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R. Srinivasan[a]; E. Ruckenstein[a]
[a] Department of Chemical Engineering, State University of New York at Buffalo, Buffalo, NY

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ROLE OF PHYSICAL FORCES IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY

R. Srinivasan and E. Ruckenstein*
Department of Chemical Engineering
State University of New York at Buffalo
Buffalo, NY 14260
(*Correspondence should be addressed to E. Ruckenstein)

ABSTRACT

Hydrophobic interaction chromatography (HIC) is a new non-biospecific liquid chromatography method for the separation of proteins, and other biological macromolecules; the mobile phases are aqueous and the adsorbents are agarose beads coated with ionogenic, or non-ionogenic, hydrocarbonaceous ligands. A non-traditional interpretation is given here for the mechanisms of retention and elution in HIC in terms of the several physical forces between the protein and the adsorbent, chiefly the van der Waals attraction (comprising dispersion, orientation and induction) and the electrostatic double layer interaction. From a qualitative analysis of the hydrogen-bond and the structural features of water, it is shown here that the role of the alkyl ligands on the adsorbent, the lyotropic salt effects and the effect of additives to the mobile phase such as ethylene glycol can all be unifyingly represented in the Hamaker coefficient of the van der Waals attraction between protein and adsorbent in water. An increase in the number and length of alkyl ligands, and the addition of structure-making ("salting-out") salts at high ionic strengths increase the latter attraction while the addition of structure-breaking "salting-in" salts or organic solvents such as ethylene glycol decreases the attraction. The potentials corresponding to the different physical forces add up to the total interac-

tion potential. The shapes of the total interaction potential relevant to HIC are identified. Coefficients of adsorption and desorption are shown to be related to this potential and the high sensitivity of the latter to its parameters such as the Hamaker coefficient, is illustrated. Retention, elution and the natures of the different fractions into which a protein mixture may be separated by HIC are visualized in terms of the interaction potential.

Numerous experimental reports in HIC are classified, tabulated, and in certain cases, discussed in detail. Experimental evidence is presented for the application of ionic strength manipulations, in the low ionic strength range (electrostatic effects), in the high ionic strength range (lyotropic salt effects) as well as for the combined use of low and high ionic strength effects, retention onto adsorbents with no ligands as well as onto adsorbents with alkyl ligands of various chain lengths and number densities, temperature effects, and the effects of heterogeneities of proteins and adsorbents. Applications and variants of HIC are cited. In total, the paper summarizes various types of HIC experimental facts and explains most of them in a simple, unifying fashion.

CONTENTS

1. INTRODUCTION

Traditionally, proteins and other biological macromolecules have been separated by ion-exchange, gel-permeation or affinity chromatrography. A recent addition to this list of liquid-chromatography methods employs aqueous solutions and adsorbents which consist of polysaccharide matrices carrying hydrocarbonaceous ligands. Retention is not bio-specific and is a function of physiochemical variables such as pH, ionic strength and the content of organic solvents in the mobile phase. Experimental research on this method has been reviewed by Ochoa[1] and others[2-7]. The consensus in the literature is that a major contribution to retention in this chromatography comes from the "hydrophobic interaction" between the solute and the hydrocarbonaceous ligands on the adsorbent. This is reflected in the name hydrophobic interaction chromatography (hereafter abbreviated as HIC) by which the method is known.

Hydrophobic interaction is a term for the association of nonpolar entities in aqueous media. Earlier theories[8-13] visualized this interaction as follows: When a nonpolar solute is introduced in water, adjacent water molecules form an ice-like structure that is more ordered than the structure of water. The reduction in entropy corresponding to this "ice-berg" formation makes a dominant contribution to the free energy change and hence renders the dissolution unfavorable. Hence, the association of nonpolar solutes in water has been seen as an entropically driven process that results in contact of the entities with complete elimination of the intervening water.

Recent computer-simulation studies[14], however, indicate that the preferred configuration of two associated nonpolar molecules contains a water molecule in between them. In addition, from a thermodynamic analysis of the solubility of hydrocarbons in water, Shinoda[15,16] arrived at conclusions different from the above: When an apolar solute is introduced in water, the breakage of hydrogen-bonds of water that occurs results in a positive enthalpy change. "Ice-berg" formation results in a considerable negative entropy

change as well as a negative enthalpy change, which largely cancel each other. Thus the dissolution of nonpolar entities in water is essentially enthalpic and is controlled by the former effect of hydrogen-bond breaking. This implies that the association of nonpolar entities in water also is enthalpically driven. The latter view is consistent with earlier theories[17,18] which stress the importance of the energy of forming a cavity in the liquid to accommodate the solute, to the dissolution process as well as to the interaction between entities in a solvent.

In the present paper, the traditional view of the hydrophobic interaction is not made use of. Chromatographic retention is seen as the outcome of an adsorption-desorption process due to the interplay of several physical forces, especially van der Waals attraction. (Although the van der Waals attraction between small molecules is short range, between macromolecules and the macroscopic adsorbent, it attains a long range effectiveness due to the cooperation of numerous intermolecular van der Waals interactions[19,20].) The special role played by the mobile phase is recognized and an interpretation is presented here for the way in which the van der Waals forces between protein and adsorbent are affected by the intervening solvent-medium.

As is well-known, van der Waals interactions are of three kinds: namely, dispersion, orientation, and induction. The dispersion interactions among water molecules are, in general, weaker than those among nonpolar molecules[68b]. The orientation and induction, however, are much stronger in water as a result of the highly ordered structure of water. This is shown to lead to the van der Waals attraction between nonpolar entities being stronger in water than in a nonpolar medium. Hence, proteins, significant extents of their content being nonpolar, are attracted by a nonpolar, hydrocarbon-coated adsorbent when water is the mobile phase. An increase in the structural order of the aqueous medium (by the addition of "salting-out" salts such as NaCl) enhances the van der Waals attraction between protein and adsorbent across the medium. Similarly, a

breakdown of this structure (by the addition of "salting-in" salts such as NaSCN or organic solvents such as ethylene glycol) reduces the attraction between protein and adsorbent. In the present paper, all these effects, namely, the role of the alkyl ligands on the adsorbent, lyotropic salt-effects, and the action of organic solvents, are unifyingly reflected in the Hamaker coefficient for the (total) van der Waals attraction between protein and adsorbent across the medium.

HIC adsorbents and solutes are usually charged, and establish electrical double layers around themselves by attracting from solution ions which are oppositely charged relative to the surface charge. Consequently, electrostatic double layer interactions arise, which are attractive or repulsive depending on the signs of the net charges on the protein and the adsorbent. At very small distances between the surfaces of the latter, there occur short range repulsive forces such as Born repulsion which arises from the overlap of molecular orbitals, and the hydration repulsion which stems from the difficulty in eliminating water from the hydration layers around polar or charged groups on the surfaces.

This paper seeks to explain the various experimental observations in HIC in a simple and unitary fashion, basing the interpretation on the above-mentioned physical forces. To understand the combined effects of these forces, the approach would be similar to that of colloid stability theory. While the influences of double layer and van der Waals interactions have long been recognized in quantitative accounts of colloid stability[21,22], it is only recently that they have been used in the interpretation of chromatographic separations[23,26]. The approach here would be that of Ruckenstein, et al., who computed the total interaction potential comprising the van der Waals, double-layer, and Born interactions, and analyzed the implications which the shape of this potential has for deposition, adsorption, and desorption of globular proteins, cells, and colloidal particles[23-30]. In this context, it is noteworthy that theories of HIC, different from the present one, exist in the liter-

ature. Carbonell, et al.[31,32], and Koller, et al.[33], following early models of protein solubility[34], used a modified version of the Debye-Hückel theory (for predicting activity coefficients) in deriving expressions for the equilibrium constant for the complex formation between protein and adsorbent. Horvath, et al., arrived at similar expressions for the equilibrium constant in a more fundamental fashion, by applying Sinanoğlu's solvophobic theory to the chromatography of small molecules[35,36] and proteins[37]. These expressions consist of a term representing electrostatic interactions (which depend on the ionic strength as in the Debye-Hückel theory) and a term for non-electrostatic interactions (which is linear in ionic strength).

The present interpretation is similar to these theories in terms of the nature of the mechanisms that are taken into account. Yet, while the latter are of a thermodynamic flavor, the present interpretation is more a kinetic approach.

This paper is organized in the following manner. Initially, some structural aspects of proteins and HIC adsorbents are briefly reviewed. Subsequently, the interaction forces are examined and expressions are given for the interaction potentials. The effect of changing certain properties of the mobile phase (e.g., ionic strength) are analyzed qualitatively. Various shapes are possible for the way in which the sum of the interaction potentials depends on the distance between the surfaces of the adsorbate and the adsorbent. The total interaction potential profiles relevant to HIC are identified. Coefficients for adsorption and desorption are shown to depend on the total interaction potential profile. The sensitivity of the latter to its parameters is shown by citing the results of a parametric study. A visualization is presented of chromatographic retention and elution in terms of the various potential profiles.

Experimental evidence is presented in several categories, basing the classification on the parameter central to each experiment. For example, some experiments concern the effect of the alkyl chain length of the ligands on retention, while others concern

lyotropic salt effects and so on. While most of the experimental facts are listed in tabular form, some are discussed in detail in the light of the present interpretation.

In total, the paper serves a two-fold purpose. It reviews many experimental reports on HIC, in some detail. It also provides a simple and unifying interpretation of the various HIC experimental facts. Thus, HIC is placed in perspective with other types of chromatography.

2. HIC ADSORBENTS AND SOLUTES

A. Adsorbents

In general, HIC adsorbents consist of a hydrophilic, water insoluble matrix onto which hydrocarbonaceous ligands are attached.

The matrix is typically (with a few exceptions[38-40]) a polysaccharide such as agarose. In most cases, the matrix is activated by treatment with cyanogen bromide[41,42]. Ligands such as alkylamines that possess one or two amino groups[3,7], or amino acids[43,44] are then coupled to the activated matrix. The ligands may be further modified by treatments such as acetylation[45,46]. This type of adsorbent acquires charges through the dissociation of basic NH_2 groups ($pK_b \simeq 9.5$) and acidic COOH groups if any ($pK_a \simeq 4.6$). The agarose matrix itself is known to carry negative charges[47] and CNBr activation introduces positive charges[48].

In other HIC adsorbents, the chances of the adsorbent being charged are minimized by either (a) avoiding CNBr activation and coupling nonionogenic ethers or alcohols[49-51] or (b) coupling alkyl hydrazides ($pK_b \simeq 4$) to CNBr activated agarose, thus ensuring that the adsorbent will be uncharged at neutral pH[52]. Charged adsorbents have been made neutral also by means of acetylation[45].

Ochoa[1] and Yon[6] have tabulated several types of HIC adsorbents.

B. The Biological Macromolecule

The transfer of a nonpolar molecule from water to a nonpolar environment is accompanied by a negative free energy change[8]. Therefore, the nonpolar side chains are likely to stay in the inter-

ior of the three-dimensional structure of a protein molecule, whereas the hydrophilic polar or charged groups would prefer to stay outside, when the environment is aqueous. Whether such tendencies materialize or not is answered by x-ray crystallography. Protein crystals are heavily hydrated with approximately half of their volumes occupied by water. Thus, the ambience and structure are expected to be similar in crystal as in aqueous solution[53]. While x-ray crystallography generally confirms the external location of most of the polar and charged side chains, the complementary expectation that all the nonpolar side chains are buried in the interior, has been discounted. Lee and Richards[54], from a survey of single crystal x-ray diffraction data on proteins, concluded that about 40 to 50% of the accessible surface area of proteins is nonpolar. Klotz[55] arrived at a similar conclusion. Accordingly, the extent to which the accessible surface of a protein is nonpolar, termed "surface-hydrophobicity" of the protein, is non-negligible. Estimating the surface-hydrophobicity as well as the total nonpolar content of proteins has been the aim of several investigations[37,56-58].

A protein that is endowed with a large number of nonpolar resiues on its accessible surface has an appreciable surface-hydrophobicity. Naturally, some proteins (e.g., membrane proteins) are more hydrophobic than others (e.g., serum proteins). In this context, it is noteworthy that proteins as a class are much less hydrophobic than inorganic colloids such as metal or oxide sols, and hence, are known as "hydrophilic colloids".

3. THE INTERACTION FORCES

A. The van der Waals Interaction

The incessant motion of electrons creates an instantaneous dipole moment in each atom of any material. Such an instantaneous dipole moment generates an electric field which polarizes another atom inducing in it a dipole moment. The interaction between these two dipoles results in the dispersion or London attraction between the two atoms[59,60]. This is one in a set of van der Waals interac-

tions. The others are the orientation or Keesom interaction[61] between permanent dipoles, if any, of different molecules, and the induction or Debye interaction[62] between the permanent dipole of a molecule with an induced dipole, caused by it in another molecule. In general, the dispersion effect is stronger than the other two, except between molecules of highly polar materials such as water.

Orientation and induction account for about 80% of the van der Waals attraction between water molecules[63,64] and are experimentally and theoretically difficult not to be considered as the major components of hydrogen-bonding[65]. Table I lists the approximate contributions of various kinds of interactions to the hydrogen-bond, as estimated by Coulson[66]. Noting that both covalent interaction (-8.0 kcal/mol) and overlap repulsion (+8.4 kcal/mol) are contact interactions, it can be inferred from Table I that they more or less cancel each other out. Thus, it can be concluded that orientation and induction form the major long-range components of the hydrogen bond.

The London interaction, to a good approximation, is additive[63]. Hence, when macromolecules or large bodies are in London-van der Waals interaction, one should consider an atom of one body interacting with one of the other, and do this pair by pair in an additive fashion. Thus, although the London interaction potential between small molecules decays with the sixth power of intermolecular distance, between larger bodies, it attains a longer range in effectiveness due

TABLE I

Contribution of Various Intermolecular Interactions to the Hydrogen Bond, as estimated by Coulson[66]

Interaction	Energy in kcal/mol
Dispersion	-3
Electrostatic (i.e., orientation + induction)	-6
Delocalization (Covalent)	-8
Overlap Repulsion	+8.4
Overall Energy of the H-Bond	-8.6

to the cooperation of numerous intermolecular dispersion interactions. For example, it has been indicated that between particles of colloidal sols, the dispersion interaction may be effective for distances of the order of colloidal dimensions[19]. Orientation and induction effects, however, are not additive, due to their vectorial nature[67]. Nevertheless, they are of considerable importance when water is the medium across which the interactions take place. In the present analysis, all three kinds of van der Waals interactions are taken into account as would be described shortly, in a manner suggested by Nir[68a].

The potential of the van der Waals interaction between bodies of materials 1 and 2 across a medium 3 is given by the product of a geometric term that is influenced by the size and shape of each body, and a "Hamaker coefficient" A_{132} that reflects the chemical natures of the materials 1 and 2 and the medium 3. A_{132} is given by

$$A_{132} = A_{12} + A_{33} - A_{13} - A_{23}, \quad (1)$$

where A_{ij} is the Hamaker coefficient for the van der Waals attraction of materials i and j across a vacuum. (N.B.: A list of nomenclature is provided at the end of the paper.) A_{132} can be estimated by dividing it into contributions from dispersion, and orientation and induction, i.e.,

$$A_{132} = \bar{A}_{132} \text{ (dispersion)} + \hat{A}_{132} \text{ (orientation + induction)}. \quad (2a)$$

Equation (2a) can be rewritten as suggested by Nir[68a] as,

$$A_{132} = \{\bar{A}_{12} + \bar{A}_{33} - \bar{A}_{13} - \bar{A}_{23}\} + \frac{3}{4} kT \frac{(\varepsilon_1-\varepsilon_3)(\varepsilon_2-\varepsilon_3)}{(\varepsilon_1+\varepsilon_3)(\varepsilon_2+\varepsilon_3)}, \quad (2b)$$

where the term in curly brackets denotes the contribution from dispersion to A_{132}; $\bar{A}_{ij}$ denotes the dispersion component of Hamaker coefficient A_{ij} (defined above) and can be estimated from spectroscopic data[68a,68b]. The static dielectric permittivity (dielectric constant) of medium i is denoted by ε_i and the term involving ε_i in Eq. 2b represents the contribution from orientation and induction to A_{132}. Table II lists a few examples for such an estimation of A_{132}.

TABLE II

Estimated[68b] Values for the Hamaker Coefficient A_{132} - Examples

A_{132} (in units of 10^{-14} ergs)

Material 1	Medium 3	Material 2	Dispersion	Orientation + induction	Total
Carbon di sulfide	Decane	Carbon di sulfide	4.023	0.011	4.034
Carbon di sulfide	Water	Carbon di sulfide	9.863	2.769	12.631
Pentane	Water	Bovine Albumin	1.940	2.293	4.234
Hexane	Water	Bovine Albumin	2.689	2.290	4.979
Heptane	Water	Bovine Albumin	3.272	2.284	5.556
Octane	Water	Bovine Albumin	3.688	2.284	5.972
Nonane	Water	Bovine Albumin	4.104	2.284	6.388
Decane	Water	Bovine Albumin	4.354	2.282	6.636
Bovine Albumin	Water	Bovine Albumin	7.728	1.858	9.585

For the interaction of two bodies of the same material 1 across a medium 3, Eq. 2b simplifies to

$$A_{131} = (\sqrt{\bar{A}_{11}} - \sqrt{\bar{A}_{33}})^2 + \frac{3}{4} kT \left(\frac{\varepsilon_1 - \varepsilon_3}{\varepsilon_1 + \varepsilon_3}\right)^2 \qquad (2c)$$

when use is made of the Berthelot's rule, $\bar{A}_{ij} = \sqrt{\bar{A}_{ii}\,\bar{A}_{ij}}$.

The cohesiveness of the liquid medium 3, which is reflected in the value of the interfacial tension at the interface of the medium with a vacuum (usually approximated by the surface tension γ_{lv} of the liquid-vapor interface) determines A_{33}. The large surface tension of water indicates that A_{33} for the van der Waals attraction among water molecules is high. As discussed earlier, a large

contribution to A_{33} stems from the strong orientation and induction interactions among water molecules. In this context, it should be mentioned that the calculational procedure on which Eq. 2b is based underestimates for aqueous media, the contribution from orientation and induction to A_{33} and hence, underestimates A_{33}. For example, it is known that the contributions from dispersion and from interactions other than dispersion to the surface tension $\gamma_{\ell v}$ of water are approximately 22 dynes/cm and 50 dynes/cm, respectively[70]. While one would expect a parallel division for A_{33}, the said calculations predict the contribution from dispersion to be 35 x 10^{-14} ergs and that from other interactions to be only 3 x 10^{-14} ergs[68b]. This discrepancy probably results from the fact that the calculational procedure does not adequately take into account the importance of water structure and hydrogen-bonding (as discussed in the present paper) to A_{33}. This, however, does not affect the validity of the conclusions reached here since the present qualitative discussion does take into account the role of water structure and hydrogen-bonding. Let us now compare the interaction of nonpolar materials in water with that in a nonpolar medium.

Water can interact with nonpolar materials only <u>via</u> dispersion. Consequently, the contribution from orientation and induction to A_{13} and A_{23} would be negligible. In this situation, it can be seen that strong orientation and induction, being present in the interactions among water molecules, and being absent in the interactions between water and nonpolar moecules, indirectly enhance A_{132}. <u>Besides</u>, the strength of the dispersion interactions among nonpolar molecules ($\bar{A}_{11}$ or $\bar{A}_{12}$), when compared with that among water molecules ($\bar{A}_{33}$), is much larger (as in carbon disulfide) or a little larger (as in the lower alkanes)[68a,68b]. Consequently, dispersion will make a significant contribution to A_{131} or A_{132} for the interaction of nonpolar materials in water, as can be checked by setting $\bar{A}_{11} \geq \bar{A}_{33}$ in Eq. 2c.

In a medium less polar than water, orientation and induction occur to a lesser extent than in water. (In fact, these are absent in hydrocarbons.) Hence, the contribution of these interactions to

A_{132} or A_{131} in a nonpolar medium would be small (as can be checked by setting $\varepsilon_1 \simeq \varepsilon_3$ in Eq. 2c). Besides, the difference between $\bar{A}_{11}$ or $\bar{A}_{12}$ and $\bar{A}_{33}$ will be lesser when the medium is nonpolar than when the medium is water, for the interaction of nonpolar materials. Again, from Eq. 2c, it can be seen that the dispersion contribution to A_{131} (or A_{132}) will be smaller in a nonpolar medium, compared with an aqueous one.

In total, two nonpolar materials will undergo a larger van der Waals attraction with each other when water is the intervening medium than when the medium is nonpolar. This is supported by the A_{131} values listed in Table II (e.g., A_{131} for carbon disulfide in water is about three times larger than A_{131} for carbon disulfide in decane).

In this context, it is worthwhile to note that while A_{131} for the interaction of two bodies of the same material 1 across any medium 3 would always be positive[69] as indicated by Eq. 2c, the Hamaker coefficient for the interaction of dissimilar materials can become negative when the strength of the van der Waals interactions among the molecules of the medium lies between those of interactions among the molecules of each material, i.e.,

$$A_{132} < 0 \quad \text{when} \quad A_{11} < A_{33} < A_{22} \quad \text{or} \quad A_{11} > A_{33} > A_{22} \tag{3}$$

This possibility has been recognized earlier[68a,68b,71-75].

In order to derive simple expressions for the interaction potentials, the protein-HIC adsorbent system is represented here schematically as shown in Figure 1. The protein and the adsorbent matrix are approximated by a sphere and a semi-infinite plate, respectively. The hydrocarbonaceous ligands are construed as forming a film-like coating on this plate. An expression for the van der Waals interaction between a sphere and a film-coated semi-infinite plate has been derived[26,69,76a] as

$$\phi_{vdW}(h) = A_{P32} \quad F\left(\frac{h+\delta}{a_p}\right) + A_{F32}\left[F\left(\frac{h}{a_p}\right) - F\left(\frac{h+\delta}{a_p}\right)\right] \tag{4a}$$

with the function F given by

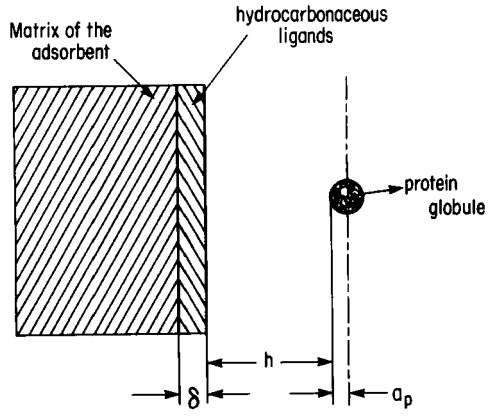


FIGURE 1

Schematic of the protein - hydrophobic interaction chromatography adsorbent system.

$$F(x) = \frac{1}{6} \ln\left(\frac{x+2}{x}\right) - \frac{1}{3} \frac{(x+1)}{(x+2)(x)} \qquad (4b)$$

where δ is the film thickness, a_p is the radius of the sphere, and h is the distance between the surfaces of the sphere and the film. The subscripts P and F denote the plate and the film, respectively. Thus, A_{P32} is the Hamaker coefficient of the interaction between the sphere 2 and the plate P across the medium 3, while A_{F32} is that for the interaction of the sphere 2 with the film F across the medium 3. Some comments regarding these Hamaker coefficients are in order. It was shown that nonpolar entities undergo strong van der Waals attraction with each other, in water. Thus proteins, with an appreciable number of nonpolar residues both on the surface and in the interior would be attracted by the nonpolar hydrocarbon-coated adsorbent when water is the mobile phase. It should be noted, however, that the adsorbate does not necessarily have to be highly nonpolar (i.e., "hydrophobic") to sustain a significant van der Waals attraction with the adsorbent. This is so since the van der Waals attraction between a hydrophilic material and a hydrophobic material

across water is significant, albeit weaker than the corresponding interaction of two hydrophobic materials[75a,75b]. That A_{F32} is larger than A_{P32} follows as an a posteriori inference from experimental observations that proteins are, in general, retained more on hydrocarbon-coated agaroses rather than on the agarose matrix itself (at various ionic strengths). As would be discussed in later sections, these Hamaker coefficients can be altered by changing the alkyl chain length and the number density of the alkyl ligands on the adsorbent as well as by changing the nature of the aqueous medium (via addition of certain salts and organic solvents).

It should be mentioned that dispersion interactions begin to be retarded at large distances between the interacting bodies, due to the finite velocity of electro-magnetic waves[77]. Since the present paper concerns events that occur when the surfaces of the protein and the adsorbent are separated by distances less than 100 Å, the retardation effects need not be taken into account.

Lifshitz[78] has developed a theory of van der Waals interactions based on quantum electrodynamics. For distances h less than 100 Å, the predictions of the Hamaker approach and the Lifshitz theory have been found to be close to each other[68-72]. Since the Hamaker approach is simpler in form, it has been adopted for use in this paper.

Further details on van der Waals interactions can be found in the many existing reviews[68b,72,79,80].

B. Electrostatic Double Layer Interactions

Solid surfaces in contact with aqueous media can become electrically charged due to several mechanisms[81]. In general, proteins and HIC adsorbents acquire their surface charges through the dissociation of acidic and basic surface sites, if any. In situations where ionic detergents have been used to solubilize the solutes (e.g., membrane proteins), the surfaces can become charged by the adsorption of surfactant ions.

An electrically charged surface attracts to its vicinity, ions that are oppositely charged relative to the net surface charge (counter-ions). Due to thermal motions, however, the counter-ions tend to drift away to the bulk where they are less concentrated. As

a result of these opposing forces, the distribution of counter-ions in the liquid is diffuse, resembling a cloud. The charged surface and the diffuse layer of counter-ions constitute the electrical double layer. A characteristic length associated with the double layer, $1/\kappa$, is known as the "Debye length" or the "double-layer thickness" and is defined by

$$\kappa^2 = \frac{8\pi e^2}{\epsilon kT} \left(\frac{1}{2} \sum_{i=1}^{I} n_i z_i^2\right) \quad (5)$$

where the solution has I different kinds of ions, n_i and z_i being the bulk-concentration and valence, respectively, of ions of type i. ϵ is the dielectric constant of the solution, e is the charge of a proton, k is the Boltzmann constant and T is the absolute temperature in degrees Kelvin. The quantity in parenthesis can be recognized as the ionic strength μ of the solution. As the ionic strength is raised, κ^{-1} decreases, i.e., the diffuse layer is compressed towards the surface.

As two surfaces carrying electrical double layers approach each other, when the diffuse layers begin to overlap, the counter-ion distribution is altered. The accompanying free energy change, with electrical as well as chemical components, manifests itself as the double layer interaction. This interaction has been estimated assuming constant surface potentials[82,83] or constant surface charges[84]. The former is appropriate when the surface charges arise from the adsorption of ions, if the approach of the layers is slow enough to allow equilibration of the counter ion distributions. The latter is appropriate when the surface charges arise from the dissociation of strongly acidic or basic sites. In the present situation, the surface charges may originate from the dissociation of weakly acidic or basic groups and hence, neither surface potentials nor surface charges will strictly remain invariant as the surfaces approach. In this general case, the surface charge can be derived as a function of pH as follows[26,27]:

The dissociation equilibria are represented by

$$G_{ai}H(\text{surface}) \overset{K_{ai}}{\rightleftharpoons} G_{ai}^{-}(\text{surface}) + H^{+}(\text{aqueous}) \qquad (6a)$$

$$i = 1,2,\ldots,m_a$$

$$G_{bj}H^{+}(\text{surface}) \overset{K_{bj}}{\rightleftharpoons} G_{bj}(\text{surface}) + H^{+}(\text{aqueous}) \qquad (6b)$$

$$j = 1,2,\ldots,m_b$$

Denoting by N_{ai} (N_{bj}) the total number of surface sites of type ai (bj) per unit area, by K_{ai} (K_{bj}) the corresponding dissociation-equilibrium constant, and by quantities in brackets the corresponding concentrations, one has

$$[G_{ai}^{-}][H^{+}] = K_{ai}[G_{ai}H] \qquad (7a)$$

and

$$[G_{bj}][H^{+}] = K_{bj}[G_{bj}H^{+}], \qquad (7b)$$

where

$$[G_{ai}^{-}] + [G_{ai}H] = N_{ai} \qquad (8a)$$

and

$$[G_{bj}] + [G_{bj}H^{+}] = N_{bj}. \qquad (8b)$$

The charge per unit area of the surface, σ', is proportional to the total number of positively charged sites less the total number of negatively charged sites, i.e.,

$$\sigma' = e\left(\sum_{j}^{m_b} [G_{bj}H^{+}] - \sum_{i}^{m_a} [G_{ai}^{-}]\right) \qquad (9)$$

Combining Eqs. 7 to 9 and noting that when the surface potential is ψ_o, the hydrogen-ion concentration near the surface $[H^{+}]$ is related to that in the bulk $[H^{+}]_b$ via the Boltzmann equation,

$$[H^{+}] = [H^{+}]_b \exp(-e\,\psi_o/kT), \qquad (10)$$

one obtains

$$\sigma' = e\left(\sum_{j=1}^{m_b} \frac{[H^{+}]_b N_{bj}}{[H^{+}]_b + K_{bj}\exp(e\,\psi_o/kT)} - \sum_{i=1}^{m_a} \frac{K_{ai}N_{ai}}{K_{ai}+[H^{+}]_b\exp(-e\psi_o/kT)}\right) \qquad (11)$$

At a particular pH in the bulk, known as the isoelectric point pI or the point of zero charge of the surface, the two terms on the right hand side of Eq. 11 cancel each other rendering the net surface charge to be zero. For pH > pI, the net charge of the surface is negative and for pH < pI, the net surface-charge is positive.

A method has been described elsewhere[26,27] for calculating ϕ_{DL}, the potential for the double layer interaction between a sphere and a semi-infinite plate, with the surface charge of each given by an expression such as Eq. 11. In this general case, a closed form expression for ϕ_{DL} is unavailable. Such is possible, however, when certain approximations are made. For example, ϕ_{DL} for the interaction of a sphere, and a semi-infinite plate, at constant surface potentials ψ_{S1} and ψ_{S2} under certain conditions, is given by[82,83]

$$\phi_{DL}(h) = 16\varepsilon\left(\frac{kT}{ze}\right)^2 a_p \tanh\left(\frac{ze\psi_{S1}}{4kT}\right)\tanh\left(\frac{ze\psi_{S2}}{4kT}\right)\exp(-\kappa h) \qquad (12)$$

where z is the valence of the counter-ion, and κ is defined by Eq. 5.

The adherence of particles which are dissimilar in size as well as in electrical and chemical characteristics (just as in the case of the adsorption of proteins onto HIC adsorbents), termed "heterocoagulation" by Derjaguin[71] has features that are distinct from the coagulation of identical particles[86-89]. The possibility of repulsive van der Waals forces, mentioned earlier, is one such feature. Situations have been identified[26,88] wherein the double layer interaction between two electrically nonidentical surfaces can change over from repulsion to attraction or vice versa as the distance between the surfaces becomes smaller. For example, it has been shown that[88] a strongly acidic particle could be effectively prevented from depositing on a strongly basic collector by double layer repulsion, even though the charges on the two surfaces have opposite signs, provided the ratio of the two surface potentials at infinite separation is significantly different from -1.

C. Short Range Repulsive Forces

(a) Steric Repulsion. Adsorbed macromolecules have been known to generate a stabilizing repulsion between particles of colloidal

sols[90]. When two surfaces carrying macromolecules approach each other, as the distance between the surfaces becomes smaller than the extended lengths of the macromolecules, the number of conformations permissible to the macromolecules decreases. The accompanying reduction in entropy causes a repulsion between the surfaces. Besides, the overlap of the polymeric chains is tantamount to an increase in the free energy (via the increase in the concentration of macromolecules). This osmotic effect also entails in a repulsion. Steric repulsion falls off rapidly with increasing distance between the surfaces and rises in proportion to the chain length of the adsorbed molecules[91-93]. The latter dependence on chain length suggests that the short-range steric repulsion is negligible in HIC, because the ligands on the adsorbent are too short to behave like adsorbed macromolecules. Steric repulsion may become important, however, when the adsorbent is covered by previously adsorbed protein.

(b) Hydration Repulsion. Around polar or charged surfaces in aqueous media, a thin layer of water is likely to be held strongly through dipole-dipole or ion-dipole interactions of water with the surfaces. When two such surfaces approach each other, the work required to remove the water of hydration manifests itself as a repulsion[81]. This hydration repulsion has been tentatively assigned as the source of a strong repulsive force between lecithin bilayers that has been experimentally detected[94-96]; the repulsion approximately decays exponentially with distance.

(c) Born Repulsion. The interacting surfaces experience a repulsion when they approach each other within distances of the order of atomic dimensions, due to the fact that the overlap of electron orbitals is forbidden[97].

For the purpose of illustration, these different types of short range repulsion are combined into a single repulsive potential which is assumed to decay with the twelfth power of distance as in the empirical Lennard-Jones 6-12 potential. By linear superposition of the 12th order term in the Lennard-Jones potential, an expression has been derived for the short range repulsion potential correspon-

ding to the interaction between a sphere and a film-coated semi-infinite plate[26] as,

$$\phi_{SR}(h) = \left(\frac{\sigma}{a_p}\right)^6 \frac{1}{7560} \{A_{P32} G\left(\frac{h+\delta}{a_p}\right) + A_{F32}[G\left(\frac{h}{a_p}\right) - G\left(\frac{h+\delta}{a_p}\right)]\} \quad (13a)$$

with the function G given by

$$G(x) = \frac{(x+8)}{(x+2)^7} + \frac{(6-x)}{x^7} \quad (13b)$$

where σ is the collision diameter and A_{P32}, A_{F32} are Hamaker coefficients defined earlier.

Equation 13 has been used earlier[23,26] to represent only the Born repulsion. If σ is treated as an adjustable parameter, however, it may in addition represent the other types of short range repulsion.

D. The Effect of Eluant Composition on the Interaction Forces

(a) Ionic Strength. As noted earlier, the distribution of counter-ions near a charged surface is the outcome of a competition between the disrupting influence of random thermal motions and the orienting effect of the surface charge. When the ionic strength is raised, the latter orienting effect extends lesser into the solution, due to screening by the higher concentration of the ions. Thus, the counter-ion layer becomes more compact, i.e., the double layer thickness is reduced, when the ionic strength is raised (Eq. 5). Consequently, the interacting surfaces can approach each other closer without experiencing the double layer force. In effect, a raise in the ionic strength enhances the net attractive force when the double layer forces are repulsive and decreases it when the double layer forces are attractive. A reduction in the ionic strength does the opposite.

Usually, the double layer interaction potential is derived assuming that ions are point charges and that charged surfaces interact with counter-ions in a non-specific manner. Relaxing these assumptions, as has been done by Stern in colloid stability theory[98] can explain ion-specific effects, if any, concerning double layer interactions. Ion-specific effects in HIC, however, have been observed mostly at high ionic strengths (with exceptions).[152]

Since the effect of salts on double layer interactions should reach a saturation at a relatively low ionic strength (< 1M), these salt-specific "lyotropic" effects result from other mechanisms, which are discussed below.

(b) Lyotropic Effects of Neutral Salts at High Ionic Strengths. The effect of high concentrations of salts, on the solubility of proteins in water, as well as on the interactions between proteins and HIC adsorbents in aqueous media (which determine chromatographic retention) has been the subject of numerous investigations[10,37,99-108]. In the following, we examine in the light of the present interpretation, how a salt solution differs from water as regards these interactions: In water, strongly hydrogen-bonded three-dimensional assemblies of water molecules exist. These structures are not taken to be permanent and rigid but as "flickering clusters" of very short half-lives. Nevertheless, at any instant, considerable proportions of water molecules participate in such three-dimensional structuring[108,109]. These molecules are highly hydrogen-bonded. As discussed in the context of Table I, more hydrogen-bonding is tantamount to more of orientation, induction and dispersion interactions. In other words, water molecules in three-dimensional assemblies are more cohesive than free molecules, and hence, contribute more to the high surface tension of water.

Cations are in general smaller than, and hence, create stronger electric fields than anions. The strong electric field of the cations, coupled with their tendency to associate with the oxygens, results in the normal structure of water being broken in the presence of cations. The three-dimensional structures that result from the strong orientation of water molecules on the cations, however, form a structure that is more organized that the normal water structure. Hence, these cations are termed "structure-making". Similar to the normal water assemblies, the cation-caused structures also have their hydrogens oriented outward. Anions readily associate with the available hydrogens of normal water assemblies. The anion-associated structures have their oxygens outward (p. 35 in Ref. 108). Cations and anions together form multi-ionic assemblies in water which are

more hydrogen-bonded than the structures in pure water. Thus, according to the present interpretation, a solution of structure making ions can be seen as held together by stronger orientation, induction and dispersion than water, and hence, characterized by a larger value for A_{33} (see Eq. 1).

Some ions, on the other hand, are "structure-breaking". Large anions, due to their size, break up the normal structure of water but are unable to form their own assemblies since their electric fields are weak. As a result, large anions such as ClO_4^- are structure-breaking. Structure-breaking also occurs when the ions (e.g., guanidinium or thiocyanate) consistently promote two-dimensional assemblies of water molecules as dictated by resonance structures of the ions (p. 37 in Ref. 108) rather than three-dimensional assemblies as sodium ions would promote, for example. Thus, the solutions of structure-breaking ions have a lower proportion of three-dimensional assemblies than water, and according to the present interpretation, would be characterized by a smaller A_{33} than water.

It is appropriate to stress here the parallelism between the above-mentioned salt effects on the cohesiveness of the medium, and the effects of these salts on the surface tension $\gamma_{\ell v}$ of the medium (Table III lists a few examples). That a structure-breaking salt such as guanidinium chloride reduces the cohesiveness of the aqueous

TABLE III

Surface Tension ($\gamma_{\ell v}$) Values for a few Aqueous Salt-Solutions (at 25°C)[108]

Salt	Concentration	$\gamma_{\ell v}$ dynes/cm
$(NH_4)_2SO_4$	3M	78.4
NaCl	3M	76.9
Water	--	72.0
Tetraethyl Ammonium Chloride	1M	57.0
Guanidinium Chloride	4M	51.0

medium is supported by the fact that a solution of this salt has a surface tension $\gamma_{\ell v}$ lower than that of water. Similarly, a structure-making salt such as ammonium sulfate increases the cohesiveness of the aqueous medium as indicated by the larger surface tension of a solution of this salt, compared with water.

In addition to the above-discussed salt effects on A_{33} via salt-induced changes in the structure of the aqueous medium, salts may also affect A_{13} and A_{23} which correspond to the interaction of the medium with the adsorbent and protein, respectively.

For example, since proteins (which behave as neutral dipoles at high ionic strengths) have much larger dipole moments than water, ion-dipole interactions between the ions of the salt and the protein-dipoles may tend to keep the protein in solution, i.e., increase A_{23}.

Also, A_{23} or A_{13} may be increased via dispersion interactions between the ions and the protein or the ions and the adsorbent if the ion has a higher dispersion coefficient (a quantity proportional to the product of the polarizability of the molecule and the number of molecules per unit volume) than water. It can be inferred that this effect may be important for ions such as tetra alkyl ammonium but not for small inorganic ions such as sulfate.

If the overall effect of an ion or a salt is an increase in A_{132} (the subscripts 1, 3, 2 denoting the adsorbent, the aqueous medium, and the protein, respectively), the attractive force between protein and adsorbent across the medium will increase. Correspondingly, the salt or ion will also increase A_{232}, rendering the protein less soluble in water. Accordingly, the salt or ion is considered the "salting-out" type. Small inorganic ions such as Na^+, NH_4^+ or $SO_4^=$ belong to this category. For these ions, since the mentioned dispersion effects (which increase A_{13} or A_{23}) are negligible, the "structure-making" effect of the ions dominates and thus the consequent enhancement of A_{33} increases A_{132} or A_{232}.

Similarly, a salt or ion that reduces A_{132} will decrease the attractive force between protein and adsorbent across the medium. It will also reduce A_{232}, thus increasing the solubility of the pro-

tein in the aqueous medium. This salt or ion is of the "salting-in" type. Ions such as guanidinium, SCN^- or ClO_4^- which reduce A_{132} *via* structure-breaking (and the consequent reduction in A_{33}) or ions such as tetra alkyl ammonium which reduce A_{132} *via* enhancing A_{13} or A_{23} (by dispersion effects) belong to this "salting-in" category.

In summary, the present interpretation has shown that a "salting-out" salt at high ionic strengths will increase the van der Waals attraction between protein and adsorbent across the medium, while a "salting-in" salt will reduce it. While the present discussion has used some suggestions of Lewin[108], especially the two-dimensional structure promotion by certain salting-in salts, the analysis presented here in terms of the Hamaker coefficient is new. Several other views of the phenomena can be found elsewhere[10,37,99-108].

(c) Changes in the pH. When the surfaces acquire charges through the dissociation of acidic or basic groups, the influence of pH is felt *via* the dissociation equilibria (Eq. 6). The double layer interaction is affected, depending on the nature of the dissociating groups. For instance, if both adsorbate and adsorbent carry only strong acid groups, the surface charges and hence the double layer interaction will remain essentially unaffected by pH. With surfaces that carry weakly acidic and weakly basic groups, however, the pH effect is significant. For example, with a positively charged adsorbent, at a pH above the isoelectric point of the protein, the double layer force is attractive. As the pH is reduced, the double layer attraction will decrease and the force will become repulsive for pH < pI.

The above mentioned pH effect concerns electrostatic double layer interactions between protein and adsorbent. In addition, intramolecular electrostatic interactions between the charges on the protein may be affected by pH-induced changes in the extent of dissociation of its surface sites. Such changes in the intramolecular electrostatic interactions alter the conformation of the proteins[99]. Consequently, the number and orientation of nonpolar groups on the accessible surface as well as the overall shape of the

protein will change. The changes in the nonpolar character of the surface will alter the Hamaker coefficients A_{F32} and A_{P32} by affecting the interaction of the surface with the aqueous medium. As will be discussed later, the van der Waals interaction is very sensitive to the Hamaker coefficient, and hence, will be much affected by (such pH-induced) conformational changes in the protein. The van der Waals attraction is also sensitive to changes in the shape of the interacting bodies. For example, a plate-shaped solute having its larger dimensions parallel to the adsorbent will sustain a stronger van der Waals attraction with the adsorbent, than a spherical solute of equivalent volume, the comparison being made at the same value of h, the distance between the surfaces of adsorbent and adsorbate[76b].

It is noteworthy that double layer interactions between protein and adsorbent, and pH effects on these interactions, will be annulled at high ionic strength. On the other hand, the intramolecular electrostatic interactions can continue to be affected by pH alterations even at high ionic strengths if the spacing of charges on the protein is less than or comparable to the sizes of the counter-ions in solution. In other words, a protein of high charge density can undergo pH-induced conformational changes even at high ionic strengths since the close spacing of its surface charges does not permit the intramolecular electrostatic interactions to be screened by the ions in solution.

(d) Addition of Certain Organic Solvents to the Eluant.

Ethylene glycol (EG) is most often added to the mobile phase in order to elute proteins from HIC adsorbents. Occasionally, another solvent such as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF) or propanol is also used. Table IV lists some properties of these solvents.

In general, such additives lower the dielectric constant of the medium. Thus, as Eqs. 12 and 5 indicate, electrostatic double layer interactions may be enhanced by such additives. On the other hand, the extent to which the surfaces are charged may be reduced,

TABLE IV

Some Properties of Solvents Used in HIC
(All data from Ref. 111 except surface tension data[108,193,196]; all data at 25°C unless mentioned otherwise.)

Solvent	Viscosity (centipoise)	Dielectric Constant	Dipole Moment (Debye units)	Surface Tension (dynes/cm)
Water	0.89	78.3	1.85	72.00
Ethylene glycol	16.90	40.7	2.28	46.70
Dimethyl Sulfoxide	1.96	46.7	3.96	43.54
Dimethyl Formamide	0.796	36.71	3.86	36.76 (20°C)
n-propanol	2.00	20.33	1.68	23.71

since a reduction in the dielectric constant of the medium will decrease the dissociation of weakly acidic surface groups as well as cause pairing between counter-ions and surface charges. Due to these opposing effects, the double layer interactions may not be strongly affected by these additives.

The van der Waals attraction between proteins and adsorbent is expected to be substantially reduced by these additives. The possible mechanisms can be qualitatively discussed as follows.

Protic solvents such as ethylene glycol and propanol are hydrogen-bonded, yet to a lesser extent than water. Aprotic solvents such as dimethyl sulfoxide or dimethyl formamide are not hydrogen-bonded at all[111]. Besides, mixtures of such solvents with water, especially at high volume fractions of the organic solvent, would lack the special structural features of water. Indeed, mixtures of ethylene glycol and water show a partial break-down of the water structure[109]. Thus, as with structure-breaking salts, adding such solvents to the aqueous medium will reduce the extent of orientation and induction interactions among molecules of the medium. Consequently, A_{33} is decreased (despite the fact that the dispersion attraction among molecules of such organic solvents is stronger than

that among water molecules). Such a reduction in the cohesiveness of the aqueous medium upon addition of an organic solvent such as ethylene glycol is attested to by the observed reduction in the surface tension $\gamma_{\ell v}$ of the medium, e.g., water and a 50 vol% aqueous mixture of ethylene glycol have surface tensions of 72 dynes/cm and about 50 dynes/cm, respectively[74]. These organic solvents have higher dispersion coefficients (quantities which are proportional to the product of the polarizability and the number of molecules per unit volume) than water. Hence, in an aqueous mixture of such a solvent, the dispersion attraction between adsorbent or protein and the medium will be larger than the corresponding interactions in water. Thus, the contributions from dispersion to A_{13} and A_{23} will be larger than the corresponding contributions in water. If dispersion were the only van der Waals attraction between the medium and the adsorbate or adsorbent (for example, when the latter are completely nonpolar) A_{13} and A_{23} will be unequivocally increased upon the addition of an organic solvent to the aqueous medium. When the adsorbate or adsorbent has polar as well as nonpolar characteristics, however, orientation and induction interactions between these entities and the medium become important and may be reduced when an organic solvent is added (due to the increased structural disordering of the medium). Hence, depending on the relative extents of polarity and nonpolarity of the adsorbate or adsorbent, the net effect of an organic solvent additive may either be a reduction or an enhancement of A_{13} or A_{23}. In some cases for instance, A_{13} may be reduced while A_{23} is enhanced.

The effect of the additives on the Hamaker coefficient A_{132} depends on the changes in A_{33} and on changes in A_{13} or A_{23}. Recalling the larger contribution ($\simeq$ 50 dynes/cm) from orientation and induction than the one from dispersion ($\simeq$ 22 dynes/cm) to the surface tension of water and hence to A_{33}, one can expect the effect of organic solvent-additives on A_{33} to form a dominant component of the effect of the additive on A_{132}. Indeed, the reduction in A_{33} (due to the smaller extent of orientation and induction among molecules of the organic solvent-water mixture) parallels the net

effect of the organic solvent additive on A_{132}, namely the experimentally observed reduction in the (van der Waals) attraction between protein and adsorbent. Van Oss et al.[73a], have suggested that such additives may even render this van der Waals interaction repulsive.

(e) Addition of Detergents. At concentrations below the critical micelle concentration (CMC), the detergent will compete with the adsorbate for sites on the adsorbent thus facilitating elution. At concentrations larger than the CMC, an additional effect occurs in that proteins begin to be adsorbed on the detergent-micelles. This additional effect also enables the proteins to elute. Apart from participating in the above effects, ionic detergents can affect the electrostatic double layer interactions between protein and adsorbent.

4. THE TOTAL INTERACTION POTENTIAL AND CHROMATOGRAPHIC RETENTION

A. The Total Interaction Potential and Its Shapes

The total interaction potential ϕ represents the combined influence of the different physical forces discussed in the previous section.

$$\phi(h) = \phi_{vdW}(h) + \phi_{DL}(h) + \phi_{SR}(h) \qquad (14)$$

For the interaction between a sphere and a film-coated semi-infinite plate, Eqs. 4, 12 and 13 provide expressions for the potentials on the right hand side. While deriving these expressions, for the sake of simplicity, several effects have been omitted. These include solvation of the surfaces, surface roughness of the order of σ, the importance of the topology of the surfaces at an atomic level for the van der Waals interactions when the surfaces are within atomic distances, and the specific adsorption of ions. Nevertheless, the present approach to adsorption or deposition is independent of the particular expression one assumes for the interaction potential ϕ; when more realistic expressions for ϕ_{vdW}, ϕ_{DL} and ϕ_{SR} are obtained, they can be readily incorporated into Eq. 14 for the total inter-

action potential and in the subsequent analysis. Depending on the values of the parameters of Eq. 14, plots of the total interaction potential ϕ with the distance between the surfaces, h (potential profiles) can have several shapes. Figure 2 schematically depicts some of these shapes that are possible in HIC.

Curve A: The double layer force is either attractive or non-existent. This, in combination with the van der Waals attraction, produces a potential profile that has a minimum as its only extremum.

Curve B: The double layer force is repulsive and dominates at intermediate distances, while the van der Waals attraction dominates at long and short distances. Thus, the potential profile consists of a deep primary minimum at $h = h_{pmn}$, separated from a shallower

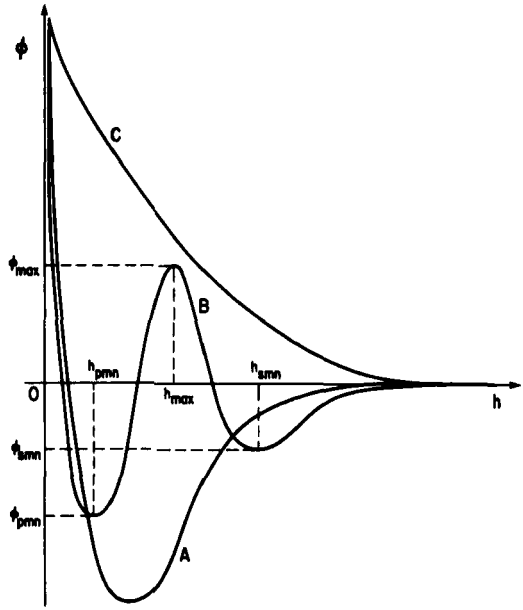


FIGURE 2

Shapes of the total interaction potential profile possible in hydrophobic interaction chromatography.

secondary minimum at $h = h_{smn}$ by a maximum at $h = h_{max}$, with $h_{pmn} < h_{max} < h_{smn}$.

Curve C: This represents situations wherein the repulsive forces dominate at all distances.

In the event that the van der Waals forces are made repulsive, the potential profile may be devoid of minima as in curve C (when the double layer forces are also repulsive or only weakly attractive), or may have a potential barrier and minima (if the double layer forces are strongly attractive).

B. Coefficients for Adsorption and Desorption

If the total interaction potential profile has a potential barrier, the effect of interaction forces on the adsorption rate is considerable even when they act over distances much smaller than the diffusion boundary layer thickness. In an approximate analysis of this situation[112,113], fluid convection is neglected inside a thin layer near the adsorbent, and the interaction forces are neglected outside this interaction force boundary layer. This approach leads to expressions for the coefficients of adsorption and desorption as[25b]

$$k_a = \frac{D_{f\infty}\, h_{max}}{a_p} \frac{(\gamma_{max}\, \gamma_{smn})^{1/2}}{2\pi kT} \exp\left(-\frac{\phi_{max} - \phi_{smn}}{kT}\right) \qquad (15)$$

and

$$k_d = \frac{D_{f\infty}\, h_{max}}{a_p} \frac{(\gamma_{max}\, \gamma_{pmn})^{1/2}}{2\pi kT} \exp\left(-\frac{\phi_{max} - \phi_{pmn}}{kT}\right), \qquad (16)$$

where

$$\gamma_{pmn} = + \left.\frac{d^2\phi}{dh^2}\right|_{h=h_{pmn}}, \quad \gamma_{max} = - \left.\frac{d^2\phi}{dh^2}\right|_{h=h_{max}},$$

and

$$\gamma_{smn} = + \left.\frac{d^2\phi}{dh^2}\right|_{h=h_{smn}}$$

It is noteworthy that the expressions are of the Arrhenius form with $(\phi_{max}-\phi_{smn})$ as the activation energy for adsorption and $(\phi_{max}-\phi_{pmn})$ as that for desorption. The flux J of adsorbate towards the adsorbent is given by

$$-J = K_m(C_\infty - C_i) = k_a\, n_{smn} - k_d\, n_{pmn} \tag{17}$$

where K_m is the mass transfer coefficient, C_∞ and C_i are respectively, the concentrations of adsorbate in the bulk and at the start of the interaction force boundary layer; n_{pmn} and n_{smn} are the number of adsorbate entities per unit area near the primary minimum and that near the secondary minimum, respectively. When the secondary minimum is absent, i.e., when $\phi_{smn} \simeq 0$ (e.g., Figure 6 corresponds to this situation), Eqs. 15 and 17 change to

$$k_a' = \frac{D_{f\infty}\, h_{max}}{a_p} \left(\frac{\gamma_{max}}{2\pi kT}\right)^{1/2} \exp\left(-\frac{\phi_{max}}{kT}\right) \tag{15a}$$

and

$$-J = K_m(C_\infty - C_i) = k_a'\, C_i - k_d\, n_{pmn} \tag{17a}$$

Note that the units of k_a and k_a' are (sec^{-1}) and (cm/sec), respectively. Equations 15-17 have been used in analyses of adsorption and desorption in chromatography[24,25b].

If the potential profile has no maximum and only a minimum, adsorption into the energy well is rapid and convective diffusion is the rate determining step for adsorption. Under these conditions and if the energy well is sufficiently deep, Eq. 17a simplifies to

$$-J = K_m\left[C_\infty - n_{pmn}\left(\frac{\gamma_{pmn}}{2\pi kT}\right)^{1/2} \exp\left(\frac{\phi_{pmn}}{kT}\right)\right]. \tag{18}$$

For large, negative values of ϕ_{pmn}, the second term on the right hand side of Eq. 18 becomes practically negligible.

This type of profile (curve A, Figure 2) occurs in many experimental situations in HIC and it is noteworthy that desorption of proteins much affected by the depth of the energy well is the central phenomenon in these experiments. When Eq. 18 is applied to the desorption stage, it becomes evident that as long as the energy well is deep, (i.e., ϕ_{pmn} is large and negative), even if the number of adsorbed entities n_{pmn} is quite high, the rate of desorption (J) would be negligible. The magnitude of ϕ_{pmn} has to be reduced, i.e., the energy well has to be sufficiently lifted so that desorption can

occur by thermal motions. As would be discussed later, such modifications of the potential profile are often required in HIC.

C. Results from a Parametric Study

The enormous sensitivity of the total interaction potential to its parameters was demonstrated in an earlier parametric study[23], which assumed the surface potentials to be constant and did not consider the presence of alkyl ligands on the adsorbent. The indicated trends are illustrative for the present paper.

Figure 3 illustrates the effect of changes in the Hamaker coefficient A_{132} on the potential profile for the interaction of a sphere and a semi-infinite plate. It can be inferred that (even small) changes in A_{132} brought about by lyotropic salt effects or the addition of organic solvents, radically alter the shape of the potential profile.

Similar sensitivity to ionic strength is indicated in Figure 4, which shows how double layer repulsion can be effectively suppressed by raising the ionic strength.

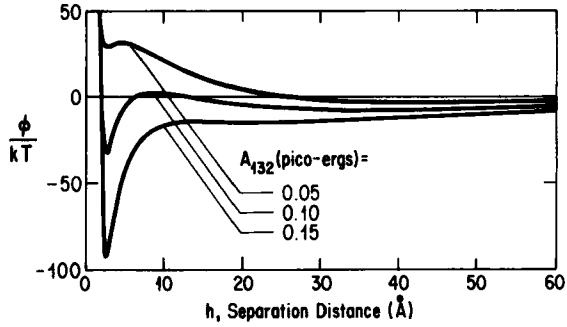


FIGURE 3

Effect of the Hamaker coefficient A_{132} on the total interaction potential profile. The other parameters are $\psi_{s1} = \psi_{s2} = 25$ mV, $\sigma = 5$A, $\mu = 0.1$M, $a_p = 0.1\mu$m, $\varepsilon = 74.3$ and T = 300°K, (subscript 1 ← adsorbent (semi-infinite plate); subscript 2 ← protein (sphere); and subscript 3 ← the aqueous medium).

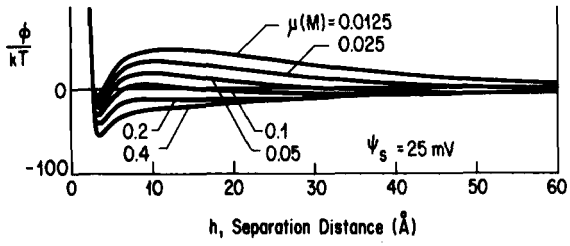


FIGURE 4

Effect of ionic strength on the total interaction potential profile when the surface potentials are held fixed. Parameters are the same as the ones in Figure 3 and $A_{132} = 10^{-13}$ erg, (subscript 1 ← adsorbent (semi-infinite plate); subscript 2 ← protein (sphere); and subscript 3 ← the aqueous medium).

Thus, the energy well in the potential profile can be made shallow by reducing A_{132} (via the addition of salting-in salts or organic solvents or via the removal of salting-out salts) as indicated in Figure 3, by lowering the ionic strength when the double layer forces are repulsive (Figure 4), or by raising the ionic strength when the double layer forces are attractive (not shown in the figures).

Figure 5 shows that larger particles experience larger potential barriers and sustain deeper minima in their interaction potential profile than smaller particles. For the situations represented in Figure 5, potential barriers exist and it can be inferred that larger particles will find it more difficult to adsorb as well as desorb than smaller ones. Similarly, when the potential profile does not have a maximum (as in curve A, Figure 2), larger particles will desorb with more difficulty.

Figure 6 is from another study[26], which considered the presence of the alkyl ligands on the adsorbent and that the surface charges arise from the dissociation of surface groups. It shows that if the Hamaker coefficient for the film-sphere interaction A_{F32} is greater than that of the adsorbent matrix-sphere interaction A_{P32},

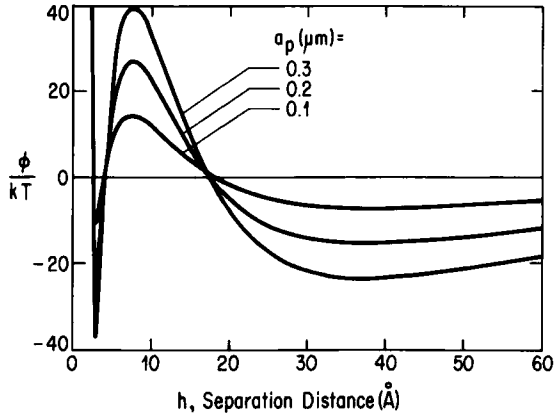


FIGURE 5

Effect of the radius of the (protein) sphere on the total interaction potential profile. All parameters as in Figures 3 and 4 except $\psi_{s1} = \psi_{s2} = 28mV$.

the adsorption-coefficient increases appreciably with chain length while the desorption-coefficient decreases. Although Figure 6 corresponds to the case in which a potential barrier to adsorption exists, the extension to the situation where such a barrier is absent is simple. In the latter case, it will be more and more difficult for a protein to desorb as longer alkyl ligands are used. Figure 6 indicates that no further changes occur, however, as the film thickness (or chain length) becomes very large, since the adsorbent begins to behave as if it were made entirely of the film material.

Increasing the alkyl chain length has an additional effect. From the values of the Hamaker coefficient A_{132} for the interaction of bovine albumin with several alkanes, listed in Table II, one can conclude that A_{F32} increases with the alkyl chain length and tends to a saturation (e.g., from pentane to hexane, A_{F32} increases by 0.745 x 10^{-14} ergs, while from nonane to decane, it increases by a lesser amount, namely 0.248 x 10^{-14} ergs).

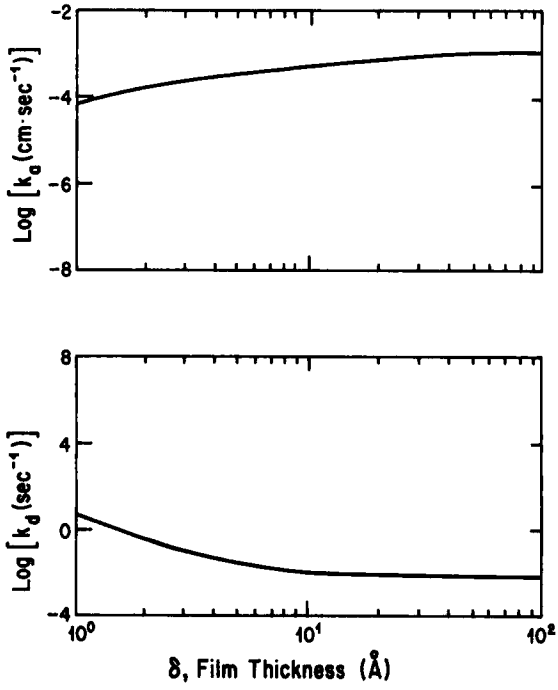


FIGURE 6

The effect of the length of the alkyl chains of the adsorbent on the coefficients of adsorption and desorption. Attached chains are modeled as a continuous film (see Figure 1). Hamaker coefficients for the film-sphere and adsorbent matrix-sphere interactions are, respectively, $A_{F32} = 1.5 \times 10^{-13}$ erg, and $A_{P32} = 1.0 \times 10^{-13}$ erg. The adsorbent and the (protein) sphere are taken to be strongly acidic, each carrying a single acidic site.

Characteristics of the adsorbate (sphere): $N_a = 1.147 \times 10^{13}$ cm^{-2}, $N_b = 0$, $pK_a = 0$, $\psi_{s2} = -25$ mV.

Characteristics of the adsorbent (semi-infinite plate): $N_a = 2.576 \times 10^{13}$ cm^{-2}, $N_b = 0$, $pK_a = 0$, $\psi_{s1} = -50$ mV.

Other parameters are: $\mu = 0.1$M, $pH_{bulk} = 7$, $\sigma = 2$Å, $a_p = 100$Å, $\varepsilon = 73.4$, $T = 300$°K.

In total, increasing the alkyl chain length of the ligands results in a larger film thickness as well as a large Hamaker coefficient A_{F32} thus enhancing the van der Waals attraction between protein and adsorbent, the enhancement tending to a saturation as the chain length is increased.

D. Chromatographic Retention and Elution

If a pulse of a mixture of different proteins is introduced into an HIC column, it can be expected that the mixture will be resolved into several fractions during the subsequent elution development. Occasionally, even a sample containing protein molecules all having the same primary structure can elute in more than one fraction. This may be caused by heterogeneities of the protein sample or of the adsorbent. Such heterogeneity is discussed separately in a later section. Here, the nature of various fractions that elute from the column (whether they originate from a single protein sample or from a mixture of proteins) is discussed in terms of the underlying interaction potential profiles. The fractions have been named A, B, C and so on merely to facilitate the recall of the discussion of each fraction given in this section, when these names appear as identifiers in later sections wherein numerous experimental reports are summarized.

Fraction A: Some proteins may elute ahead of an unretained micromolecular tracer (i.e., $V_R/V_0 < 1$, where V_R is the retention volume of the protein while V_0 is that of the unretained micromolecular tracer). The corresponding potential profile is either devoid of extrema (curve C, Figure 2) or has a potential barrier that is substantially higher than the one in curve B. Thus, the proteins are excluded from the region very near the wall, where the carrier solution moves slower than in the bulk. The unretained micromolecular tracer, however, is not excluded from this region. As a result, the tracer lags the proteins. While this mechanism affects only a minor fraction of the eluting proteins in HIC, it is a major effect in "hydrodynamic chromatography"[114,115]. Within this fraction, the larger proteins elute earlier since a larger particle experiences a

larger repulsion, all the other parameters remaining the same (Figure 5).

Fraction B: Another fraction of proteins will elute with a residence time equal to or slightly larger than that of an unretained micromolecular tracer ($V_R/V_0 \geq 1$). This fraction has been held in either an energy well that is much shallower than the one in curve A (Figure 2) or in the shallow secondary minimum beside a high potential barrier similar to the one in curve B.

Fraction C: The retention times of the above fractions are less than or not very different from that of an unretained micromolecular tracer. Following these fractions, some proteins may elute, their retention being governed by rates of adsorption and desorption which are comparable. The corresponding potential profile has a moderately high potential barrier as well as a moderately deep energy well, as in curve B (Figure 2). The retention times will be quite higher than that of the tracer and will be very sensitive to the characteristics of the proteins. This fraction corresponds precisely to the "Potential Barrier Chromatography" suggested by Ruckenstein, et al.[24], who predicted that particles differing only 5% in size, or 1% in surface potential, or 1% in Hamaker coefficient elute at appreciably different times and hence could be separated. Although adsorption and desorption at comparable rates as in PBC promise fine separations, this does not occur often in protein chromatography[116]. Usually, the range of parameters (such as pH, ionic strength) over which the proteins are very tightly held is so narrow that most of the bound protein will not elute unless the composition of the eluant is changed. In other words, the potential profile favoring retention is such that, adsorption-desorption equilibrium is driven too far towards adsorption, and desorption is possible only if the profile is altered suitably.

In HIC the proteins introduced are often bound very tightly. The corresponding profiles have energy wells deeper than the ones shown in Figure 2. Desorption is facilitated by means of changes in the eluant composition which: (a) lift the energy well to a

level whence desorption can occur by thermal motions; (b) introduce a maximum in the profile at a distance smaller than h_{pmn} of the profile which was responsible for retention; or (c) eliminate all minima and introduce a profile as in curve C (Figure 2).

The energy well can be lifted by weakening the attractive forces, or by strengthening the repulsive forces. If the double layer force is attractive, it can be weakened by raising the ionic strength or by changing the pH (e.g., if initially the adsorbent is negatively charged and the protein is positively charged, an increase in pH is appropriate). With repulsive double layer forces, retention would have occurred at high ionic strength and elution can be achieved by reducing the ionic strength. The van der Waals attraction between protein and adsorbent can be decreased by adding an organic solvent such as ethylene glycol or a salting-in salt such as NaSCN to the eluant.

A potential barrier can be generated by strengthening double layer repulsion as discussed above, or by causing a change-over from double layer attraction to repulsion *via* an alteration in the pH (e.g., if retention occurred on a positively charged adsorbent at a pH above the isoelectric point pI of the protein, elution can be triggered by lowering the pH to a value less than pI). Rendering the van der Waals forces repulsive (possibly with an eluant of high ethylene glycol content) is sure to introduce a maximum in the potential profile, or eliminate all minima depending on whether the double layer forces are attractive or repulsive. The fractions that would elute only if the mobile phase is modified can be divided into two types.

Fraction D: A fraction can be eluted by manipulating the double layer interactions *via* changes in the ionic strength or pH. For example, the appropriate procedure would be a reduction in ionic strength if the double layer forces are repulsive and a raise in the ionic strength if the forces are attractive.

Fraction E: Subsequent to the above fractions, there may be proteins which are held *via* strong van der Waals forces (that had

caused retention independent of attractive double layer forces or despite repulsive double layer forces). These cannot be eluted by merely modifying the double layer interaction. In addition, the van der Waals attraction has to be reduced. This can be achieved by adding organic solvents such as ethylene glycol or a salting-in salt, or by reducing the concentration of salting-out salts in the eluant, if any are present. van oss, et al.[73a], have suggested that such additives cause elution by rendering the van der Waals forces repulsive. In the view of the present interpretation, however, it can be seen that while making the van der Waals forces repulsive is a sufficient cause of elution, it is not a necessary condition; if the attractive forces are adequately reduced (as discussed in the context of Eq. 18), the energy well can be lifted to a level whence elution can occur by thermal motions. In other words, while desorption of proteins (upon introducing additives such as ethylene glycol) can certainly stem from the involvement of repulsive van der Waals forces, elution can occur even with attractive van der Waals forces prevailing, provided these are made sufficiently small.

The strong attractive forces in the vicinity of the adsorbent can drastically change the conformation of some proteins, thus increasing the strength of the binding. Such proteins require drastic elution procedures (high concentrations of ethylene glycol or detergents) and quite possibly elute only in a denatured form (fraction F). Table V summarizes the characteristics of the different fractions.

The required changes in the composition of the mobile phase in order to cause elution, can be done either in steps (i.e., by operating with a different isocratic eluant) or continuously (in a gradient elution).

5. EXPERIMENTAL EVIDENCE

Experimental evidence in HIC can be divided into several categories depending on the ways in which the characteristics of the

TABLE V

Shapes of the Total Interaction Potential Profile and the Corresponding Elution Behavior in HIC

Fraction	Change in the Eluant-Composition Required for Elution	Profile in Figure 2 Responsible for Retention	$\frac{\text{Retention Volume of the Fraction}}{\text{Retention Volume of an Unretained Tracer}} = \frac{V_R}{V_0}$
A	None	Curve C	< 1
B	None	Shallow minima in Curve A or Curve B	≥ 1
C	None	Moderate extrema in Curve A or Curve B	> 1 (adsorption and desorption at comparable rates)
D	Change ionic strength or pH (both in the low ionic strength range 0 to 1M)	Deep minimum in Curve A	∞ (i.e., strong binding and elution is possible only by changing the composition of the eluant)
E	Reduce Hamaker coefficient [(i) add ethylene glycol, dimethyl sulfoxide, etc., (ii) add a salting-in salt such as NaSCN, or (iii) reduce the concentration of salting-out salts, e.g., NaCl]	Deeper minimum in Curve A	∞ (i.e., strong binding and elution is possible only by changing the composition of the eluant)
F	Add detergent or a high concentration of ethylene glycol	Very deep minimum in Curve A	∞ (protein is likely to be eluted in a denatured form)

mobile phase or the adsorbent have been manipulated in each experiment.

An important characteristic of the mobile phase is its ionic strength. Ionic strength effects fall into two types. The first concerns the effects that occur in the low ionic strength range (< 1M) (Table VI) and are shown by only adsorbents that are charged. Electrostatic double layer forces prevail and at least some of the adsorbed proteins can be eluted, by raising the ionic strength if these forces are attractive and by reducing the ionic strength if the forces are repulsive. The mechanism of any pH effect in this ionic strength range is also likely to be electrostatic. The second type of effect is observable only at high ionic strengths and is strengthened by raising the ionic strength and vice versa. This lyotropic salt effect is exhibited with adsorbents carrying hydrocarbonaceous ligands[43] as well as adsorbents devoid of such ligands[110], and whether the adsorbents carry ionogenic groups[43] or not[50]. Any pH effect in this range is probably due to conformational changes in the proteins and the consequent changes in the van der Waals interaction (as discussed in Sect. 3.D.c). Some experiments[154] have evidence for both the low ionic strength electrostatic effects and the high ionic strength lyotropic effects.

In many experiments, apart from modifications of the mobile phase, the characteristics of the adsorbent have also been altered by changing the alkyl chain length and the number of the alkyl ligands on the adsorbent. Some experiments concern temperature effects while others show direct evidence for the heterogeneity of proteins and adsorbents. Some recent experiments[73-75] have elaborated on the parallelism between the effect of additives on the surface tension $\gamma_{\ell v}$ of the aqueous medium and the effect of these additives on protein-retention in HIC.

In this section, experimental evidence is classified in accordance with the above discussion. While most of the results are summarized in tables (with identification of the various fractions discussed in Section 4.D), some are discussed in detail.

A. Low Ionic Strength Range - Attractive Double Layer Forces

HIC adsorbents carrying alkylamines (coupled to the adsorbent matrix by CNBr activation) are in general positively charged around neutral pH, due to the charging of the (α and ω, if any) amino groups. (By α and ω amino groups, we mean the amino group that connects the alkyl chain with the adsorbent and the amino group present at the distal end of the alkyl chain, respectively.) Acidic proteins (pI < 7) are negatively charged at this pH. Hence, the electrostatic double layer interaction between the protein and adsorbent is attractive.

The importance of electrostatic (double layer) interactions in HIC was demonstrated by Wilchek and Miron[45] who compared α-alkylaminoagaroses (butyl and hexyl) which were positively charged at pH = 8 with acetylated aklylamino agaroses which were uncharged at that pH. While negatively charged ovalbumin and α-lactalbumin were retained on the alkylamino agaroses (and eluted upon raising the ionic strength), they were unretained on the acetylated adsorbents. The retention of these proteins had been dominantly caused by double layer forces. Bovine serum albumin, on the other hand, was strongly retained on both charged and uncharged adsorbents. The van der Waals attraction in this case is strong enough to cause retention even in the absence of attractive double layer forces.

Identical behavior of these proteins was observed in a study by Jost, et al.,[52] who compared charged alkylaminoagaroses with uncharged alkyl hydrazide agaroses. While ovalbumin and α-lactalbumin were retained only on the charged adsorbents, bovine serum albumin was retained on both the charged as well as the uncharged adsorbents.

In other instances, retention is mostly due to nonelectrostatic (van der Waals) attractive forces rather than electrostatic (double layer) forces. Shaltiel, et al.,[7] observed that the elution profiles of hemoglobin, phosphorylase b, lysozyme and erythrocytes on charged alkyl amino agaroses were largely identical with the elution profiles on uncharged acetylated alkylamino agaroses. Similarly, the elution profile of erythrocytes on alkylamino agaroses was essentially un-

TABLE

Experimental Evidence - Section 5A & B --

(*N.B.: For the explanation of the

EG - ethylene glycol;

Entry #	Ref.	Adsorbent/Protein(s)	Fraction D and its Elution Procedure* (Additives or pH manipulations required to cause elution by manipulating the electrostatic double layer interaction)
1.	120	phenylbutylamine agarose/α-chymotrypsin	absent
2.	120	phenylbutylamine agarose/serum albumin	(1M NaCl)
3.	120	phenylbutylamine agarose/β-lactoglobulin	(1M NaCl)
4.	120	phenylbutylamine agarose/γ-globulin	absent
5.	120	phenylbutylamine agarose/ovalbumin	(1M NaCl)
6.	120	phenylbutylamine agarose/chymotrypsinogen	absent
7.	120	phenylbutylamine agarose/chymotrypsinogen + OV + serum albumin	(0 to 1M NaCl gradient) ovalbumin + serum albumin
8.	120	A-ACTME (see text for formula)/chymotrypsin, γ-globulin, bovine serum albumin (low ionic strength)	(1M NaCl)

VI

-Effects in the Low Ionic Strength Range

different fractions A to E, see Table V

TEAC-tetraethyl ammonium chloride)

Fraction E and its Elution Procedure* (Additive required to cause elution by reducing the van der Waals attraction)	Details and Remarks
(1M TEAC or 33V% EG + 1M NaCl)	Does not bind to agarose without ligands, whether CNBr activated or not; Fractions A to C absent.
(1M NaCl + 50V% EG)	Does not bind to agarose without ligands, whether CNBr activated or not; Fractions A to C absent. (See Sect. 5H on Heterogeneity).
(1M NaCl + 50V% EG)	See Sect. 5H on Heterogeneity and note [Fraction D] > [Fraction E].
(1M NaCl + 50V% EG)	Binds to CNBr activated agarose but can be eluted without changing the eluant (Fraction B); see also Sect. 5.C.c.
absent	Binding mostly caused by electrostatic double layer attraction.
absent	Can be eluted in a broad peak without modifying eluant (Fraction C), see text.
(50V% EG + 1M NaCl) serum albumin + ≈ ovalbumin	See discussion on heterogeneity in Sect. 5H; chymotrypsinogen was unretained.
see remarks	Elution occurs better with the salting-in salt 1M TEAC (than with 1M NaCl).

TABLE VI

Entry #	Ref.	*Adsorbent*/Protein(s)	Fraction D and its Elution Procedure* (Additives or pH manipulations required to cause elution by manipulating the electrostatic double layer interaction)
9.	119	*polyaminomethyl styrene*/a mixture of several dehydrogenases and human serum albumin 0.067M, pH 8	lactate and alcohol dehydrogenases and serum albumin (pH 8 to 5, NaCl 0 to 0.2M)
10.	127	α,ω *diamino decyl agarose*/hepatitis B surface antigen (0.01M Tris buffer pH 7.2)	(0.8M NaCl)
11.	128	α,ω *diaminohexyl agarose*/extract containing glycogen phosphorylase; low ionic strength, pH 7.2	(0.2M Na_2SO_4)
12.	129	α,*amino hexyl agarose*/tryspin treated extract of acetyl choliesterase	(0.4M saline)
13.	130	α *amino propyl agarose*/potato juice containing potato phosphorylase	(0.3M NaCl)
14.	47	α *amino alkyl agarose*/A-chymotrypsinogen, chymotrypsin and lysozyme pH 8, low ionic strength	absent

Continued

Fraction E and its Elution Procedure* (Additive required to cause elution by reducing the van der Waals attraction)	Details and Remarks
not tested	3 hydroxy butyrate hydrogenase could be eluted in an inactivated form at 2.5M NaCl, pH 3 (in general, 40 to 70% recovery of activity); malate and isocitrate dehydrogenase were unretained.
4M NaSCN	Note that a salting-in salt is used for elution.
not tested	Proteins were unretained but the enzyme was retained and eluted in Fraction D.
0.2M tetraethyl iodide	Note that a salting-in salt is used for elution.
not tested	Proteins were unretained but the enzyme was retained and eluted in Fraction D.
absent	At pH 8, the adsorbent and proteins are positively charged. A large fraction is unretained due to double layer repulsion. In octyl agarose, the van der Waals attraction overcomes the repulsion to some extent and causes weak binding.

TABLE VI

Entry #	Ref.	*Adsorbent*/Protein(s)	Fraction D and its Elution Procedure* (Additives or pH manipulations required to cause elution by manipulating the electrostatic double layer interaction)
15.	131	*ω carboxy, α amino agarose*/lipoamide dehydrogenase; pH 7.2, low ionic strength	absent
16.	132-135	*N-(3-carboxy propionyl) amino decyl agarose CPAD-a*/asparate transcarbamoylase, membrane proteins of the human erythrocyte "ghost," etc.	see remarks
17.	136	*alkyl amino glycyl, agaroses & 6-amino caproyl amino alkyl agaroses*/β-hydroxy butyrate dehydrogenase (pH 8.0 low ionic strength)	not observed

altered when the surface charge of the cells was considerably reduced by treatment with neuraminidase[118].

The importance of nonelectrostatic (van der Waals) attraction is highlighted by the observation that proteins which are electrically

Continued

Fraction E and its Elution Procedure* (Additive required to cause elution by reducing the van der Waals attraction)	Details and Remarks
absent	Double layer repulsion dominates over van der Waals attraction and prevents binding; thus, all of the enzymes were unretained in the propyl to heptyl adsorbents.
see remarks	Discussed in detail in Sect. 5.B.
not observed	Repulsive double layer forces prevent strong retention; eluted in a single fraction which shifted from A to C from the C_3 to the C_7 absorbent; could not be eluted from the C_{18} adsorbent since the van der Waals attraction is very strong.

not much different, e.g., ribonuclease (pI = 9.7), cytochrome C (pI = 9.8) and trypsin (pI = 10.8) show quite different extents of binding on polyaminomethyl styrene[119].

Having noted that both electrostatic and nonelectrostatic interactions contribute towards retention, some experimental results can now be discussed in detail.

Hofstee[120] studied the binding of many proteins to 4-phenyl butyl amine agarose (A PBA) and ε-Amino-Caproyl-D-Tryptophan Methyl Ester agarose (A-ACTME), as well as to both untreated and CNBr-activated forms of the agarose matrix carrying no ligands.

For both A-PBA and A-ACTME, binding was in general stronger than either to the untreated or the activated forms of the agarose matrix. In the absence of the additional van der Waals attraction and the double layer attraction that the ligands introduce, the matrix is unable to create an energy well adequately deep for strong retention.

From A-PBA, for most of the proteins, a fraction eluted upon raising the ionic strength (Fraction D of Table V), the remainder being eluted by supplementing the higher ionic strength with ethylene glycol or a salting-in salt, tetraethyl ammonium chloride (Fraction E). The amount eluting in the presence of ethylene glycol was smaller when a high ionic strength was not maintained simultaneously. This shows that not only the van der Waals attraction has to be reduced by addition of ethylene glycol, but the double layer attraction if any, has to be annulled by keeping a high ionic strength, in order to elute the strongly retained Fraction E, in this particular case.

With A-ACTME, for the majority of the proteins, the amount of protein eluting upon raising the ionic strength (relative to the amount eluting upon adding ethylene glycol) is higher than with A-PBA. This reflects that the double layer attraction has a larger contribution than the van der Waals attraction towards protein binding to A-ACTME.

While chymotrysin shows the highest affinity for A-PBA, chymotrypsinogen, the precursor to the enzyme, shows the lowest. Hofstee[120] explained this on the basis of the proposition that the site which binds nonpolar molecules is unmasked only after the activation of the zymogen[121,122]. The essential outcome of the

exposure of such nonpolar sites can be seen as an increase in the van der Waals attraction with the adsorbent since the surface of the adsorbate becomes less hydrophilic and thus, its attraction with the aqueous medium is reduced; in addition, the activation of chymotrypsinogen is known to involve the formation of a buried internal ion-pair[123] indicating that the enzyme possibly has a lesser positive charge than the zymogen and hence, sustains a smaller double layer repulsion with the positively charged adsorbent.

Similar to the above-cited effect of enzyme activation, pH changes can also modify protein binding in an un-straightforward manner.

Positively charged lysozyme (pI = 10) emerges unbound from positively charged α-amino decyl agarose as well as from the uncharged acetylated version of this adsorbent at pH = 7. Upon reducing the pH to 2, however, it has a much larger residence time on both adsorbents, despite its higher positive charge at this pH. As the experimenters[7] inferred, a conformational change caused by the reduction in pH is possibly responsible for this observation. At pH = 7, the Hamaker coefficient for the total van der Waals interaction of lysozyme with the adsorbent is probably very small or even negative. At pH = 2, more nonpolar residues are exposed to the medium, rendering the surface less hydrophilic thus, reducing its attraction with water. Consequently, A_{F32}, A_{P32}, and the van der Waals attraction between the protein and adsorbent are increased. The citation by Shaltiel[7] that at pH = 2, lysozyme increasingly binds hydrocarbons[124] supports this interpretation.

Several such experiments (entries 1 to 13 in Table VI) demonstrate the cooperation of electrostatic double layer forces with the nonelectrostatic van der Waals attraction in causing retention of proteins on HIC adsorbents. The recurrent observation is that, while the presence of hydrocarbonaceous ligands is necessary for binding to occur, at least a fraction of the bound protein can be eluted by increasing the ionic strength.

B. Low Ionic Strength Range - Repulsive Double Layer Forces

As evident from the above discussion, the charges if any, on HIC adsorbents are in general positive, arising from the dissociation of amino groups. Yon[132] synthesized adsorbents that carry long (C_{10}) alkyl ligands and become negatively charged through the dissociation of carboxyl groups. Operating at a pH at which both the protein and the adsorbent are negatively charged, it is then possible to control the extent of binding by tuning the electrostatic double layer repulsion.

By coupling in sequence diamino decane and succinic anhydride to CNBr activated agarose, Yon[132] prepared N-C_3 carboxy propionyl amino decyl agarose (CPAD-a) which is negatively charged[134] above pH= 8.0. The binding of bovine serum albumin (pI = 4.9) was attempted at various pH (5 to 9.5), at low ionic strength. For pH < 8, the protein was bound and a minor amount (Fraction D) eluted upon addition of 0.5M NaCl, the rest eluting when a detergent was added. For pH ≥ 8, the amount of protein that emerges unbound increased, at the expense of the detergent-eluted fraction. For pH ≥ 8, raising the ionic strength did not elute any protein.

For pH between 5 and 8, BSA is negatively charged while the adsorbent is positively charged. Hence, electrostatic double layer attraction cooperates with the van der Waals attraction to cause binding. The latter attraction, however, is by itself strong enough to keep the protein bound. Hence, only a minor amount elutes when the double layer attraction is curtailed by raising the ionic strength.

At pH ≥ 8, both protein and adsorbent are negatively charged. The ensuing double layer repulsion generates an insurmountable potential barrier for a fraction (A) of the protein which emerges unbound. (Such elution of a single protein in different fractions is caused by possible heterogeneities in the dissolved protein and in the adsorbent, which are discussed in Section 5.H). For some other fraction, the van der Waals attraction is strong enough to moderate the potential barrier and cause retention. As the pH is raised, the

repulsion gains strength thus increasing the fraction that emerges unbound. Raising the ionic strength would only eliminate the repulsion and strengthen the retention. Yon used this approach to purify the enzyme aspartate transcarbamoylase[132] which is negatively charged for pH $\geq$ 8. A crude extract of the enzyme was applied to a column of CPAD-a at pH 8. Most of the unwanted proteins emerged unbound (Fractions A and B). Another fraction which was possibly positively charged, eluted upon adding 0.2M NaCl. Purified enzyme was eluted by resuming the low ionic strength and raising the pH (thus strengthening the double layer repulsion) or by adding sodium deoxycholate or acetone.

Yon[133] obtained better purification of aspartate transcarbamoylase by passing its extract, in sequence, through N-pyromellityl aminodecyl agarose (PMAD-a) and CPAD-a, the former adsorbent bearing a higher magnitude of negative charge. While the unwanted proteins, possibly positively charged, were retained, the enzyme emerged unbound from PMAD-a due to the insurmountable potential barrier. On the CPAD-a, however, the enzyme was retained since the potential barrier was moderate. Elution was achieved by raising the pH (strengthening the double layer repulsion) and by adding a detergent (which facilitates elution *via* mechanisms discussed in Section 3.D.e).

Yon and Simmonds[134] compared the binding of this enzyme to three adsorbents: α-amino Dodecyl agarose (DS-a), α,ω-diamino decyl agarose (ADS-a), and CPAD-a. DS-a and ADS-a are positively charged at pH 8.5; the large van der Waals attraction, in cooperation with the double layer attraction sets up a very deep energy well. Hence, at this pH, the enzyme is bound to DS-a and ADS-a extremely, defying all methods of elution. On CPAD-a, however, the binding was moderate and reversible, due to reasons discussed above, especially repulsive double layer forces.

Yon and Simmonds[134] observed that the amount of enzyme bound, as well as the strength of binding to CPAD-a were higher in the presence of 0.7M sodium phosphate or 2M sodium acetate; in the absence of these salts (at low ionic strength) raising the pH

could elute the enzyme, but in their presence, the pH change had no appreciable effect. This observation is explained by the fact that the double layer repulsion between adsorbent and protein, as well as pH effects on this repulsion, are effectively annulled at the high ionic strength. In the presence of 2M sodium acetate, a lyotropic salting-out effect may also have been responsible for the increased binding.

Simmonds and Yon[135] studied the binding of membrane proteins from the human erythrocyte "ghost" to CPAD-a, with 0.33V% sodium dodecyl sulfate in the eluant. Elution was attempted by increasing the pH from 4 to 12 in a gradient.

Glycophorin, largely a carbohydrate, sustained a weak van der Waals attraction with the hydrocarbonaceous adsorbent. The presence of detergent further weakened this attraction and hence glycophorin emerged unbound. Protein E (the name of this protein is not to be confused with "Fraction E" of the present paper), containing a relatively high proportion of nonpolar residues, eluted with difficulty. The strength of the double layer forces in the interaction of protein E with the adsorbent is apparently insignificant compared to that of the nonelectrostatic van der Waals attraction. This is indicated by the fact that protein E remained bound at quite alkaline pH values despite the prevalence of repulsive double layer forces. (For pH > 8, the CPAD-a adsorbent will be negatively charged and the amino acid composition of protein E indicates that it is an acidic protein[135] and hence would also be negatively charged.) Elution of spectrin was distributed over the entire pH range (indicating either heterogeneity of the protein or that spectrin's attachment to other proteins in the mixture was too tenacious to be disrupted by the adsorbent or the other features of the experiment).

Some other experimental results (entries 14 to 17 in Table VI) also illustrate the possibility of repulsive double layer forces which oppose the van der Waals attraction and thus mitigate the binding. In the low ionic strength range, a characteristic of these experiments is that a reduction in binding occurs upon lowering the ionic strength.

C. High Ionic Strength Range - Lyotropic Salt Effects

The numerous reports of HIC separations based on lyotropic salt effects are summarized in Table VII. Some features, however, merit a detailed discussion.

(a) Adsorbents Carrying Ionogenic Ligands. The typical procedure consists of adsorbing the proteins at high concentrations of a salting-out salt such as ammonium sulfate and achieving differential elution by lowering the concentration of this salt in a linear gradient (entries 1 to 13 in Table VII). This procedure is not of recent origin, however, and dates back to the "salting-out chromatography" of Tiselius[141] and has been applied in affinity chromatography[142]. At the high ionic strengths employed, double layer interactions are annulled and the mechanism of retention rests in the modifications of the mobile phase due to the presence of salting-out salts which enhance the van der Waals attraction between the protein and adsorbent, as discussed in Section 3.D.b. As the salt concentration is reduced, this van der Waals attraction is progressively reduced, causing differential elution.

(b) Adsorbents Carrying Non-ionogenic Ligands. This type of adsorbent was introduced by Porath, et al.,[48,49] and Hjertén, et al.,[50] and the reports are listed as entries 14 to 21 in Table VII. Here, a typical experiment is examined in detail.

Hjerten, et al., synthesized HIC adsorbents by coupling aliphatic alcohols[50] to unactivated agarose. The neutral adsorbents were used in the chromatography of plasma proteins, STN virus and yeast cells. Retention occured at quite high ionic strengths (4M NaCl) at pH = 6.8. Major fractions were eluted by reducing the ionic strength down to 1M. Further fractions were eluted by reduction of the ionic strength to 0.01M. At this low ionic strength, a minor fraction was eluted by a raise in the pH to 9.6. The most strongly held fraction was eluted by addition of 50V% propanol.

One may be led to ascribe the elution of major fractions during the ionic strength reduction from 4 to 1M, to a rejuvenation of repulsive double layer forces between proteins and adsorbent which had

been suppressed at the high ionic strength. Two facts, however, stand against this view.

(i) Although the proteins may be charged, the adsorbent may not carry any significant charge since the ligands are not ionogenic and CNBr activation was not used. A surface charge arising from the adsorption of ions is not certain.

(ii) If electrostatic double layer repulsion is somehow possible, it will be annulled even at the lower ionic strength 1M. Hence, what happens between this high ionic strength and even higher ionic strengths (4M) cannot be adequately explained based on double layer interactions.

A salt-induced enhancement of the van der Waals attraction, however, offers a plausible explanation. Thus, a gradual decrease in the ionic strength differentially elutes the proteins, just as with ionogenic adsorbents. The effect of pH at high ionic strength[50] may be explained by pH induced conformational changes in the protein (see discussion in Section 3.D.c). Elution of minor fractions at very low ionic strength by a raise in the pH possibly results either from enhancement of double layer repulsion, if any, or by pH-induced conformational changes of the protein that reduce its van der Waals attraction with the adsorbent, (as discussed in Section 3.D.c).

(c) Adsorbents Without any Ligands. As summarized in entries 22 to 25 in Table VII, quite a few reports exist of protein binding to the agarose matrix with no ligands, at high concentrations of phosphates or chlorides. With the exception of a suggestion by von der Haar[110], the mechanisms of protein adsorption onto uncoated agarose and onto hydrocarbon coated agarose are thought to be different[147,150] From the discussion in this paper, however, it follows that the difference is probably only a matter of degree than kind. The total (dispersion + orientation + induction) van der Waals attraction between protein and adsorbent, when sufficiently increased by modifying the solvent medium with the addition of salting-out salts, can cause retention even on adsorbents without alkyl ligands. Alkyl ligands, however, offer an additional means of increasing this attraction.

That the adsorption mechanisms are perhaps the same in the two cases is supported by an observation due to Hofstee[147] that ethylene glycol at 50V% can elute γ-globulin from both uncoated agarose and agarose coated with butylamine, the protein having been retained at high salt concentrations.

Such retention on adsorbents without any ligands is similar to the retention of hydrophilic proteins (e.g., serum proteins) on adsorbents with alkyl ligands (in that in both cases the binding is not as strong as that for hydrophobic proteins on hydrocarbon-coated adsorbents). In the absence of alkyl ligands, the van der Waals attraction between protein and adsorbent has to be increased via the addition of salting-out salts, while with the hydrophilic serum proteins, this attraction is just sufficient to cause retention[74].

A noteworthy element in the experiments summarized in Table VII is that retention of a protein occurs at a concentration (of a salting-out salt) less than that required for precipitating the protein out of solution. Complementing an existing explanation by von der Haar[110], recalling the discussion on lyotropic salt effects in Section 3.D.b, a mechanism for this phenomenon can be proposed in the light of the present interpretation as follows:

A salting-out salt increases the Hamaker coefficient A_{132} corresponding to the protein-adsorbent interaction as well as A_{232} for the protein-protein interaction. The net van der Waals attraction in each case, however, is given by a product of A_{132} or A_{232} and the corresponding geometric term (Eq. 4). This term is larger for the interaction of a (protein) sphere with a semi-infinite plate (adsorbent) than for the interaction of two such spheres (protein molecules). Hence, less of the salting-out salt is required to cause retention than for precipitation out of solution.

(d) Illustration of Salt-Specific Effects. The early study by Hofmeister[107] on the relative abilities of different ions in precipitating euoglobins from aqueous solutions led to the arrangement of ions in the order of increasing "salting-out potential". This is known as the Hofmeister or lyotropic series:

TABLE

Experimental Evidence; Section

(Concentration (not ionic strength) of

Entry #	Ref.	*Adsorbent*/Proteins	Retention Occurring in the presence of
1	43	*L-valine agarose*/cell free extract from E. Coli strain 12	1M K_2PO_4
2	137	*α-amino methyl agarose*/ phosphorylase b	1.1M $(NH_4)_2SO_4$
3	44	*L-phenylalanine agarose*/ human placental alkaline phosphatase	1.25M $(NH_4)_2SO_4$
4	138	*caprylyl hydrazide agarose*/ovalbumin + β-lactaglobulin + bovine serum albumin	1.0M Na_2SO_4
5	139	*α-aminopropyl agarose*/ tryptophanase	1.2M $(NH_4)_2SO_4$
6	139	*α-aminopropyl agarose*/ asparto kinase I - homoserine dehydrogenase I	0.5M KCl + 0.5M $(NH_4)_2SO_4$
7	139	*α-aminopropyl agarose*/ β-galactosidase	0.5M NaCl + 0.4M $(NH_4)_2SO_4$
8	140	*10 carboxy α-amino decyl agarose (possibly negatively charged at pH 8.5)*/cell free extract containing alcohol dehydrogenase pH 8.5	1.0M K_2HPO_4/KH_2PO_4
9	145	*daunomycin-ω-amino carboxy pentyl agarose*/nonhistone proteins from an extract of rat lukemia cells	4M NaCl

VII

5C - Lyotropic Salt Effects

salts is given, unless mentioned otherwise)

Elution was Achieved by	Remarks and Details
gradient to 0.1M K_2PO_4	Proteins eluted in decreasing order of the amount of $(NH_4)_2SO_4$ needed to precipitate them out of aqueous solution.
step to 0.0M $(NH_4)_2SO_4$	The enzyme was unretained when introduced without any $(NH_4)_2SO_4$.
in steps to 0.05M $(NH_4)_2SO_4$	The enzyme eluted between 0.75M and 0.5M.
in steps to 0.0M Na_2SO_4	The proteins eluted separately; OV (1M), BLG (0.75M) and BSA (0.0M).
gradient to 0.0M $(NH_4)_2SO_4$	5 fold purification was obtained.
gradient to 0.5M KCl	
gradient to 0.5M NaCl	See Sect. 5.C.d. for discussion of salt-specific effects.
in steps to 0.067M K_2HPO_4/KH_2PO_4	Apart from lyotropic salting-out effects, suppression of electrostatic double layer repulsion at the higher ionic strengths may have been responsible for retention, especially of the fraction that eluted at very low ionic strength.
in steps to 1.0M NaCl	Further fractions eluted by adding 20% glycerol, pH 7.5.

TABLE VII

Entry #	Ref.	*Adsorbent*/Proteins	Retention Occurring in the presence of
10	146	*Speron - 300 (synthetic polymer)*/Lysozyme	2.5 mol/lit KCl pH 6.2
11	146	*Speron - 300 (synthetic polymer)*/human serum proteins	36 W% $(NH_4)_2SO_4$
12	146	*Speron - 300 (synthetic polymer)*/extract of hog pancreatic amylase	20 W% $(NH_4)_2SO_4$
13	146	*Speron - 300 (synthetic polymer)*/human serum albumin + chymotrypsinogen + lysozyme, pH 5.65	0.75 mol/lit KCl
14	49	*Benzyl ether agarose*/kidney bean extract	3M NaCl, pH3
15	50	*pentyl ether agarose*/plasma proteins	4M NaCl, pH 6.8
16	50	*hexyl ether agarose*/extract of β-glucosidase and CM-cellulase from Trichoderma viride	4M NaCl
17	50	*dodecyl ether agarose*/satellite tobacco necrosis virus	4M NaCl
18	50	*napthyl ether agarose*/Baker's yeast	4M NaCl
19	154	*octyl ether agarose*/hisidine decarboxylase	0.6M K_2PO_4

Continued

Elution was Achieved by	Remarks and Details
in a step to 0.0M KCl	Elution could also be due to repulsive double layer forces
gradient to 0% $(NH_4)_2SO_4$ (simultaneous gradient of 0 to 50 V% EG)	
gradient to 0% $(NH_4)_2SO_4$ (the latter half of this gradient accompanied by a gradient of t-butanol from 0 to 60 V%)	
gradient to 0.0M KCl	Possibly, repulsive double layer forces are involved; good separation resulted.
(i) raise pH to 7.5; (ii) switch to 0.0M NaCl; (iii) add 50 V% EG; (iv) add 50 V% glycerol	Each step eluted a fraction; the pH effect at high ionic strength is probably due to a conformational change of proteins (Sect. 3.D.c).
in steps to 0.0M NaCl	Further fractions eluted by raising pH to 9.8 and adding propanol (see text).
gradient to 0.0M NaCl	
gradient to 0.0M NaCl	Note that the adsorbate particles are much larger than proteins.
gradient to 0.0M NaCl	Note that the adsorbate particles are much larger than proteins.
steps to 0.005M K_2PO_4	Further fractions were eluted by adding 50 V% ethylene glycol.

TABLE VII

Entry #	Ref.	*Adsorbent*/Proteins	Retention Occurring in the presence of
20	143	*butyl and pentyl ether agaroses*/phosphoprotein phosphatases from rat-liver cell-sap	3M NaCl pH 7.5
21	144	*phenyl ether agarose*/ snake venom containing cardiotoxin and phospholipase A2	1.5M $(NH_4)_2SO_4$
22	110	*agarose (no ligands)*/ a mixture of aminoacyl t-RNA synthetases	50 wt% $(NH_4)_2SO_4$
23	149	*agarose (no ligands)*/ t-RNA of E. Coli	1.3M $(NH_4)_2SO_4$, pH 4.5
24	148	*agarose (no ligands)*/ a natural mixture of halophilic bacterial proteins	2.5M $(NH_4)_2SO_4$
25	147	*CNBr activated agarose (no ligands)*/ γ-globulin	3M or 0.3M NaCl

$$Li^+ < Na^+ < K^+ < NH_4^+ < Rb^+ < Cs^+$$
$$SCN^- < I^- < NO_3^- < Br^- < Cl^- < F^- < SO_4^= \qquad (19)$$

A characteristic feature of this series is the specificity exhibited by different ionic species that have the same valence and sign.

Continued

Elution was Achieved by	Remarks and Details
steps to 0.0M NaCl at pH 7.5	
gradient to 0.2M $(NH_4)_2SO_4$	Cardiotoxin eluted between 0.8M and 0.4M and thus was separated from phospholipase A2, which did not elute even at 0.0M $(NH_4)_2SO_4$.
gradient to 30% $(NH_4)_2SO_4$	Elution and presumably adsorption occur at a $[(NH_4)_2SO_4]$ about 10% lower than that required for precipitation from solution.
gradient to 0.0M $(NH_4)_2SO_4$, pH 4.5	At pH 7.5, no binding occured even at 1.3M $(NH_4)_2SO_4$, possibly due to conformational changes of the adsorbate (Sect. 3.D.c).
gradient to 0.75M $(NH_4)_2SO_4$	The enzymes malate dehydrogenase glutamate dehydrogenase, and aspartate amino transferase eluted in the decreasing order of the amount of $(NH_4)_2SO_4$ needed to precipitate them out of aqueous solution.
adding 50 V% EG	See discussion in Sect. 5.C.c.

Similar arrangements of ions have been observed in HIC and are briefly noted here.

Raibaud, et al.,[139] studied the effect of various potassium salts on the binding of β-galactosidase on α-amino butyl agarose, in batch experiments. At concentrations of 0.4M, anions could be

arranged in the order of the amount of enzyme bound in their presence (with the additional presence of 0.1M NaCl) as

$$SCN^- \simeq I^- < Cl^- < CH_3COO^- < C_6H_5O_7^{3-} \quad (20)$$

A one-to-one correspondence between salting-out and retention-enhancement, the mechanism of which was discussed in Section 3.D.b is indicated by a comparison of the inequalities 19 and 20.

Påhlman, et al.,[151] studied the influence of salts on the binding of human serum albumin (HSA), phycoerythrin and ovalbumin (OV) to alkyl hydrazide agaroses in batch experiments. They also studied the circular dichroism spectra of HSA and OV in the salt solutions, in order to assess the extent of salt-induced conformational changes in the proteins.

At ionic strength = 3M, the salts could be arranged in the order of the amount of protein bound in their presence as,

$$NaSCN < NaBr < NaCl < Na_2SO_4 \quad \text{(for HSA on pentyl agarose)} \quad (21)$$

and

$$NaBr < NaCl < NaSCN < Na_2SO_4 \quad \text{(for OV on decyl agarose)} \quad (22)$$

In addition, less HSA bound to pentyl agarose in the presence of NH_4^+ than in that of Na^+ (in discord with the Hofmeister series).

Salt-induced conformational changes, as indicated by the circular dichroism spectra, were observed in the presence of NaBr or NaSCN but not in the presence of NaCl or Na_2SO_4. Also, except for ovalbumin in NaSCN, an increased change in the circular dichroism spectra corresponded to a reduced binding.

NaSCN is known to salt-in proteins. Hence, the increased binding of OV in the presence of NaSCN, relative to that in NaCl, seems anamolous. Påhlman, et al., suspected that NaSCN caused severe structural changes in the protein. Such structural changes can modify the van der Waals attraction considerably as discussed in the context of pH effects in Section 3.D.c.

While the above two studies concentrated on salt effects on strong retention of proteins (Fraction E), Nishikawa and Bailon[138]

studied salt effects in a situation wherein the proteins were not strongly retained (Fractions A-C of Table V). In the absence of any salt, β-lactoglobulin was retarded on capryl hydrazide agarose and eluted without any modifications of the eluant. At concentrations > 2M, salting out salts such as NaCl or Na_2SO_4 caused the protein to be severely retained (in Fraction E) while salting-in salts such as guanidinium chloride, KSCN and tetrethyl ammonium chloride accelerated the movement of the protein through the HIC column.

Jennissen and Heilmeyer[152] studied how the desorption of phosphorylase kinase from α-amino ethyl agarose is affected by a linear increase in the concentration of different salts in the eluant. The ionic strengths required for elution followed the series

$$KSCN < CaCl_2 < NH_4Cl < NaCl < Na_2SO_4 \qquad (23)$$

and ranged from 0.098M to 0.45M. The low ionic strength range in question (compared with the multimolar levels cited in Table VII) indicates that these salt effects are primarily electrostatic. The series is perhaps the arrangement of the different salts in the decreasing order of their efficacy in suppressing the double layer attraction. Such specificity in the low ionic strength range may be explained using the modifications that Stern[21,98] introduced in the theory of electrostatic double layer interactions.

D. Combination of Lyotropic and Electrostatic Effects.

Jennissen[153] observed that the extent of binding of phosphorylase b on α-amino methyl or butyl agaroses exhibited a minimum when recorded as a function of the concentration of ammonium sulfate in the mobile phase. The binding is appreciable in the absence of salt, lower in the presence of a small concentration of the salt, and above a certain ionic strength, is larger again. According to the present interpretation, the suppression of the double layer attraction (between enzyme and adsorbent) as the salt is added causes the reduction in binding at low ionic strength. Above a certain ionic strength, the lyotropic salting-out effect begins to enhance

the binding. As would be discussed later, this (change-over from the reduction of electrostatic attraction to an enhancement of non-electrostatic attraction as the ionic strength is progressively raised) may sometimes cause a single protein to elute in more than one peak[3].

Hammar, et al.,[154] observed that phycoerythrin adsorbed to pentylamino agarose both at high ionic strength (4M) and at low ionic strength (0.004M). The protein could be eluted in both cases by introducing a buffer of intermediate ionic strength (0.5M). A similar observation was made by Morrow, et al., [31]. β-galactosidase was retained on 3,3'-diamino dipropyl amine agarose in the absence of any salt as well as in the presence of 1.8M phosphate buffer; in the former case, elution was achieved by raising the ionic strength to 1M NaCl, and in the latter, by reducing the concentration of phosphate in a linear gradient. Each of these two studies[31,154] is a demonstration on a single adsorbent of lyotropically caused binding, (elution caused by lowering the ionic strength) and binding caused by electrostatic double layer attraction (elution achievable by raising the ionic strength). Koller, et al.,[33] studied the binding of lipoproteins HDL, LDL and VLDL onto α-amino butyl agarose. The proteins were retained at low ionic strength and were eluted in the order HDL, LDL and VLDL upon gradually raising the concentration of NaCl to 0.5M. When the proteins were introduced at high ionic strength (4M NaCl), VLDL emerged unbound. Reducing the ionic strength in steps eluted LDL and HDL, rather poorly resolved.

The reversal in elution order, when results from the low ionic strength (electrostatic) and the high ionic strength (lyotropic) ranges are compared, may be explained as follows: Among the three proteins, VLDL has the potential for the strongest electrostatic double layer attraction and the weakest van der Waals attraction; exactly the reverse holds for HDL, whild LDL is of intermediate strength in both interactions. In addition, the van der Waals attraction between the proteins and the adsorbent is larger at multimolar salt concentrations due to lyotropic salting-out effects of NaCl than at low ionic strength.

At low ionic strength, the double layer attraction is largely responsible for the retention and as the ionic strength is raised, the proteins elute in the increasing order of their potential for this interaction (HDL, LDL, VLDL).

For VLDL, the multimolar NaCl concentration eliminates the double layer attraction, but the lyotropic salt effect has not raised the van der Waals attraction much. Hence it elutes unretained. LDL and HDL, the double layer attraction having been annulled, elute in the increasing order of their potential for van der Waals attraction with the adsorbent.

The latter elution order occurred also on an uncharged dodecyl agarose adsorbent in a gradient of 3 to 0.0M NaCl. This supports the above explanation since, with this adsorbent, the proteins interact essentially by the non-electrostatic (van der Waals) attraction.

E. The Effect of Varying the Alkyl Chain Length of the Ligands of The Adsorbent.

In order to assess the importance of non-electrostatic interactions relative to that of electrostatic interactions towards protein retention, Shaltiel[5] synthesized HIC adsorbents each of which had the same charge characteristics as the others but carried a different member of a homologous series of alkyl ligands. Some other researchers[155], noted that an adsorbent carrying alkyl ligands bound more protein than the adsorbent matrix itself, and synthesized such homologous adsorbents, expecting the higher members to have larger affinities toward the protein. Table VIII summarizes the results of numerous such studies. In general, a minimum alkyl chain length is required to bring about retention. The adsorbent with the longer chain length binds more protein; also, the strength of the binding is larger, as indicated by the enhancement of the strongly retained fraction. In some cases, it was observed[38,117] that above a certain alkyl length, the chain length effect reached a saturation, i.e., the marginal increment in binding brought about by lengthening the alkyl ligands diminished above a certain length.

Complementing previous explanations[5,52], the present interpretation attributes the chain length effect to the enhancement of

TABLE

Experimental Evidence - Section

*N.B.: Fractions D and E are eluted by interactions,

Notation: EG ← ethylene glycol; DMF ← det ← detergent; Agr ← ionic strength).

Entry #	Ref.	Protein(s)/Adsorbent	Number of Methyl Groups Required for Retention, n
1	47	ovalbumin/Agr-NH-$(CH_2)_n$-H	n≥ 4
2	47	bovine serum albumin, and β-lactoglobulin (separately)/ Agr-NH-$(CH_2)_n$-H	n≥ 4
3	52	ovalbumin, and α-lactoglobulin (separately)/Agr-NH-$(CH_2)_n$-H	not tested
4	156	phosphorylase a /Agr-NH-$(CH_2)_n$-H	n≥ 1
5	156	phosphorylase b /Agr-NH-$(CH_2)_n$-H	n≥ 4 (pH=7)
6	157	phosphorylase b /Agr-NH-$(CH_2)_n$-H	n≥4 (pH=7)
7	158	catalase T/Agr-NH-$(CH_2)_n$-H	n≥4 (at 1M NaCl)
8	158	catalase T/Agr-NH-$(CH_2)_n$-H	n≥1 (at 0.02 M NaCl)
9	158	horse hemoglobin/Agr-NH-$(CH_2)_n$-H	n≥1
10	158	bovine hemoglobin/Agr-NH-$(CH_2)_n$-H	n≥6

VIII

5E - Alkyl Chain Length Effects

manipulating the double layer and van der Waals

respectively. (See Table V.)

dimethyl formamide; DB ← deforming buffer[157];

Agarose; the upward arrow ↑ indicates a raise (in the

Elution Procedure*		
Fraction D	Fraction E	Remarks
ionic strength ↑ 0 to 1M NaCl	not tested	
ionic strength ↑ 0 to 1M NaCl	absent for n=4; for n=8, add 1M NaCl+50V% EG	
ionic strength ↑ 0 to 1M NaCl	not tested	The amount eluted by raising ionic strength decreased with n.
ionic strength ↑ 0 to 0.5M NaCl	absent for n=3; n=4, add DB	
lower pH by adding acetic acid		
not tested	n=4, add DB at pH 7; n=6, add DB+ acetic acid	
not tested	not tested	Weak binding for n=3 and 4.
ionic strength ↑ to 0.05M NaCl	not tested	Compare with the previous entry (attractive double layer forces).
n=1 to 4, ionic strength ↑ to 1M NaCl; absent for n=6	absent for n=1 to 4; n=6, add detergent	
absent	add detergent	

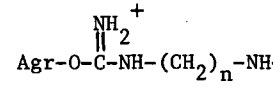
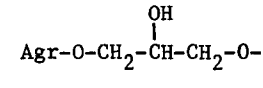
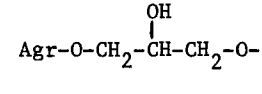
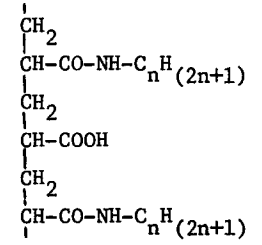
TABLE VIII

Entry #	Ref.	Protein(s)/Adsorbent	Number of Methyl Groups Required for Retention, n
11	152	phosphorylase kinase and other enzymes/Agr-NH-$(CH_2)_n$-H	not tested
12	159	DNA polymerase from R-Mu leukemia virus/Agr-NH-$(CH_2)_n$-H	$n \geq 6$
13	131	lipoamide dehydrogenase/Agr-NH-$(CH_2)_n$-H	not tested
14	139	tryptophanase and homoserine-kinase/ Agr-NH-$(CH_2)_n$-H	not tested
15	139	β-galactosidase and aspartokinase/ Agr-NH-$(CH_2)_n$-H	$n \geq 3$
16	160	glycogen synthetase/Agr-NH-$(CH_2)_n$-H	$n \geq 4$
17	160	glycogen synthetase/Agr-NH-$(CH_2)_n$-NH_2	$n \geq 6$
18	117	bovine serum albumin/Agr-NH-$(CH_2)_n$-NH_2	not tested
19	161	L-histidinol phosphate amino transferase/Agr-NH-$(CH_2)_n$-NH_2	100% activity retention for $n \geq 6$
20	127	extract of serum proteins containing hepatitis B antigen/Agr-NH-$(CH_2)_n$-NH_2	not tested

Continued

Elution Procedure*		
Fraction D	Fraction E	Remarks
ionic strength ↑ 0 to 1M NaCl	not tested	The number density of alkyl ligands required for retention was lower for larger n.
not tested	n=8, add 2M EG; n= 10, add 8M EG+det	
ionic strength ↑ 0 to 30mM phosphate buffer followed by 0 to 1M KCl	n≥6, add concentrated urea or guanidinium chloride (elutes in denatured form)	Binding becomes stronger as n is increased and cannot be reversed by ionic strength changes for n≥ 6.
not tested	not tested	The larger the n, the more retarded are the enzymes.
not tested	not tested	
n=4, ionic strength ↑ 0 to 0.6M NaCl; absent for n=6	absent for n=4	From n=6, the enzyme could be eluted only in denatured form.
ionic strength ↑ 0 to 0.6M NaCl	not tested	Compare with the previous entry.
not tested	not tested	The amount retained increased with n and saturated above n=8.
ionic strength ↑ 0 to 0.4M NaCl	not tested	The amount retained increased gradually from 0% at n=2 to 100% for n≥6.
n<8, ionic strength ↑ 0 to 1M NaCl; absent for n>8	not tested for n<8; for n>8, 25v% EG or 40v% DMF or 0.1% detergent unsuccessful; but 4M NaSCN eluted the antigen	(1) Elution shifted to higher [NaCl] as n increased; (2) A "salting-in" salt is more potent an eluant for fraction E than an organic solvent.

TABLE VIII

Entry #	Ref.	Protein(s)/Adsorbent	Number of Methyl Groups Required for Retention, n
21	135	membrane proteins of the human erythrocyte "ghost"/adsorbent: 	$n \geq 4$
22	162	phycoerythrin/adsorbent: 	$n \geq 5$ (at 3M NaCl)
23	154	crude extract of histidine decarboxylase (at 1.1M)/adsorbent: 	not tested
24	38	mitochondrial membrane proteins/adsorbent: (alkyl ligands attached to polyacrylic acid) 	not tested
25	136	β-hydroxybutyrate dehydrogenase/alkyl amino glyceryl agaroses	not tested

Continued

Elution Procedure*		
Fraction D	Fraction E	Remarks
raise pH from 4 to 12 in a gradient	add 1-butanol	For larger n: (a) elution occurred at higher pH, and (b) the amount eluting upon adding 1-butanol was larger.
absent	(a) lower ionic strength; (b) add EG or glycerol	Organic solvents had to be added for elution only with large n.
absent	lower ionic strength and add EG	26, 32 and 47% of the applied protein was retained on and 6, 12 and 27% eluted in the EG fraction from n=6, 8 and 12, respectively.
ionic strength ↑ 0 to 2M KCl	add detergent	(1) From n=0 and n=4, most of the protein could be eluted without detergent; (2) The number of peaks (indicating either separation efficiency or adsorbent heterogeneity) increased with n and reached a saturation above n=12.
not tested	not tested	The double layer forces were repulsive and prevented strong retention. Elution occurred without changing mobile phase (Fractions A, B, C) and the retardation increased with n.

TABLE VIII

Entry #	Ref.	Protein(s)/Adsorbent	Number of Methyl Groups Required for Retention, n
26	136	β-hydroxybutyrate dehydrogenase/6-amino caproyl alkyl agarose	not tested

the van der Waals attraction that entails from an increase in the alkyl chain length. As noted earlier (Section 4.C), increasing the alkyl chain length is tantamount to having a thicker film of the material which sustains a stronger van der Waals attraction with the protein than does the adsorbent matrix (i.e., $A_{F32} > A_{P32}$). In addition, A_{F32} increases with chain length (Table II). These enhance the attraction between protein and adsorbent (Eq. 4). Above a certain length, however, the film is thick enough for the adsorbent to behave as if it were made entirely of the film material. Besides, the increase in A_{F32} with chain length tends towards saturation (Table II). Hence, further increases in the chain length no longer alter the attractive force. Figure 6 illustrates these points.

While Table VIII offers a general survey of the experiments that concern chain length effects, some of these experiments need to be discussed in detail. These experiments support the general conclusions reached above and in addition illustrate the interplay of the chain length effect with some of the other effects discussed earlier, such as the lyotropic salt-effects.

Hofstee[155] observed that CNBr-activated agarose carrying no ligands or carrying hydroxylamine ligands does not bind several

Continued

Elution Procedure*		
Fraction D	Fraction E	Remarks
not tested	not tested	With n=12, adsorption and desorption possibly occured at comparable rates (Fraction C); from n=18, enzyme activity could not be recovered (Fraction E and F).

(negatively charged) proteins. Agarose carrying the smallest hydrocarbon (methyl amine agarose), however, formed an effective adsorbent. Noting that CNBr-activated agarose carries positive charges, Hofstee postulated that the small hydrocarbon groups prevent the quenching effect of water on these positive charges, thereby permitting electrostatic attraction to cause retention. Alternatively, it is proposed here that the increment in van der Waals attraction brought in by the hydrocarbon moiety, in cooperation with the double layer attraction, is a sufficient cause for the binding. While this van der Waals attraction is necessary for binding onto adsorbents carrying short alkyl ligands ($< C_6$), it is not sufficient to prevent the reversal of retention when the double layer attraction is reduced by raising the ionic strength; for instance, the salt concentration at which the binding was reversed was about the same for proteins such as ovalbumin and serum albumin, with very different extents of nonpolarity.

With adsorbents carrying homologs higher than the hexyl ligand, the van der Waals attraction is dominantly responsible for the binding as indicated by the stability of the binding in the presence

of high ionic strength, as well as the stronger binding experienced by proteins with a higher extent of nonpolar character[155]

Shaltiel, et al., noted that in the α-amino alkyl agarose series [Agarose-NH-$(CH_2)_n$-H], ligands with four methylene groups (n=4) can retain phosphorylase b[156], while ligands of six methylene groups were required for retaining this enzyme on the α,ω-diamino alkyl agarose series [Agarose-NH-$(CH_2)_n$-NH_2][160]. A similar effect was noted by Visser and Strating[131] who compared the binding abilities of α-amino butyl agarose and α,ω-diamino butyl agarose for the enzyme lipoamide dehydrogenase. The enzyme bound to both adsorbents at low ionic strength and pH = 7.2, and eluted in two fractions, one upon introducing 30 mM phosphate buffer of pH = 7.2, and the other during a linear increase in the concentration of KCl from 0 to 1M. While the first fraction eluted at about the same time from both adsorbents, the second fraction eluted from the α,ω-diamino adsorbent at a lower KCl concentration than from the α-amino adsorbent. In accordance with the present interpretation, the following explanation is proposed for these effects.

The presence of the distal amino groups on the diamino adsorbent, while increasing the double layer attraction, reduces the extent to which the adsorbent is nonpolar, making it more hydrophilic. To compensate the ensuing reduction in the Hamaker coefficient A_{F32} of the van der Waals attraction, longer alkyl chain lengths are required for binding phosphorylase b. With the enzyme lipoamide dehydrogenase, for the same reason, the total attractive force and hence the concentration of salt for elution of the second fraction are lower for the diamino adsorbent of equal alkyl chain length. In other words, since the double layer attraction is accompanied by a smaller van der Waals attraction in the case of the diamino adsorbent (than in the case of the α-amino adsorbent), it needs to be reduced by a smaller extent in order to lower the total attractive force to a level that would permit elution of the enzyme. Such a reduction in affinity can also be achieved by introducing hydrophilic groups other than NH_2, e.g., hydroxyl groups at the distal

end of the ligands. Indeed, the extent of binding of lipoamide dehydrogenase on the ω hydroxy α amino hexyl agarose is about the same as on the α-amino butyl agarose[131].

Shaltiel and Halperin[7] observed that at 0.05M ionic strength and pH = 8, negatively charged ovalbumin was retained on positively charged n-alkyl α-amino adsorbents with $4 \leq n \leq 8$ or $n=12$ but was unretained when n=9 or 10. Upon doubling the ionic strength or upon acetylation of the adsorbents, however, retention occured above a certain n (here $n>11$) and increased monotonously with the alkyl chain length.

As discussed earlier, the adsorption of ovalbumin on alkylamino agaroses is dominantly caused by electrostatic (double layer) attraction[45]. In addition, Shaltiel[7] has shown by potentiometric titration that the net positive charge on these adsorbents decreases with increasing chain length (perhaps due to incomplete dissociation of the weakly basic groups). Combining these facts, an explanation along the lines of the present interpretation for the above mentioned behavior can be put forward:

While the nonelectrostatic (van der Waals) attraction between protein and adsorbent increases with n, the charge on the adsorbent and hence the electrostatic (double-layer) attraction, decrease. If the increase in the van der Waals attraction more than compensates for the decrease in the double layer attraction for $n > 11$ and if the reverse is true for $n < 9$, the net attractive force goes through a minimum (for n = 9 or 10) at which retention fails to occur. Acetylation of the adsorbent or raising the ionic strength eliminates the double layer attraction; the van der Waals attraction still monotonically increases with chain length and retention occurs when this attraction is sufficiently strong ($n > 11$).

A similar observation was made by Shaltiel, et al.,[163]. When the osmotic shock fluid from salmonella typhimurium was subjected to binding with α,ω-diamino alkyl derivatives of agarose, negatively charged protein J emerged unbound even from the C_{10} adsorbent. Probably that in this case, the van der Waals interaction between protein and adsorbent is either very weakly attractive or even

repulsive (A_{F32} and $A_{P32} < 0$), rendering retention unlikely, despite the attractive double layer forces.

A variation of the chain length effect - experiments listed in Table VIII was discussed by Hofstee and Otillio[147] who studied the adsorption of serum proteins onto a set of α-amino alkyl agarose columns connected in a series of increasing alkyl chain length. The adsorbents with $n \leq 4$ were unable to retain even the most nonpolar protein in the sample (γ-globulin) which was, however, retained on the pentyl adsorbent. The effluent from this adsorbent contained proteins (of lesser nonpolar content than γ-globulin) which bound to a larger extent on octyl agarose than on hexyl agarose. Thus, when the columns were disconnected and the amounts of protein retained on them were compared, the pentyl and octyl adsorbents exhibited maxima.

An interplay of the chain-length effect with lyotropic salt effects is illustrated by a study of Peeters, et al.,[117] of the binding of bovine serum albumin (BSA) to α,ω-diamino n-alkyl agaroses. At pH = 6.8, BSA is negatively charged while the adsorbents are positively charged, i.e., the double layer forces are attractive. At this pH and at a low ionic strength (0.001M), the amount of protein bound increased with increasing chain length and reached a saturation for $n \geq 8$. At 0.5M ionic strength, the binding was much lower (than that at 0.001M) for all chain lengths, possibly due to the suppression of attractive double layer forces. At higher ionic strengths, the binding was larger than that at 0.5M. For adsorbents with $n < 8$, this lyotropic effect could not raise the binding back to its level at the very low ionic strength of 0.001M. When $n \geq 8$, however, the binding in the presence of 3M NaCl was larger than that at 0.001M. The lyotropic effect is the contribution from the solvent medium towards the enhancement of the van der Waals attraction, while the alkyl chain length effect is the contribution from the adsorbent; the retention is large when both effects are simultaneously operating at significant levels.

While the general result from chain length studies is that the amount of protein bound monotonically increases with increasing alkyl chain length, exceptions to this rule have been observed.

Halperin and Shaltiel[164] studied the binding of Guinea pig red cells to α amino n-alkyl agaroses. The amount of cells bound increased with n till n = 7, where it dropped to a lower value from which it increased again for n > 7. This apparently contradicts the chain length effects discussed above, but turns out to be an experimental artifact. The number of microequivalents of positive charge per 0.84 ml of adsorbent determined by potentiometric titration[164] was 11.8 for n = 2 to 5, and for reasons unknown, dropped to 4.5 for n ≥ 7, all at pH 7.4. Around this pH, the cells are negatively charged. The lower charge density with n ≥ 7 causes a lower double layer attraction on these adsorbents. This reduction, superimposed on the van der Waals attraction which monotonically increases with n, explains the observed discontinuity at n = 7 in the extent of binding as a function of chain length.

On α amino alkyl agaroses, Hofstee[155] noted that γ-globulin bound in larger amounts to adsorbents with n = 3 or 5 in comparison with those having n = 2, 4 or 6. He compared this oscillation between odd and even number C-chains to the effect of substrate C-chain length on the activity of certain enzymes[165]. At this stage, we are unable to explain this phenomenon using the present interpretation.

F. The Influence of the Number Density (≡ Number per Unit Volume of the Adsorbent) of Alkyl Ligands

Jennissen and Heilmeyer[152] studied protein binding to α-alkyl amino agaroses, as a function of the alkyl chain length and the number density ρ of the ligands, the latter estimated by radioactive labelling.

In general, the amount of protein bound per unit volume of adsorbent exhibited a sigmoidal dependence on the density ρ of the ligands. The binding occured above a certain density, and as the

density was raised, increased slowly and then increased exponentially over a narrow range of the density, and reached a saturation. With ligands of longer alkyl chain length, the exponential increase occured at a lower density. The strength of the binding also increases with density as indicated by the observation that at larger densities, the fraction of protein which is retained and can be eluted by raising the ionic strength is larger while the fraction that is mildly retained and elutes with the 50 mM buffer is smaller. The following offers an explanation of this behavior based on the present interpretation.

As the density of alkyl ligands is increased, the nonpolar character of the adsorbent is enhanced. Consequently, the adsorbent sustains a larger van der Waals attraction with proteins, at higher densities. With ionogenic ligands, the number of charged groups on the absorbent also increases with density. In the experiment cited[152] the double layer forces are attractive. Hence, the increase in the number of charged groups is reflected in an increased double layer attraction. Binding occurs at a density at which the net attractive force is strong enough to retain the protein.

The observed saturation in binding at higher densities can be understood by analogy with the Langmuir adsorption isotherm. The ratio of the fraction θ of the adsorbent surface that is occupied by the protein to θ_{max}, the maximum possible value of θ, at adsorption equilibrium is given by

$$\frac{\theta}{\theta_{max}} = \frac{k_a(\rho)C_\infty}{k_a(\rho)C_\infty + k_d(\rho)} \tag{24}$$

where k_a and k_d are coefficients for adsorption and desorption respectively (both being functions of the ligand density ρ), and C_∞ is the concentration of protein in the bulk solution. Assuming that the ligands are uniformly distributed over the adsorbent matrix, for a given area of the adsorbent-surface, enlarging the density of ligands increases the attractive force; as a result, the coefficient for adsorption k_a increases while that for desorption,

k_d, decreases. At a particular density, k_d becomes negligible in comparison with the product of k_a and C_∞. At this point θ tends to θ_{max} and the amount of protein bound saturates. For any ligand density, the van der Waals attraction is larger with ligands of longer alkyl chain length. Consequently, the exponential increase and the saturation in binding occur at lower densities for longer ligands.

The enhancement of the total attractive force explains the reduction in the amount of protein weakly held as the density is raised; the enhancement of the double layer attraction results in the enlargement of the fraction that requires a raise in the ionic strength for elution, i.e., a reduction in the weakly held fractions (A-C) and an increase in the more strongly held Fraction D (Table V) occur as ρ is increased.

The role of the contribution from the adsorbate towards the attractive force is illustrated by an example from this study[152]. The higher the molecular weight of the protein, the larger its size and hence, the larger the strength of its interaction with the adsorbent as discussed in the context of Figure 5 (Section 4.C). This, combined with the above inference (that the binding of the protein that sustains a larger attractive force with the adsorbent commences and saturates at values of the density which are smaller than the corresponding values for a protein that sustains a smaller attractive force) explains the following observation: Phosphorylase kinase of molecular weight 1.3×10^6 amu can be retained on an ethylamine agarose which has a density of ligands less than that needed to bind phosphorylase b of molecular weight 2×10^5 amu. Also, the binding of phosphorylase b begins at the density of ligands at which the amount of phosphorylase kinase bound levels off into a plateau.

Rosengren, et al.,[162] in batch experiments, studied the influence of ligand density on the binding of phycoerythrin to aryl and alkyl hydrazide agaroses, estimating the density by NMR. Their results are similar to the ones discussed above. The amount of

protein bound increased and tended to saturate as the number density of ligands was increased. The strength of the binding was larger at higher densities, as indicated by the increase in the fraction that requires addition of ethylene glycol for elution, i.e., an increase in the strongly held Fraction E (Table V) accompanied by a decrease in the weakly held Fractions A-D. Since the adsorbents are neutral in this case, raising the ligand density increases the attractive force solely by enhancing the van der Waals attraction.

Hofstee[155] used the binding of a colored dye, Ponceau S, to estimate the ligand density. His results are similar to the above. For example, the amount of bovine serum albumin that can be eluted from α amino octyl agarose by adding ethylene glycol declined as the density of ligands increased[166], i.e., a reduction in Fraction E accompanied by an enhancement of the more strongly retained Fraction F (Table V). Some other studies also[138] report similar effects of changing the ligand density.

G. The Effect of Changes in the Temperature

The influence of temperature in HIC is the outcome of a combination of different effects. As discussed in the context of Eqs. 15 and 16, activation energies may be associated with adsorption ($\phi_{max}-\phi_{smn}$) and desorption ($\phi_{max}-\phi_{pmn}$). If one assumes for the moment that these activation energies are independent of temperature, it can be seen that adsorbate entities can overcome potential barriers to adsorption and desorption with more ease at higher temperatures due to the increase in the thermal energy, kT. This effect will increase the rates of both adsorption and desorption if the potential profile has a maximum (curve B, Figure 2). If a maximum is absent (curve A, Figure 2), a rise in the temperature will facilitate the escape of adsorbate from the energy well thus favoring desorption over adsorption.

In fact, however, the said interaction energies are functions of temperature since their constituent interactions (Eq. 14) are temperature dependent. The effect of temperature on the electrostatic double layer interactions is not straightforward (see for

example, Eq. 12); the van der Waals attraction between macroscopic bodies in water is expected to increase as the temperature is raised[167]. In addition, a drastic temperature change may alter the conformation of proteins and change the van der Waals interaction.

It follows hence that depending on which of the above mentioned mechanisms dominate, different temperature effects may be experimentally observed in HIC.

On neutral adsorbents, where the non-electrostatic (van der Waals) attraction is probably the sole cause of retention, one may expect the above-cited increase of van der Waals interaction (between the macroscopic adsorbent and the macromolecular solute in water) with temperature to make a dominant contribution to the net temperature effect on retention. Indeed, such an effect is observed. Hjertén, et al.,[50] observed that 25 to 30% of the phycoerythrin bound to (neutral) pentyl hydrazide agarose is relased upon lowering the temperature from 20°C to 0°C.

With adsorbents carrying ionogenic ligands, however, the temperature effect is not predictable in a straightforward manner[168].

Hjertén observed that only 3% of the bound phycoerythrin was released upon reducing the temperature from 20°C to 0°C, from phenyl ethyl amino agarose. As Hjertén noted, this amount is not significant enough to draw a conclusion from[168].

Visser and Strating[131] observed that the binding of the enzyme lipoamide dehydrogenase to α amino butyl agarose was weaker at 25°C than at 4°C.

Similarly, Breitenbach[158] observed that hemoglobins and myoglobins consistently bound to α alkyl amino agaroses to a larger extent at 4°C than at 20°C. The dependence was reversed on α,ω-diamino alkyl agaroses, in that the binding was stronger at 20°C than at 4°C.

LePeuch and Balny[169] studied the binding of many proteins to phenyl butylamine agarose, concentrating on the interplay of the effects of ethylene glycol (EG) and temperature on the binding. α-chymotrypsin bound at 20°C and eluted, when the ethylene glycol

concentration was raised to 38v%. At cryogenic temperatures, however, the enzyme remained bound even at high volume fractions of EG. For example, retention was observed at -20°C in 50v% EG and at -15°C in 30v% EG. The mechanism is probably not electrostatic since the binding behavior was not altered by the addition of 1M KCl (which should annul any electrostatic effects). It may be tentatively proposed that the mechanism probably rests in the dependence of the van der Waals attraction (between macroscopic bodies in aqueous media) on the dielectric constant ε_3 of the medium (Eq. 2b). It is noteworthy in this regard that the dielectric constant of an aqueous medium can be lowered by adding ethylene glycol and can be restored to a high value by cooling the resultant mixture to subzero temperatures[170].

Similar results were observed for chymotrypsinogen A. The zymogen elutes unretained at 20°C in the presence of 30v% EG, but is fully retained at the same concentration of EG at -20°C. The behavior, again, is independent of the presence of 1M KCl. Ovalbumin and catalase, however, were unretained in the presence of 50v% EG, both at +20°C and at -20°C. LePeuch and Balny[169] used these results and obtained a good separation of chymotrypsin and chymotrypsinogen from each other as well as from ovalbumin and catalase, by adsorbing them at -15°C in the presence of 30v% EG and eluting the zymogen at +4°C, and chymotrypsin at +20°C, 50v% EG.

H. Effect of the Heterogeneity of Proteins and Adsorbents

A protein sample ostensibly consisting of a single protein may be heterogeneous due to the presence of impurities, or of variants of the same protein which differ slightly in the primary structure. Even a protein that is devoid of impurities and is of a single primary structure may be heterogeneous in terms of secondary and tertiary structures in an environment in which it has physiological functions. The disparate functions a single protein serves (e.g., interaction with carbohydrates on the one hand and interaction with lipids on the other) attests to this lack of homogeneity in the functional environment[171]. In a crystallized state, all heterogen-

eities except the ones due to differences in primary structure are likely to be absent. In solution the protein finds itself in an environment that is in between these two extremes. Besides, a protein in the mobile phase near a HIC adsorbent is in an environment which is much less passive than that in the crystallized state. Hence, some of the heterogeneities of the functional state become manifest and different molecules of the same protein species may differ in the extent of surface nonpolarity as well as in the overall shape and thus may differ in the strengths of the van der Waals attraction they sustain with the adsorbent (see Section 3.D.c).

It is noteworthy that since different regions in each protein molecule have different characteristics, the strength of adsorption of a particular molecule will depend on which part of the molecule is retained on the adsorbent. In consequence, even protein molecules which are identical in primary to tertiary structures can behave heterogeneously towards adsorption, one molecule retained via a certain region on its surface and the others via different types of regions.

The number density of alkyl ligands, and surface charges if any, will vary in a random fashion over the surface of a HIC adsorbent. This and variations in other characteristics such as surface roughness lend a heterogeneity to the adsorbent in terms of sites that differ in the strength of adsorption.

As a result of the possibility of such heterogeneities of proteins as well as the adsorbent, each molecule of the same adsorbate species will undergo diverse interaction profiles on its way down the column. If there is adsorption-desorption equilibrium and if the column is long enough to provide a large number of equilibrium stages, these variations will be statistically smoothed out. Otherwise, a single species can elute as several peaks, especially in a step-wise elution procedure[116].

In a simple illustrative situation the adsorbent may have two types of sites, on one of which the protein is adsorbed stronger, than on the other. At small loadings, the protein would be mostly

adsorbed on the strongly attracting site. When the loading exceeds the capacity of such sites, the weakly attracting sites will begin to bind the protein. Hence the strength of adsorption per unit load of protein would be higher at smaller loadings. This was demonstrated by Hofstee who noted[155] that the fraction of the loaded bovine serum albumin that remains bound (to phenyl butylamine agarose in the presence of 1M NaCl) increased upon a reduction in the load. Similarly[155] when 2 milligrams of ovalbumin were adsorbed on α-alkylamino agarose and the ionic strength was raised, all of it eluted at the salt concentration at which the last few milligrams of a 20 mg sample of OV were eluted.

The above examples indicate that the heterogeneity of the adsorbent is probably responsible for the elution of an electrophoretically pure protein in two fractions, one upon raising the ionic strength (by the addition of a salting-out salt such as NaCl), and the other upon adding ehtylene glycol, (e.g., entry 2 in Table VI). Hofstee[3] suggested a plausible alternative explanation for the latter observation. While a raise in the ionic strength reduces the electrostatic (double layer) attraction causing elution, above a certain ionic strength, lyotropic salting-out effects become significant and increase the non-electrostatic (van der Waals) attraction, and above a point the latter enhancement outweighs the former reduction. At this point a change in the ionic strength, either way, would only increase the total attractive force thus preventing elution. Addition of ethylene glycol, however, successfully elutes the rest of the protein by reducing the non-electrostatic (van der Waals) attraction. Heterogeneity may be reponsible for some observations in HIC such as the following:

Robillard, et al.,[172] studied the binding of an E. Coli extract containing enzyme 1 (E_1) to α-amino octyl agarose. E_1 distributed itself into two fractions, one eluting at high (70v%) ethylene glycol concentrations and the other upon addition of the detergent sodium deoxycholate. When either fraction was recycled, again two such fractions resulted. Upon increasing the loading of the extract

to the column, the detergent-eluted fraction of E_1 first increased and then decreased to zero. After a pretreatment of the column (which consisted essentially of leaving the detergent-elutable fraction still bound), E_1 eluted in a single fraction upon adding ethylene glycol. As Robillard, et al., recognized, all these observations can be attributed to the presence of two classes of sites on the adsorbent one type more attractive than the other to E_1 and the proteins, and is blocked by the pretreatment process. The authors, however, were not sure about the mechanism since they also observed that only E_1 redistributes itself into two fractions, all the (other) proteins in the detergent eluted fraction staying only in that fraction upon rechromatography on adsorbents without pretreatment. Here, we propose that this does not contradict the two site mechanism, since it is possible that the proteins which are retained sustain a stronger attractive force than E_1 with the adsorbent and their concentration is smaller than both the concentration of E_1 and the binding capacity of the strongly attracting sites. If such is the case, they would always be found in the most strongly retained fraction. Thus, they would elute from the unmodified adsorbent in the detergent-eluted fraction and from the pretreated adsorbent at higher concentrations of ethylene glycol than that required to elute E_1. This is supported by the observation by Robillard, et al.,[172], that E_1 eluting from a pretreated octyl adsorbent, upon rechromatography on another pretreated octyl adsorbent, eluted at a concentration of ethylene glycol (67v%) higher than that required for elution from the first adsorbent (47v%), i.e., the strongly adsorbing proteins were left out on the first adsorbent.

Marcus, et al.,[159] reported an apparently anamolous effect when comapring stepwise and gradient elution procedures. In the presence of 1M KCl, R-Mulv proteins were strongly retained on α-amino decyl agarose. Upon raising the concentration of ethylene glycol in the eluant from 0 to 8.5M in a single step, a fraction eluted. Subsequent addition of a detergent eluted another fraction. Both fractions contained polypeptides as well as DNA polymerase. In

a separate experiment, the concentration of ethylene glycol was raised in steps of 2M and the enzyme activity failed to elute even above 8.5M EG. The enzyme eluted in a single fraction, however, upon addition of a detergent. Marcus, et al., postulated that "shocking" the adsorbed enzyme by a sudden raise from 0 to 8.5M EG is more potent an elution procedure than gradually raising the concconcentration of EG. Here, an alternative mechanism is suggested.

The fact that polymerase as well as the peptides eluted, each in two fractions in the first experiment suggests that the adsorbent may be heterogeneous. In addition, the more strongly held fraction contained more of polypeptides than polymerase implying that in a competition between the two for the strongly attractive sites on the adsorbent, the polypeptides would be favored. These offer an explanation, if in the second experiment (compared with the first one):

(a) the particular batch of the adsorbent had a larger number of strongly attracting sites;

(b) the protein sample had a lesser amount of polypeptides, or

(c) the protein sample was lower in polymerase content, resulting in the retention of all the polymerase in the strongly attracting sites, permitting elution only by addition of a detergent.

I. Experiments that Relate Interfacial Tensions to Retention in HIC

van Oss, et al.,[73-75] have done experiments that elaborate on the parallelism between the effect of additives on the surface tension $\gamma_{\ell v}$ of the medium, and the effect of these additives on HIC retention.

In addition to recording the retention behavior of proteins or cells on HIC adsorbents (or variants thereof) these experimenters:

(i) measured the surface tension $\gamma_{\ell v}$ of the eluants (mixtures of organic solvents and water), and

(ii) estimated the interfacial tensions γ_{p-sw} and γ_{a-sw} (at the interface of the protein or the adsorbent with saline water, respectively), via measuring the contact angles of the protein or adsorbent with sessile drops of saline water.

Proteins or cells bound to the adsorbent in the presence of water (with or without salts) and eluted upon gradual increase of the concentration of organic solvents in the aqueous eluant. This type of study was done on the binding of:

(a) bovine serum albumin, ovalbumin, and human γ-globulin on α-amino octyl agarose or phenyl agarose, and human serum proteins on the latter adsorbent, with aqueous mixtures of ethylene glycol as eluants[74]; (the "surface tensions" γ_{a-sw} of the agarose-based octyl and phenyl adsorbents could not be measured and were approximated by the surface tensions $\gamma_{\ell v}$ of octanol and phenol, respectively);

(b) γ-globulin on polystyrene latex particles, with water-dimethyl sulfoxide mixtures as eluants[195]; and

(c) live human granulocytes on nylon fibers, with mixtures of water and dimethyl sulfoxide or dimethyl acetamide as eluants.[194]

van Oss, et al., noted that in general, when retention occurs, the surface tension $\gamma_{\ell v}$ of the aqueous medium is higher than the "surface tension" γ_{p-sw} of the adsorbate which in turn is larger than the "surface tension" γ_{a-sw} of the adsorbent. Elution of the adsorbate commences when the surface tension $\gamma_{\ell v}$ of the eluant is lowered to a value just below the "surface tension" γ_{p-sw} of the adsorbate, i.e., retention corresponds to

$$\gamma_{a-sw} < \gamma_{p-sw} < \gamma_{\ell v} \tag{25}$$

and elution occurs when

$$\gamma_{a-sw} < \gamma_{\ell v} \leq \gamma_{p-sw} \tag{26}$$

In a related experiment, van Oss, et al.,[75b] studied the adsorption of human serum proteins onto surfaces of different polymers, of various "surface tensions" γ_{a-sw}, from aqueous media of various surface tensions $\gamma_{\ell v}$. These authors noted that the surface tension of the medium from which a dissolved protein adsorbs (to any of the solid surfaces studied) is just about equal to the "surface tension" γ_{p-sw} of the protein as estimated by the contact angle method.

By means of certain approximations that are outlined elsewhere,[73a] van Oss, et al., have inferred that the inequality, Eq. 26, leads in many cases to negative Hamaker coefficients, A_{132}, i.e., the van der Waals interaction (between adsorbate and adsorbent in the presence of the eluant) becomes repulsive when elution of the adsorbate occurs. In the view of the present interpretation, however, elution can occur not only with repulsive van der Waals forces but even with attractive van der Waals forces prevailing, provided these are made sufficiently small. (See also the discussions in Section 4.D, and in the context of Eq. 18).

J. Applications

(a) Biological Separations. The research reports discussed so far (Table VI to VIII) illustrate the fact that HIC has been used successfully for separating several biological macromolecules. Most of these reports, however, concern the development of HIC as a separation method. In recent years, the method has been incorporated as a step in several separation schemes, e.g., for membrane proteins[50,173-175], histones[176], interferons[177,178], bacteria[179], immunoglobulins[180], enzymes[181], and hormones[125,126]. Recently[194] a novel variant of HIC has been used for the isolation of live human granulocytes. The procedure uses nylon fibers as adsorbents and aqueous mixtures of dimethyl sulfoxide or dimethylacetamide for eluting the bound cells without damage. HIC has also been combined with procedures such as electrophoresis to give newer methods[182].

(b) Enzyme Reactors. The strong retention that is possible with HIC adsorbents has been used to advantage in a new type of immobilized enzyme reactor[50,183,184]. Traditionally, the enzyme is coupled covalently to the support in a more or less irreversible fashion. When the enzyme is strongly bound to a HIC adsorbent, however, it is possible to reverse the binding (by adding ethylene glycol, for instance) when desired (e.g., if the enzyme loses its activity) and regenrate the bed by applying a fresh load of the enzyme.

(c) High Pressure HIC. The HIC systems discussed in this paper in general involve compressible adsorbent matrices and hence, are not

used in the high pressure chromatographic equipment designed for "High Performance Liquid Chromatography".

Adsorbents made by coupling alkyl silanes (e.g., octyl or octadecyl silanes) via siloxane linkages[185] have been traditionally used for separating small molecules in (high-pressure) "Reverse Phase Liquid Chromatography"[186,187]. In such applications, the chromatographers have been advised to scrupulously remove any proteins present in the sample since proteins "stick" (or bind very strongly) to the highly nonpolar (C_{18}) adsorbent thus hindering the separation of small molecules.

Recently, however, such reverse phase adsorbents are being used for the separation of proteins[189,192]. High pressure systems are more efficient and faster than low pressure systems. On the other hand, the small pore size of the high pressure adsorbents renders them to be of low capacity when very large proteins are to be separated[191]. Besides, there is a pH limitation on these adsorbents since the silica matrix becomes unstable at alkaline pH.

6. SUMMARY AND CONCLUDING REMARKS

Hydrophobic interaction chromatrography (HIC) is a recently developed procedure for the separation of biological macromolecules; the mobile phases in this method are aqueous and the adsorbents in general consist of an agarose matrix onto which hydrocarbonaceous ligands are attached. Often, these ligands possess ionogenic groups.

Here, the cause of retention in HIC is ascribed to physical forces between protein and adsorbent, namely the van der Waals attraction (with contributions from orientation, induction and dispersion interactions), the electrostatic double layer interaction (which is repulsive or attractive depending on whether the net charges on protein and adsorbent are alike or opposite in sign) and short range repulsive forces such as Born repulsion.

The physical forces between protein and adsorbent are much affected by the intervening solvent medium. Because water is highly structured, cohesive, and polar, water molecules strongly attract

one other. This, coupled with the fact that the dispersion interaction among water molecules is in general weaker than that among nonpolar molecules, is shown to lead to the van der Waals attraction between two nonpolar entities in water being stronger than the corresponding interaction in a nonpolar medium. Thus, proteins having an appreciable nonpolar content, are attracted by a nonpolar-hydrocarbon-coated adsorbent when the environment is aqueous. Note is taken of the fact that even hydrophilic proteins can undergo a significant van der Waals attraction with the adsorbent (which would albeit be smaller than the corresponding interaction of a more hydrophobic protein). For similar reasons, additives which increase the structuring of the aqueous medium (e.g., salting-out salts such as $(NH_4)_2SO_4$) are shown to augment the van der Waals attraction between protein and adsorbent across the medium. Additives which break down this structure (e.g., salting-in salts such as guanidinium chloride and solvents like ethylene glycol) make the medium more lipophilic and thus reduce the attraction between protein and adsorbent. Apart from the cited lyotropic salt effects which occur at high ionic strengths, salts suppress the double layer interaction at quite low ionic strengths (< 1M).

Expressions are given for the various interaction potentials. The sum of these is the total interaction potential, which can vary in several ways as a function of the distance between the surfaces of protein and adsorbent. Such shapes of the potential profile as are relevant to HIC are identified. Results are cited from a parametric study which shows the sensitivity of the potential profile to its parameters such as the ionic strength. Expressions are given for the coefficients of adsorption and desorption. Chromatographic retention and elution in HIC, and the ways of controlling them, are shown to be related to the potential profiles.

Experimental evidence is classified by grouping the experiments to which variation of a single parameter (e.g., temperature) has been central. Numerous experimental reports are listed in tabular form and several of the results are discussed in detail. Applications

and variants of HIC are cited. The present interpretation is able to explain the various aspects of HIC (such as the increase in the strength of binding with increasing alkyl chain length of the ligands on the adsorbent) as well as certain general phenomena (e.g., the mechanism behind the fact that proteins begin to be retained on an adsorbent at salt concentrations lower than those required for precipitating the proteins out of solution). The paper offers a unifying interpretation of apparently distinct phenomena (such as retention on adsorbents coated with hydrocarbon ligands versus retention on the adsorbent matrix itself) and provides a detailed review of this new separation procedure.

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NOTATION

a_p	radius of the solute particle (cm)
A_{ijk}	Hamaker coefficient for the van der Waals interaction between bodies i and j across medium k (ergs)
$\bar{A}_{ijk}$	the contribution from dispersion to A_{ijk} (ergs)
$\hat{A}_{ijk}$	the contribution from orientation and induction to A_{ijk} (ergs)
A_{ij}	Hamaker coefficient for the van der Waals attraction between materials i and j across a vacuum (ergs)
$\bar{A}_{ij}$	contribution from dispersion to A_{ij} (ergs)
C	local concentration of solute particles (particles/cm^3)
C_i	concentration of solute particles at the outer periphery of the interaction force boundary layer (particles/cm^3)
C_∞	concentration of solute particles in the bulk (particles/cm^3)
D	local diffusion coefficient of adsorbate (cm^2/sec)
$D_{f\infty}$	diffusion coefficient of adsorbate in the bulk of the liquid (cm^2/sec)
e	protonic charge (stat coulomb)

h	distance between the surfaces of the adsorbent and the (protein) solute (cm)
h_{max}	position of the maximum in the potential ϕ vs. h profile
h_{pmn}	position of the primary minimum (cm)
h_{smn}	position of the secondary minimum (cm)
$[H^+]$	concentration of hydrogen ions (moles/litre)
$[H^+]_b$	value of $[H^+]$ in the bulk (mole/litre)
J	adsorbate flux (particles/cm^2-sec)
k	Boltzmann constant (erg/°K)
k_a	adsorption coefficient, defined by Eq. 15 (sec^{-1})
k_a'	adsorption coefficient, defined by Eq. 15a (cm/sec)
k_d	desorption coefficient, defined by Eq. 16 (sec^{-1})
K_{ai}, K_{bj}	dissociation constants of the acidic and basic sites of the surface respectively (moles/litre)
K_m	mass transfer coefficient (cm/sec)
n_i	concentration of ions of kind i (ions/cm^3)
n	number of methyl groups in a ligand on the adsorbent
n_{pmn}	number of adsorbate entities in and near the primary minimum (particles/cm^2)
n_{smn}	number of adsorbate entities in and near the secondary minimum (particles/cm^2)
N_{ai}, N_{bj}	number of acidic and basic sites per unit area of surface respectively (sites/cm^2)
t	time (sec)
T	absolute temperature (°K)
V_R	retention volume of the (protein) adsorbate (cm^3)
V_0	retention volume of an unretained micromolecular tracer (cm^3)
z	valence of the counter-ion (Eq. 12)
z_i	valence of ion type i

Greek Letters

γ_{a-sw}	the interfacial tension at the adsorbent-saline water interface, estimated from contact angle measurements[73a] (dynes/cm)
$\gamma_{\ell v}$	interfacial tension at the interface of a liquid and its vapor (dynes/cm)

γ_{max} $= -\left(\frac{d^2\phi}{dh^2}\right)_{h=h_{max}}$ (erg/cm^2)

γ_{pmn} $= +\left(\frac{d^2\phi}{dh^2}\right)_{h=h_{pmn}}$ (erg/cm^2)

γ_{p-sw} the interfacial tension at the adsorbate-saline water interface, estimated from contact angle measurements[73a] (dynes/cm)

γ_{smn} $= +\left(\frac{d^2\phi}{dh^2}\right)_{h=h_{smn}}$ (erg/cm^2)

δ film thickness of hydrocarbonaceous film ≡ alkyl chain length of the ligands on the adsorbent [Figure 1] (cm)

ε dielectric constant of the mobile phase

ε_i dielectric constant of material i

θ fraction of the surface of the adsorbent which is covered by protein

θ_{max} maximum value of θ

κ reciprocal Debye length (defined in Eq. 5) (cm^{-1})

μ ionic strength of the solution (M)

ρ density (number per unit volume of adsorbent) of ligands (cm^{-3})

σ collision diameter (Eq. 13) (cm)

σ' surface charge per unit area (Eq. 11) (stat coul./cm^2)

ϕ total interaction potential (Eq. 14) (erg)

$\phi_{pmn}, \phi_{max}, \phi_{smn}$ values of ϕ at the primary minimum, maximum and secondary minimum respectively (erg)

ϕ_{DL} double layer interaction potential (Eq. 12) (erg)

ϕ_{SR} short range repulsion potential (Eq. 13) (erg)

ϕ_{vdW} van der Waals interaction potential (Eq. 4) (erg)

ψ electrostatic potential (stat. volt)

ψ_{si} electrostatic potential at the i-th surface (stat. volt)

ψ_o electrostatic surface potential (Eq. 11) (erg)

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