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MECHANISM OF PROTEIN RETENTION IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention of a number of proteins by alkylsilicas with phosphate buffer–isopropanol, buffer–ethanol and buffer–detergent mobile phases (all pH 2.1) was investigated. In each case the percentage of organic component was varied over a wide range without causing appreciable retention. However, a solution composition was reached where very small changes, less than 1 % for the alcohols, caused dramatic increases in protein retention. Retention of proteins decreased with increased temperature. The mobile-phase composition was found to be characteristic of each protein at a given temperature so that separations could be achieved by careful selection of temperature and of buffer–organic component ratios. Calculations of the energy of interaction from surface tension and contact angle measurements supported the concept that protein retention by alkylsilicas may be explained by Van der Waals interactions, and that the interactions may be attractive or repulsive depending on the surface tension of the mobile phase.

INTRODUCTION

The potential of high-performance liquid chromatography (HPLC) for rapidly separating and/or characterizing mixtures of biomacromolecules such as proteins is slowly being realized, as discussed recently in a review by Regnier and Gooding[1]. We envision that a "multidimensional" chromatographic approach with various types of columns will be needed to resolve protein isolates from complex biological matrices. Therefore we have been developing information to understand better the interactions of proteins in chromatographic systems[2,3]. Hearn *et al.*[4], Revier[5] and Mönch and Dehnen[6] have used hydrophilic counterions to form ion-pairs with peptides and proteins and have explored conditions for elution from alkylsilica columns. In general, severe conditions, which may induce structural changes, were necessary for elution of proteins. The present study was undertaken to elucidate further the mechanisms of retention in these systems so that less stringent chromatographic conditions may be developed.

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Theory

The general elution expression for chromatography may be written as:

$$V_R = V_M + V_S K \tag{1}$$

where V_R is retention volume, V_M is the mobile phase or interstitial volume (m), V_S is the volume of stationary phase(s), and K is the distribution coefficient. For considerations of protein–alkylsilica–aqueous mobile phase interactions:

$$-\Delta F_{smp} = RT \ln K \tag{2}$$

where ΔF_{smp} is the free energy of binding of the protein ion-pair (p) to the alkylsilica (s) in the presence of mobile phase (m). It may be assumed that under conditions of chromatography all of the protein exists as the ion-pair. Then the capacity factor, k', a measurable quantity, is given by

$$\ln k' = \ln K + \ln \varphi = (-\Delta F_{smp}/RT) + \ln \varphi \tag{3}$$

The value of φ is a column constant and reflects V_S/V_M. Horváth *et al.*[7] have proposed that the magnitude of the interaction energy is dominated by solute–solvent interactions when hydrocarbonaceous supports are used, and have developed expressions to describe solute retention in such systems. The model is based on comparisons of the energies required to form cavities in the mobile phase for accommodation of the solute and solute–hydrocarbon complex. Analysis of the theory reveals that the magnitude of the non-polar contact area plays a paramount role in retention. Assumptions concerning the relative sizes of the interacting species, particularly that the area of the solute be much less than that of the hydrocarbon, may limit its application to protein containing chromatographic systems.

Van Oss and Gillman[8] have proposed that Van der Waals attractions or repulsions control many biological interactions, particularly those between hydrophobic and hydrophilic molecules. The concept of Van der Waals repulsion emerges from the microscopic treatments of Fowkes[9], and the macroscopic treatments of Israellachvilli[10], and Good and Elbing[11] of the intermolecular forces at interfaces and is shown shematically in Fig. 1. The hydrocarbonaceous surface of the support is viewed as interacting with solute (protein) across a film of mobile phase. Expressions for calculating the forces between two surfaces from considerations of dielectric permittivity were developed, with the general result that the free energy of interaction between substances i and k immersed in a liquid j is expressed by:

$$\Delta F_{ijk} = -A_{ijk}/12\pi D^2 \tag{4}$$

where A_{ijk} is the Hamaker constant of interaction and D is the distance between surfaces, *i.e.* between phases considered as continuous dielectric media. Furthermore:

$$A_{ijk} = \pm B \sqrt{A_{iji} A_{kjk}} \tag{5}$$

where B is constant ($0 \leqslant B \leqslant 1$)[10] for a particular system and was found to be close

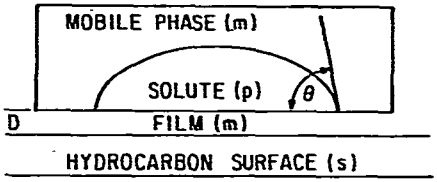

Fig. 1. Schematic of interaction of protein (p) at mobile phase (m)–alkylsilica (s) interface.

to unity for many systems. Thus, the energy of interaction between species i and k acting across medium j may be either attractive or repulsive. The interaction energy is also given by:

$$\Delta F_{ijk} = \gamma_{ik} - \gamma_{ij} - \gamma_{jk} \tag{6}$$

where the γ's are the designated surface tensions. Neuman *et al.*[12] showed that for solids an equation of state may exist between the solid–vapor, liquid–vapor, and solid–liquid interfacial tensions. These authors developed tables for obtaining the solid–vapor and solid–liquid values from the measure of the contact angle (σ) with a liquid of known surface tension. Thus, it is possible to calculate ΔF for the system shown in Fig. 1, where i = s, j = m, and k = p, and the solute is taken as solid, and to predict conditions for either retaining or eluting proteins from alkylsilica supports.

EXPERIMENTAL

Two chromatographs were used in these studies. One was assembled from components as described elsewhere[2] and operated at ambient temperatures. The other was a Spectra-Physics Model 8000B* operated at 25°C unless specified otherwise. Sample sizes were 25–100 μg. Flow-rates were 1 ml/min. In the chromatographs the fixed-wavelength detectors (254 nm) were used in series with a Schoeffel Model 770 fluorescence module equipped with a 370-nm emission filter. Excitation was at 280 nm. The presence of protein in collected fractions corresponding to the chromatographic peaks was confirmed by a dye-binding test[3].

Bovine serum albumin (BSA) and hemoglobin were purchased from Sigma (U.S.A.). The other proteins studied were from Polysciences, (U.S.A.). Their approximate molecular weights were confirmed by size-exclusion chromatography. Protein solutions prepared in mobile phase (1 mg/ml) were filtered through 0.45-μm bacteriological filters.

Mobile phases were prepared with ACS reagent-grade potassium dihydrogen phosphate and 85% phosphoric acid, HPLC grade organic solvents, and water purified with a Continental (U.S.A.) ion-exchange-carbon cartridge circulating system. The pH of 0.05 *M* KH_2PO_4 solutions was adjusted to 2.1 with 85% H_3PO_4 in the presence of the organic component. The non-ionic surfactant used was an ethoxylated alcohol (Neodol 91-6) obtained from Shell (U.S.A.). Sodium azide (0.02%) was added to aqueous solutions as a bacteriostat.

Surface tension measurements were made with a Du Nouy Tensiometer (Cen-

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

tral Scientific, U.S.A.) and the appropriate ring corrections applied. Tensions so determined for a number of liquids agreed with published values to within ±1.5 dynes/cm^2.

A goniometer (Gaertner Scientific, Chicago, IL, U.S.A.) was used to measure contact angles of sessile drops of mobile phases on layers of BSA. Surface tension of the octadecylsilica was approximated from contact angles on layers of octadecane, which was used to simulate the column support. Protein or octadecane layers of increasing thickness were prepared on glass slides by applying 500 µl of solutions containing 20, 30, 35 and 40 mg BSA/ml to clean microscope slides and drying at room temperature.

Partial specific volumes of BSA were calculated from densities[13] measured in a mechanical oscillator density meter (Model DMA02D, A. Paar, K. F. Graz, Austria). Concentrations of 5 mg/ml were used for determinations.

Columns used in these studies were: (I) Partisil-10 ODS, 250 × 4.6 mm I.D. (Whatman, U.S.A.); (II) LiChrosorb RP-8, 250 × 4.6 mm I.D. (Spectra-Physics, U.S.A.); (III) methylsilica, prepared in house by reaction of methyllithium with silica-chloride[14], 500 × 4.2 mm I.D.; (IV) Supelcosil LC-18, 150 × 4.6 mm I.D. plus guard column, (LC-18 Pellicular Packing) (Supelco, U.S.A.).

RESULTS AND DISCUSSION

First studies were conducted with phosphate buffer and isopropanol mixtures (pH 2.1) as mobile phases to obtain data which could be compared with earlier reports[6]. The data (Fig. 2) show that no proteins are retained at isopropanol concentrations above 40%. As the concentration of the alcohol is reduced a composition is reached below which protein cannot be observed to elute. This composition was different for each protein studied. The capacity factor (k') increases from zero to a very large number over a very narrow range of mobile-phase compositions. The

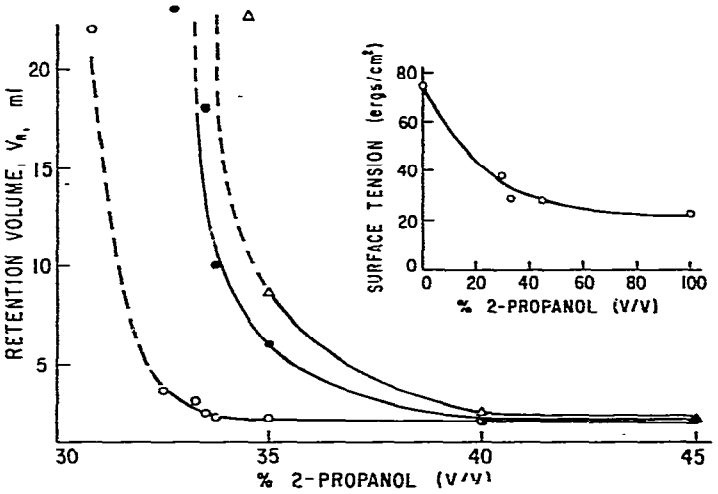

Fig. 2. Effect of isopropanol concentration and surface tension on retention of several proteins: ○, bovine serum albumin; ●, β-lactoglobulin; △, hemoglobin. Mobile phase: 0.05 *M* phosphate buffer–isopropanol (pH 2.1). Column: C_{18} (I).

addition of other additives to the mobile phase to bring about elution was not required, in contrast to other reports. This retention behavior was not peculiar to this particular reversed-phase column but was observed with another octadecylsilica column from a different manufacturer (IV) and with octyl silica (II) and methylsilica (III) columns from other sources (Fig. 3).

Elution of minor non-proteinaceous components of samples and appearance of "solvent peaks" often gave ambiguous results when only UV detection was used. The use of a fluorescence detector with excitation at 280 nm and with a 370-nm long-pass emission filter reduced much of the ambiguity. Fractions of mobile phase corresponding to observed peaks were collected, and the presence or absence of protein was confirmed by a dye-binding test[2].

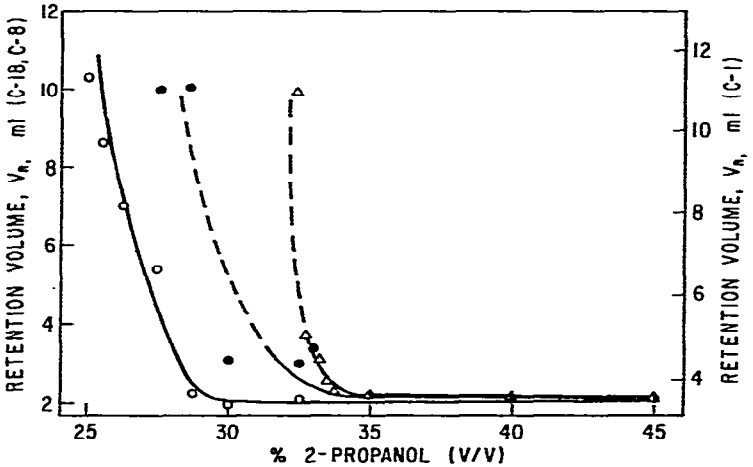

Fig. 3. Variation of retention of BSA with selected reversed-phase columns: △, C_{18} (I); ●, C_8 (II); ○, C_1 (III).

The sudden change in retention characteristics with increasing alcohol concentration could conceivably be the result of an alcohol-dependent change in conformation (denaturation) of the protein molecule. To test this hypothesis, partial specific volumes of BSA in solutions of varying isopropanol concentration were measured. The partial specific volume of BSA increased, but only slightly, over the range of concentrations used in the chromatography experiments (0.72 ± 0.01 ml/g at 40% isopropanol to 0.67 ± 0.01 ml/g at 30% (n = 2)). However, considering the pore diameter of the packings studied (less than 60 Å) and the buffer–isopropanol ratios coincident with the sudden change in retention, elution by a size-exclusion mechanism did not seem likely. Furthermore, elution order of proteins in this and subsequent studies did not correlate with molecular size (see Table I).

Little correlation of elution order with average hydrophobicity was found. The hydrophobicity scale shown in Table I (average hydrophobicity) is one of several in common use. It is based on the number of residues of each amino acid and the experimentally determined free energies of transfer of amino acid moieties from water to ethanol[15]. The average hydrophobicity has correlated well with protein properties such as solubility, aggregation phenomena, structural characteristics and thermal stability.

TABLE I

PROPERTIES OF PROTEINS

	Molecular weight (kD)[15]	*Average hydrophobicity (cal/residue)*[15]	*pI*[16]
Lysozyme (LYZ)	15	970	11[17]
Carbonic anhydrase (CAR)	30	1060	7.3
Bovine serum albumin (BSA)	67	1120	4.8
Hemoglobin (Hb)	68	1120	7.0
β-Lactoglobulin (β-LAG)	17[18]	1230	5.2

Plots of surface tension *vs.* mole fraction of alcohol in the buffer–alcohol mixture were linear over the range used in chromatography. However, when viewed with concentration expressed as volume percent (as usually done in chromatography), greater changes in surface tension were observed in the concentration range where large changes in protein retention occurred. Therefore, surface properties of the system seemed to be important factors in retention. When ethanol replaced isopropanol a larger proportion of ethanol was needed for BSA elution (Fig. 4). Nevertheless, elution in both cases occurred at about the same surface tension of mobile phase (*ca.* 33 ergs/cm^2). Buffer salts precipitated when methanol was used.

Free energies of interaction (ΔF_{smD}) of the mobile phase, stationary phase and BSA systems were estimated from contact angle measurements as proposed by Van Oss *et al.*[19] using the equation-of-state approach and the table based on it as published by Neuman *et al.*[12]. The results of the contact angle and surface tension measurements and the values derived from them are given in Table II. The ΔF_{smp} values predicted that BSA would be eluted when the mobile phase contained less than *ca.* 49% buffer when ethanol was the organic component. This same elution composition was determined by chromatography. Layers of octadecane were used to simulate

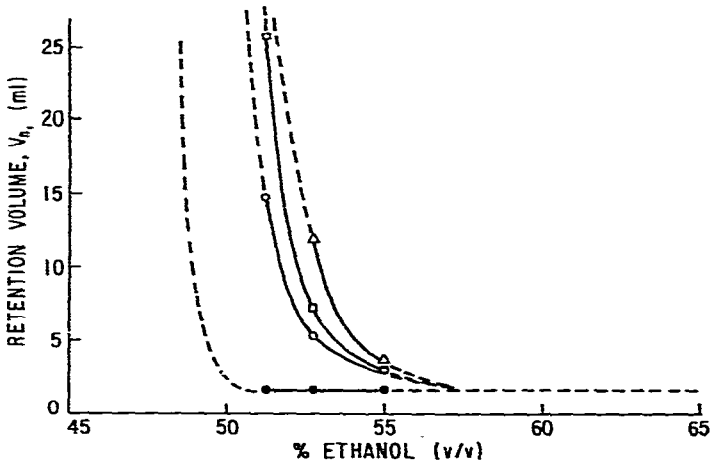

Fig. 4. Effect of ethanol concentration and surface tension on retention of several proteins: ●, bovine serum albumin; □ and ○, lysozyme (two peaks observed); △, carbonic anhydrase. Mobile phase: phosphate buffer–ethanol. Column C_{18} (IV).

stationary phase in the contact angle experiments. There was no measurable difference in contact angle over the solvent compositions used. Some difficulties were encountered in depositing a smooth layer of BSA on the slide, which may account for the large average deviation of the measured angles compared with those obtained for known liquids on teflon, which agreed within $\pm 1°$ of published values. Dissolution of protein into the drop may be another source of error. The average contact angle over layers deposited from all BSA concentrations was used in calculations. Considering these factors, the thermodynamic assumptions made in the equation-of-state approach used for the calculations of ΔF_{smp} are only approximated in these experiments. Nevertheless, consideration of these data, eqn. 3, and the chromatographic result support the concept that protein retention by alkylsilicas may be explained by Van der Waals interactions, and that the interactions may be repulsive or attractive depending on the surface tension of the mobile phase.

TABLE II

ESTIMATION OF INTERACTION ENERGY IN BSA–OCTADECANE*–ETHANOL–BUFFER SYSTEM

Percent buffer in ethanol	*Contact angle BSA (degrees)*	*Surface tension mobile phase (ergs/cm²)*	*Calculated values***			
			γ_{sm} *(ergs/cm²)*	γ_{pm} *ergs/cm²)*	γ_{sp} *ergs/cm²)*	ΔF_{smp} *(ergs/cm²)*
45.6	5 ± 2	32	1.0***	0	1.3	+0.3
48.8	9 ± 3	33	1.1	0	1.1	0
52.5	12 ± 3	34	1.1	0.1	1.0	−0.2
56.3	24 ± 4	35	1.2	0.1	0.5	−0.8
60.0	29 ± 2	36	1.2	0.2	0.4	−1.0

* Contact angles of mobile phases with octadecane were 45 ± 2° over the range of mobile phase compositions studied.

** From tables of ref. 12.

*** Interpolated values.

The retention of all of the proteins studied was observed to decrease with increasing temperature (Fig. 5). It is difficult to assess experimentally the effect of temperature in eqn. 6. For many liquids, surface tension decreases by *ca.* 0.1 ergs/cm² per degree near room temperature[20]. Limited data on contact angle measurements at various temperatures suggests little variation with temperature[21]. Thus, complete reconciliation of the observed temperature effects on protein retention with the surface chemical model cannot be made at present.

Aqueous solutions of alcohols are known to denature proteins, and the kinetics of denaturation increase with increasing alkyl chain length[22]. This denaturation may be reversed in some cases. However, the use of alkylsilicas in the chromatography of proteins to solve important biochemical problems would be enhanced if less drastic mobile phases could be found. The observations reported here suggest that small amounts of surfactants could be added to buffers to lower surface tension sufficiently to bring about elution. The use of such mobile phases may permit separation and recovery of proteins in their native state. Preliminary HPLC experiments indicate retention could be controlled by addition of a non-ionic surfactant to phosphate

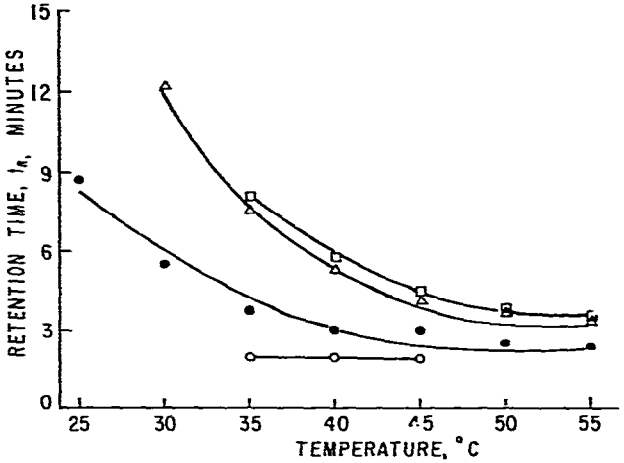

Fig. 5. Variation of protein retention with temperature. Column: C_{18} (I). Mobile phase, 33.8% isopropanol in 0.05 *M* KH_2PO_4 buffer, pH 2.1. ○, Bovine serum albumin; ●, carbonic anhydrase; △, lysozyme; □, β-lactoglobulin.

buffer. More detailed studies of the effects of surfactant, pH and other chromatography variables on the recovery and structure of proteins as well as mechanisms of retention are being conducted.

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