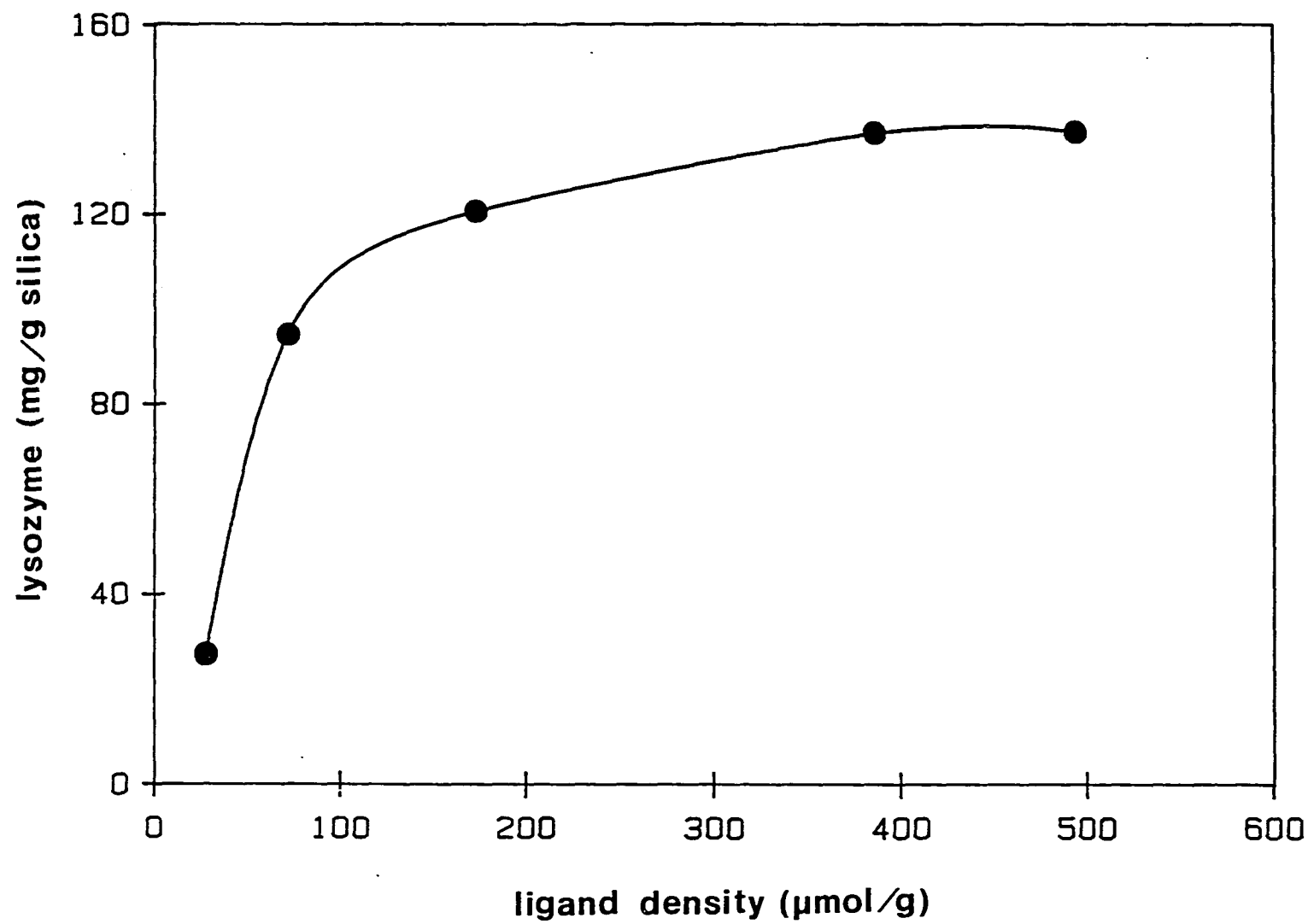Figure 2. Ion exchange capacity for lysozyme as a function of the ligand density
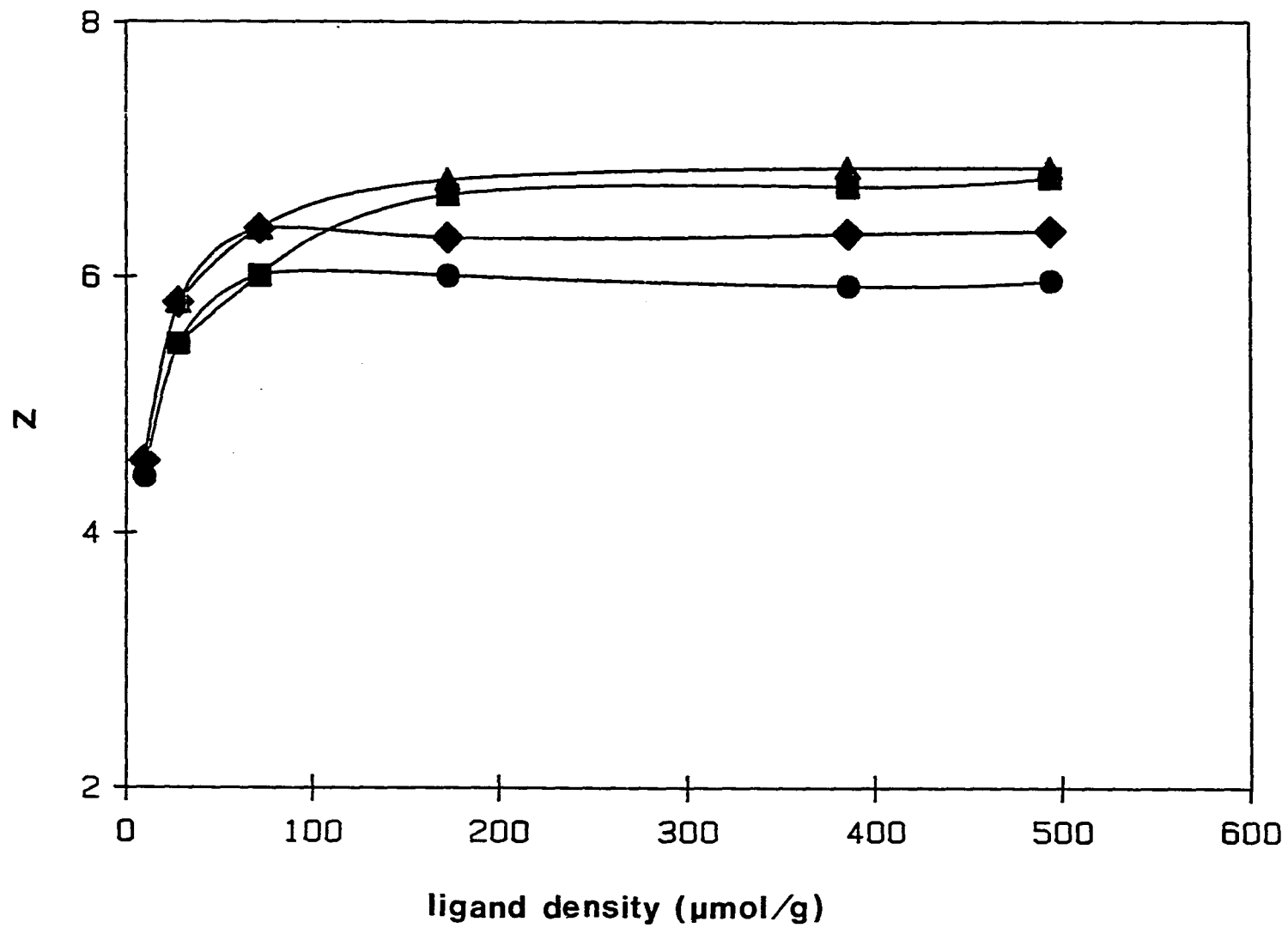
Figure 3. The Z number of cytochrome C (● , ■) and lysozyme (▲ , ◆) on various ligand density supports, as measured in the low ionic strength range (▲ , ■) and high ionic strength range (◆ , ●)

obtained for the k' range of 1-15 by changing the mobile phase strength, which reached a B% of 80% for the ligand density of 173 μmol/g and of 100% for the ligand density of 386 μmol/g or higher. Columns of 100 x 4.1 mm I.D. were used to obtain this data. To investigate the influence of ionic strength on the Z value, the upper two curves were measured in the lower ionic strength range of 30% - 60% B. One exception was the ligand density of 10 μmol/g, which was tested in the B% range of 8% - 23%. To elute proteins in a reasonably short period of time and to avoid detection limit problems at the low eluent concentration due to band-broadening, 25 x 2.5 mm I.D. columns were used for ligand densities of 173 μmol/g or higher in the k' measurement of the upper two curves. For low ligand densities, 100 x 4.1 mm I.D. columns were used. The Z number is apparently a function of ionic strength as will be discussed later.

In the low ligand density region, the Z numbers increased rapidly with ligand density. The curves were shallower at medium ligand densities and leveled off at high ligand densities. It is believed that the first increase in ligand density makes it possible for more binding sites of proteins to interact with the resin because in the low ligand density region the surface density of the protein binding sites is higher than that of ion exchange groups on the resin, allowing

proteins to recognize the ligand density increase. When the distance between the ligands matches the distance between the closest binding sites on the protein's surface, the Z number stops growing and any further increase in ligand density would only increase k'. This last case is believed to occur near 72 µmol/g (Fig. 3), where the protein diameter (approximately 20 Å) approaches the distance between 2 ligands (15 Å according to Table I).

However, it is difficult to accurately calculate the exact distance between ligands on the stationary phase because of the rough silica surface and the probable random distribution of ligands. The diameter of a protein with a molecular weight of 17,000 Daltons is about 20 Å while the 5 um silica consists of submicroparticles ranging from 1 to 100 nm (23). In other words, a protein views the silica surface as a "wrinkled" plane as long as the Z number is greater than one. In using the manufacturer's surface area data (m^2/g) obtained from Brunaner-Emmett-Tetta (BET) experiments of nitrogen adsorption to calculate the ligand distribution, it is automatically assumed that the silica surface is totally smooth. For example, on the various ligand density packings the average distance between two nearest ligands can be calculated based on the following equation:

Table I.   Distance Between Two Nearest Ligands as a Function
of Ligand Density

Ligand Density (μmol/g)	Distance Between Two Nearest Ligands (Å)
10	41
28	24
72	15
173	9.8
386	6.6
494	5.8

$$S = \left(\frac{A}{CN} \right)^{\frac{1}{2}}$$

where S is the average distance between the two nearest

ligands, C is the ligand density, N is the Avogadro's Number

and A is the surface area given by manufacturer. The results

of this calculation are listed in Table I. At a ligand densi-

ty of 10 μmol/g, the distance was calculated to be 40.7 Å, a

value greater than the diameter of cytochrome C (about 20 Å).

In this case, however, the protein had a Z number of about 4.

This discrepancy may be partially caused by the assumption

that the silica surface is totally smooth. Another possible

cause of the large Z number for the low ligand density support

may have been the presence of "cavities" in the support, into

which the protein may have fallen, resulting in the formation

of "side binding".

It is known that the three dimensional structure of

proteins is an asymmetrically charged surface. This gives

each protein a unique orientation with respect to the resin

surface, resulting in the maximum electrostatic interaction.

For example, cytochrome C has a well defined binding domain

(17). The number of binding sites is also well defined for a

particular protein, provided that the ligand density and other

experimental parameters are fixed. If the protein molecule is

rigid enough and the silica surface is homogeneous, each

molecule of a protein should interact with the resin through the same number of binding sites each time it binds to the resin. Ideally, the Z number should be some integer. However, it was experimentally observed that the Z numbers were usually random nonintegers. This may be attributed, at least in part, to heterogeneity of the silica surface.

Each time a protein molecule binds to the resin, it covers a fixed number of ligands, provided that the ligand distribution is uniform. However, the accessibility of these ligands may differ from one ligand to another as a result of different local configurations at various spots on the silica surface. A protein may bind to one area with a large Z number, where binding sites of the protein meet accessible ligands, while binding to another area with a small Z number, where it interacts with some of the less accessible ligands. The measured Z number is the average of all the possible Z numbers the protein senses as it travels down the column. Since the average number of a series of integers could well be a noninteger number, the fact that the measured Z numbers are nonintegers is certainly reasonable.

Z Number and Ionic Strength

It has been found in many systems that raising the ionic strength can decrease the electrostatic interaction between lysozyme or cytochrome C and other enzymes both kinetically and thermodynamically (24-27). Protein retention in chromatography has also been found to exponentially decay with an increase in salt concentration. However, there has been no effort made to determine whether the decrease in electrostatic interaction is caused by simultaneous weakening in the strength of all binding sites, changes in the Z number, or a combination of both. The data presented here allow a closer look at the interaction between proteins and the charged surface.

In the case of cation exchange chromatography, when a positively charged protein is placed near the stationary surface, the protein is pulled toward the resin under the electric field generated by an anionic layer (e.g., the carboxylate layer on the surface). This adsorption process is directly affected by the field strength, the charge of protein, the distance between the surface and the protein, and the nature of the mobile phase.

Dielectric media tend to mask the effective charge and shield the effective field strength. Besides simultaneously weakening all of the binding sites, this can also cause the interaction of some of the binding sites with low binding energies to cease when the ionic strength of mobile phase reaches a certain level. A significant increase in the Z number was observed on various packings of different ligand densities when the ionic strength was decreased, especially on high ligand density columns where a strong mobile phase is usually applied to elute proteins. The data are summarized in Figure 3 and Table II.

Z Number and Elution Order

Rounds and Regnier (28) found a positive correlation between the Z number and protein retention for B-LAC at different pH values. A more detailed description of such a correlation, obtained from ligand density study, is given for lysozyme and cytochrome C in Figure 4. Note, however, that this correlation is valid only for each individual protein under certain circumstances (i.e., that among different proteins a comparison of retention based on the Z number may be meaningless). Cytochrome C showed Z values smaller than those for lysozyme throughout the entire ligand density

Table II. Z Number as a Function of Ionic Strength[a]

%B	Z(lysozyme)	Z(cytochrome C)
60 - 80	6.34	5.93
30 - 60	6.86	6.71
25 - 35	8.59	7.72

[a]Obtained from a 386 μmol/g column at 1.0 ml/min.

region studied even though its k' values were greater when the ligand density was 72.0 μmol/g or higher (see Figures 3 and 4). It was therefore concluded that the electrostatic attraction of proteins to the resin depends on the summation of contributions made by individual binding sites rather than on just the number of binding sites.

The Z number can also be referred to as the "number of ions needed to displace a protein molecule" (29). In this respect, each ion can be visualized as an independent unit displacing a particular protein-resin bond. If these ions are viewed as a whole group (i.e., that the energy left over from one ion displacing a protein-resin bond is viewed as being carried on to another displacing process), one would conclude that a protein having a large Z number will bind more tightly to the resin than one with a small Z number, which is contradictory to the above discussion. It is probably more accurate to define the Z number as the number of interaction sites between the protein and the stationary phase.

The elution order reversal of lysozyme and cytochrome C is clearly seen in Figure 5. Lysozyme eluted before the cytochrome C when the ligand density was higher than 72.0 μmol/g but after the cytochrome C when the ligand density was lower than 72.0 μmol/g. To examine the possible connection between the Z number and the elution order as the ligand density is

Figure 4. Capacity factors obtained from isocratic elution with 50% B vs. the Z
number measured in the fixed B% range of 30% to 60% for the cytochrome
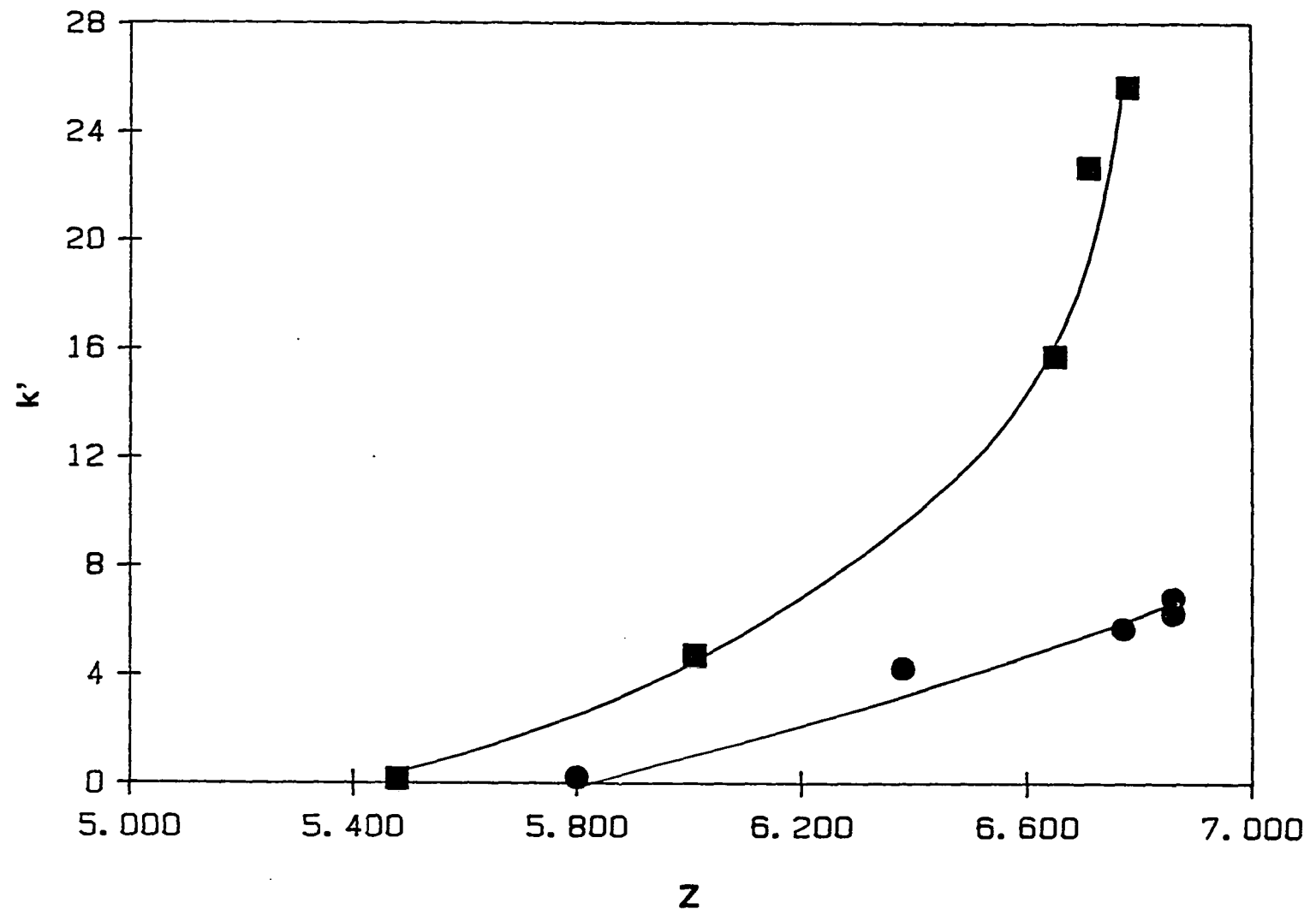C (■) and (●)

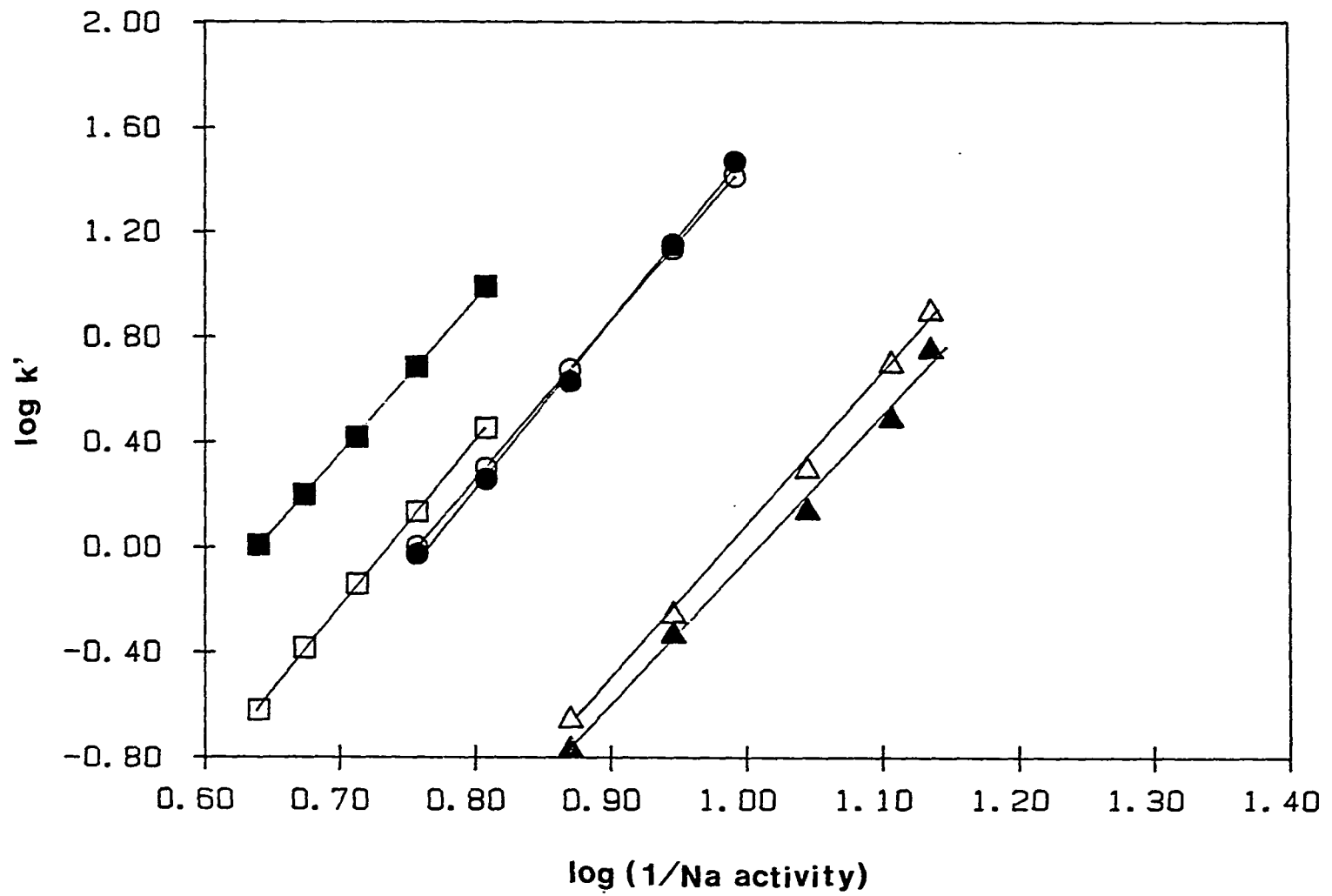Figure 5.   A plot of logarithm of capacity factor vs. logarithm of the reciprocal
            of sodium activity for the cytochrome C (▲ , ◯ , ■) and lysozyme
            (△ , ● , ▢) on the supports of ligand density of 28.2 μmol/g (△ , ▲),
   72.0 μmol/g (◯ , ●)   and 386 μmol/g (▢ , ■)

changed, relative Z numbers, such as delta Z or Z of lysozyme
- Z of cytochrome C and the ratio of Z(lysozyme) / Z(cyto-
chrome C), were calculated at different ligand densities.
These are summarized in Table III. It was found that these
parameters were fairly constant over the entire ligand density
range, with the exception of the lowest ligand density at 10
$\mu mol/g$, indicating that the elution order reversal is not
necessarily related to the Z numbers of the proteins. If
this were the case, then the relative Z number would have
altered in parallel with the elution order. A likely explana-
tion for this, based on the three dimensional structures of
proteins, is that the heterogeneity of the binding strengths
of individual sites on a protein caused variations in the
difference of binding strength among these proteins as the
ligand density and the Z number were changed, resulting in the
elution order reversal. In other words, those binding sites
of cytochrome C effective only at high ligand densities must
have had stronger attractions for the resin than those of
lysozyme. On the other hand, for the sites bound in the low
ligand density supports, the lysozyme had stronger or compara-
ble affinity relative to that of cytochrome C. This can also
be demonstrated by the Z number dependence of k', as illus-
trated in Figure 4, where the k' of cytochrome C increases
more rapidly with an increase in the Z number than that of

Table III. Ratio and Difference of Z Numbers of Cytochrome C
            and Lysozyme at Different Ligand Densities

Ligand Density (μmol/g)	Z(lysozyme) / Z(cytochrome C)	Z(lysozyme) - Z(cytochrome C)
10	1.03	0.12
28	1.06	0.32
72	1.06	0.37
170	1.05	0.30
390	1.07	0.41
490	1.07	0.39

lysozyme at high ligand density. This means that cytochrome C
was more cooperative in multipoint binding. If the curves
were extrapolated toward the smaller Z region, an intersection
of the curves would be expected for the elution order rever-
sal. Finally, the intersection I of a log k' vs. log (1/Na
activity) plot, which is equal to the log k' at unit salt
concentration, may serve as a good indication of the binding
strength of proteins at the corresponding Z number calculated
from the same curve. An examination of I as a function of Z,
as shown in Figure 6, reveals the change in the binding
strength difference of these proteins or the change in selec-
tivity or elution order with Z values. Again, the cytochrome
C increased in its binding strength more rapidly than lysozyme
when the Z number was increased, as illustrated by the I of
cytochrome C minus that of lysozyme at a given Z number. This
value changed in sign from negative to positive at a Z number
of approximately 5, corresponding to the elution order rever-
sal, and kept increasing with the Z number, in coincidence
with the better resolution observed between the proteins at
higher ligand density. The general trend of this data is in
good agreement with the elution order reversal shown in Figure
5. Note that I was based on the unit salt concentration,
which is higher than the actual salt concentration applied to
the Z value measurement. Thus, the I values may differ from

Figure 6.　Intersection I as a function of the Z number for cytochrome C (●) and lysozyme.(■)
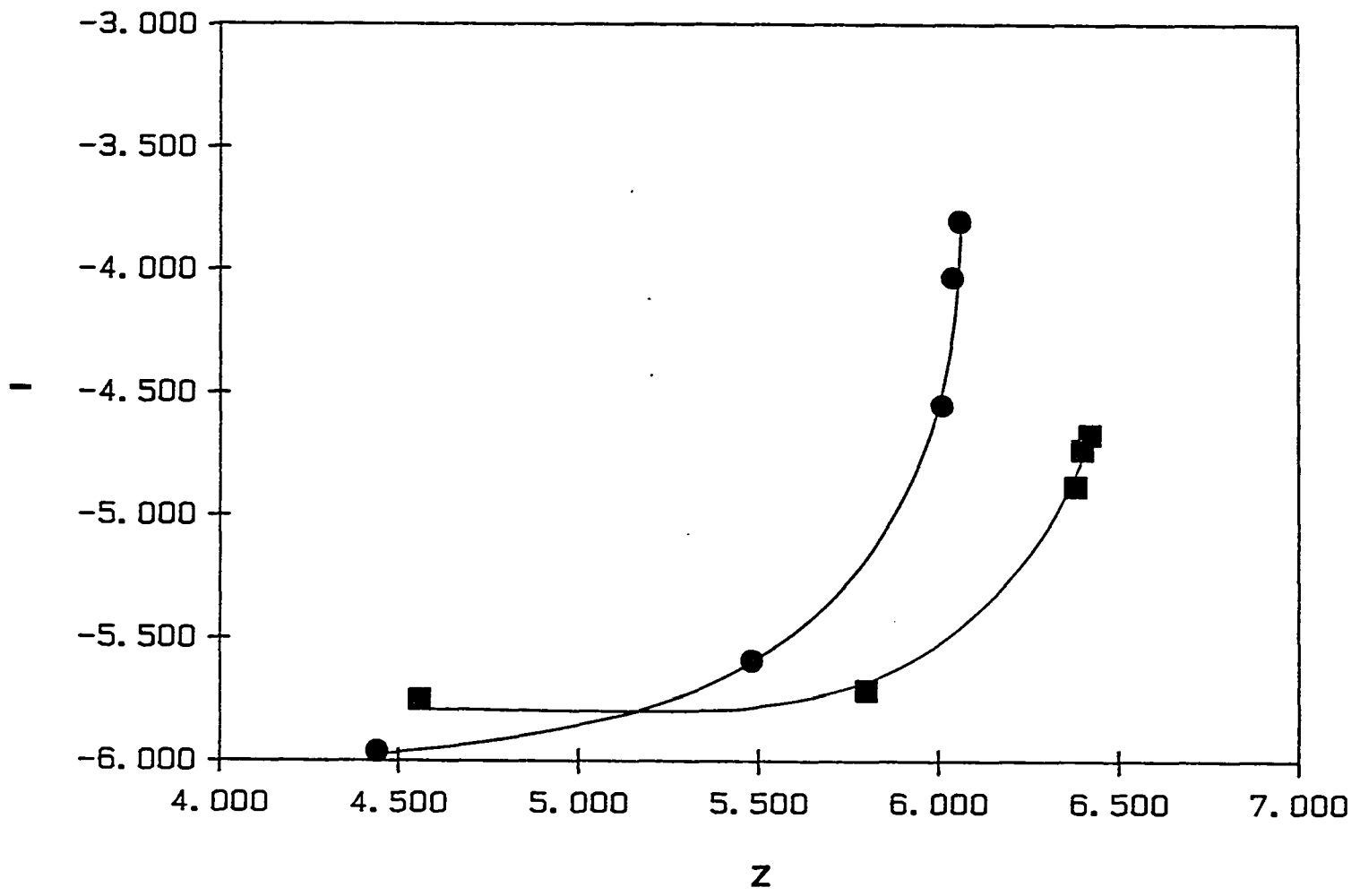
the log k' values to different extents under actual experimental conditions depending on the B% range used.

The chromatographic data seems to be in good agreement with the molecular parameters of these proteins. From Figure 3, the Z numbers of both proteins started leveling off at the same ligand density (high ionic strength: 72 μmol/g; low ionic strength: 180 μmol/g) implying that these proteins have very close sizes and charge distributions. This is not surprising since these proteins have very similar molecular weight and amino acid composition.

The log k' vs. log (1/Na activity) plots for various columns of different ligand densities, as shown in Figure 5, all gave linear regression coefficients greater than 0.999. Since the retention model (15) was derived on the assumption of purely electrostatic interaction, the good linearity obtained over such a broad ionic strength and ligand density range serves to confirm that hydrophobic or other types of nonspecific interactions were essentially insignificant on this packing material.

Note from Table II that on the same columns, when B% shifted from one region to another the cytochrome C and lysozyme showed different changes in their Z numbers. As the B% shifted from 60% - 80% to 25% - 35%, the Z number of lysozyme increased by 2.25 while that of cytochrome C

increased by 1.79. This implies that these proteins had different sensitivities to ionic strength changes, with the cytochrome C being less sensitive than lysozyme, providing additional evidence that the binding sites of cytochrome C only effective at high ligand densities had higher strength of interaction than those of lysozyme.

Cytochrome C (MW 13400 (25, 26)) and lysozyme (MW 13900 (30)) have very close molecular weights and isoelectric points (10 for cytochrome C (31), 10.4 for lysozyme (32)). Their chromatographic separation is made possible by the difference in their charge distributions, which is sensitive to the resin surface.

Band-Broadening

Plate heights were measured at different k' values as a function of ligand density for both the proteins and benzylamine in order to investigate the effect of ligand density on band-broadening. When the proteins were eluted, the plate height rose with k' in the small k' region and leveled off as a certain k' value was reached, as illustrated in Figures 7 and 8. One exception to this was at very low ligand density, where the plate heights corresponding to large k' values were not measured due to detection problems caused by the severe

Figure 7. Plate height vs. capacity factor for the cytochrome C on supports of ligand density of 28.2 μmol/g (●), 72.0 μmol/g (■) and 386 μmol/g (▲)
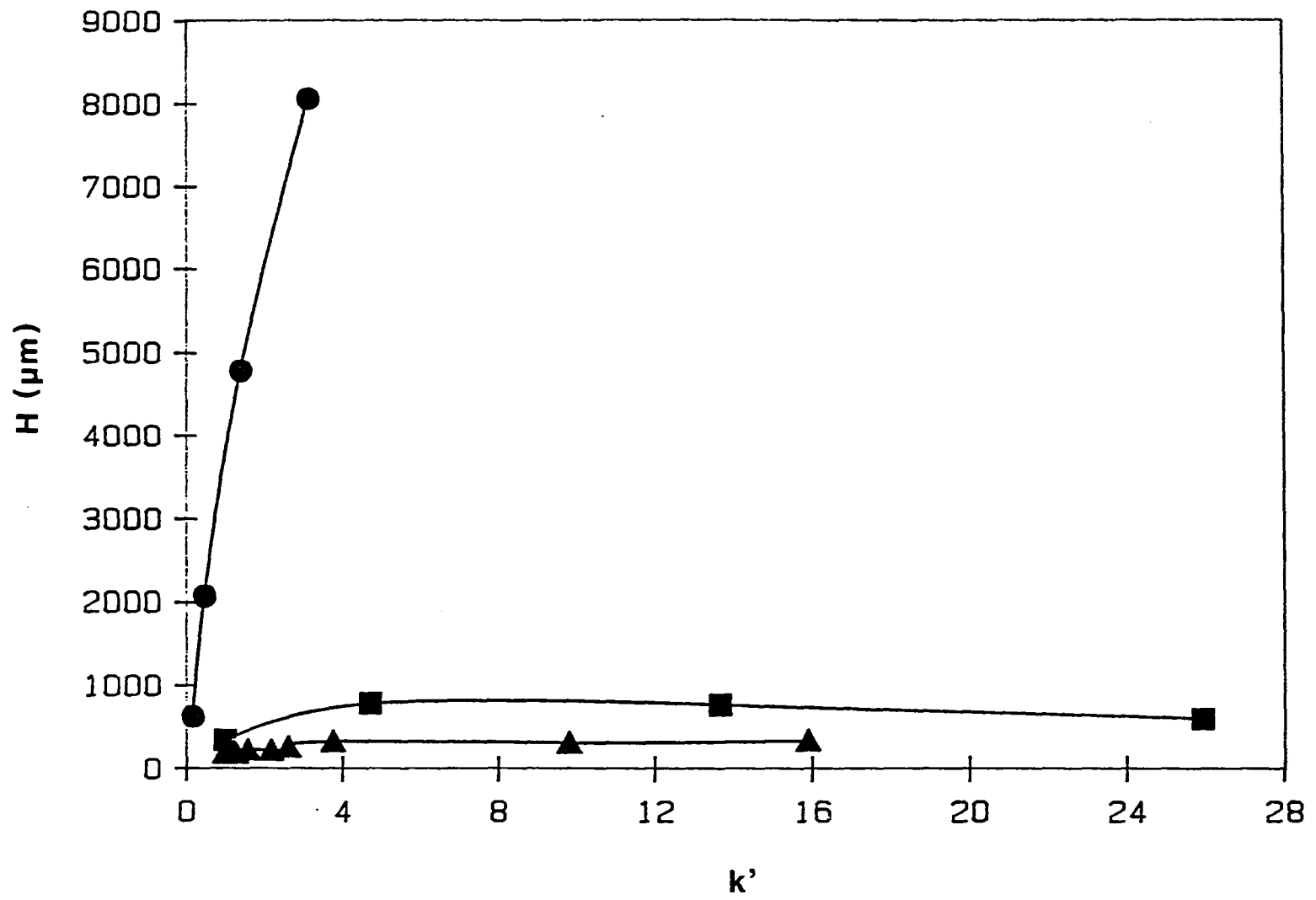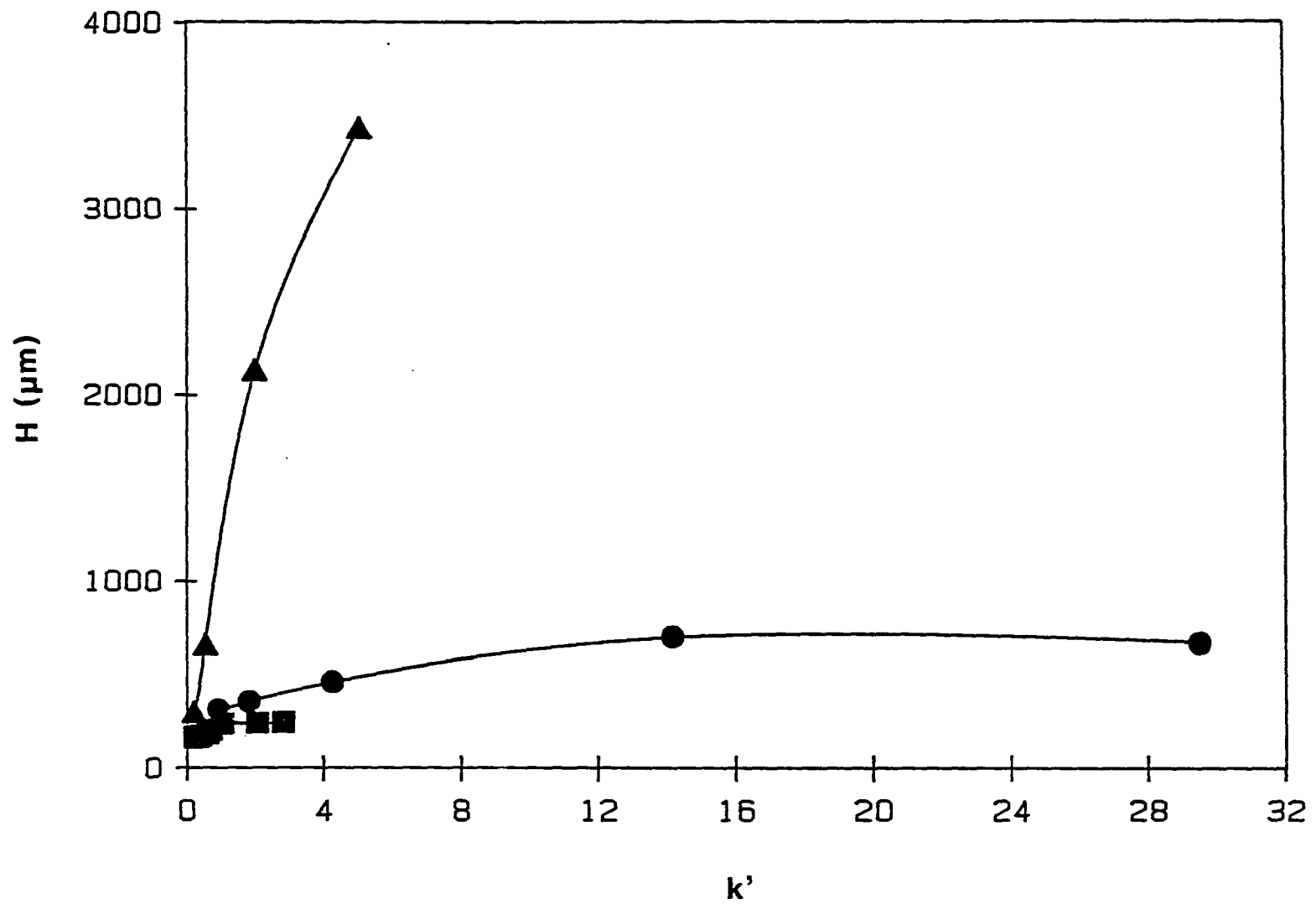
Figure 8. Plate height vs. capacity factor for lysozyme on supports of ligand density of 28.2 μmol/g (▲), 72.0 μmol/g (●) and 386 μmol/g (■)

band-broadening. In the case of benzylamine, plate heights varied with k' in a similar manner to that seen for the proteins, as shown in Figure 9, but with a much smaller change in the plate height being observed as the ligand density was varied. Overall, the plate heights were large at low ligand densities for both the proteins and benzylamine.

Horvath and Lin have derived an equation for the evaluation of the individual plate height contributions (33). They found that kinetic band-broadening can be given by:

$$H(kinetics) = \frac{2\ (k')^2 u_e}{(1+k_o)\ (1+k')^2\ k_a\ \varphi} = \frac{2\ (k')^2\ u_e}{(1+k_o)(1+k')^2 k_d} \quad (1)$$

where k' is the capacity factor, u_e is the linear velocity of an excluded solute, k_o is the ratio of the intraparticulate void volume to the interstitial void volume in the column, k_a is the adsorption rate constant and φ is the so-called phase ratio, which is further defined by:

$$\varphi = \frac{k_o\ S(p)\ C}{(1+k_o)\ V_p\ E_i} \quad (2)$$

where E_i is the intraparticulate porosity, $S(p)$ is the total surface area of the stationary phase, V_p is the pore volume

Figure 9. Plate height vs. capacity factor for benzylamine on the supports of ligand density of 28.2 µmol/g (■) and 386 µmol/g (●)
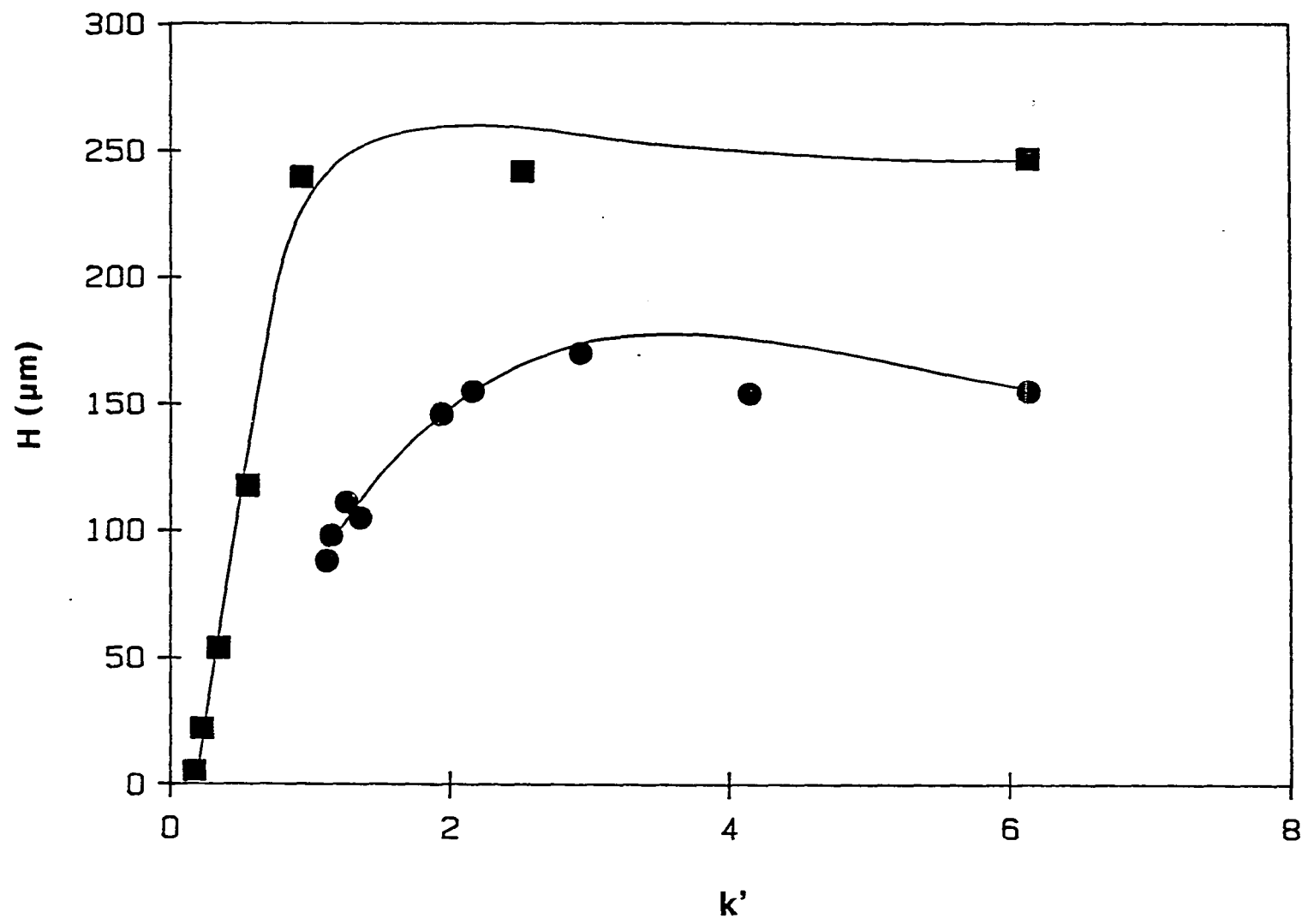
and the C is the surface concentration of the ligands (i.e., the ligand density).

From the above equations, it is apparent that the kinetic plate height is inversely proportional to the ligand density, provided that the adsorption process is the same (i.e., the adsorption rate constant is constant) at different ligand densities. This may not actually be true but does not affect the final results qualitatively, as will be shown shortly. Thus, the overall larger band broadening of both big and small solutes at lower ligand density may be partially explained by the kinetic plate height and its phase ratio contribution.

In view of the size difference between the proteins and the benzylamine, it appears possible that the larger band-broadening of the proteins may be due to their smaller diffusion coefficients and the larger physical resistance to their mass transfer in the pores. An independent study performed on a diol-bonded column rules out this argument by revealing that these effects only account for a plate height difference of 60 - 70 μm between the proteins and benzylamine (in contrast to 7,750 - 3,750 μm at low end of ligand density range), as summarized in Table IV.

It is likely that the proteins and benzylamine underwent different adsorption-desorption mechanisms. On the high coverage column (386 μmol/g), the plate height difference

Table IV. Comparison of Plate Height Between Big and Small
Solutes on a Diol Bonded Column

Solute	Retention Time (sec.)	H (μm)
acetone	31.7	65.7
benzylamine	31.6	61.2
cytochrome C	27.6	123
lysozyme	28.6	135

between the proteins (Figures 7 and 8) and the benzylamine
(Figure 9) was approximately the same as that obtained from
the diol column (Table IV), suggesting that the larger band-
broadening of proteins on that column can be explained in
terms of their size (i.e., that the kinetic plate heights and
rate constants for the benzylamine and the proteins were
similar). However, on the low coverage column (10 μmol/g)
approximately a 200-fold increase in plate height relative to
that on the high coverage column was found for both lysozyme
and cytochrome C, as opposed to a 1.5-fold change for the
benzylamine. The large difference in band-broadening between
the proteins and benzylamine at low ligand density implies,
according to equations (1) and (2), that the adsorption rate
constants of proteins changed with ligand density, since the
rest of the parameters in these equations are essentially
constant for different solutes at a given ligand density.
Note that k_d is equal to the product of k_a and $\mathscr{P}$. Even if k_a
does not change with ligand density, k_d is still subject to
change through $\mathscr{P}$. On the other hand, since $\mathscr{P}$ and k_a both
decrease as a result of ligand density decrease, k_d decrease
more rapidly than k_a. In this respect, k_d makes more
contributions to the kinetic plate height than does k_a.

The rate constants for the adsorption-desorption process
may be affected by the ligand density in the following way.

Both proteins were found to have a varying number of binding sites (different Z numbers) on materials with varying ligand densities, as discussed earlier, indicating that the adsorption-desorption process was recasted every time the ligand density was changed. Consequently, the activation energy of the adsorption-desorption process may have varied with the ligand density, affecting the rate constants. This is a unique characteristic of proteins due to their multivalent binding.

Furthermore, the double layer may have contributed to the binding process. Charged surfaces are often referred to as being an electrical "double layer", which is basically a potential gradient extended from an "inner Helmholtz plane" (IHP) to a plane where the potential is equal to that of the bulk solution. The thickness of the double layer is proportional to the ligand density (34) and is typically on the order of 5 Å (15, 34). Since the dimensions of benzylamine are smaller than that of the double layer, it is conceivable that such a small molecule is entirely engulfed by the double layer when it is retained on the stationary phase. On the other hand, cytochrome C and lysozyme have diameters of about 20 Å, which are substantially greater than the double layer dimensions. Thus, when they come in contact with the stationary phase, only a small portion of the total protein

surface probably encounters the double layer. This would allow the proteins to rotate or tilt on the surface, leading to a distribution of their relative positions with respect to the stationary phase when they are adsorbed. This motion may complicate the adsorption-desorption process and affect the rate constants, especially at low ligand density where the dimensions of the double layer are small.

Finally, the distribution of ligands on the silica surface could be nonuniform to some extent. Spots of different ligand densities would have different Z numbers and different energies of interaction with a given protein and such a dispersion in interaction energy could give rise to a dispersion in the elution band. This could be especially important in the low ligand density region, since proteins recognize the difference in ligand density more easily in that region, as discussed previously.

Resolution

Even though cytochrome C and lysozyme could be separated on either high or low coverage columns, the band-broadening for these was severe in the low ligand density region, as discussed earlier. It is obvious that higher ligand density supports exhibit more favorable chromatographic resolution in

terms of either a larger k' ratio (Figures 4 and 5) or nar-
rower peaks (Figures 7 and 8).

One of the disadvantages associated with the use of high
ligand density supports in chromatographic separations is that
a stronger mobile phase must be used to elute the solutes.
This might be destructive to the protein structure, depending
on the chromatographic mode. In ion exchange chromatography,
however, this is not a problem because proteins can maintain
their native structures in 0.5 M sodium salt, which is common-
ly used as strong mobile phase. Thus, high ligand densities
are advisable in ion exchange chromatography.

Phosphate Bound to Cytochrome C

Phosphate is known to bind to cytochrome C and alter its
retention on cation exchangers (35). Both phosphate and
acetate buffers of the same concentration and pH were used to
measure the Z number of cytochrome C on a 6.2 X 2.1 mm I.D.
column with a ligand density of 386 μmol/g. It was found that
the Z numbers were essentially the same for these different
buffers (phosphate, 6.71; acetate, 6.69) but the k' values
were markedly greater in the case of acetate, as shown in
Table V. The fact that the Z number did not change with k'

Table V . Effect of Phosphate Buffer on Capacity Factor
of Cytochrome C

%B	k'(phosphate)	k'(acetate)	Δ% in k'
30	477	612	21.9
40	79.5	115	30.9
50	33.6	37.4	10.1
60	11.2	16.2	30.5

further demonstrates that there is no inherent correlation between k' and the Z number, even for a given protein.

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SECTION III. STATIONARY PHASE LIGAND DENSITY IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) has demonstrated enormous resolution power for closely related proteins. For example, different kinds of cytochrome C of various origin whose sequences differ by between two and eight out of 104 amino acid residues have been successfully separated in 10 minutes (1). Other examples of RP-HPLC separations of polypeptides and proteins include the separation of human collagen from mixtures (2), separation of neurophysin I from neurophysin II (3), separation of the normal α- and β-chains of human hemoglobin from their respective mutant chains which differ by only a single amino acid residue (4), separation of membrane or virus proteins (5), purification of mouse interferon (6) and many more (7-9).

However, it has been difficult to achieve satisfactorily reproducible results for labile proteins such as ovalbumin and papain because of relatively low mass recovery and denaturation. Many studies have been performed in order to enhance the recoveries of both proteins and their biological activities. For example, the influence of n-alkyl chain length (10) and synthesis chemistry (11) on protein recovery and resolution have been explored; mobile phase effects such as flow rate, gradient slope, salt concentration, organic modifier,

and pH, etc. on retention and recovery have been extensively investigated (12-13). In addition, studies have been undertaken to examine the mechanisms by which the proteins lose their biological activities (14-19).

Another possible variable which could be optimized to improve the RP-HPLC of proteins is the surface concentration of alkyl chains. Although retention is directly related to the hydrophobicity of the stationary phase, which is in turn affected by ligand density, this parameter has not been thoroughly studied due to the unavailability of supports of broadly variable ligand densities (15). In this study, a C_8 RP-HPLC support was prepared by the attachment of octylamine to carboxylated silica using EDC (1-Ethyl-3-(2-morpholino-ethyl) carbodiimide) as activation reagent. Ligand densities ranging from a few percent of a monolayer to a monolayer were obtained by using the controlled reaction of diglycolic anhydride with diol-bonded silica (20). The mass recovery, denaturation, loading, and resolution of several proteins were studied with respect to ligand density.

EXPERIMENTAL

Materials

1-Ethyl-3-(2-morpholinoethyl) carbodiimide (EDC), papaya latex papain, bovine hemoglobin, soybean trypsin inhibitor (STI), horse heart cytochrome C, bovine pancreas ribonuclease A, and egg albumin (ovalbumin) were from Sigma (St. Louis, MO). Octylamine was from Aldrich (Milwaukee, WIS). Nucleosil 300-5 silica was from Alltech (Deerfield, IL).

Chromatography

A model 344 gradient liquid chromatograph (Beckman, Berkeley, CA) was used. Absorbance was monitored at 280 nm by a V4 variable-wavelength absorbance detector (ISCO, Lincoln, NE). Data were collected and processed on an Apple IIe computer via an ADALAB interface board (Interactive Microware, State College, PA).

For the resolution study of protein mixtures and the papain denaturation determination, mobile phase A was 9 mM phosphoric acid (pH 2.6) and mobile phase B was 9 mM phosphoric acid in acetonitrile/water (1:1 v/v). In the denaturation study of STI (Fig. 1), mobile phase A consisted of 10 mM

phosphoric acid (pH 2.2) and mobile phase B of 1-propanol/ water (45/55 v/v) with an overall phosphoric acid concentration of 10 mM. In another STI denaturation study (Table II), acetonitrile/water (1:1 v/v) was used in the place of 1-propanol/water. Samples were prepared in mobile phase A. 10 ul injection of 10 mg/ml protein was employed in both the denaturation and resolution studies.

The application buffer in breakthrough curve measurements was 0.01 M sodium phosphate, pH 7.0, and the elution solvent was 2-propanol. The protein was dissolved in application buffer at a concentration of 0.02 mg/ml.

Preparation of Stationary Phase

Silica of varying surface concentration of carboxyl groups was prepared using diol-bonded silica and diglycolic anhydride, and quantitated as described previously (20). A 0.5 gram sample of carboxylated silica was suspended in 25 ml of 2-propanol and degassed by sonication under aspirator vacuum for 10 min. Added to this was 0.72 g of EDC and 4.09 g of octylamine. The pH was adjusted to 4.3 - 4.5 using hydrochloric acid. The suspension was shaken for 9 hours at room temperature. A 0.72 g of EDC was added after 3 and 6

hours of reaction. The support was washed with 2-propanol followed by methanol.

Glucosamine immobilization and assay were performed (20) to assess the completion of octylamine immobilization. It was found that the amount of glucosamine immobilized was virtually zero. Therefore, the octylamine coupling was assumed to be quantitative and surface concentration of carboxyl groups measured by ester assay (20) before octylamine coupling was used as the surface concentration of the C_8 phase.

An n-octyldimethylchlorosilane bonded reversed-phase support was prepared according to a published procedure (21). The endcapping was done by the addition of trimethylchloro-silane after 24 h of reaction and allowing the reaction to run for another 24 h.

RESULTS AND DISCUSSION

Hydrophobicity of the Stationary Phase

To compare the hydrophobicity of the octylamine phase
with a phase of the same alkyl chain length and similar ligand
density but prepared by different synthesis chemistry, the
small solutes aniline and benzene were injected into columns
containing the octylamine and n-octyldimethylchlorosilane
supports, then eluted with 0.01 M sodium phosphate, pH 2.8.
The data are summarized in Table I. The benzene showed com-
parable capacity factors (k') on different supports, indicat-
ing that those supports prepared by different synthesis
chemistry had similar hydrophobicities. When chromatographed
on the octylamine bonded support (or on n-octyldimethylchloro-
silane supports), benzene was retained more strongly than
aniline, indicating that the electrostatic interaction between
the charged analyte (e.g., aniline) and the stationary phase
was not significant under the experimental conditions.

Denaturation

In hydrophobic interaction chromatography (HIC), Cohen
et al. (16, 22) found that papain eluted as 2 peaks and STI as

Table I. The Capacity Factor of Small Solutes on Different
         C_8 Stationary Phases

Synthesis of Stationary Phases	k' (aniline)	k' (benzene)
octylamine coupling	0.1	3.3
n-octyldimethylchlorosilane coupling (without endcap)	0.2	2.1
n-octyldimethylchlorosilane coupling (with endcap)	0.5	4.2

2 groups of peaks corresponding to the native form (first or first group) and denatured form (second or second group) of the proteins. The peak assignment was confirmed by reinjection of the collected eluent fraction of the individual peaks and spectroscopic identification (16, 22). Also, protein molecules were proved to be denatured on the stationary phase (14) rather than in the mobile phase. In this regard, a decrease in the hydrophobicity of the stationary phase by using a low ligand density would reduce the degree of denaturation and accordingly, the native peak(s) should grow at the expense of the denatured ones as the ligand density decreases. In this study, elution profiles similar to those in previous studies (16, 22) were obtained for papain and STI (see Fig. 1 for STI) on C_8 reversed phase columns when the ligand density was moderately high (e.g., higher than 40 μmol/g). The identities of the peaks were assumed to be the same as in the published studies (16, 22). At extremely low ligand density, the peaks were broader but the general pattern remained. The relative peak areas of native and denatured forms were examined as a function of ligand density.

STI and papain were injected into octylamine bonded columns of ligand densities ranging from 320 to 10 μmol/g. At the ligand densities greater than 28 μmol/g, a 20 minute and a 10 minute gradient (0% - 100% of B) were used to elute STI and

Figure 1. Chromatography of STI at ligand density of 386
          umol/g (A), 40 umol/g (B), and 10 umol/g (C).
          The first group of peaks correspond to the native
          form of the protein and second one the denatured
          form. The peak area ratio was 1.57 (A), 1.89
          (B), and 3.34 (C). The gradient was 20 min (A,
          B) and 75 min (C) from 0 to 100% B at 1 ml/min
          with 2-propanol/water used as the strong mobile
          phase
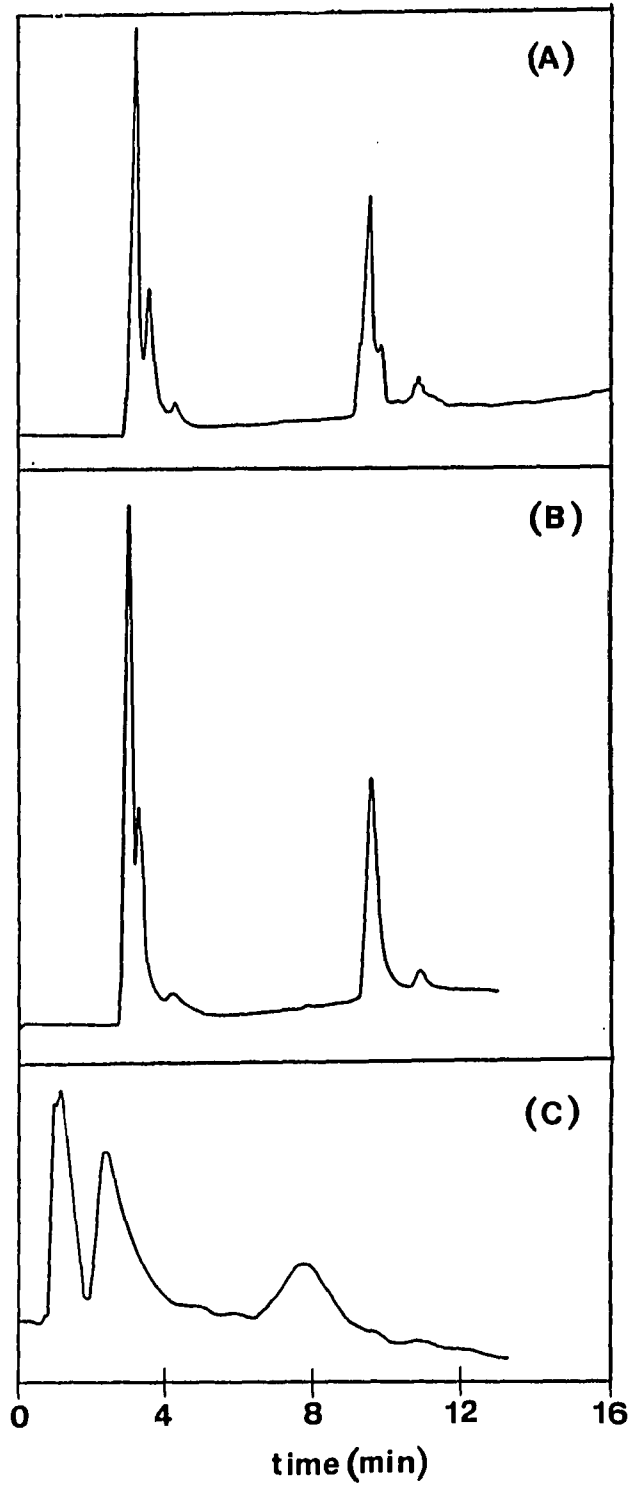
papain, respectively. For both proteins, the native and the denatured forms were eluted from different ligand density columns at essentially the same retention volume. At a ligand density of 28 μmol/g or lower, the gradient slope was reduced so that the native and denatured peaks were separated with good resolution and eluted at essentially the same %B ratio as compared to the chromatograms obtained from higher ligand density columns. In this way, the difference in peak area at different ligand density due to protein absorbance which is a function of %B would be canceled out by taking the peak area ratio of native / denatured. In other words, the peak area ratio becomes a true measurement of relative amount of native and denatured protein. The data are summarized in Table II and Fig. 1 (note that different organic modifiers were used for STI in Fig. 1 and Table II, see "EXPERIMENTAL" for details). The amount of native form grew at the expense of denatured form when the ligand density was decreased, as indicated by the change of peak area ratio. It is apparent that the low hydrophobicity of stationary phase corresponding to low ligand density in this study would minimize the denaturation of those proteins. Furthermore, the mobile phase seemed to contribute to the denaturation process also. In the ligand density range of 28 - 320 μmol/g, the peak area ratio of STI increased by 9.2 fold (Table II) when water/acetoni-

Table II. Protein Denaturation[a] as a Function of Ligand
           Density

Ligand Density (μmol/g)	Protein	$\frac{\text{Area(native)}}{\text{Area(denatured)}}$
320	papain	0.6
50	papain	0.9
40	papain	1.8
320	STI	2.6
50	STI	2.7
40	STI	5.3
28	STI	23.8

[a]A 20 min gradient and a 10 min gradient, both from 0 to
100% B, were used for STI and papain respectively. The flow
rate was 1.0 ml/min.

trile was used as strong mobile phase; while in a larger
ligand density range - 10 to 386 μmol/g, this ratio showed
only 2.1 fold increase when 2-propanol/water was used as
strong mobile phase. It appeared that more native STI would
be obtained with an acetonitrile/water system.

It was noted that the peaks obtained from extremely low
ligand density were significantly broader (Fig. 1). To
investigate the loading effect on the band broadening, the
following examinations were carried out. For a column with a
ligand density of 10 μmol/g, the amount of ligand present is
approximately one thousand times that of protein molecules
injected, which should have eliminated the possibility of
overloading. Injections were also made with the sample size
reduced to 1/4 and 1/8 of the original. Similar elution
profiles as that from full sample size injection were obtained
with respect to retention time and peak shape. Therefore, the
overloading did not seem likely to occur, and the cause of
band-broadening at low ligand density is not well understood
at this time.

Mass Recovery

One of the major drawbacks of RP-HPLC of proteins is the
loss of protein molecules through irreversible adsorption on

the stationary phase. This has been observed with many supports of various bonded phases (11, 12, 23). The primary reason for the strong interaction between proteins and the support is the availability of the large number of hydrophobic sites on the protein surface together with high ligand density of stationary phase usually employed. This leads to a high degree of multivalent adsorption (24). The Z number has been found to be 208 when bovine serum albumin was eluted with methanol (25), in contrast to approximately 7 when cyt C was chromatographed by IEC (26). In this regard, low ligand density would reduce the hydrophobic interaction between proteins and stationary phase and enhance the mass recovery.

In order to study the amount of protein irreversibly adsorbed at various ligand densities, breakthrough curves (27) for ovalbumin were measured on columns of different ligand densities. Each breakthrough was followed by 3 gradient cycles of fast flow rate and steep slope (5 min from 0 to 100% B at 2 ml/min) to ensure the removal of the reversibly adsorbed ovalbumin before the next run. It was found that for most ligand densities, the second breakthrough capacity dropped down significantly due to the presence of irreversibly adsorbed protein molecules and the consequent decrease in available support surface area. The third curve generally gave the same value as the second one, indicating that the

irreversible adsorption sites had been saturated after the
first breakthrough curve (Table III). The amount of decrease
in breakthrough capacity is a reflection of the degree to
which the irreversible adsorption takes place. The mass
recovery, in this study, was defined as the average capacity
of second and third breakthrough curves divided by the
capacity of the first one. This ratio increased from 22% to
100% as ligand density decreased from 320 to 28 μmol/g (Table
III).

To examine the possible nonspecific interactions between
the ovalbumin and residual silanol and carboxyl groups possi-
bly left over from stationary phase synthesis, ovalbumin was
injected into a high coverage carboxylated silica column (300
μmol/g) and eluted with the application buffer of the break-
through curve measurements. A sharp and nonretained peak was
obtained indicating that there was no significant nonspecific
interaction in the breakthrough curve measurement and the
irreversible adsorption could be attributed to strong hydro-
phobic interaction.

It was also noted that the mass recovery in this study
was based upon the breakthrough curve measurement corres-
ponding to an extremely large sample size. Since the mass
recovery is a function of loading (28), different results may

Table III.   Mass Recovery as a Function of Ligand Density

| Ligand Density (μmol/g) | Column Dimensions (mm) | Breakthrough Capacity (mg ovalbumin per column) | | | Recovery (%)[a] |
		1st Breakthrough	2nd Breakthrough	3rd Breakthrough	
14	50x4.1	0.0390	0.0391		100
35	6.2x2.1	0.1523	0.0952	0.0975	64
150	6.2x2.1	0.1897	0.1143	0.1121	60
220	6.2x2.1	0.1950	0.0440	0.0400	22
320	6.2x2.1	0.1997	0.0427	0.0465	22

[a] $Recovery = \dfrac{2nd + 3rd}{2 \times 1st} \times 100.$

be expected using other methods where a small sample size is applied.

Capacity

From Table III, when the ligand density increased from 14 μmol/g to 35 μmol/g, there was a 76.5-fold increase in overall capacity (including the contribution of both irreversible and reversible sites as indicated by the first breakthrough) for ovalbumin, while above 35 μmol/g a 9.1-fold increase in ligand density (35 - 320 μmol/g) caused only a 1.3-fold change in that capacity. It is obvious that the protein could not utilize the ligand density increase at high ligand density because most of the binding sites had been bound to stationary phase already.

After the first breakthrough when the irreversible sites had been saturated, the capacity of reversible sites peaked at a ligand density of 150 μmol/g, rather than continuously increasing over the whole ligand density range. Apparently, as the ligand density increased in the high ligand density region (150 μmol/g), more and more reversible sites changed to irreversible sites by binding to protein. In addition, each time a protein molecule is irreversibly bound to the stationary phase it covers a large number of ligands which may

not bind to the protein at all. Effectively, the population of irreversible sites appears to grow faster than the rate at which reversible sites would be generated, leading to the decrease in capacity of reversible sites when the ligand density is increased.

Resolution

A mixture of three proteins was chromatographed on three columns of different ligand densities, as shown in Fig 2. Essentially the same chromatograms were obtained for ligand densities of 320 and 50 μmol/g while the chromatogram for the 40 μmol/g column was shifted toward the void volume but with similar resolution. Such a comparison of resolution indicates that under the experimental conditions the higher ligand density has little or no further effect on the binding of these proteins as long as the ligand density is kept above certain limit (e.g., 40 μmol/g). Although the extent of multivalent adsorption and the strength of hydrophobic inter-action should be dependent upon the ligand density, once all of the protein hydrophobic sites are in contact with the alkyl chains, an increase in ligand density would not have any further positive effect.

Figure 2.   Chromatography of ribonuclease A (R), cytochrome
             C (C), and hemoglobin (H) at ligand densities of
             320 umol/g (A), 50 umol/g (B), and 40 umol/g
             (C). The gradient was 20 min from 0 to 100% B
             at 1 ml/min with water/acetonitrile used as the
             strong mobile phase
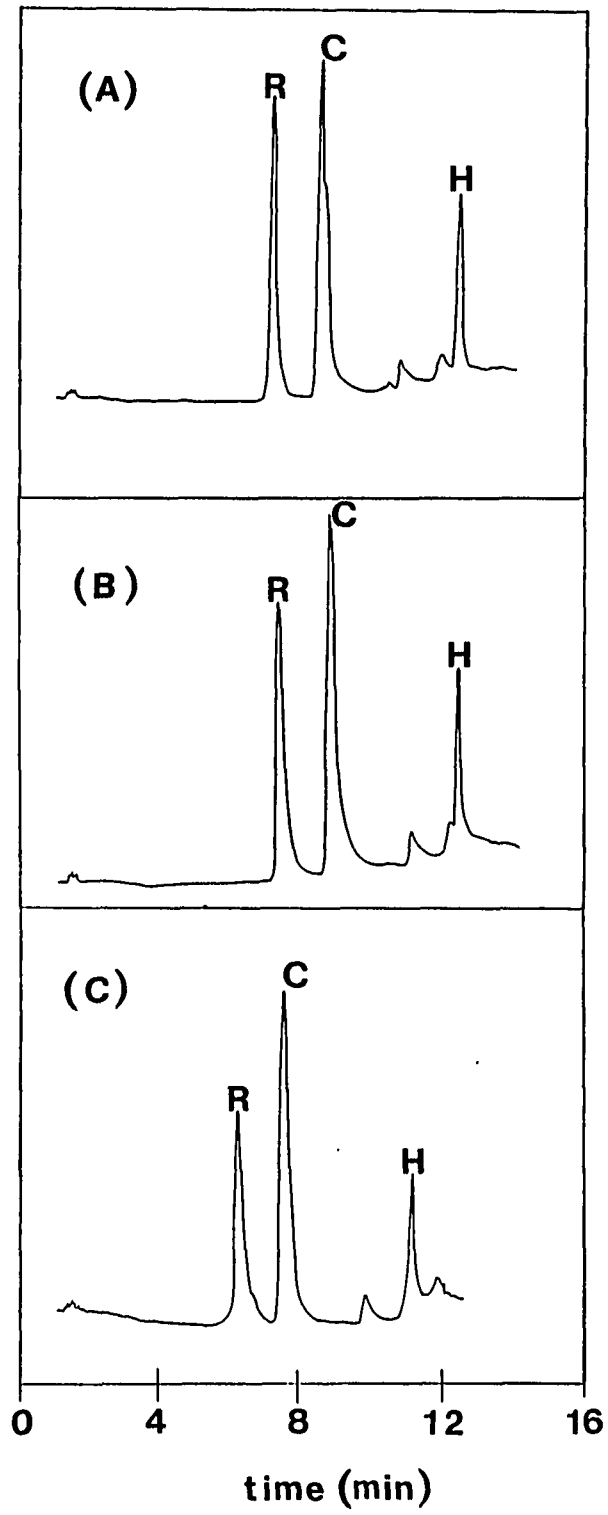
A similar phenomenon has also been observed with proteins chromatographed on degraded reversed phase columns (29), where a 50% loss of alkyl chain bonded phase had little effect on the resolution.

Note that the lowest ligand density in Fig. 2 was 40 μmol/g. If a protein mixture is injected into a column of even lower ligand density (e.g., 10 μmol/g), large band-broadening as that in Fig. 1 (c) may be expected and resolution may decrease.

Retention of Small Solutes

The k' of anisole was measured at varying ligand density in order to examine the dependence of retention of small solutes on the ligand density. It was found that the k' increased concomitantly with the ligand density (Table IV), in contrast to what has been observed with proteins in which the retention was insensitive to ligand density. Apparently, the multivalent interaction does not occur with a small solute like anisole and, according to theory, the k' should be proportional to ligand density. In other words, the remarkable difference in the retention behavior of large and small solutes with respect to ligand density can be attributed to

Table IV. Capacity Factor for Anisole as a Function of Ligand
Density

Ligand Density (μmol/g)	k'^{a}
14	0.6
40	2.0
50	2.8
150	4.2
320	4.9
n-octyldimethylchlorosilane with endcap	4.5

[a]Mobile phase: water; 5 μl of 10^{-4} M anisole in methanol/
water (40/60 v/v) injected.

the valency through which the analytes interact with the
stationary phase.

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SECTION IV.   PROTEIN IMMOBILIZATION ON SILICA

SUPPORTS: A LIGAND DENSITY STUDY

INTRODUCTION

Immobilized enzyme technology has been growing rapidly
over the past several decades (1). The major advantage of
using immobilized enzyme systems in the place of soluble
enzymes is the increased stability of immobilization which
allows reuse of the enzymes and thus greater economy. Recent
advances in the application of this technique have been found
in: [A] Enzyme engineering where the immobilized enzymes are
used as the catalyst to carry out conversions of industrial
interest; [B] Organic synthesis where the discrimination
between enantiomers is desired; [C] in vivo pharmaceutical
systems; [D] basic biochemical research where immobilized
enzymes are used as a model of intracelluar membrane-bound
enzymes; and [E] chemical analysis, e.g., affinity chromato-
graphy and enzyme electrodes.

The most common questions about immobilized enzymes
relate to their activity. Immobilized enzymes generally show
lower specific activities than the soluble ones, primarily due
to multipoint attachment which causes distortion of the three
dimensional structure of the immobilized enzyme and steric
hindrance of substrate binding site by spacer arms (2). By
using a low surface concentration of spacer arms, the multi-
point attachment would be avoided and the steric hindrance

diminished to some extent, so that the specific activity of immobilized enzymes could be significantly increased. This has been observed for several proteins immobilized on hydroxyalkyl methacrylate gels (3), Sepharose CL (4), and aminoethylcellulose (5).

Silica gel has drawn much attention in the field of immobilized enzymes, especially in the application of affinity chromatography since 1969 when Weetall first immobilized enzymes on silica (6). Advantages of silica include high mechanical and chemical stability and resistance to microorganisms. Although silica gel has been extensively studied in terms of pore size, particle size, synthesis chemistry, etc., the effect of spacer arm concentration or ligand density on the activity of immobilized proteins has not been systematically examined.

In a previous report (7), the silica was silanized to produce diol-bonded silica and further derivatized with diglycolic anhydride to yield a matrix of variable ligand density, i.e., variable surface concentration of spacer arms. This has made it possible to investigate the effects of spacer arm concentration on the activity of immobilized proteins over a very broad concentration range. In this study, several proteins were immobilized to silica supports of varying spacer arm concentration by use of N-hydroxysuccinimide as the acti-

vating reagent. The specific activities of these immobilized proteins were consequently studied as a function of spacer arm concentration. The influence of condensing reagent, pore size, and pH on the immobilization yield were also examined.

Note the "ligand" in this study is defined as the spacer arm, rather than the immobilized proteins.

EXPERIMENTAL

Reagents

1-Ethyl-3-(2-morpholinoethyl) carbodiimide (EDC), eel acetylcholinesterase, acetylcholine iodide, soybean trypsin inhibitor (STI), bovine pancreas trypsin, bovine pancreas α-chymotrypsin, N-succinyl-1-alanyl-1-alanyl-1-prolyl-1-phenyl-alanine-p-nitroanilide, porcine stomach mucosa pepsin, N-acetyl-1-phenylalanyl-3,5-diiodo-1-tyrosine, staphylococcus aureus protein A, rabbit immunoglobulin G (IgG), antihuman albumin (anti-HSA, developed in goat), and human albumin (HSA) all were from Sigma (St. Louis, MO). 1-Cyclohexyl-3-(3-dime-thylaminopropyl) carbodiimide metho-p-toluenesulfonate (CMC), dithio bis-2-nitrobenzoic acid (DTNB), ninhydrin, hydrindan-tin, and 2-methoxyethanol were from Aldrich (Milwaukee, WI). N,N'-dicyclohexylcarbodiimide (DCC), Triton X-100, and phenol reagent solution were purchased from Fisher (Fair Lawn, NJ). Nucleosil 1000-5 and Nucleosil 300-5 were obtained from Alltech (Deerfield, IL). Lichrospher SI-4000 and SI-1000 were from Rainin (Woburn, MA). N-hydroxysuccinimide (NHS) was from Eastman Kodak (Rochester, NJ).

Methods

Carboxylated silica of variable ligand density was prepared and quantitated as described previously (7). N-hydroxysuccinimide (NHS) ester was synthesized according to a published procedure (8) with the following modifications: the silica was suspended in dioxane and degassed by sonication under aspirator vacuum for 10 minutes prior to reaction and the N-hydroxysuccinimide ester silica was dried under vacuum and room temperature overnight. One hundred mg of the activated matrix was suspended in 2 ml of the coupling buffer (0.1M phosphate of the desired pH) and degassed for 10 minutes. 5 - 30 mg of protein was then added and the suspension was clamped in a mechanical shaker. In the pH study, the trypsin and acetylcholinesterase suspensions were shaken at 4 °C for one day, STI at 4°C for 6 hours, and anti-HSA at room temperature for 18 hours. In the pore size study, the suspensions of STI (pH 3.0) and anti-HSA (pH 5.0) were shaken at room temperature for 18 hours. In the activity study, the suspensions of α-chymotrypsin (pH 4.0), pepsin (pH 4.0), Rib A (pH 5.0), anti-HSA (pH 7.0), acetylcholinesterase (pH 6.0), and STI (pH 3.0) were shaken at 4°C, and protein A (pH 5.0) at room temperature. The reaction times ranged from 24 hours for anti-HSA and 18 hours for acetylcholinesterase and protein A,

to five days for STI and 7 days for α-chymotrypsin, pepsin, and Rib A, respectively. The support was washed with 2 M sodium chloride followed by water in the pH and pore size studies and 1 M sodium chloride in 0.1 M phosphate buffer of the appropriate pH in the activity study.

Immobilized glucosamine was quantitated according to a previous procedure (7). Immobilized protein was determined by Lowry protein assay (9). In all of these assays, blank values were determined using diol-bonded silica samples. Also, the silica was removed by centrifugation prior to absorbance measurement.

The activities of immobilized acetylcholinesterase and pepsin were determined by enzyme assays (10, 11). To determine the activities of immobilized α-chymotrypsin, a published enzyme assay procedure (12) was used with the modification that sodium arsenate was used as buffer rather than triethanolamine. The activities of STI, protein A, and anti-HSA were determined from chromatographic breakthrough curves (13) of trypsin, IgG, and HSA, respectively.

RESULTS AND DISCUSSIONS

Use of Condensing Reagents

It has been reported in the case of succinylated agarose support that some of the carbodiimide condensing reagents were more effective than others in the formation of the NHS ester (14). To examine this effect on carboxylated silica, activation using N,N'-dicyclohexylcarbodiimide (DCC), 1-cyclohexyl-3-(3-dimethylaminopropyl)-carbodiimide (CMC), and 1-ethyl-3-(2-morpholinoethyl)-carbodiimide (EDC) were performed followed by glucosamine coupling and ferricyanide assay (7). In this way, one can determine the effectiveness of the condensing reagent by measuring the amount of glucosamine immobilized which is proportional to the surface concentration of NHS ester formed during the activation step. DCC was found to be more than twice as effective as CMC or EDC in terms of the glucosamine yield (Table I) and therefore chosen as condensing reagent during the course of this study.

### Effect of pH on the Immobilization of Proteins

With different proportionalities for different proteins, the amount of protein immobilized was found to be inversely

Table I. Effectiveness of Condensing Reagents

Carbodiimide	Glucosamine Immobilized (μmol/g)
DCC	124 ± 10
CMC	61 ± 5
EDC	54 ± 5

proportional to the pH of the reaction media as indicated in Fig. 1. It is well known that hydrolysis of the ester bond of the activated silica is substantially faster at higher pH so that coupling of proteins would decrease with pH. On the other hand, higher pH values would promote the deprotonation of amino groups of proteins, which would favor the nucleophilic attacking reaction and thus produce higher protein yield (14). Among these two conflicting factors, hydrolysis was obviously more predominant in determining the final protein content, while deprotonation of amino groups led to different slopes for the curves in Fig. 1 due to different pKa values of amino groups which vary from one protein to another as well as the three dimensional distribution of amino groups on each protein. In contrast to what has been observed in this study, free amino acids showed an optimum in plots of immobilization content vs. pH (14). This is primarily due to the fact that the N-terminal group of a peptide chain is a much weaker base than the α-amino group of a free amino acid. Therefore, deprotonation becomes the controlling factor for free amino acids in the low pH range.

Figure 1. Protein coupled as a function of the pH of reaction mixture. The
          proteins are anti-HSA (■), acetylcholinesterase (▲), STI (●), and
          trypsin (◆)
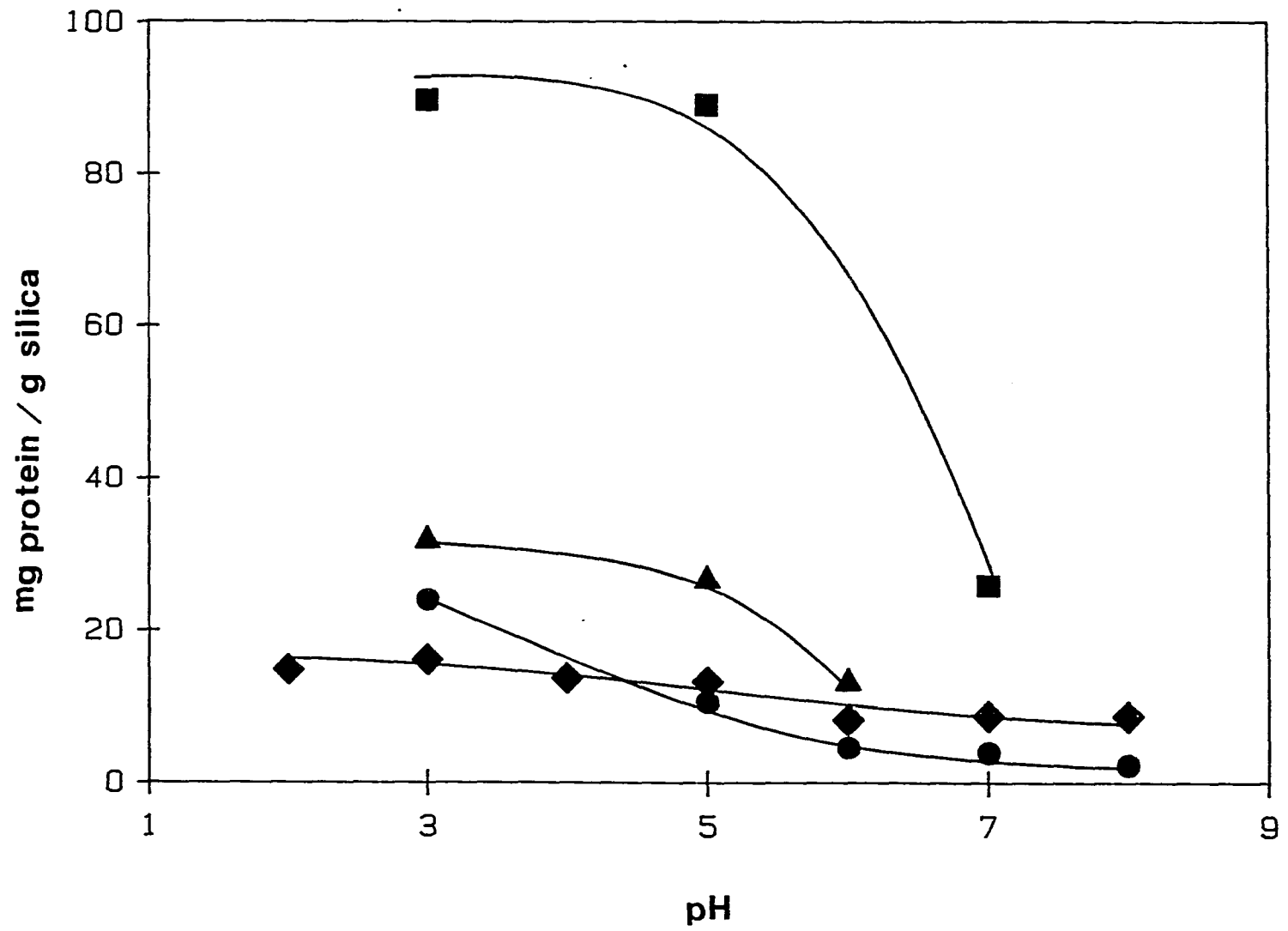
Pore Size and the Efficiency of NHS

The immobilization of antiHSA using the N-hydroxysuccin-imide (NHS) ester resulted in higher yields as compared to the CDI or Schiff-base method on 300 Å silica and slightly lower yield as compared to CDI method on 4000 Å silica (Table II). Since both the CDI and Schiff-base methods have been demonstrated to yield reasonably high protein content (15-17), the NHS is also concluded to be an efficient activating agent for protein immobilization.

To examine the pore size effect on the protein coupling yield, silica supports of pore size 300 Å, 1000 Å and 4000 Å were used to attach the anti-HSA and STI. The coverage of both proteins with respect to surface area of silica was found to increase significantly when the pore size was increased (Table II). The pore size effect was further confirmed by coupling anti-HSA to 300 Å and 4000 Å silica using the CDI method, which showed the same bulk increase in coverage, as summarized in Table II. It is possible that the internal surface of silica pores is not a smooth surface, but rather with cavities distributed around. The dimensions of these cavities could be too small for the protein to get into, but large enough for small molecules like nitrogen to penetrate through. So the surface area obtained from BET measure-

Table II. Protein Immobilization Dependent upon Pore Size and Coupling Chmistry

Protein	Pore Size (Å)	% of a Monolayer[a]			Protein Content (mg /g silica)		
		NHS	CDI	Schiff-base	NHS	CDI	Schiff-base
STI	4000	145			15		
STI	1000	117			39		
STI	300	23			38		
anti-HSA	4000	248	262		30	32	
anti-HSA	1000	198			80		
anti-HSA	300	40	36	33	81	71	66

[a] Based on the Stoke's diameter of proteins and surface area of silica.

ment, which is based upon the monolayer of nitrogen molecules, would be in fact larger than the "effective" surface area that the protein molecules can really "feel", leading to the seemingly lower protein coverage. This effect appeared to be more influential with the smaller pore size, as indicated by the protein coverage as a function of pore size in Table II.

The optimal pore size for obtaining the maximum protein content is a function of the increasing surface coverage but decreasing surface area as the pore size increases. The data in Table II indicate that the optimal pore size is approximately 1000 Å.

Protein Content

Discussion of both protein content and specific activity as a function of ligand density is difficult unless the data for proteins of variable size are normalized to account for the fact that larger proteins will cover a larger area of the silica surface.

Specific Activity

The specific activities of immobilized proteins, expressed as either the ratio of activity of immobilized protein

over that of soluble protein or the ratio of mg test solute
over the mg immobilized protein, are summarized in Table III.
The specific activities of STI, protein A, anti-HSA, and
acetylcholinesterase were essentially independent of ligand
density over the range 2.62 - 48.4 µmol/g.

The data for α-chymotrypsin and pepsin in Table III
indicate that the specific activities of the two proteins fell
off considerably as the ligand density increased. The cause
can be attributed to the multiple covalent attachment of the
enzyme molecule, which disturbs the tertiary structures and
modifies the conformation. This effect has been demonstrated
by a fluorescence study of chymotrypsin immobilized on Sepha-
dex G-200 (18). Also, the enzyme molecule can be fixed on the
silica surface in an orientation that would render certain
parts of the molecule less accessible to substrate, i.e., the
covalent bonds formed during the coupling reaction may be
located close to or in the active site. This is particularly
important at the lowest ligand density, where multiple bonding
was excluded because the distance between two nearest ligands
was greater than the diameters of those proteins. Therefore,
the steric hindrance caused by orientation of fixed protein
molecules may be the primary reason leading to less than 100%
specific activity at low ligand density.

Table III. Specific Activities of Immobilized Proteins at Different Ligand Densities

| Protein | Specific Activity (immobilized / native) | | | Substrate |
	Ligand Density 48.4 (µmol/g)	Ligand Density 11.8 (µmol/g)	Ligand Density 2.62 (µmol/g)	
∝-chymotrypsin	0.19	0.25	0.39	suc-(ala)-pro
pepsin	0.42	0.51	0.59	ac-phe-dit
acetylcholin-esterase	1.0	1.0	0.97	acetylcholine

| Protein | Specific Activity (mg test solute / mg ligand) | | | Test Solute |
	Ligand Density 48.4 (µmol/g)	Ligand Density 11.8 (µmol/g)	Ligand Density 2.62 (µmol/g)	
STI	0.92		0.92	trypsin
protein A	3.6	3.5	3.4	IgG
anti-HSA	41×10^{-3}	41×10^{-3}	44×10^{-3}	HSA

It should be pointed out, regardless of the ligand density, the restricted diffusion of small substrates did not contribute to the low specific activities of ∝-chymotrypsin or pepsin. This is illustrated by the 100% retention of specific activity by immobilized acetylcholinesterase enzymatically assayed with acetylcholine as the substrate. Acetylcholine has a similar molecular weight to that of the substrates for ∝-chymotrypsin and pepsin.

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DISCUSSION

The present work presents an in depth study to date concerning the examination of the influence of stationary phase ligand density on the performance of HPLC of proteins. Studies involving three separation modes (ion exchange, RP, affinity), nine proteins and a 38.6-fold change in ligand density were performed. The broad extent of this work allowed for adequate examination of protein retention and other variables in HPLC. As a result, conclusions could be made with respect to the validity of popularly circulating theories and recently proposed retention mechanisms.

There is direct evidence in ion exchange chromatography (sec. II) and hydrophobic interaction chromatography (1) suggesting that changing the stationary phase ligand density of the support changes its selectivity. It was suggested in HIC that proteins may be grouped according to the critical ligand density at which selectivity begins to vary (2). This phenomenon was explained in terms of solute ligand density. As long as the stationary phase ligand density is greater than the density of the complementary groups on a solute's surface, there is little change in selectivity. When support ligand density falls below that of complementary groups on one of the solutes, however, selectivity begins to vary. This hypothesis

basically attributes the selectivity to the Z number. This seems to be contradictory to what has been discovered in the ion exchange chromatography of this work, in which selectivity was found to be independent of the Z number. It has been discovered from this work that it was the binding strength differences in individual binding sites of a protein molecule that led to the changes in selectivity.

It is possible that both conclusions are correct since they differ from each other in the nature of different separation modes from which they were derived, both being valid in their respective individual cases. There has been no sufficient evidence at this point to determine, for all proteins, which hypothesis more accurately describes reality. However, the conclusion drawn from this work appears more general and complete because the finding in HIC can always be viewed as a special case under the general conclusion.

The influence of stationary phase ligand density on reversed phase chromatography of proteins has been investigated in terms of mass recovery, denaturation, resolution, and loading capacity. The general finding indicates that most of the variables were sensitive to ligand density and can be improved by using low ligand density. However, protein retention time was found to be independent of ligand density over a broad range (see sec. III). This is advantageous from a practical

point of view since it means that low ligand densities can be used without sacrificing resolution power. Moreover, this sort of "unusual behavior" may reveal some fundamental information concerning the stationary phase configuration.

A phenomenon central to the stationary phase is the dynamic reorganization of the alkyl chains via solvation by mobile phase components (3-5). Solvents compatible with the nonpolar alkyl surface solvate the chains and promote their extension away from the surface. On the other hand, in this "brushlike" or extended chain arrangement there is penetration of the solvent molecules into the stationary phase. "Hostile" solvents that are not compatible with the alkyl chains promote collapse of the chains. It has been postulated that the same solvophobic effect invoked for solute-mobile phase interaction would cause the alkyl chain to aggregate in order to reduce the total surface exposed to the polar solvent (3). If this is true, the stationary phase then can be viewed as a "liquid-crystalline-like" layer. It is suggested from the data presented in this work (sec. III) that changes in the ligand density would change the thickness of the "liquid crystalline" layer but not the hydrophobicity density of this layer. In other words, the density of hydrophobic moities, which may be an association of alkyl chains and the organic solvent, has reached the maximum even at low ligand density as a result of

the collapsed state of the ligands. If this is the case, it then becomes apparent from experimental observations that the hydrophobic interaction between protein and stationary phase is only related to the hydrophobicity density as far as the retention is concerned. Therefore, the protein retention is not affected by the ligand density within a certain ligand density range. This is also supported by the evidence that there is little or no change in protein retention time when alkyl chain length altered from C_{22} to C_2 at a given ligand density (6).

HPLC has demonstrated tremendous separation power in discriminating closely related proteins like lysozyme and cytochrome C in this work. Moreover, in the HPLC application to bioengineering, where derivatized proteins to be separated are often just slightly different in their structures, it has been shown that the ionization of a single amino acid in a polypeptide of several hundred residues can be easily detected (2). However, one must keep in mind that this amino acid must lie within the chromatographic contact region of the protein, which is usually a small spot on the protein surface, otherwise the stationary phase will not recognize the difference. It is clear that some of the modifications introduced during biosynthesis and purification of a polypeptide will lie outside the chromatographic contact region. Even if each chroma-

tographic mode has a different chromatographic contact region, there is still a high probability that LC systems will not detect all structural variants of a protein.

The influence of ligand density on HPLC separations studied in this work leads to the conclusion that there may be differences in selectivity (IE), mass and activity recovery (RP), and specific activity (affinity chromatography) in columns with identical stationary phases but different ligand densities. Column manufacturers have made no efforts to standardize ligand density, so it is likely that one brand of column is better for a particular separation than another. The problem is further compounded by the fact that small chemical differences in stationary phase also contribute to those parameters mentioned above. It is especially disturbing that there is no way to judge a priori which column will be the best for a given separation.

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SUGGESTIONS FOR FUTURE WORK

One of the major questions remaining unanswered in this study is how the ligands are distributed on the silica surface. This can be investigated by attaching a fluorescent molecule to the carboxylated silica, followed by measuring the fluorescence intensities of the monomer and excimer emission.

Good candidates for such probe molecules are pyrene derivatives. The photophysics of these molecules has been extensively studied and pyrene has been shown to exhibit short range (3 - 5 $\overset{\bullet}{A}$) excimer formation. Pyrene in dilute solution reveals a structured fluorescence emission band, with a 0-0 transition characteristic of the excited monomer at approximately 27000 cm^{-1} (1). As the monomer concentration of pyrene is increased, the fluorescence quantum yield of the monomer is seen to decrease. The concentration quenching of the monomer fluorescence is accompanied by the appearance of a new, broad, and structureless fluorescence band at approximately 21000 cm^{-1}. This new band is attributed to the presence of excited state dimers (excimers) formed by collisional interaction of an excited molecule with another molecule in its ground electronic state within the fluorescence lifetime of the excited species. Excimer formation has a critical distance of 3 to 8 $\overset{\circ}{A}$ but is actually expected to be closer to 3 $\overset{\circ}{A}$ (2).

The observation of monomer and "excimer-like" emission of pyrene coated or adsorbed on silica gel has been reported by Hara et al. (3) and Baner et al. (4). The excimer of "excimer-like" emission from silica gel derivatized with pyrene functionality implies that covalently bonded pyrene molecules must be within the critical interaction distance for excimer formation. A study of monomer and excimer fluorescence intensities at different ligand densities should permit a relative determination of the distribution of surface carboxyl groups.

Lochmuller et al. used [3-(3-pyrene] dimethylchlorosilane (3PPS) to examine the surface distribution of silanol groups by attaching 3PPS directly to bare silica (5). They found the "cluster" formation even at very low ligand density (0.13 $\mu mol/m^2$). Since carboxyl groups used in this work are coupled to the diol silica rather then directly to the bare silica, it is anticipated that the ligand distribution for this work should be more even in this study than reported in 3PPS study. Further experiments must be done to see if this is indeed the case.

Regarding the preparation of pyrene bonded phase, a pyrene-alkyl-amine can be coupled to the carboxyl groups through EDC activation. The same experimental conditions used for octylamine coupling (sec. III) can be used.

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