

## On the Adsorption of Protein to Alkylsilica: Relevance to Reversed-Phase and Hydrophobic Interaction Chromatography

### 1. PROTEIN ADSORPTION PHENOMENA

During the past decade, enlightenment of factors that influence the sorption of proteins to surfaces has been the thrust of much research (1). Most of the effort has been directed toward studies of blood proteins as related to biocompatibility of prosthetic devices and materials used in the medical field. Some of the general concepts and observations, however, are relevant to chromatography.

The proteins sorbed at any interface—water/air, water/oil, or water/solid—may be perceived depending on concentration and on resistance of the biopolymer to structural perturbations, as shown schematically in Fig. 1. At low surface concentrations many proteins will spread at an air or oil interface with water (2). Spreading is often accompanied by changes in secondary protein structure to permit loops of apolar and polar amino acids to partition into phases of similar polarity. At solid interfaces, the orientations may be in the surface plane or directed away from it into the solution but usually involve multiple contacts with the surface. As surface concentration increases, higher packing density inhibits spreading and promotes further looping of segments into the solution phase. Finally, with further increase in concentration multiple layers form often. These structural alterations are influenced by factors such as pH, ionic strength, and number of protein disulfide bridges (2). The last of these factors imparts rigidity in a fashion similar to increasing cross-links in organic polymers. Electrostatic interactions are important and, owing to electric double

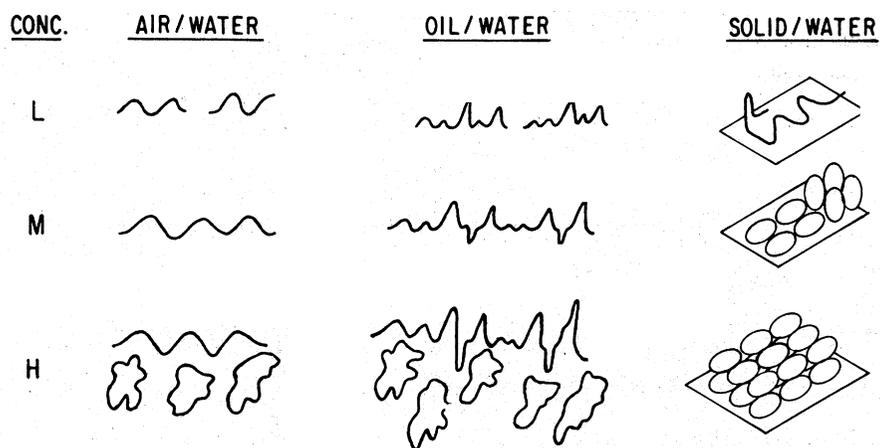


Fig. 1. Schematic of protein orientation at interfaces.

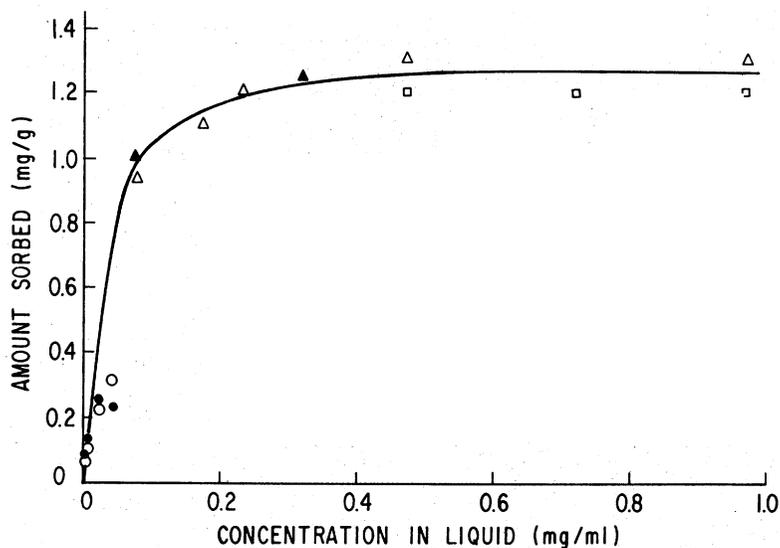
layer repulsion, tend to promote expansion of sorbed biopolymer at hydrogen ion concentrations removed from the isoionic point of a protein (2).

Sorption of proteins to ionic as well as neutral surfaces exhibit Langmuir or at least pseudo-Langmuir behavior (3, 4). Adsorption that is described by the Langmuir isotherm is analogous to an ideal solution, that is, there is no interaction between adsorbed molecules. Moreover, adsorption ceases when monolayer coverage is achieved. Just as in solution chemistry, deviations from ideality are observed often in adsorption. Nevertheless, the Langmuir equation is an often used starting point.

The Langmuir equation is

$$S = S_p K C_m / (1 + K C_m) \quad (1)$$

where  $C_m$  is the protein concentration at equilibrium,  $S_p$  is the apparent binding capacity of the sorbent,  $S$  is the mg protein sorbed/g sorbent, and  $K$  is the desorption constant, which is related to the binding energy. This expression may be derived from both kinetic and statistical thermodynamic considerations and assumes a uniform surface with sorption limited to a monolayer. An example is given in Fig. 2 (5); which shows the isotherm for sorption of BSA onto highly substituted alkylsilicas and demonstrates that there was little difference in affinity of BSA for either the octyl- or octadecyl-derivatized surface. By inverting both sides of Eq. 1 and rearranging, a linear form results so that when  $C_m/S$  is plotted against  $C_m$ , the sorbent binding capacity  $S_p$  and desorption constant  $K$  can be obtained. These are shown in Table 1 for sorption of BSA from a number of different solvents. The higher capacity at pH 2 could reflect the fact that BSA is more flexible and elongated at lower pH and therefore has access to a larger fraction of sorbent pores. High affinity of the alkylsilicas for the



**Fig. 2.** Static sorption of bovine serum albumin onto alkylsilicas. Solvent: 2-propanol (40%)/0.05 M phosphate, pH 2.1. ( $\blacktriangle$ ) 500-Å pore, data using  $C_8$  and  $C_{18}$  alkyl groups superimposable; ( $\triangle$ ) 100-Å pore, data using  $C_8$  and  $C_{18}$  alkyl groups superimposable; ( $\circ$ ) 100-Å pore,  $C_{18}$ ; ( $\bullet$ ) 100-Å pore,  $C_8$ ; ( $\square$ ) concentration in solvent determined by UV measurement, other data by fluorescence.  $T = 23 \pm 1^\circ\text{C}$ .

**Table 1.** Sorption  $a$  of BSA to Ocytl- and Octadecyllkylsilica

| Solvent                            | Apparent Binding Capacity ( $S_p$ ) (mg/g) | Desorption Constant ( $K$ ) [(g/mL) $\times 10^3$ ] |
|------------------------------------|--|---|
|                                    | <i>pH 2.1</i>                              |   |
| 0.05 M Phosphate (27) <sup>b</sup> | 3.2  | 5   |
| 30% 2-Propanol (29)                | 3.2  | 68  |
| 40% 2-Propanol (32)                | 1.9  | 130   |
| 0.01% Neodol (21)                  | 2.6  | 11  |
| 1.0% Neodol (18)                   | 2.6  | 110   |
|                                    | <i>pH 7.0</i>                              |   |
| 0.05 M Phosphate (8)               | 1.3  | 30  |
| 0.01% Neodol (13)                  | 1.2  | 40  |
| 1.0% Neodol (22)                   | 1.5  | 300   |

<sup>a</sup>One hour contact time at  $23 \pm 1^\circ\text{C}$ .

<sup>b</sup>Number of experiments in parentheses.

protein is shown for all solvents except 1% neodol, a polyethoxylated alcohol surfact, and 40% isopropanol in phosphate buffer.

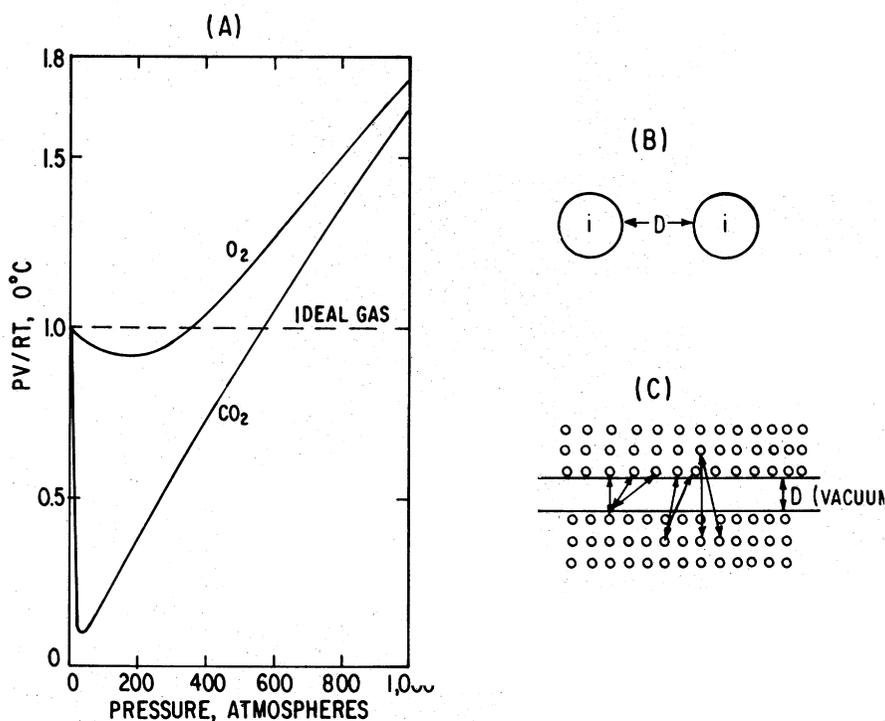
Spectroscopic techniques are used to probe the nature of species sorbed on solids (6). Infrared measurements showed, for example, that about 10% of the serum albumin molecule is in contact with the silica particle to which it is bound (7) and that this percentage is the same regardless of whether native or cross-linked protein is used in the experiment, indicating that major unfolding does not occur upon sorption. When just one disulfide of the native protein was broken before equilibration, however, the number of contacts increased by 70%. Other infrared studies demonstrated that albumin molecules attached to the surface were more ordered than molecules absorbed onto other albumin molecules (8). Studies (9) revealed that serum albumin remained immunologically active upon sorption to polystyrene. Fluorescence data support the hypotheses that ribonuclease sorbs onto this same substrate with only minor structural change (10). Sorption of fibronectin, a plasma protein consisting of two long chains of 220 kilodalton molecular weight connected by disulfide bridges at one end, on silicas has been characterized by ellipsometry (11). These studies revealed that the protein conformations were different on alkylated silica as compared to unmodified silica. Partial desorption from the hydrophilic surface was accomplished upon solution dilution but not from the hydrophobic one. Desorption upon dilution is referred to commonly as reversible desorption.

Recently it was demonstrated through the use of a novel technique for measuring surface tensions of sorbed proteins (12) that the hydrophobicity of serum albumin, immunoglobulin, and fibrinogen sorbed onto polytetrafluoroethylene (PFTE), polyvinyl chloride (PVC), and nylon (N-6,6) particles increased with decreasing solution concentration over a 1–25 mg/ml range. This change was related to alterations in protein structure that were inhibited spatially with the higher number of protein molecules on the surface. The magnitude of the effect increased with increasing hydrophobicity of the polymer particle (PFTE > PVC > N-6,6).

Not all proteins are as robust as those mentioned in the previous paragraphs and may exhibit much greater structural alterations. Studies based on chromatography, for example, have shown that papain (13) and alpha-lactalbumin (14) undergo structural alteration on hydrophobic silicas and that the degree is related to contact time. Spectroscopy also has demonstrated that changes in structure continue after sorption (8).

## 2. SORPTION FORCES

Having considered protein sorption at interfaces from a phenomenological perspective, let us now consider the forces acting between a protein molecule and a surface across a liquid. We assume that under the conditions usually employed in chromatography of proteins that the mobile phase ionic strength is sufficiently high that the effects due to electrostatic double layer are small



**Fig. 3.** Intermolecular forces: (a) Attraction between molecules accounts for deviation from ideality; (b) mutual attraction between two molecules in a vacuum (Eq. 3); (c) interaction of molecules in solids (Eq. 4).

when modified silicas are the sorbents. The number of ionizable silanols is small in such materials and, considering the bond lengths of covalently bound organic groups as well as their concentration, have minimal interaction with proteins. We may, therefore, consider the interaction in terms London-van der Waals forces.

The concept of cohesive forces had its origins in van der Waals attempt to explain deviations from the ideal gas law at high pressures:

$$\left(p + \frac{a}{V^2}\right)(V - b) = RT \quad (2)$$

where the correction term,  $a/V^2$ , accounts for the added contraction of volume due to the attraction between molecules. Examples of the effect show in Fig. 3a.

With the development of quantum mechanics, London (15) was able to quantitate van der Waals observation for molecules without permanent dipoles:

$$F = \frac{-3}{4} hf \frac{P_2}{D^6} = \frac{-B_{ii}}{D^6} \quad (3)$$

$F$  is the mutual attraction energy between two molecules of species  $i$  under vacuum,  $h$  is Planck's constant,  $f$  is the characteristic frequency of oscillation of the charge distribution,  $P$  is the polarizability, and  $D$  is the separation distance (Fig. 3b).

Hamaker extended this equation to consider the attraction of assemblies of molecules in a solid with other assemblies (Fig. 3c). Then the interaction energy is the sum of interaction energies of all molecules present, which results in a pressure of attraction  $P$ :

$$P = \frac{-A_{ii}}{6\pi D^3} \quad (4)$$

where  $A_{ii}$  is the Hamaker constant,  $\pi^2 q_i^2 B_{ii}$ , is the number of atoms per cubic centimeter (16).

This approach to calculating the interaction between bodies from molecular properties has limitations in that it does not take into account the screening effect of surface molecules on interactions between molecules in secondary levels of the two bodies. It has been shown as a consequence of this effect that the predominant contribution to van der Waals forces comes from those parts of the bodies equal to the separation distance between them (17). This observation demonstrates the importance of surface layers on particles, around cells, or in structured macromolecules on the overall interaction between them.

A number of workers (18-20) have sought to overcome these limitations through consideration of macroscopic properties of materials. Such approaches lead to complex expressions for  $A$  that use measurable quantities such as dielectric constant, spectral absorbance, and surface tension. The observation that comparable values of  $A_{ii}$  are found when these various approaches are applied substantiates the validity of the concept, as well as some conclusions drawn from them. Hamaker constants for some polymers (21), normalized to that of PFTE, are given in Table 2. In general, there is good agreement between those calculated from spectral information and those determined from surface tension measurements.

The discussion in the previous paragraph and references contained in it leads us to three points for our consideration of liquid chromatography and proteins. First, van der Waals forces and the concepts evoking them are operable at distances to 100 Å. Such separation distances are not unrealistic for our system considering the sizes of protein molecules, high-performance supports, and the interstitial spaces between support particles. Second, the rule for Hamaker constants that describe interaction between two different species (1 and 2) is valid for many systems to within 5%. Thus,

$$A_{12} \cong A_{11} A_{22} \quad (5)$$

**Table 2. Hamaker Constants**

| Polymer      | Relative Hamaker Constant (Calculated) | Relative Hamaker Constant (from Surface Tension Measurements) |
|--------------|--|---|
| PFTE         | 1.0                                    | 1.0   |
| Parafin      | 1.2                                    | 1.2   |
| Polyethylene | 1.3                                    | 1.8   |
| Polystyrene  | 1.6                                    | 2.2   |
| PMMA         | 2.1                                    | 1.9   |
| PVC          | 2.6                                    | 2.2   |
| Nylon        | 2.6                                    | 2.2   |
| Polyester    | 2.9                                    | 2.2   |

for two materials interacting across a gap filled with a third, we may sum the interactions and write:

$$A_{132} = A_{12} + A_{33} - A_{13} - A_{23} \quad (6)$$

By substitution

$$A_{132} = (\sqrt{A_{11}} - \sqrt{A_{33}})(\sqrt{A_{22}} - \sqrt{A_{33}}) \quad (7)$$

Examination of this expression reveals that when  $A_{33}$  falls between  $A_{11}$  and  $A_{22}$ ,  $A_{132}$  is negative. Third, from Eq. 4, we reach the often-overlooked conclusion that the van der Waals energy of interaction between two dissimilar materials in the presence of a third medium can be *repulsive*.

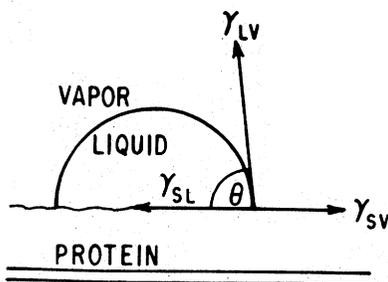
For this discussion, we have considered that van der Waals interactions result from forces between (a) permanent dipoles, (b) dipoles induced by dipoles, and (c) statistical dipoles resulting from random motion of electrons. These forces operate at relatively long distances from the interface. It has been proposed recently that at distances less than 2 Å beyond normal bond length need to be considered in systems consisting of polar liquids, apolar sorbents, and polymers (22). Since the supporting literature is presently small, the effect is not included in the subsequent discussion.

### 3. THERMODYNAMICS OF PROTEIN SORPTION

With this background we consider the Helmholtz free energy of adhesion (23) of a particle (protein, p) and a solid (s) in a liquid (l):

$$\Delta F_{slp} = \gamma_{ps} - \gamma_{pl} - \gamma_{sl} \quad (8)$$

$\gamma$  refers to the respective interfacial tensions. The expression therefore reflects



**Fig. 4.** Contact of liquid with thick protein layer contained on membrane.  $\theta$  is contact angle,  $\gamma_{sv}$  and  $\gamma_{lv}$  are solid and liquid surface tensions, respectively;  $\gamma_{sl}$  is interfacial tension.

the difference in energy required to bring a protein from solution to the solid interface relative to the energy involved in returning solvent molecules to the bulk.

From the discussion in the previous section the interaction energy is described also by  $A_{slp}$  where  $s = 1$ ,  $l = 3$ ,  $p = 2$  and that van der Waals repulsion is expected when  $A_{ss} < A_{ll} < A_{pp}$ . It has been demonstrated (24) that  $A_{ii} \sim \gamma$ ; thus, from Eqs. (7) and (8) that sorption of protein onto the surface will be favored when the surface tension of the liquid is either above or below the surface tensions of both other components and desorption is favored when it falls between them.

### 3.1. Surface and Interfacial Tension Measurement

Measurement of surface tensions of liquids by DuNouy tensiometer or Wilhelmy balance is described in every text on surface chemistry and will not be discussed here. Surface tension determination for solids and proteins is less well known and will be described briefly.

Several approaches have been proposed for evaluating protein surface tension. One is to measure the incremental change as a function of protein concentration in solution. Because proteins may undergo major conformational change at the air/solution interface, low values result.

Another method uses the measurement of the angle of contact a sessile drop of liquid makes with a surface (Fig. 4). For proteins, thick layers are deposited from solution on ultrafilter membranes. The surface thus produced is in the hydrated, native state (23). Contact angles are measured with a goniometer or on enlarged photographs of the drop.

The relationships between the respective surface tensions are deduced from an equation of state (25) that is adopted from the Gibbsian dividing surface approximation. When this equation is combined with Young's equation, two basic equations result:

**Table 3. Surface Tensions of Proteins**

| Protein                           | Method   |  |
|-----------------------------------|--|--|
|                                   | Contact Angle <sup>a</sup><br>(mJ/m <sup>2</sup> ) | Sedimentation <sup>b</sup><br>(mJ/m <sup>2</sup> ) |
| Human Serum Albumin               | 70.2   | 69.7   |
| Immunoglobulin G                  | 67.7   | 67.8   |
| Bovine Serum Albumin              | 70.3   | —  |
| Bovine Serum Albumin <sup>c</sup> | 35.0   | —  |
| Ovalbumin                         | 68.8   | —  |

<sup>a</sup>Liquid was saline (30).<sup>b</sup>Reference (12).<sup>c</sup>Liquid was EtOH/buffer, pH 2 (29).

$$\gamma_{sl} = \frac{[(\gamma_{pv})^{\frac{1}{2}} - (\gamma_{lv})^{\frac{1}{2}}]^2}{1 - 0.015 (\gamma_{pv} \gamma_{lv})^{\frac{1}{2}}} \quad (9)$$

$$\cos \theta = \frac{(0.015\gamma_{pv} - 2.00) (\gamma_{pv} \gamma_{lv})^{\frac{1}{2}} + \gamma_{lv}}{\gamma_{lv} [0.015(\gamma_{pv} \gamma_{lv})^{\frac{1}{2}} - 1]} \quad (10)$$

These equations may be solved through the use of published computer programs (26) or tables (27) such that a protein's surface (tension  $\gamma_{pv}$ ) can be obtained from a contact angle determined with a liquid of known surface tension ( $\gamma_{lv}$ ). This liquid is generally physiological saline.

The final technique to be described is based on the observation (28) that when similar masses of packing are dispersed in liquids of differing  $\gamma_{lv}$ , the bed volumes after settling will reach a minimum or maximum value at  $\gamma_{sv} = \gamma_{lv}$ , i.e.,  $A_{ii} = 0$ . Whether a minimum or maximum is reached depends on the extent to which the particles agglomerate in early stages of sedimentation. If particles are coated with thick layers of protein absorbed from solution,  $\gamma_{sv} = \gamma_{pv}$ . Thinner layers may have substantially lower  $\gamma_{pv}$ , probably reflecting conformational change (12). Some  $\gamma_{pv}$  are given in Table 3.

#### 4. SURFACE TENSIONS OF LC PACKINGS

The sedimentation technique previously described, can be used in the absence of protein solution, to determine surface tensions of LC packings (31). A table of surface tensions for chemically modified silicas is presented in Table 4. In general, the hydrocarbonaceous packings (reversed phase) are in the 32–37 mJ/m<sup>2</sup> range, whereas those with carbon–oxygen functionality are much higher.

#### 5. RELEVANCE TO CHROMATOGRAPHY

We now examine the chromatography of proteins from consideration of van der Waals attraction/repulsion concepts as shown in Eq. (8) and of measured

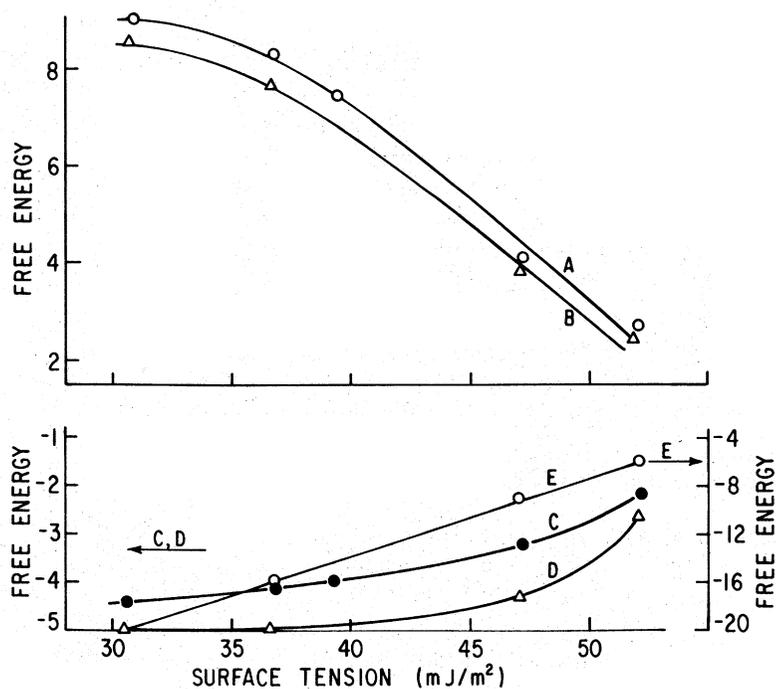
**Table 4. Surface Tension ( $\gamma_{sv}$ ) of Derivatized Silica LC Packings<sup>a</sup>**

| Organo Group                 | $\gamma_{sv}$<br>(mJ/m <sup>2</sup> ) | Organo Group                  | $\gamma_{sv}$<br>(mJ/m <sup>2</sup> ) |
|------------------------------|---------------------------------------|-------------------------------|---------------------------------------|
| Polyamidopropyl <sup>b</sup> | 53                                    | <i>n</i> -Butyl (deactivated) | 41                                    |
| Diether                      | 52                                    | <i>n</i> -Butyl               | 36                                    |
| Diol                         | 47                                    | Octadecyl                     | 37                                    |
| <i>t</i> -Butyl              | 39                                    | <i>t</i> -Butylphenyl         | 35                                    |
| Diether (deactivated)        | 39                                    | <i>n</i> -Hexyl               | 32                                    |

<sup>a</sup>Determined by sedimentation method (31).

<sup>b</sup>From SynChrom, Inc.; others supplied by Supelco, Inc.

surface tensions of column packings (s), proteins (p), and mobile phases (m). Plots of  $\Delta F_{smp}$  against surface tensions of some modified silicas (Table 4) are given in Fig. 5 for BSA and OVAL with two hypothetical mobile phases. The higher  $\gamma_{mv}$  is analogous to that of water containing  $\sim 0.1$  M NaCl or Na<sub>2</sub>SO<sub>4</sub>.



**Fig. 5.** Effect of support and mobile phase on protein sorption. (●-●) BSA; (Δ-Δ) Oval; (O-O) solvent denatured BSA.  $\gamma_{mv} = 60$  mJ/m<sup>2</sup> for curves, A, B, E;  $\gamma_{mv} = 73$  mJ/m<sup>2</sup> for curves C and D

With such mobile phases sorption of proteins is favored on all packings although binding affinity is reduced greatly at higher  $\gamma_{sv}$ . Packings with such surface tensions are used commonly for size-exclusion chromatography or "hydrophobic interaction" chromatography (HIC). The latter are similar chemically to the former but lightly alkylated. The energy of interaction becomes similar for the proteins as  $\gamma_{sv}$  increases but is negative unless the surface tension of the mobile phase is reduced until it is less than that of the protein. Ideally, then, determination of molecular size should be conducted with such mobile phases to prevent sorption. However, the use of buffer additives to achieve this may be precluded because the additives could alter protein size or shape or both also.

Further addition of salt increases  $\gamma_{mv}$  and promotes sorption. For the lightly alkylated supports, then, that protein retention can be manipulated by varying salt concentration is predicted. We may relate  $F_{smp}$  to a common measure of chromatographic retention,  $k'$ :

$$k' = \frac{V_R - V_m}{V_m} = K \left( V_s/V_m \right) \quad (11)$$

and,

$$\Delta F = -RT \ln K \quad (12)$$

so that,

$$k' = e^{(-\Delta F/RT)(V_s/V_m)} \quad (13)$$

where  $V_s/V_m$  is the phase ratio,  $K$  is the distribution coefficient, and  $V_R$  is the protein retention volume. A strategy, then, for HIC is to introduce protein mixture with high surface tension (salt) mobile phase and reduce surface tension, eluting each protein as  $\gamma_{mv}$  passes that of the protein. Reduction of surface tension may be accomplished by reducing salt concentration or by addition of a modifier such as ethylene glycol.

Alternatively, retention may be increased by reducing the surface tension of the packing, which is accomplished by increasing the length or surface concentration of alkyl groups. This effect, as well as the salt concentration effect, were observed experimentally (32, 33). They were examined in the framework of solvophobic theory. The conclusions drawn in this report do not evoke concepts of repulsion by the solvent but concept of van der Waals attraction/repulsion of the protein and the surface that are accounted for by thermodynamic considerations. It should be noted, considering the amphiphilic nature of proteins, that the van der Waals attraction between an apolar species and a polar one in water has been shown to be significant (34).

As the surface alkyl group density is increased further or is the only surface modification,  $\gamma_{sv}$  is substantially lower and  $\Delta F$  becomes much more negative.

ADSORPTION OF PROTEIN TO ALKYL-SILICA

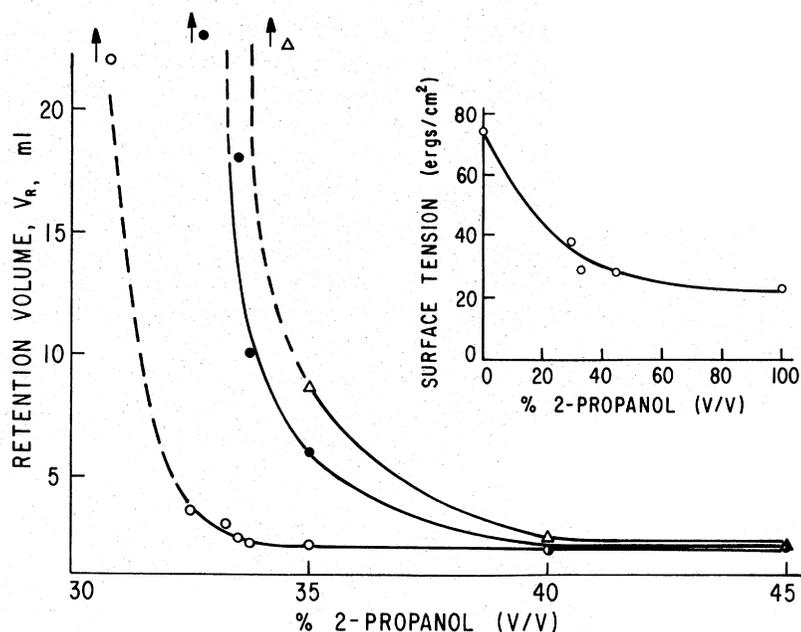


Fig. 6. Relation of surface tension to retention of (○) BSA, (●) beta-lactoglobulin, and (△) hemoglobin on a reversed-phase column. Mobile phase: phosphate buffer/2-propanol (pH 2.1)  $1 \text{ erg/cm}^2 = 1 \text{ mJ/m}^2$  (29).

Mobile phases containing very large proportions of organic additive in buffer are required to desorb proteins. This is shown in Fig. 6 for BSA, beta-lactoglobulin, and hemoglobin (29). The inset indicates the mobile phase surface tension that correlates with the percentages of 2-propanol that brought about elution of the three proteins from a reversed-phase column. It is seen readily that small changes in eluant composition caused large effects on retention. At 40°C, increasing 2-propanol percentage by only 3.75% reduced retention by a factor of 12 (Fig. 7). Compared to the large effect on retention due to mobile phase composition, the effect of temperature is slight but tends to decrease as temperature increases. A decrease is predicted from consideration of  $\Delta F_{\text{smp}}$ , since  $\gamma_{\text{mv}}$  decreased with temperature increase. When ethanol replaced 2-propanol, elution of BSA occurred at the same surface tension value, although ~50% ethanol was needed to achieve 35 mJ/m<sup>2</sup> as compared to ~33% 2-propanol. Such proportions of organic modifier can alter the secondary structure of many proteins as observed by spectroscopic methods (35) and as evidenced by lower measured surface tensions. Solvent denatured BSA, for example, has a surface tension of ~35 mJ/m<sup>2</sup>, whereas the value of native protein is ~70 mJ/m<sup>2</sup> in buffer. As seen in Fig. 5, the solvent denatured BSA has much greater attraction to a reversed-phase column than the native protein.

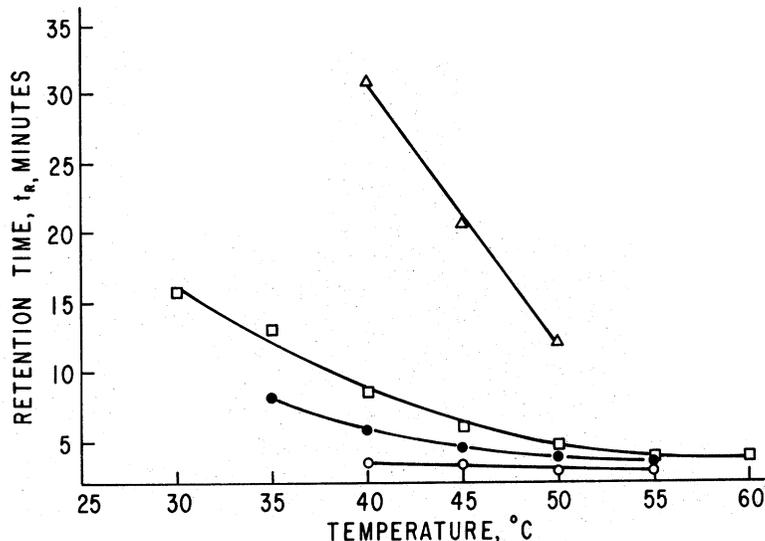


Fig. 7. Mobile phase composition and temperature effects on beta-lactoglobulin retention mobile phase and column as in Fig. 6: ( $\Delta$ - $\Delta$ ) 31.25% 2-propanol; ( $\square$ - $\square$ ) 33.25%; ( $\bullet$ - $\bullet$ ) 33.75%; ( $\circ$ - $\circ$ ) 35.00%.

## 6. CONCLUSIONS

The general view of a protein is that of a molecule of complex chemical nature with a high proportion, but not all, of the apolar amino acids arranged inside of its three-dimensional structure and a high proportion, but not all, of polar amino acids on the surface. Moreover, because proteins contain large numbers of ionizable groups so that a high electrostatic potential may occur depending on solution pH and ionic strengths. Therefore, proteins may interact with surfaces through a number of chemical functionalities. Specific interactions of proteins are mediated by many effects that influence their surface composition, structure, and the orientation of interacting groups. Furthermore, in chromatography, fluid dynamics and adsorption kinetics may be important. Nevertheless, protein adsorption and, therefore, retention in liquid chromatography may be approximated by considerations of van der Waals attractive and repulsive forces as manifested by the free energy required to bring a protein molecule from solution to an equilibrium position at the interface. Nonpolar/nonpolar, nonpolar/polar, and polar/polar interactions are considered and arguments based on repulsion by the solvent need not be evoked. Surface energies of proteins and of chromatographic packings can be measured and used to guide development of separations by chromatography.

Size-exclusion, "hydrophobic interaction," and reversed-phase siliceous packings represent a continuum of materials with decreasing surface tension.

Retention of proteins is decreased by decreasing surface tension of the mobile phase.

For "hydrophobic interaction" packings, retention of native proteins may be influenced by number and size of alkyl groups and may be manipulated by salt concentration of aqueous mobile phase or through addition of small concentration of organic modifier. Sorption is at one or a few alkyl sites, so that protein structural changes due to sorption are minimal. Some alteration may occur, and the degree may be influenced by contact time. However, for many preparative applications where high recoveries of protein with native structure or activity or both are required, the conditions of HIC offer advantages. For highly alkylated (reversed-phase) packings, high percentages of organic modifier are required to desorb protein. Such mobile phases induce structural alterations in many proteins. Moreover, multisite attachment tends to induce structural change. Alkyl chain length has little effect on sorption, but number of chains could, as they reflect unreacted silanol groups. These could act as secondary binding sites. Aryl-modified silicas have about the same surface tensions as alkyl silicas but are capable of specific  $\pi$ -interactions. Recoveries of protein from reversed-phase packings are improved by increasing temperature (36), use of silanophilic blocking agents (37), or protein derivatization (38) or all three. Recoveries tend to increase with sample load as expected from surface tension measurements. All of these may induce structural changes but these not likely to be important for analytical chromatography.

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