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Repulsive van der Waals Forces. II. Mechanism of Hydrophobic Chromatography

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Repulsive van der Waals Forces. II. Mechanism of Hydrophobic Chromatography

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Abstract

When two materials with different interfacial free energies are immersed in a liquid with an interfacial free energy intermediate between those of the two materials, the net van der Waals forces between these two materials are repulsive. Thus by lowering the interfacial free energy of the liquid medium, solutes or particles previously adsorbed onto low energy surfaces can be readily eluted from such surfaces. This is demonstrated by the coupling to and subsequent elution from Octyl Sepharose and Phenyl Sepharose of serum and other proteins. The elution of all proteins commenced when the surface tension of the eluting liquid was decreased to a point just below that of the protein in question. The eluted serum proteins successively emerged from the column in the exact decreasing order of their own interfacial free energies. In hydrophobic chromatography, coupling is favored when the van der Waals forces between solutes (or particles) and ligand are attractive (and maximum, frequently through the admixture of salts); elution is brought about by causing the van der Waals interaction between each solute (or particle) and ligand successively to become repulsive (by gradually lowering the surface tension of the eluting liquid).

INTRODUCTION

We have recently shown theoretically and experimentally that the sign of the net van der Waals interaction between two different solid bodies (*1*), or between two different dissolved polymers (*2*), in liquids in many cases is negative, i.e., their interaction is repulsive. This possibility is implicit in Hamaker's classical paper on van der Waals-London interactions (*3*), and has been more recently formulated explicitly by Visser (*4*): "when two materials are immersed in a liquid medium, and the interactions of each of these materials with that of the liquid medium is larger than the interaction between these materials themselves, spontaneous separation can occur due to dispersion forces only." Fowkes showed such a repulsive interaction for the system (poly-(tetrafluoroethylene)-glycol–iron oxide (*5*).

Briefly, the van der Waals interaction between the two different substances, 1 and 2, that are immersed or dissolved in liquid 3 will be *repulsive* when the Hamaker coefficient A_{132} of the expression for the free energy ΔF of the interaction:

$$\Delta F = -A_{132}/12\pi d^2 \tag{1}$$

(where d is the distance between two particles and/or molecules, taken to be semi-infinite homogeneous slabs), is *negative*. It is easily shown that this is always the case when the individual Hamaker coefficients of the two substances, A_{11} and A_{22}, have values that straddle that of the liquid, A_{33} (*4*):

$$A_{11} > A_{33} > A_{22} \tag{2}$$

$$A_{11} < A_{33} < A_{22} \tag{3}$$

Thus, in a liquid 3, substances 1 and 2 will undergo a van der Waals repulsion when the interfacial free energy of substance 1 is smaller and that of substance 2 is larger than that of the liquid, or vice versa (*1*, *2*); see also Ref. *6*.

Virtually all biological substances (such as proteins, polysaccharides, and other biopolymers) have an interfacial free energy that is lower than (although it can fairly closely approach) that of water, the liquid in which they are normally dissolved or suspended. They all will therefore more or less strongly undergo a van der Waals attraction to low surface energy ("hydrophobic") surfaces in water. This van der Waals attraction can be enhanced in the case of strongly hydrophilic biopolymers by raising the interfacial free energy of water through the addition of salts, which

increases the difference in interfacial free energy between biopolymer and the liquid medium. The van der Waals interaction between biopolymers and hydrophobic adsorbents in water can be made repulsive by lowering the interfacial free energy of water (through the addition of water-miscible low energy liquids) to a value below that of the biopolymer, but still well above that of the hydrophobic adsorbent, thus bringing about desorption or elution. This, essentially, is the mechanism of the separation method that has come to be called hydrophobic chromatography, as we shall endeavor to demonstrate and illustrate quantitatively with a number of experiments with a few separate proteins, and on the fractionation of human serum proteins, below.

MATERIALS AND METHODS

As adsorbents for hydrophobic chromatography, Octyl Sepharose CL-4B (batch 01–900–1–2818–01) and Phenyl Sepharose CL-4B (batch 01–900–1–2735–01) (Pharmacia Fine Chemicals, Piscataway, New Jersey), each with a ligand concentration of 40 μmole/ml of gel, were used (*7*). Ethylene glycol (EG) (Fisher Scientific, Rochester, New York) was used at various concentrations, dissolved in 0.01 *M* phosphate buffer (pH 6.8, consisting of 0.005 *M* Na_2HPO_4 and 0.005 *M* KH_2PO_4). Surface tensions of the various EG and other solutions were determined by the pendant drop method as described by Padday (*8*); see also Ref. *9*. Briefly, a drop of the liquid of which the surface tension is to be measured is extruded downward through a small tube by means of a syringe. The drop is then photographed and on an enlargement of the photograph the widest diameter d_e and the diameter d_s at a height d_e above the lowest point of the drop are measured. The surface tension (γ) of the liquid is then expressed as

$$\gamma = \Delta\rho g d_e^2/H$$

where H (which is a function of d_e/d_s) may be found in published tables (*8*, *9*). $\Delta\rho$ is the difference in densities between that of the liquid and of air, and g is the gravitational constant ($= 980$ cm sec^{-2}). Figure 1 shows the surface tensions of solutions EG vs the EG concentration at 20°C.

Whole human serum was obtained from normal donors; antisera against particular human serum components were purchased from Behring Diagnostics, Somerville, New Jersey, and rabbit-anti-whole human serum was obtained from GIBCO, Grand Island, New York. Immunodiffusion and immunoelectrophoresis analyses were performed in the traditional

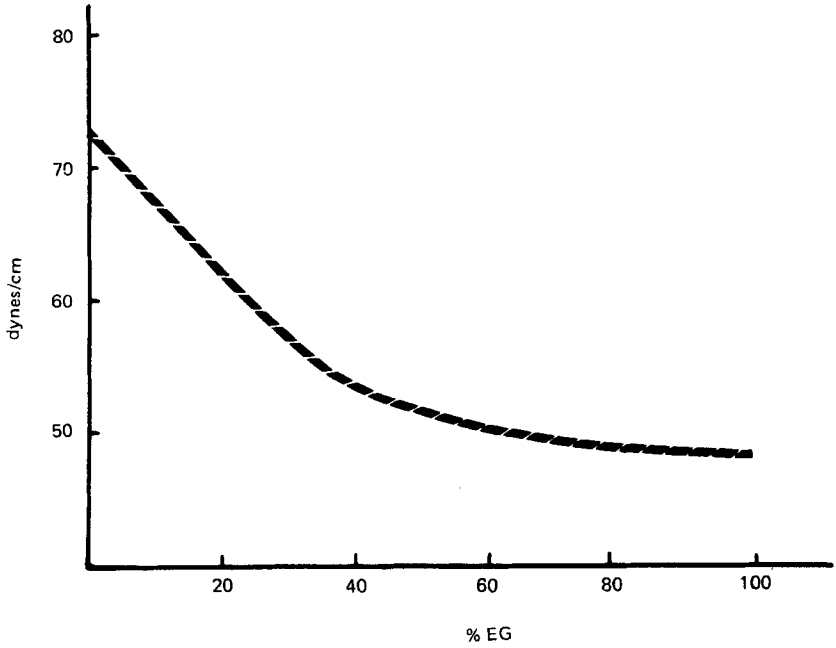

FIG. 1. Graph of the surface tensions of ethylene glycol (EG) solutions in 0.01 *M* phosphate buffer vs the EG concentration, in % (v/v).

manner, see, e.g., van Oss et al. (*10*). The gels were made with 1 % Ionagar No. 2 (Colab Laboratories, Chicago Heights, Illinois). Bovine serum albumin, ovalbumin, and human gamma globulin (IgG) were obtained from Miles Laboratories, Elkhart, Indiana. All salts and other chemicals were obtained from Fischer Scientific (Rochester, New York).

Chromatography was done in 10 ml disposable plastic pipettes, provided at the lower inside extremity with a porous siliconized glass-wool plug and at the lower outside with a short pliable plastic tube and a clamp. The Sepharose 4B gel beads were successively washed with 3 bed volumes of distilled water, 3 bed volumes of *n*-butanol, 2 bed volumes of ethanol, 2 bed volumes of distilled water, and extensively with the coupling buffer (see below). Four milliliters of washed gel beads were then mixed with 6 ml of protein solution (1 % protein dissolved in the appropriate coupling buffer), allowed to stand at 20°C for 1 hr, and then centrifuged at ≈ 500 *g* for 5 min. The A_{280} value of the supernatant was then determined with a UV spectrophotometer (Gilford Spectrophotometer 230, Oberlin,

Ohio). The pellet was resuspended in 5 ml of the appropriate buffer, allowed to stand for 10 min at 20°C, and again centrifuged at $\approx 500\,g$ for 5 min after which the A_{280} values of the supernatants were determined once more. This operation was repeated a third time. Thereafter the gel bead slurry was mixed with 1/2 of its volume of Sephadex G-10 (Pharmacia) to ensure a satisfactory flow rate. The slurry was then packed in a 10-ml disposable pipette (see above) with the bottom outlet open during the process of packing to ensure an even distribution of the two gel types. The column was then washed with the appropriate buffer until the eluate had an $A_{280} < 0.001$. Once that value was reached, elution was commenced. For coupling, the 0.01 *M* phosphate buffer (see above) was either used as is or with an additional 1 *M* $(NH_4)_2SO_4$. For elution, gradients of increasing EG concentration (in 0.01 *M* phosphate buffer as solvent) were used. Elution of proteins was monitored by A_{280} readings. In the case of whole normal human serum the various fractions were characterized by immunoelectrophoresis; also, a larger column was used: 8 ml of the Phenyl Sepharose gel was mixed with 12 ml of normal human serum (7 times diluted in buffer).

Contact angles with sessile drops of saline water were measured according to the method described by van Oss et al. (*11*); see also van Oss (*12*). The interfacial free energies were derived from these contact angles via the approach of Neumann et al. (*13*); see also Ref. *11*.

RESULTS

Influence of Salt on Protein Binding to the Ligand

Bovine serum albumin (BSA) was coupled to Octyl as well as Phenyl Sepharose, and dissolved in phosphate buffer as well as dissolved in phosphate buffer containing 1 *M* $(NH_4)_2SO_4$. Table 1 shows the coupling efficiency in these four cases.

TABLE 1

Influence of 1 *M* $(NH_4)_2SO_4$ on the Coupling of BSA to the Ligand

Ligand	% of initial BSA bound	
	In phosphate buffer	In phosphate buffer with 1 *M* $(NH_4)_2SO_4$
Octyl Sepharose	74	80
Phenyl Sepharose	69	73

Clearly, the presence of 1 *M* salt ($(NH_4)_2SO_4$) gives rise to 6 to 8% more efficient coupling of BSA, and Octyl Sepharose binds 7 to 9.5% more BSA than Phenyl Sepharose. Similar results were obtained with ovalbumin (OA) and human gamma globulin (IgG). Once bound, the BSA and also OA and IgG could not be further eluted to any significant extent from either Octyl or Phenyl Sepharose with phosphate buffer, with or whithout 1 *M* $(NH_4)_2SO_4$.

Elution of Three Proteins with an EG Gradient; Influence of Salt

BSA, OA, and IgG were each coupled to Phenyl and also to Octyl Sepharose, and then eluted with an EG gradient of increasing concentration (from 0 to 50% EG). Each protein was coupled and eluted separately,

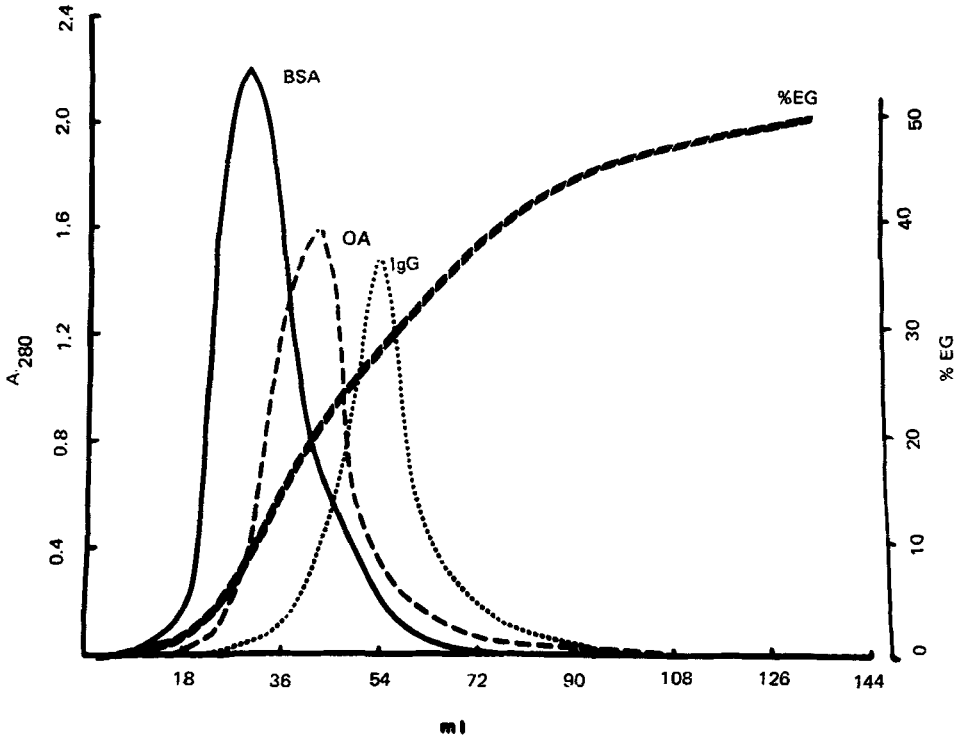

FIG. 2. Elution of bovine serum albumin (BSA), ovalbumin (OA), and human immunoglobulin G(IgG) from Phenyl Sepharose columns, with EG gradients of increasing EG concentration.

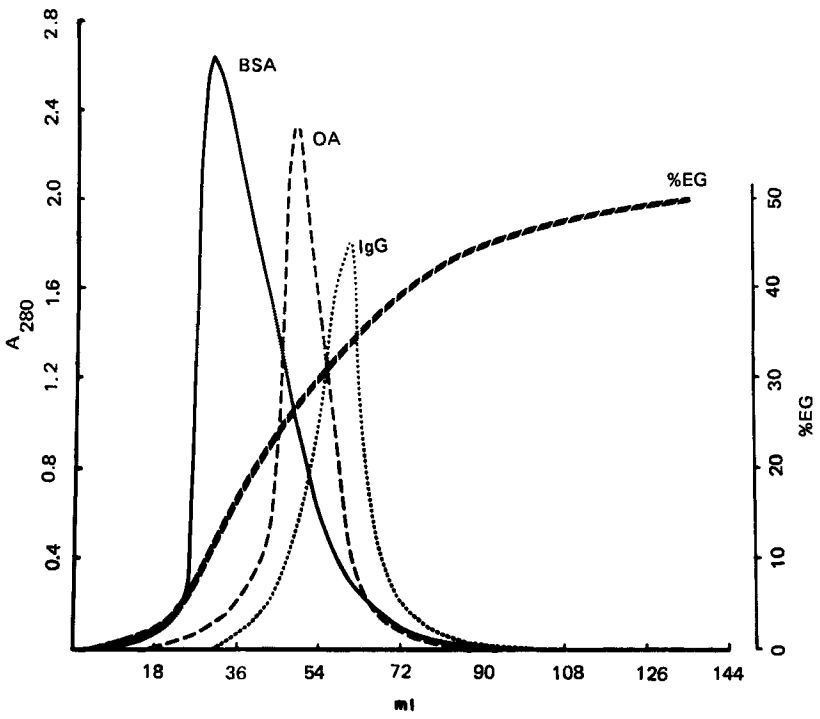

FIG. 3. Elution of BSA, OA, and IgG from Octyl Sepharose columns, with EG gradients of increasing EG concentration.

but in the graphic representation the results of identical treatments of BSA, OA, and IgG are grouped together; see Figs. 2 and 3. In all cases the three proteins eluted in the following order with increasing EG concentration: first BSA, then OA, then IgG. All three proteins eluted from Phenyl Sepharose at somewhat lower EG concentrations than from Octyl Sepharose (Figs. 2 and 3).

Separation of Serum Proteins

Whole normal human serum was coupled to Phenyl Sepharose and eluted with a gradient of increasing EG concentration. Six major fractions appeared (see Fig. 4). The composition of each was tentatively determined by immunoelectrophoresis against goat anti-whole human serum (see Fig. 5) and confirmed by immunodiffusion against antisera that react

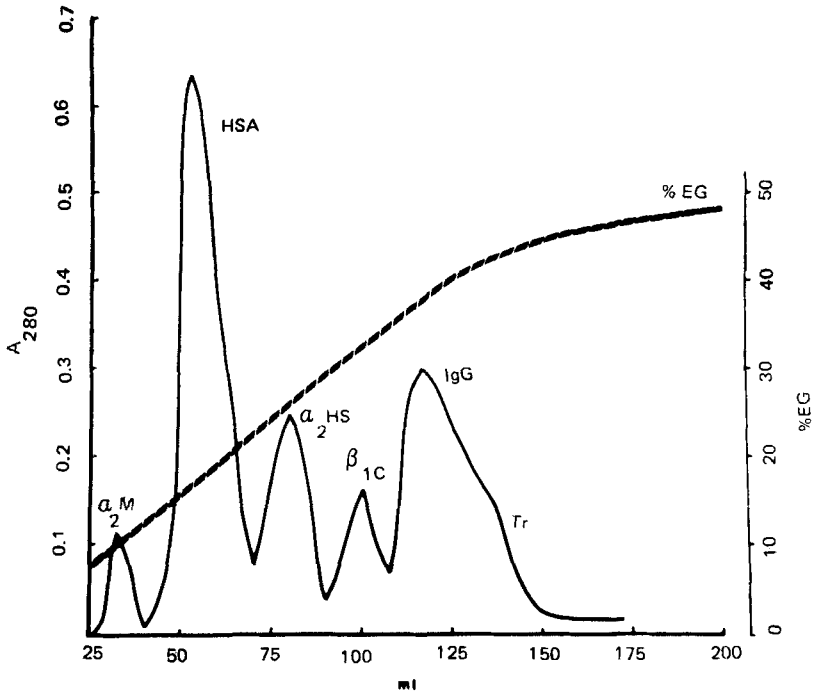

FIG. 4. Elution of human serum proteins from a Phenyl Sepharose column, after adsorption of whole human serum, with an EG gradient of increasing EG concentration. The following fractions were analyzed for content and purity and were found mainly to contain: 30–35, α_2M; 50–62.5, BSA; 75–85, α_2HS; 95–102.5, β_{1C}; 112.5–122, IgG; 135–140, Tr. For the explanation of these abbreviations for the various serum fractions, see Table 2.

monospecifically with only one given serum component. The serum proteins that could be eluted under our experimental conditions were, successively: α_2 macroglobulin (α_2M), albumin (HSA), α_2 HS glycoprotein (α_2HS), $\beta_{1C} - \beta_{1A}$ or C3 complement component (β_{1C}), IgG, and transferrin (Tr); see Fig. 4. These are six of the 11 major serum protein components; five others (IgA, IgM, haptoglobin, α_1 antitrypsin, and α_1 acid glycoprotein) were not isolated under the conditions described above. The six proteins that were eluted came off the column in the order of decreasing hydrophilicity, see Table 2.

The values found for α_2M, HSA, and IgG agree closely with those found earlier for the pure components (*11*, *12*). The value found for BSA is very

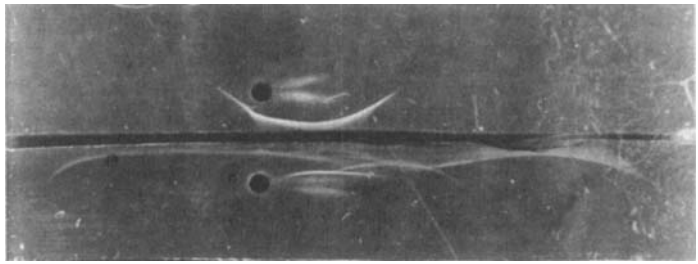

FIG. 5. Immunoelectropherogram of the Transferrin fraction (fraction 135–140, see Fig. 4): Tr (top) and normal whole human serum (bottom). The precipitate lines were obtained by immunodiffusion with rabbit anti-whole human serum. The cathode was on the left and the anode on the right. The top half of the figure shows that Tr was the only immune precipitate detectable. In addition to Tr, however, a shallow-tail shaped precipitate of nonimmunological origin is visible to the right of the wells (both top and bottom of the figure), caused by traces of β-lipoprotein interacting with the agar gel. This effect was also detectable in most of the other fractions, with the exception of the fraction containing albumin.

TABLE 2

Contact Angles with Sessile Drops of Saline Water (*11*, *12*) and Interfacial Free Energies (*11*, *12*) of the Various Protein Fractions Isolated (see Fig. 4) and of BSA and OA (see Figs. 2 and 3)

Protein	Contact angles in degrees $\pm$ S.E.[a]	Interfacial free energy ΔF (in ergs/cm^2)	Corresponding to a "surface tension" γ (in dyn/cm)[b]
α_2 Macroglobulin (α_2M)	13.5 $\pm$ 0.3	-141.1	70.6
Bovine serum albumin (BSA)	14.4 $\pm$ 0.3	-140.6	70.3
Human serum albumin (HSA)	14.6 $\pm$ 0.6	-140.4	70.2
Ovalbumin (OA)	19.2 $\pm$ 0.2	-137.6	68.8
α_2 HS glycoprotein (α_2HS)	20.8 $\pm$ 0.4	-136.2	68.1
β_{1C}–β_{1A} Globulin (C3) (β_{1C})	21.6 $\pm$ 0.3	-135.6	67.8
Immunoglobulin G (IgG)	23.2 $\pm$ 0.3	-134.4	67.2
Transferrin (Tr)	23.7 $\pm$ 0.3	-133.5	66.8

[a]Measured at 20°C.
[b]According to $\Delta F = -2\gamma$.

close to that of HSA, as should be expected. The value found for the contact angle of the protein fraction containing mainly α_2HS is somewhat lower than that found earlier with purified (*14*) α_2HS (*12*, *15*); this may be an indication that the protein fraction containing α_2HS may also contain other proteins in amounts too small to be detected by immunoelectrophoresis but large enough to influence the interfacial free energy of the total proteins in the fraction.

DISCUSSION

Hydrophobic chromatography (HC) is a method with a largely empirical basis that developed as an outgrowth of affinity chromatography. It was described, almost simultaneously, by a number of authors, the first of whom appears to have been Hofstee, who at first called the approach "Hydrophobic Affinity Chromatography of Proteins" (*16*); see also Refs. *17–20*. At first "salt effects" were deemed of the utmost importance in HC (*17–21*), but it became evident that especially the elution of proteins from hydrophobic adsorbents was enhanced by the presence of "nonpolar" liquids such as EG (*16*, *22*) and even of detergents (*21*). Hofstee, in 1976, emphasized that "true" hydrophobic bonding is stabilized by increasing salt concentration (*23*). Indeed, elution from hydrophobically linked carriers is now more and more routinely done with eluants containing either detergents (*24*) or other surface-tension covering solutes such as EG (*25*).

We have recently demonstrated that a variety of antigen–antibody complexes whose interactions are solely of the van der Waals type can be completely dissociated by simply lowering the surface tension of the liquid medium (*26*). The results reported in the present paper point to the same simple mechanism for the elution of adsorbed proteins in HC.

First, one must not lose sight of the fact that, while there are a number of categories of strong as well as of weak chemical bonds, "hydrophobic bonds" are not chemical bonds, and should not (and generally are not) called by that name. The "hydrophobic effect" (*27*) is an interaction in a (generally) aqueous medium between macromolecules (and/or particles) by which some of the more hydrophobic sites, upon approaching one another, also preferentially interact with each other, frequently by van der Waals interactions, which tend to be attractive between low surface energy materials (or sites) immersed in high energy liquids such as water. In the process some fairly organized interstitial water molecules tend to get expelled into the bulk liquid and to become more randomized, thus

often giving rise to an increase in entropy, which is an accompanying (and occasionally even a contributing) effect, but not a cause, of the bonding (*28*).

The results described above show that the couplings as well as the elution characteristics of a number of typical instances of HC of proteins closely conform to the van der Waals attraction or repulsion requirements under the experimental conditions of, respectively, coupling or elution.

1 *M* $(NH_4)_2SO_4$, which further increases the surface tension of water [by $\approx$2.2 dyn/cm (*29*)], enhances the coupling of proteins to both hydrophobic ligands (Table 1). Also, Octyl Sepharose, which is even more hydrophobic than Phenyl Sepharose, bonds protein more strongly than Phenyl Sepharose (Table 1 and Figs. 2 and 3). It is not yet feasible to make direct measurements of the interfacial free energies of Octyl or Phenyl Sepharose, but we know that the surface tension at 20 °C of octanol is 27.5 and that of phenol 40.9 dyn/cm (*30*). Hofstee also already remarked upon the enhanced protein bonding by more hydrophobic ligands (*31*).

The elution of all proteins clearly commenced when the surface tension of the eluting liquid (compare Fig. 1, for the surface tensions of EG, with Figs. 2–5) was lowered to a point just below that of the protein in question (see Table 2 for the "surface tensions" of the proteins). Indeed, the eluted blood serum proteins came off the column, with decreasing surface tension of the eluant, in the precise decreasing order of their own interfacial free energies (compare Fig. 4 with Table 2). Given the lower interfacial free energy of Octyl Sepharose as compared with Phenol Sepharose, it is quite reasonable that proteins (Figs. 2 and 3) eluted at slightly lower EG concentrations from Phenyl than from Octyl Sepharose, as their van der Waals energy of bonding to the phenyl ligand was somewhat smaller.

Thus, HC is a category of liquid chromatography in which coupling between solutes (and/or particles) and the ligand surface occurs by van der Waals attraction under conditions where the interfacial free energy of the liquid medium is higher* than the interfacial free energies of both solutes (and/or particles) and the ligand surface. Elution is effected by changing the van der Waals attraction into a repulsion by lowering the interfacial free energy of the liquid to a value intermediate between that of the solute(s) (and/or particles) and the ligand surface.

*In principle, for coupling the interfacial free energy of the liquid medium may also be *lower* than that of *both* solutes (and/or particles) and the ligand surface; elution can then be effected by *raising* the interfacial free energy of the liquid to a value intermediate between that of the solute(s) (and/or particles) and the ligand surface.

In polar liquids such as water, electrokinetic interactions will, of course, also play a certain role, and result in a hybrid methodology that is principally based on HC, but for a smaller part also on ion-exchange chromatography in which pH as well as salt effects remain of some importance, in addition to the influence the latter parameters may have on interfacial free energies.

The importance of van der Waals interactions in enzyme–substrate reactions, as well as in antigen–antibody binding (*26*), makes the considerations that have been outlined here of equal relevance to affinity chromotography and hydrophobic chromatography.

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