Liquid Chromatography of Ionogenic Substances with Nonpolar Stationary Phases

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The effect of solute ionization on the retention of weak acids, bases, and ampholytes on octadecylsilica was investigated both theoretically and experimentally. The retention was attributed to a reversible association of the solutes with the hydrocarbonaceous ligand of the stationary phase. A phenomenological treatment of the corresponding equilibria was developed for various types of ionogenic substances. The energetics of the association process was analyzed in a rigorous fashion in the light of the solvophobic theory and a semi-empirical extension of the Debye–Hückel theory to high ionic strength. The predicted effect of solute ionization on the capacity factors was substantiated by experimental data. The observed dependence of the capacity factors on the ionic strength of the eluent and the hydrophobic surface of the solute molecules showed good agreement with the theory. The advantages of the technique in the separation of biological substances are illustrated.

The great majority of biological substances contain ionogenic functions such as carboxylic or amino groups. In most instances, ion exchange chromatography has been the method of choice for the separation of such compounds having similar chemical structure (*1, 2*). Recent developments in high performance liquid chromatography, however, have demonstrated that columns packed with a nonpolar stationary phase, such as octadecylsilica, are also eminently suitable for the separation of weak acids and bases (*3*).

Most effort has been focused on the use of "reversed phase" chromatography with eluents containing anionic or cationic surfactants in hydroorganic solvent mixtures; the technique is frequently called "ion-pair" (*4, 5*) or "soap" (*6*) chromatography. Recently it has been demonstrated (*7*) that octadecylsilica columns with neat aqueous eluents, which do not contain organic solvents, can also be successfully employed for the separation of polar organic compounds.

We have shown (*3*) that the physicochemical phenomena underlying the chromatographic process with nonpolar stationary phases can be readily interpreted in the light of the "solvophobic theory" (*8–15*) and the factors determining solute retention are amenable to an exact theoretical treatment. In this study, the theory is extended to account for the effect of solute ionization on chromatographic retention, both phenomenologically and by a rigorous treatment of the interaction between ionic solutes and the eluent.

THEORY

The chromatographic process is viewed as a reversible association of the solute, S, with the hydrocarbonaceous ligand, L, such as an octadecyl function covalently bound to the surface of the stationary phase:

$$S + L \rightleftarrows SL \tag{1a}$$

The equilibrium constant for the association, K_{assoc}, is given by

$$K_{assoc} = \frac{[SL]}{[S][L]} \tag{1b}$$

It is assumed that the equilibrium constant of the process with both neutral and ionized solutes is solely determined by solvophobic interactions (*3*), that is, no ionic or hydrogen bonding occurs between the solute and the stationary phase.

In this section, we first will present a phenomenological treatment of the association equilibria involved in the chromatography of monoprotic acids and bases, diprotic acids, and zwitterionic solutes. As a result of this analysis, the capacity factors of the neutral and ionized species are defined and equations are derived to express the capacity factors under conditions of partial solute dissociation. Subsequently, the free energy change associated with the process is analyzed on the basis of the solvophobic theory in order to estimate the relative magnitude of the capacity factors for the ionized and neutral forms of a solute as well as the effect of the ionic strength of the eluent on solute retention.

Equilibria. *Monoprotic Acids and Bases.* The dissociation of a monoprotic acid, HA, in the mobile phase is governed by the following equilibrium:

$$HA \rightleftarrows A^- + H^+ \tag{2a}$$

where A^- is the dissociated acid and H^+ is the solvated proton. The equilibrium constant is the acid dissociation constant in the eluent proper, K_{a_m}, and is given by

$$K_{a_m} = \frac{[H^+]_m[A^-]_m}{[HA]_m} \tag{2b}$$

where $[H^+]_m$, $[A^-]_m$, and $[HA]_m$ are the concentrations of the solvated proton, the dissociated, and undissociated acid in the mobile phase, respectively.

In the chromatographic process under consideration, solute retention is assumed to occur because of a reversible association between the dissociated and/or undissociated acid and the hydrocarbonaceous ligand, L, of the stationary phase. The binding of the acid is determined by the equilibrium

$$HA + L \rightleftarrows LHA \tag{2c}$$

with the equilibrium constant K_{LHA} which is given by

$$K_{LHA} = \frac{[LHA]_s}{[HA]_m[L]_s} \tag{2d}$$

where $[LHA]_s$ and $[L]_s$ are the concentrations of the complex, LHA, and the ligand of the stationary phase, respectively.

The interaction between the anion and the ligand results in the formation of the complex LA^- according to the following equilibrium

$$A^- + L \rightleftarrows LA^- \tag{2e}$$

and the corresponding equilibrium constant K_{LA^-} is given by

$$K_{LA^-} = \frac{[LA^-]_s}{[A^-]_m[L]_s} \tag{2f}$$

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Table I. Equilibria and Constants Used in the Derivation of Equations 5 and 6

	Diprotic Acids	Zwitterions
Dissociation equilibria in the mobile phase	$H_2A \rightleftarrows HA^- + H^+$ $HA^- \rightleftarrows A^{2-} + H^+$	$H^+BA^- \rightleftarrows BA^- + H^+$ $H^+BAH \rightleftarrows H^+BA^- + H^+$
Acid dissociation constants in the mobile phase	$K_{a_{1,m}} = \frac{[HA^-]_m[H^+]_m}{[H_2A]_m}$	$K_{a_{1,m}} = \frac{[H^+]_m[BA^-]_m}{[H^+BA^-]_m}$
	$K_{a_{2,m}} = \frac{[A^{2-}]_m[H^+]_m}{[HA^-]_m}$	$K_{a_{2,m}} = \frac{[H^+]_m[H^+BA^-]_m}{[H^+BAH]_m}$
Equilibria in the solute–ligand binding proess	$H_2A + L \rightleftarrows LH_2A$ $HA^- + L \rightleftarrows LHA^-$ $A^{2-} + L \rightleftarrows LA^{2-}$	$H^+BA^- + L \rightleftarrows LH^+BA^-$ $BA^- + L \rightleftarrows LBA^-$ $H^+BAH + L \rightleftarrows LH^+BAH$
Equilibrium constants for the binding process	$K_{LH_2A} = \frac{[LH_2A]_s}{[H_2A]_m[L]_s}$	$K_{LH^+BA^-} = \frac{[LH^+BA^-]_s}{[H^+BA^-]_m[L]_s}$
	$K_{LHA^-} = \frac{[LHA^-]_s}{[HA^-]_m[L]_s}$	$K_{LBA^-} = \frac{[LBA^-]_s}{[BA^-]_m[L]_s}$
	$K_{LA^{2-}} = \frac{[LA^{2-}]_s}{[A^{2-}]_m[L]_s}$	$K_{LH^+BAH} = \frac{[LH^+BAH]_s}{[H^+BAH]_m[L]_s}$
Overall capacity factor	$k = \varphi\frac{[LH_2A]_s + [LHA^-]_s + [LA^{2-}]_s}{[H_2A]_m + [HA^-]_m + [A^{2-}]_m}$	$k = \varphi\frac{[LH^+BA^-]_s + [LBA^-]_s + [LH^+BAH]_s}{[H^+BA^-]_m + [BA^-]_m + [H^+BAH]_m}$
Capacity factors of the individual species	$k_0 = \varphi[L]_sK_{LH_2A}$ $k_{-1} = \varphi[L]_sK_{LHA^-}$ $k_{-2} = \varphi[L]_sK_{LA^{2-}}$	$k_0 = \varphi[L]_sK_{LH^+BA^-}$ $k_{-1} = \varphi[L]_sK_{LBA^-}$ $k_1 = \varphi[L]_sK_{LH^+BAH}$

where $[LA^-]_s$ is the concentration of the complex in the stationary phase.

The magnitude of solute retention is expressed by the capacity factor, k, which is a measure of the stoichiometric mass distribution of HA between the stationary and mobile phases. In a given column the volume ratio of the stationary and mobile phases, ϕ, is fixed so that the mass distribution ratio is simply given by

$$k = \phi\frac{[LHA]_s + [LA^-]_s}{[HA]_m + [A^-]_m} \tag{3a}$$

Expressing the species concentrations from Equations 2b, 2d, and 2f and substituting into Equation 3a, we obtain the capacity factor as

$$k = \phi\frac{K_{LHA}[L]_s + K_{LA^-}[L]_s\frac{K_{a_m}}{[H^+]_m}}{1 + \frac{K_{a_m}}{[H^+]_m}} \tag{3b}$$

It is convenient to define the capacity factor of the undissociated acid, k_0, as

$$k_0 = \phi[L]_sK_{LHA} \tag{3c}$$

and the capacity factor of the conjugate base k_{-1}, as

$$k_{-1} = \phi[L]_sK_{LA^-} \tag{3d}$$

Substituting k_0 and k_{-1} from Equations 3c and 3d into Equation 3b, we obtain for the capacity factor of a monoprotic acid the following expression

$$k = \frac{k_0 + k_{-1}\frac{K_{a_m}}{[H^+]_m}}{1 + \frac{K_{a_m}}{[H^+]_m}} \tag{3e}$$

Equation 3e represents a phenomenological relationship between the capacity factor of a partially dissociated weak acid and the hydrogen ion concentration in the eluent, with the appropriate acid dissociation constant and the two limiting capacity factors of the undissociated and fully dissociated acid as the parameters. Although it is not shown explicitly, the model also accounts for the acid dissociation equilibrium of the bound species with a dissociation constant $K_{a_m}K_{LA^-}/K_{LHA}$.

The ionization of a weak base, B, in the mobile phase takes place according to the following equilibrium

$$BH^+ \rightleftarrows B + H^+ \tag{4a}$$

where BH^+ is the protonated base. The equilibrium is conveniently characterized by the acid dissociation constant of the protonated base in the mobile phase, K_{a_m}, which is given by

$$K_{a_m} = \frac{[H^+]_m[B]_m}{[BH^+]_m} \tag{4b}$$

A derivation analogous to that given above for a monoprotic acid yields the following expression for the capacity factor of a weak monoprotic base

$$k = \frac{k_0 + k_1\frac{[H^+]_m}{K_{a_m}}}{1 + \frac{[H^+]_m}{K_{a_m}}} \tag{4c}$$

where k_0 and k_1 are the capacity factors of the neutral and fully ionized base, respectively.

Diprotic Acids and Zwitterions. Following the above procedure and considering the protonic and binding equilibria shown in Table I, we obtain for the capacity factor of diprotic acids the expression

$$k = \frac{k_0 + k_{-1}\dfrac{K_{a1,m}}{[H^+]_m} + k_{-2}\dfrac{K_{a1,m}K_{a2,m}}{[H^+]_m^2}}{1 + \dfrac{K_{a1,m}}{[H^+]_m} + \dfrac{K_{a1,m}K_{a2,m}}{[H^+]_m^2}} \quad (5)$$

where k_0, k_{-1}, and k_{-2} are the capacity factors of the undissociated, half dissociated, and fully dissociated diprotic acid and $K_{a1,m}$ and $K_{a2,m}$ are the corresponding acid dissociation constants in the mobile phase, respectively.

Similarly the capacity factor of zwitterionic substances, such as amino acids, is given by

$$k = \frac{k_0 + k_{-1}\dfrac{K_{a1,m}}{[H^+]_m} + k_1\dfrac{[H^+]_m}{K_{a2,m}}}{1 + \dfrac{K_{a1,m}}{[H^+]_m} + \dfrac{[H^+]_m}{K_{a2,m}}} \quad (6)$$

where k_0, k_{-1}, and k_1 are the capacity factors of the zwitterionic, the anionic, and the cationic forms of the ampholyte and $K_{a1,m}$ and $K_{a2,m}$ are the corresponding acid dissociation constants, respectively.

Energetics of the Chromatographic Association Process. It has been pointed out earlier (*3*) that solute retention in liquid chromatography is determined by the energy balance of the solute–stationary phase, the eluent–stationary phase, and the eluent–solute interactions. In order to put these interactions in a rigorous thermodynamic framework we have employed Sinanoğlu's "solvophobic" theory and developed an approach which allows one, in principle at least, to calculate the capacity factors of un-ionized solutes in liquid chromatography with nonpolar stationary phases from measurable properties of the solute and the eluent (*3*). After a brief review of the most important elements of this approach, we will extend it to treat quantitatively the chromatographic retardation of ionized solutes.

The reversible association of the solute with the hydrocarbonaceous ligand of the stationary phase can be divided into the following steps: the association of the solute and the ligand in the gas phase, and the transfer of the solute, the ligand, and the complex individually into the eluent. The unitary free energy change of the association, ΔF°_{assoc}, is given by

$$\Delta F^\circ_{assoc} = \Delta F^\circ_{assoc,\,gas} + \Sigma \Delta F_{solv,j} \quad (7)$$

where $\Delta F^\circ_{assoc,\,gas}$ is the unitary free energy of the association in the gas and $\Sigma\Delta F_{solv,j}$ is the net free energy change for the transfer of the species into the eluent, i.e., the free energy of the overall species–solvent interactions. The subscript j represents the solute, S, the complex, SL, and the ligand, L.

Sinanoğlu has shown that the free energy change of transfer from the gas phase into solution is the sum of the energy required to make a suitable cavity in the solvent, $\Delta F_{cav,j}$, and the free energy change arising from the interaction of the species with the surrounding solvent molecules, $\Delta F_{int,j}$, so that

$$\Delta F_{solv,j} = \Delta F_{cav,j} + \Delta F_{int,j} + RT \ln (RT/P_0V) \quad (8)$$

where the last term, which contains the mole volume of the solvent, V, and the atmospheric pressure, P_0, takes care of the entropy change arising from the change in "free volume". For the solute–ligand complex, SL, the expression of $\Delta F_{solv,SL}$, has an additional term, ΔF_{red}, which accounts for the reduction of the free energy of gas phase association owing to the direct effect of the solvent medium. The reduction term has been neglected in our previous treatment of the retardation of unionized solutes (*3*).

The terms on the RHS of Equation 8 have been evaluated for the individual species involved in the association process as follows. The cavity term is calculated by

$$\Delta F_{cav,j} = \kappa_j^c N A_j \gamma (1 - W_j) \quad (9a)$$

where N is Avogadro's number, A_j is the molecular surface area of the species, and γ is the surface tension of the eluent. W_j is a correction for the enthalpi and entropic contributions from macroscopic to molecular dimensions, but, since its value is nearly zero for polar liquids, it can be neglected. The other correction factor, κ_j^c, however, can have a significant effect. It is constant for a given species and solvent and can be approximated as

$$\kappa_j^c = 1 + (\kappa^e - 1)(V/V_j)^{2/3} \quad (9b)$$

where κ^e is evaluated for a solute equal in molecular dimensions to the solvent (*11*) and V and V_j are the molar volumes of the eluent and the solute, respectively.

The interaction term consists of a van der Waals, $\Delta F_{vdw,j}$, and an electrostatic component, $\Delta F_{es,j}$, and is given by

$$\Delta F_{int,j} = \Delta F_{vdw,j} + \Delta F_{es,j} \quad (10)$$

In our previous treatment (*3*) an exact, or at least closely proximate, method has been given for the calculation of $\Delta F_{vdw,j}$ in terms of ionization potentials, refractive indices, and other measurable properties of the solvent and the species. Since this term is not likely to change appreciably in the chromatographic systems of present interest, $\Delta F_{vdw,j}$ will not be evaluated directly.

On the other hand, the magnitude of the electrostatic term, $\Delta F_{es,j}$, largely depends on the charge distribution in the species. Therefore, this term appears to be highly significant in our analysis and it will be evaluated for the placement of both a permanent dipole and the corresponding ion into a cavity.

The energy released due to the interaction of a dipole with the solvent molecules at the wall of the cavity has been given by Sinanoğlu (*8*)

$$\Delta F_{es,j} = -\frac{N}{4\pi\epsilon_0}\left(\frac{\mu_j^2}{v_j}\right)\frac{D}{1 - (\bar{\alpha}_j/\bar{r}^3)D} \quad (11a)$$

where ϵ_0 is the permittivity constant, μ_j, v_j, $\bar{\alpha}_j$, and $\bar{r}_j$ are the dipole moment, molecular volume, average polarizability, and the average molecular radius of the species, respectively. The value of D is given by

$$D = 2(\epsilon - 1)/(2\epsilon + 1) \quad (11b)$$

where ϵ is the static dielectric constant of the solvent.

At very low ionic strength, we can estimate the energy associated with placing an ionic species into the medium, $\Delta F^z_{es,j}$, from the Debye–Hückel equation (*34*) as

$$\Delta F^z_{es,j} = \frac{Z^2e^2N}{4\pi\epsilon_0\epsilon}\left(\frac{1}{b_j} - \frac{\kappa}{1 + \kappa a_j}\right) \quad (12a)$$

where Ze is the electronic charge of the ion, b_j is the ionic radius, and a_j is the distance of closest approach. The value of κ, the Debye screening parameter, is calculated from the relationship

$$\kappa^2 = \frac{4\pi e^2 N I^2}{\epsilon RT} \quad (12b)$$

where e is the elementary charge and I is the ionic strength of the medium. In Equations 12a and 12b the dielectric constant, ϵ, of the eluent is used for the ionized solute. For the complex of the ionized solute with the fixed ligand, however, the apparent dielectric constant of the stationary phase, ϵ^*, has to be used instead of ϵ.

In view of the above discussion, the overall unitary free energy change, ΔF°_{assoc}, for the association of a solute, S, with the ligand, L, to obtain the complex, SL, is obtained as

$$\Delta F_{assoc} = \Delta F_{assoc,\,gas} + \Delta F_{red} + \Delta F_{cav} + \Delta F_{vdw} + \Delta F_{es} - RT \ln (RT/P_0V) \quad (13a)$$

where

$$\Delta F_{cav} = \Delta F_{cav,SL} - \Delta F_{cav,S} - \Delta F_{cav,L} \quad (13b)$$

$$\Delta F_{vdw} = \Delta F_{vdw,SL} - \Delta F_{vdw,S} - \Delta F_{vdw,L} \quad (13c)$$

and

$$\Delta F_{es} = \Delta F_{es,SL} - \Delta F_{es,S} - \Delta F_{es,L} \quad (13d)$$

The subscripts S, L, and SL in Equations 13b–13d refer to the species mentioned above.

The overall cavity term for the association process, ΔF_{cav}, has been expressed (*3*) as

$$-\Delta F_{cav} = [N\Delta\Phi + 4.836N^{1/3}(\kappa^e - 1)V^{2/3}]\gamma \quad (13e)$$

where $\Delta\Phi$ is the reduction of the surface area upon binding the solute to the ligand, i.e., the hydrophobic contact area.

For the association process with a solute, which is a permanent dipole, the overall electrostatic term, ΔF_{es}, has been expressed (*3*) by

$$\Delta F_{es} = \left(\frac{N}{4\pi\epsilon_0}\frac{\lambda - 1}{2\lambda}\right)\left(\frac{\mu_s^2}{v_s}\right)\left(\frac{D}{1 - (\bar{\alpha}_s/v_s)D}\right) \quad (14a)$$

where

$$\lambda = V_{SL}/V_S \quad (14b)$$

The corresponding term for an association process, which involves a solute having charge Z and a hydrocarbonaceous ligand, is denoted by ΔF^z_{es}. It can be evaluated by using Equations 12a and 13d. Since the nonpolar ligand has no charge, $\Delta F^z_{es,L} = 0$, and Equation 13d reduces to

$$\Delta F^z_{es} = \Delta F^z_{es,SL} - \Delta F^z_{es,S} \quad (15a)$$

Evaluating the two terms on the RHS of Equation 15a and correcting for the fact that the dielectric constant at the stationary phase surface, ϵ^*, is different from that of the bulk eluent, ϵ, and substituting into Equation 15a we obtain that

$$\Delta F^z_{es} = \frac{Z^2e^2N}{4\pi\epsilon_0\epsilon}\left[\frac{\epsilon}{\epsilon^* b_{SL}} - \frac{1}{b_S} - \frac{\epsilon}{\epsilon^*}\frac{\kappa}{1 + \kappa a_{SL}} + \frac{\kappa}{1 + \kappa a_S}\right] \quad (15b)$$

In Equation 15b, b_{SL} and b_S are the radii of the particles. In view of Equation 14c and assuming that the particles are spherical, we obtain that

$$b_{SL} = \lambda^{1/3} b_S \quad (15c)$$

Both a_{SL} and a_S represent the distance of closest approach, which is not expected to be different for the ionized solute and the complex. Therefore, we can assume that

$$\frac{\kappa}{1 + \kappa a_{SL}} = \frac{\kappa}{1 + \kappa a_S} \quad (15d)$$

and Equation 15b can be rewritten in view of Equations 15c and 15d as

$$\Delta F^z_{es} = \frac{Z^2e^2N}{4\pi\epsilon_0\epsilon}\left[\frac{\epsilon - \epsilon^*\lambda^{1/3}}{\epsilon^*\lambda^{1/3} b_S} - \left(\frac{\epsilon - \epsilon^*}{\epsilon^*}\right)\frac{\kappa}{1 + \kappa a_S}\right] \quad (15e)$$

Whereas Equation 15e is expected to be valid at salt concentrations up to 0.1 M, i.e., in the domain of the Debye–Hückel theory, in practice the salt concentration in the eluent can be significantly higher. For such conditions a semiempirical approximation has to be used to estimate ΔF^z_{es}. According to Lietzke et al. (*16*) the Debye term can be expressed at high ionic strength as

$$-\frac{\kappa}{1 + \kappa a_j} = \frac{4\pi\epsilon_0\epsilon RT}{Z^2e^2N}\left[\left(\frac{-S\sqrt{I}}{1 + 1.5\sqrt{I}} - BI^{1/3} - CI\right) \times \exp(-a\psi) + BI^{1/3} + CI\right] \quad (16a)$$

where I is the ionic strength, S is the Debye parameter, a and B are constants whose value depends on the charge, C is a charge-independent constant, and ψ is given by

$$\psi = \frac{V_{salt}c}{1000} \quad (16b)$$

where V_{salt} is the molar volume of the dry salt and c is the molar concentration of the salt in the eluent. Lietzke et al. (*16*) have tabulated the values of a, B, and C for a variety of aqueous salt solutions.

In order to calculate ΔF^z_{es} at high ionic strength we substitute $\kappa/(1 + \kappa a_S)$ from Equation 16a into Equation 15e and find that

$$\Delta F^z_{es} = \frac{Z^2e^2N}{4\pi\epsilon_0\epsilon}\frac{\epsilon - \epsilon^*\lambda^{1/3}}{\epsilon^*\lambda^{1/3} b_S} + \frac{\epsilon - \epsilon^*}{\epsilon^*} \times \left[\left(\frac{-S\sqrt{I}}{1 + 1.5\sqrt{I}} - BI^{1/3} - CI\right)\exp(-a\psi) + BI^{1/3} + CI\right]RT \quad (16c)$$

According to Lietzke et al. (*16*), the average value of a is about 3×10^3. On the other hand, ψ is approximately 5×10^{-2} when the salt solution is 1 M. Consequently the magnitude of the product $a\psi$ is of the order of hundred and the value of the exponential term is negligibly small under such conditions. Hence Equation 16c can be simplified to

$$\Delta F^z_{es} = \frac{Z^2e^2N}{4\pi\epsilon_0\epsilon}\frac{\epsilon - \epsilon^*\lambda^{1/3}}{\epsilon^*\lambda^{1/3}} + \frac{\epsilon - \epsilon^*}{\epsilon^*}\left(BI^{1/3} + CI\right)RT \quad (16d)$$

and when the ionic strength of the eluent is appreciable, this expression should be used instead of that given in Equation 15e.

The energetics of placing a zwitterion into solution of low ionic strength has been extensively treated by Kirkwood (*17*). An expression to calculate the corresponding ΔF^{zu}_{es} for zwitterions is conveniently obtained by taking the first three terms of the complicated series solution given in Equation 13 of the original reference.

In many cases it is of interest to evaluate the free energy difference between the formation of complexes with a given ligand by the un-ionized and ionized forms of the same solute, $\Delta\Delta F^\circ_{assoc}$, which is given by

$$\Delta\Delta F^\circ_{assoc} = (\Delta F_{assoc,\,gas} - \Delta F^z_{assoc,\,gas}) + (\Delta F_{red} - \Delta F^z_{red}) + (\Delta F_{cav} - \Delta F^z_{cav}) + (\Delta F_{vdw} - \Delta F^z_{vdw}) + (\Delta F_{es} - \Delta F^z_{es}) \quad (17a)$$

where the superscript z refers to the fully ionized form of the solute bearing charge Z. The free energy of gas phase association is not to be the same for the neutral and ionized solute because the charged solute may induce a dipole moment in the ligand. This electrostatic effect is conveniently accounted for by the term $\Delta F_{assoc,\,gas,\,es}$, and we can write that

$$\Delta F^z_{assoc,\,gas} = \Delta F_{assoc,\,gas} + \Delta F^z_{assoc,\,gas,\,es} \quad (17b)$$

On the other hand, the reduction term for the complex containing the ionized solute, ΔF^z_{red}, is approximately given by

$$\Delta F^z_{red} = \frac{1 - \epsilon}{\epsilon}\Delta F^z_{assoc,\,gas,\,es} \quad (17c)$$

The combination of Equations 17b and 17c yields the following expression

$$\Delta F^{z}_{\text{assoc, gas}} + \Delta F^{z}_{\text{red}} = \Delta F_{\text{assoc, gas}} + \frac{1}{\epsilon}\Delta F^{z}_{\text{assoc, gas, es}} \quad (17d)$$

Since the reduction term for the un-ionized complex, ΔF_{red}, is assumed to be negligibly small, the first four terms on the RHS of Equation 17a can be combined as follows

$$\Delta F_{\text{assoc, gas}} - \Delta F^{z}_{\text{assoc, gas}} + \Delta F_{\text{red}} - \Delta F^{z}_{\text{red}} = -\frac{1}{\epsilon}\Delta F^{z}_{\text{assoc, gas, es}} \quad (17e)$$

With a given eluent the difference between the two cavity terms in Equation 17a is negligibly small in view of the fact that the molecular dimensions of the ionized and neutral forms of the solute are very close and the contact area in the complex (Equation 13e) is expected to be the same for both forms. Consequently the assumption that $\Delta F_{\text{cav}} - \Delta F^{z}_{\text{cav}} = 0$ is justifiable and can be supported by a more detailed analysis as well. Similarly we can assume that the difference between the van der Waals' terms in Equation 17a is very small and $\Delta F_{\text{vdw}} - \Delta F^{z}_{\text{vdw}} = 0$, because the magnitude of these terms is mainly dependent on the size of the individual species and the molecular volumes of the ionized species and their neutral analogues are very nearly identical. With a hydrocarbonaceous ligand, if there are no charged groups in its vicinity, the term $\Delta F^{z}_{\text{assoc, gas, es}}$ is expected to be negligibly small or at least much smaller than the difference between ΔF_{es} and ΔF^{z}_{es}.

With these assumptions Equation 17a reduces to

$$\Delta\Delta F^{\circ}_{\text{assoc}} = \Delta F_{\text{es}} - \Delta F^{z}_{\text{es}} \quad (17f)$$

Theoretical Interpretation of the Capacity Factors. The equilibrium constant for the association process, K_{assoc}, is related to the free energy change by

$$\ln K_{\text{assoc}} = -\Delta F^{\circ}_{\text{assoc}}/RT \quad (18a)$$

The chromatographically measured parameter, vide supra, is the capacity factor, k, which is dependent on the equilibrium constant as

$$\ln k = \ln K_{\text{assoc}} - \xi \quad (18b)$$

where ξ is constant for a given column.

Equations 17f, 18a, and 18b allow us to estimate the relative values of the limiting capacity factors, k_0 and k_z, for a given ionogenic solute. In the first part of this section the meaning of k_0 and k_z has already been defined. Substituting the individual terms from Equations 14a and 16d into Equation 17f and using Equations 18a and 18b we obtain that

$$\ln (k_0/k_z) = -\frac{N}{4RT\pi\epsilon_0}\left(\frac{\lambda-1}{2\lambda}\right)\left(\frac{\mu_S^2}{v_S}\right)\left(\frac{D}{1-(\bar{\alpha}_S/v_S)D}\right) + \frac{Z^2e^2N}{4RT\pi\epsilon_0\epsilon}\frac{\epsilon-\epsilon^*\lambda}{\epsilon^*\lambda^{1/3}b_S} - \frac{\epsilon-\epsilon^*}{\epsilon^*}(BI^{1/3}+CI) \quad (19a)$$

In view of the previous treatment, the capacity factor of the un-ionized solute is expressed by

$$\ln k_0 = -\frac{1}{RT}(\Delta F_{\text{assoc, gas}} + \Delta F_{\text{red}} + \Delta F_{\text{vdw}} + \Delta F_{\text{es}} + \Delta F_{\text{cav}}) + \ln (RT/P_0V) + \xi \quad (20a)$$

and that of the ionized solute is given by

$$\ln k_z = -\frac{1}{RT}(\Delta F^{z}_{\text{assoc, gas}} + \Delta F^{z}_{\text{red}} + \Delta F^{z}_{\text{vdw}} + \Delta F^{z}_{\text{es}} + \Delta F^{z}_{\text{cav}}) + \ln (RT/P_0V) + \xi \quad (20b)$$

When only the ionic strength of the eluent changes, most terms of Equations 20a and 20b are assumed to be invariant and can be lumped together into a single constant. Only the electrostatic terms and the cavity terms are expected to be affected by changing ionic strength; the latter changes because the surface tension increases with the concentration of inorganic salts (*3, 7*).

Hence Equations 20a and 20b reduce to

$$\ln k_0 = \text{const} - \frac{\Delta F_{\text{es}}}{RT} - \frac{\Delta F_{\text{cav}}}{RT} \quad (20c)$$

and

$$\ln k_z = \text{const} - \frac{\Delta F^{z}_{\text{es}}}{RT} - \frac{\Delta F^{z}_{\text{cav}}}{RT} \quad (20d)$$

For un-ionized solutes, ΔF_{es}, is practically independent of the salt concentration. Hence, by substituting the cavity term from Equation 13e into Equation 20c, we obtain that

$$\ln k_0 = \text{const} + \frac{N\Delta\Phi + 4.836N^{1/3}(\kappa^e - 1)V^{2/3}}{RT}\gamma \quad (20e)$$

The surface tension can be expressed as a function of the ionic strength, I, by

$$\gamma = \gamma_0 + \sigma' I \quad (20f)$$

where γ_0 is the surface tension of pure water and σ' is a constant, the magnitude of which depends on the nature of the salt.

By substituting γ from Equation 20f into Equation 20e, we find that the logarithm of the capacity factor of an un-ionized solute is a linear function of the ionic strength of the eluent, i.e., a plot of $\ln k_0$ vs. I yields a straight line as has been shown earlier (*3*).

With ionized solutes the electrostatic term, ΔF^{z}_{es}, is a function of the ionic strength in view of Equations 16a and 16c. To evaluate the dependence of the capacity factor on the ionic strength over a wide range of salt concentration we assume that $\Delta F_{\text{cav}} = \Delta F^{z}_{\text{cav}}$, and substitute Equations 13a and 16c into Equation 20d. As a result the capacity factor of the ionized solute is expressed by

$$\ln k_z = \text{const} + \frac{\epsilon-\epsilon^*}{\epsilon^*} \times \left[\left(\frac{-S\sqrt{I}}{1+1.5\sqrt{I}} - BI^{1/3} - CI\right)\exp(-a\psi) + BI^{1/3} + CI\right] + \frac{N\Delta\Phi + 4.836N^{1/3}(\kappa^e-1)V^{2/3}}{RT}\sigma' I \quad (20g)$$

At relatively high salt concentrations, Equation 20g reduces to

$$\ln k_z = \ln k_z^{\circ} + \alpha(BI^{1/3} + CI) + \beta I \quad (20h)$$

where k_z°, α, and β are constants for a given solute, salt, and column; k_z° is the capacity factor of the solute at zero ionic strength, $\alpha = (\epsilon/\epsilon^*) - 1$ and β depends on the nature of the eluent and on the contact area between the solute and the ligand. According to the solvophobic theory, the cavity term, which entails the contact area, plays a significant role in determining the magnitude of hydrophobic interactions. More importantly, however, it is related to the nonpolar surface area of the solutes, when the hydrocarbonaceous ligand is sufficiently large.

In view of the present treatment it is possible to relate the experimentally measured capacity factors to the cavity term when the ln k values are corrected by the corresponding electrostatic term as follows:

$$\ln k_0 + \frac{\Delta F_{es}}{RT} = \text{const} - \frac{\Delta F_{cav}}{RT} \quad (21a)$$

$$\ln k_z + \frac{\Delta F^z_{es}}{RT} = \text{const} - \frac{\Delta F_{cav}}{RT} \quad (21b)$$

The appropriate electrostatic terms can be calculated from Equations 14a and 16c or 16d. In view of Equation 20e, plots of the LHS of Equations 21a and 21b vs. $\Delta\Phi$ should yield a straight line when the solvent properties are fixed. With solutions having similar molecular geometry and with ligands of relatively large molecular dimensions, $\Delta\Phi$ is likely to be proportional to the nonpolar part of the molecular surface area. Consequently, similar plots with the hydrocarbonaceous surface area rather than with $\Delta\Phi$, which is not known, should also yield straight lines under such conditions.

EXPERIMENTAL PROCEDURES AND CALCULATIONS

We used a Model 601 (Perkin-Elmer, Norwalk, Conn.) liquid chromatograph with a Rheodyne (Berkeley, Calif.) Model 7010 sampling valve with a Perkin-Elmer Model LC 55 variable wavelength detector at 254 nm and a 20-μl loop, a Perkin-Elmer Model R-56 recorder. Most experiments were performed with Partisil 1025 ODS (Whatman, Clifton, N.J.) columns. The carbon content of the octadecylsilica packing was about 5% (w/w). One of the chromatograms was obtained with a Partisil 1025 ODS2 column which had a higher concentration of the octadecyl moiety on the surface of the silica support, and the carbon content was about 17% (w/w). The sample compounds were purchased from Aldrich, (Milwaukee, Wis.) or Schwarz/Mann (Orangeburg, N.Y.) and reagent grade phosphoric acid and KH_2PO_4 was obtained from Fisher (Pittsburgh, Pa.). The acetonitrile wa "distilled in glass" from Burdick and Jackson (Muskegon, Mich.).

Retention times were measured from the distance between the injection point and the peak maximum on the chromatogram. The mobile phase hold up times were measured by injecting $NaNO_3$ and the retention time of the small peak was taken as t_0. Capacity factors have been calculated in the usual way (*2*). The chromatographic conditions are stated in the figure captions.

Titration curves were obtained with a Radiometer (The London Co., Cleveland, Ohio) titrator consisting of a ABU 1c autoburette, a PHM 26 pH meter, a TTT II titrator, and a SBR 2c titrigraph. The titrations were performed with a glass/calomel electrode in a solution having the same salt concentration (1 M Na_2SO_4) as the eluent used in the chromatographic experiments for the investigation of the effect of pH. The pK_{a_m} values have been calculated as usual (*18*).

The analysis of the data was performed on a PDP 11/10 minicomputer (Digital Equipment, Maynard, Mass.) equipped with a floppy disk unit and a Decwriter. The computer program used for parameter estimation by the least squares method was written in BASIC language.

The hydrophobic surface areas were calculated by using the group surface increments of Bondi (*19*) with the appropriate crowding corrections (*20*) and summing the area increments for carbon and hydrocarbon groups only.

The logarithm of the capacity factor ratio for the ionized and the neutral forms of a given solute at the experimental conditions (1.0 M Na_2SO_4 solution, 25 °C) was determined from Equation 19a as follows. The first term was calculated by assuming a value of two for λ (*3*) and the average refractive index to be 1.55 for the evaluation of $\bar{\alpha}_S/\bar{v}_S$ using the Clausius–Mosotti relationship (*11*). The value of $1/4\pi\epsilon_0$ was taken as $9 \times 10^9 N$ m^2/C^2. The second term was calculated with $\epsilon^* = 35$. This value was estimated from the $(\epsilon - \epsilon^*)/\epsilon^*$ ratios obtained by analyzing the dependence of the capacity factors of ionized solutes on the ionic strength and using the dielectric constant of water for ϵ. The molecular radius of the solute, b, was calculated from the density with the assumption of spherical geometry; when the density was not known b was taken to be 3 Å. The mole volume of the solute, V_S, was estimated from its density and molecular weight. When the density was not known the value of V_s was taken as 110 cm^3. Following Lietzke et al. (*16*), we used for the constants B and C, the values −0.66 and 0.1887, respectively. The value of C represents an average value for various electrolytes.

With these numerical values, Equation 19a yields, for the case when the ionized solute carries unit charge, the expression

$$\ln (k_0/k_1) = 5.79/b - 3.88\mu^2/V_S - 0.42 \quad (22)$$

where the dimensions of μ, V_S, and b are Debye, cm^3/mol, and Å, respectively.

The dependence of the capacity factor on the ionic strength was evaluated for each solute by using Equations 16b, 20g, and 20h. The best fit from the least-squares analysis of the data according to Equation 20g yielded a constant, the value of $(\epsilon - \epsilon^*)/\epsilon^*$ and the coefficient of the final ionic strength term which correspond to $\ln k^\circ$, α, and β in Equation 20h, respectively. In this calculation the following constants were used (*16*): $S = 0.5045$, $B = -0.66$, $C = 0.1887$, $a = 3.3 \times 10^3$. V_{salt}, which was used to evaluate ψ by Equation 16b, was calculated as 37.58 cm^3. Subsequently, the mean values of α (1.26 ± 0.28) and β (0.335 ± 0.034) were calculated to determine the value of $\ln k^\circ$, which best fit the data, from Equations 20g and 20h. These parameters were used in Equation 20g in the calculation of the theoretical curve for ionized solutes shown in Figure 11. The theoretical curve for un-ionized solutes was obtained from Equation 20h in a similar fashion but setting α equal to 0.

The electrostatic terms in Equations 21a and 21b for dipoles, zwitterions, and monopoles were calculated from Equations 14a, Equation 13 of reference *17*, and Equation 16d, respectively. The constants were the same as those used in the above calculation of the capacity factor ratios of the un-ionized and ionized forms of a solute.

RESULTS AND DISCUSSION

Hydrophobic Chromatography. Nonpolar stationary phases have traditionally been used with mixed solvents as the mobile phase to separate not only hydrocarbons but also ionogenic substances as illustrated in Figure 1. Although the effect of solute ionization on retention has been observed (*7, 21*), the theoretical interpretation of this phenomenon is hampered by the lack of a rigorous treatment of protonic equilibria in hydroorganic mixtures (*22*).

As seen in Figures 2 to 4, neat aqueous eluents, which contain no organic solvent component, are eminently suitable for the separation of acidic, basic, and zwitterionic substances on octadecylsilica columns. The chromatograms illustrate that the three different types of solutes can frequently be separated in a single chromatographic run; thus, for such separations the technique offers distinct advantages over ion exchange chromatography, the most popular method that employs neat aqueous eluents.

The chromatographic system employed here is probably the simplest in liquid–solid chromatography. First, protonic equilibria and the meaning of pH in aqueous solutions are fairly well understood. Second, solute retention can be interpreted in terms of hydrophobic interactions between the solute and the hydrocarbonaceous functions attached to the support surface. In view of the great importance of hydrophobic interactions in biochemistry, the subject has extensively been treated in the literature (*23*). As discussed previously, Sinanoğlu's treatment of the "solvophobic effect" has been a particularly suitable starting point of a theoretical framework for this type of chromatography.

In our experience hydrophobic chromatography is comparable to ion-exchange chromatography in terms of column efficiency. Octadecylsilica columns with varying amount of hydrocarbon per unit weight of column material are commercially available. Stationary phases of higher carbon content yield higher solute retention under otherwise equivalent conditions. The chromatogram of the nucleotides in Figure 4 shows that this technique can indeed be used for separations which had been solely carried out by ion-exchange chromatography in the past. In both techniques, solute retention is reduced by increasing column temperature. On the other hand, solute retention is usually augmented with increasing concentration of an inorganic salt in the eluent whereas in ion-exchange chromatography the opposite effect is observed. The effect of solute ionization on the retention can also be drastically different in hydrophobic and ion-exchange chromatography. Owing to the complexities of the chromatographic process in the latter technique, optimum conditions

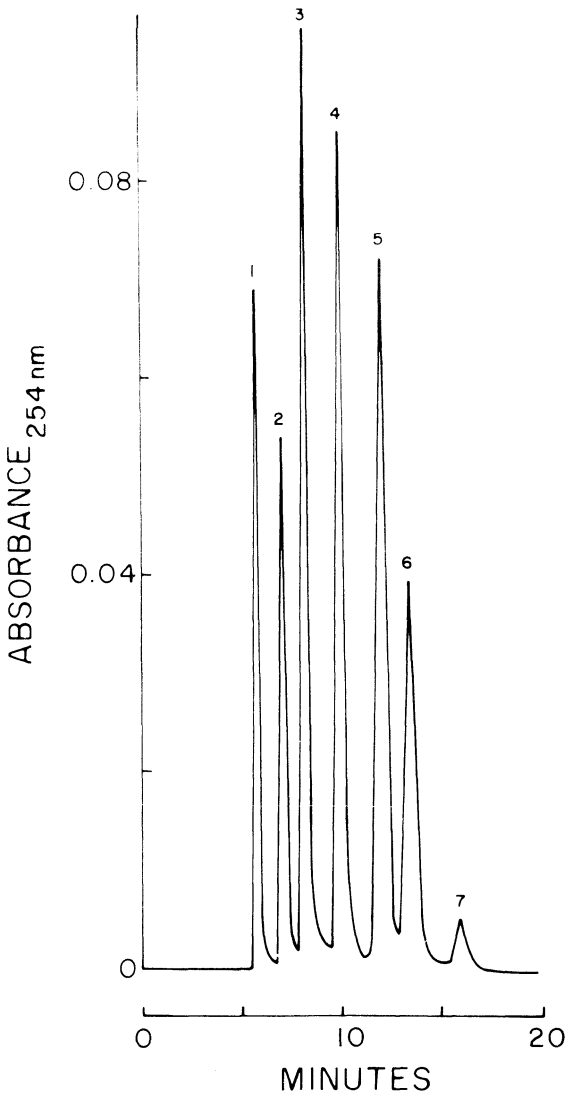

Figure 1. Chromatogram of organic acids

Column, Partisil 1025 ODS; eluent, 30% (v/v) acetonitrile and 70% (v/v) 0.05 M phosphate buffer, pH 2.7; flow rate, 1 ml/min; inlet pressure, 1000 psi; temperature, 25 °C. Sample components: (1) benzoic acid, (2) 2-methylbenzoic acid, (3) cinnamic acid, (4) 4-bromobenzoic acid, (5) 2-chlorocinnamic acid, (6) 4-chlorocinnamic acid, (7) 2,6-dichlorocinnamic acid

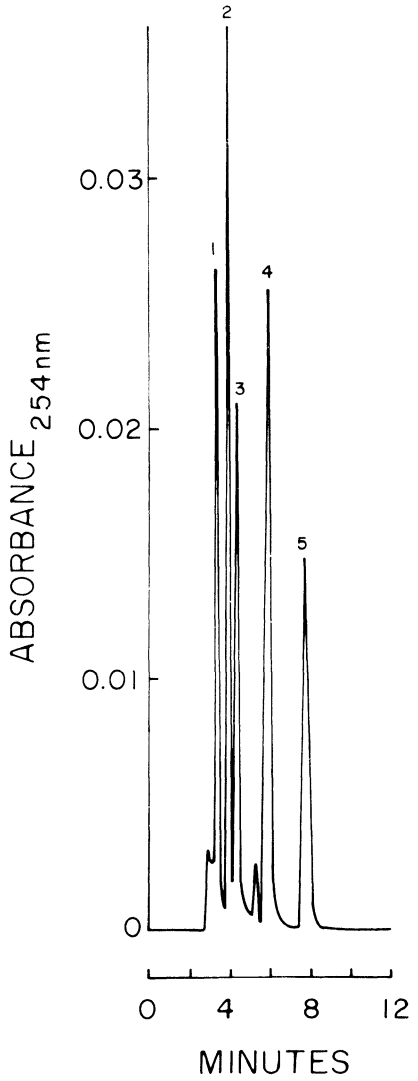

Figure 2. Chromatogram of biogenic acids and amines

Column, Partisil 1025 ODS; eluent, 0.1 M H_3PO_4–KH_2PO_4 buffer, pH 1.95; flow rate, 1.0 ml/min; inlet pressure, 750 psi; temperature 25 °C. Sample components: (1) norepinephrine, (2) 3,4-dihydroxymandelic acid, (3) dopamine, (4) vanillmandelic acid, (5) 3-methoxy-4-hydroxyphenylglycol

are selected by using certain empirical rules and a trial and error procedure. We feel that the rigorous treatment of hydrophobic chromatography presented in this study will facilitate the understanding of the various factors that govern the chromatographic process as well as the prediction of the optimum elution policy for a given separation. Furthermore the theoretical groundwork can serve as a basis for expanding the scope of hydrophobic chromatography to physicochemical measurements, particularly for characterizing the hydrophobic properties of biological substances and drugs.

At present the major limitation of the method rests with the properties of the siliceous support and the silane compounds used exclusively for the preparation of hydrocarbonaceous stationary phases. At high pH, the silanol groups provide a charged microenvironment at the surface and under such conditions the interpretation of solute–ligand interactions can be very complicated. From the practical point of view, the stability of most microparticulate siliceous column packings is unsatisfactory when they are exposed to aqueous salt solutions at pH values higher than 7 over an extended period of time. We infer that small particles which are under stress in the packing structure dissolve upon the combined effect of hydroxyl and other small monovalent anions and at a certain point the packing structure collapses. As a result of a catastrophic transmutation of the packing structure, the efficiency and often the permeability of the column are drastically reduced. Concomitantly the bonded moiety can be slowly cleaved from the silica surface upon exposure to an eluent of relatively high pH. Elevated temperature usually accelerates both effects. The potential of hydrophobic chromatography could be greatly enhanced if the silica support could be replaced by a suitable material such as zirconia, which is more stable at higher eluent pH. It is expected that octadecylsilicas, which have a high carbon content, such as Partisil ODS 2 would withstand eluents having higher pH and salt concentration over a significantly longer period of time than Partisil ODS does.

The limitations of the Partisil ODS columns used in this study precluded the extension of our investigations to study solute retention with eluents of pH above 7. Consequently it was impossible to cover the pH range in which amines are un-ionized. Nevertheless, the data obtained with acids, zwitterionic substances, and fully ionized amines have provided a satisfactory basis to test the theory presented in the previous section.

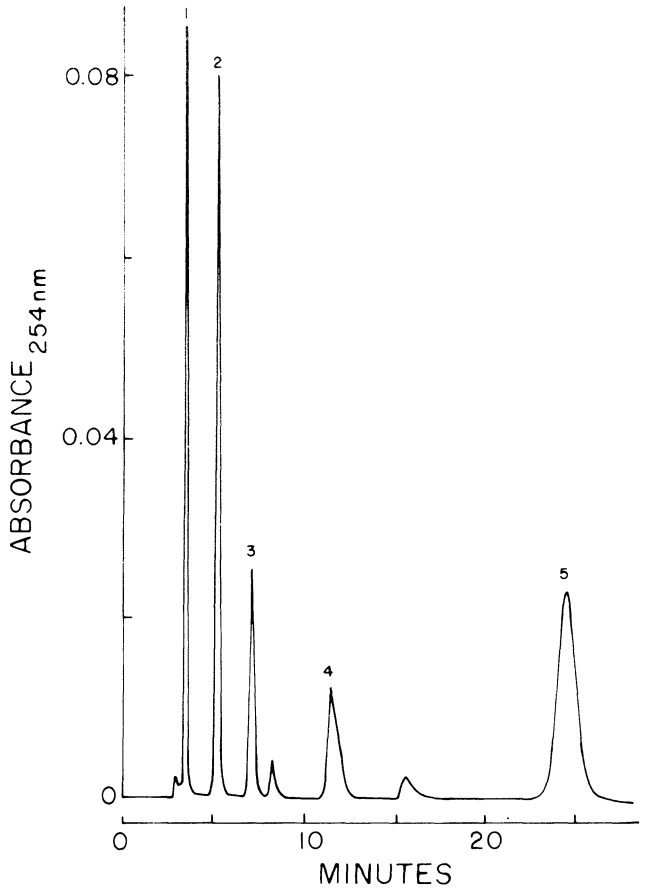

Figure 3. Chromatogram of aromatic amino acids

Column, Partisil 1025 ODS; eluent, 1.0 M Na_2SO_4 in 0.1 M H_3PO_4–KH_2PO_4 buffer, pH 2.1; flow rate, 1.0 ml/min; inlet pressure, 1000 psi; temperature, 25 °C. Sample components: (1) 3,4-dihydroxyphenylserine, (2) 3,4-dihydroxyphenylalanine (DOPA), (3) tyrosine, (4) phenylalanine, (5) tryptophan

Table II. Limiting Capacity Factors Obtained by Least Squares Analysis of the Capacity Factors of Organic Acids Measured at Different pH Values. See Experimental Conditions in Figure 6

Acid	k_0	k_{-1}
Benzoic	16.2	2.7
3,4-Dihydroxyphenylacetic	3.2	1.1
Homovanillic	14.5	4.4
p-Hydroxyphenylacetic	6.3	1.9
Mandelic	3.4	1.3
Phenylacetic	14.2	4.2
Salicylic	16.8	4.1
Vanillmandelic	2.1	0.9

Monoprotic Acids. The pH-dependence of the capacity factor for monoprotic acids and bases has been expressed by Equations 3e and 4c. In order to illustrate the effect of pH, capacity factors have been calculated according to these Equations with limiting k_0 and k_{-1} or k_1 values and plotted vs. $pH_m - pK_{a_m}$ in Figure 5. It is seen that the curves are sigmoidal and their inflection point is located at $pH_m = pK_{a_m}$, i.e., when the pH of the mobile phase and the pK_a of the solute in the mobile phase are equal. In this representation we assumed that the capacity factors of the species are the greatest when they are un-ionized. This assumption follows from the theoretical predictions given in Equation 19a and has strong experimental support, vide supra Table I.

Figure 6 shows the pH dependence of the capacity factor

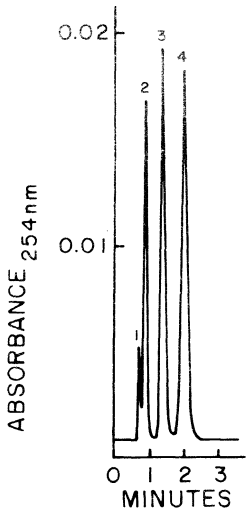

Figure 4. Chromatogram of nucleotides

Column, Partisil 1025 ODS2; eluent, 0.1 M phosphate buffer, pH 2.2; flow rate, 4.0 ml/min; inlet pressure, 2200 psi; temperature, 25 °C. Sample components: (1) 5-cytidylic acid, (2) 5-uridylic acid, (3) 5-adenylic acid, (4) 5-guanylic acid

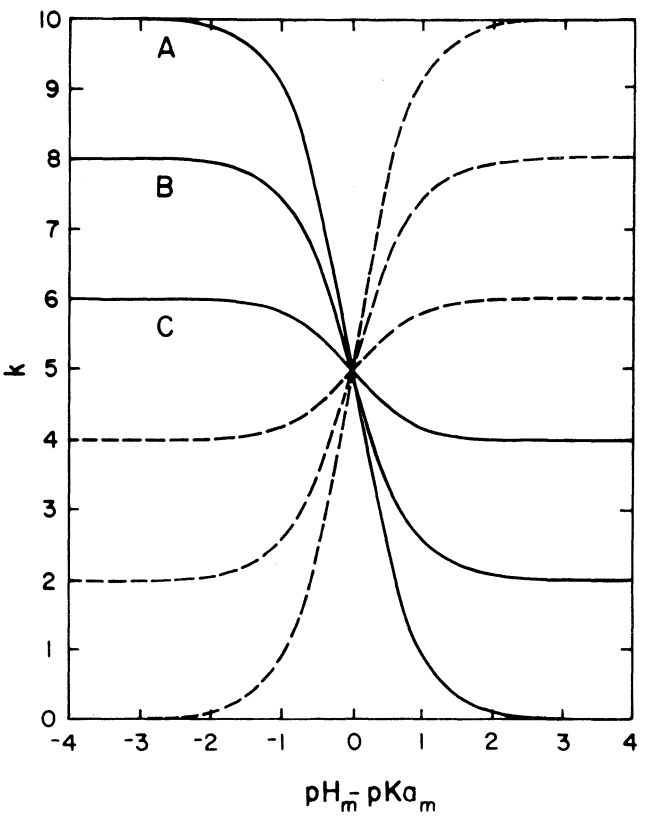

Figure 5. Plots of the capacity factor of weak monoprotic acids (solid lines) and bases (broken lines) vs. pH_m–pK_{a_m}

The curves were calculated according to Equations 3e and 4c, respectively. The limiting capacity factors k_0 and k_{-1} or k_1 are as follows: (A) 10 and 0; (B) 8 and 2; (C) 6 and 4

as measured with a number of monoprotic acids and we see that it is sigmoidal in each case. This behavior is in agreement with the expression in Equation 3e and with the observation made by Fromageot and Wurmser (*24*) that, in general, the adsorption of weak acids from aqueous solution on charcoal as a function of pH follows their dissociation curves.

The solid lines in Figure 6 have been calculated by a least-squares analysis of the data according to Equation 3e. The

Table III. Comparison of the pK_a Values of Organic Acids as Obtained by Least Squares Analysis of Chromatographic Data and by Potentiometric Titration. The Chromatographic Conditions Are Stated in Figure 6. Available Literature Data Are Also Listed

	pK_{a_m}		pK_a	
Acid	Chromatography	Titration	Literature	Ref.
Benzoic	3.93	3.78	4.19 (I = 0)[a]	(26)
3,4-Dihydroxyphenylacetic	4.20			
Homovanillic	4.29			
p-Hydroxyphenylacetic	4.30			
Mandelic	3.49	3.46	3.37	(27)
Phenylacetic	4.14	4.10	4.31	(28)
Salicylic	2.84	2.92	1.88 (I = 3)	(29)
Vanillmandelic	3.25			
o-Phthalic	3.44	3.20	2.95 (I = 0)	(28)
	5.10	4.79	5.41 (I = 0)	(28)
Anthranilic	2.21		2.09 (I = 0)	(30)
	5.30	4.74	4.79 (I = 0)	(30)

[a] I = ionic strength.

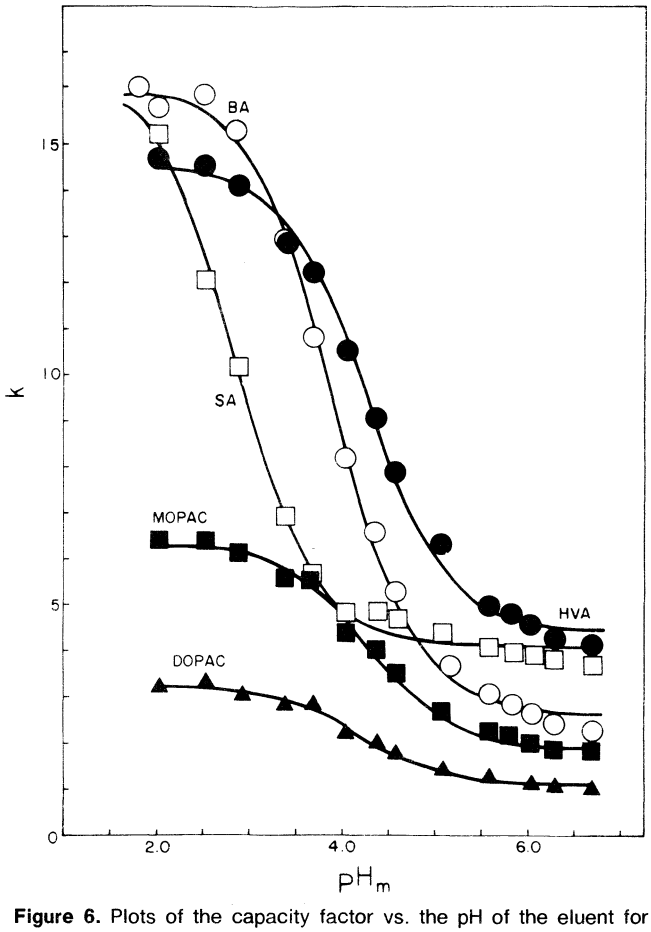

Figure 6. Plots of the capacity factor vs. the pH of the eluent for monoprotic acids

Column, Partisil 1025 ODS; eluent, 1.0 M Na_2SO_4 in 0.05 M phosphate buffers; temperature, 25 °C. The acronyms are: BA, benzoic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MOPAC, parahydroxyphenylacetic acid; SA, salicylic acid. The solid lines were obtained by fitting the experimental data to Equation 3e. The parameters obtained by the least squares method are shown in Table I

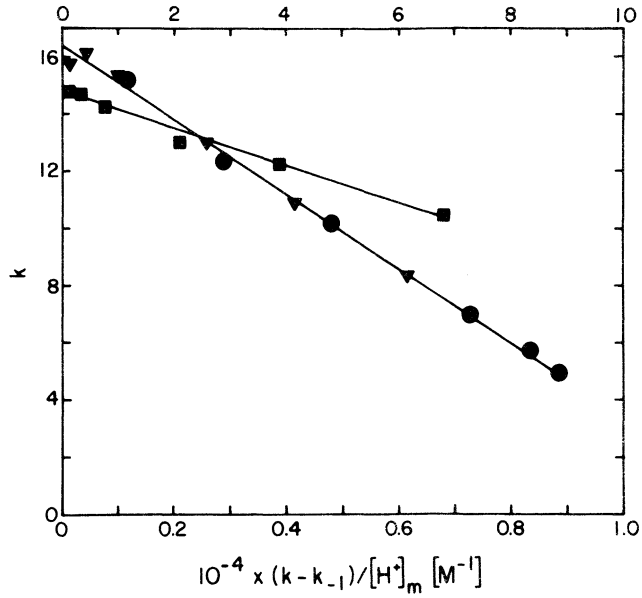

Figure 7. Plot according to Equation 23 to estimate the parameters which govern the pH dependence of the capacity factor of weak monoprotic acids

Slope: K_{a_m}, intercept: k_0. The data for salicylic acid (●) correspond to the bottom scale and those for homovanillic acid (■) and benzoic acid (▼) to the scale at the top. The pK_{a_m} values calculated from the slope are 2.88, 3.88, and 4.26 for salicylic acid, benzoic acid, and homovanillic acid, respectively

limiting capacity factors obtained from the parameter estimation for the acids illustrated and several others are listed in Table II. If Equation 3e is correct, the pK_{a_m} values obtained from the curve fitting should be the same as the pK_a values of the solutes in the mobile phase. Due to the high ionic strength of the eluent employed and the unreliability of the literature data (25), the pK_{a_m} values have been calculated from titration curves obtained in the eluent proper. The same pH meter and electrodes were used in the measurement of the eluent pH and in the titration experiments. The pK_{a_m} values obtained from chromatographic data by parameter estimation and from the titrimetric data are listed together with pK_a values from the literature in Table III. It is seen that the chromatographic and titrimetric pK_{a_m} values are in good agreement and randomly deviate from each other less than 5%.

Equation 3e can be written in a linearized form as

$$k = k_0 - (k - k_{-1})K_{a_m}/[H^+]_m \qquad (23)$$

which facilitates a graphical evaluation of two of the three

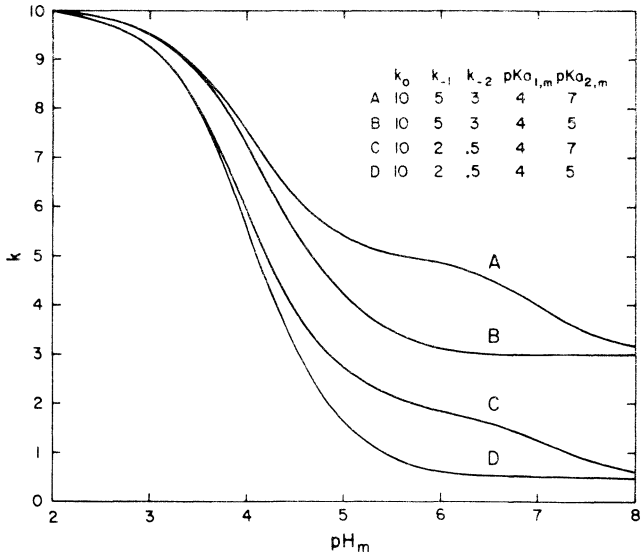

Figure 8. Plots of the capacity factor of diprotic weak acids vs. the pH of the mobile phase. The curves were calculated from Equation 5 with the parameters shown on the graph

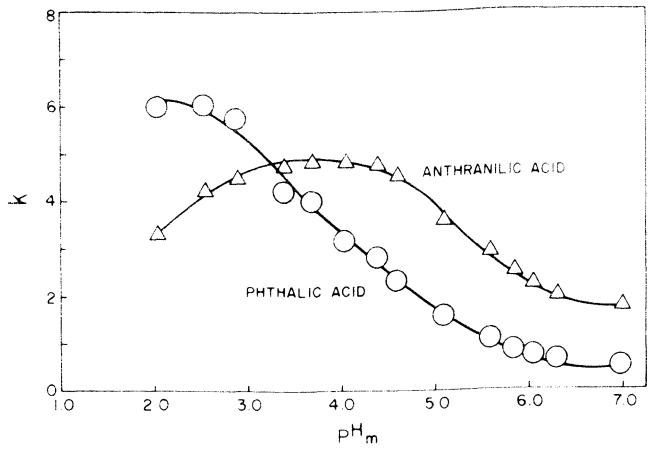

Figure 9. Plots of the capacity factor vs. the pH of the eluent for phthalic acid and anthranilic acid

Column, Partisil 1025 ODS; eluent, 1.0 M Na_2O_4 in 0.05 M phosphate buffer; temperature, 25 °C. The solid curves have been calculated by fitting the experimental data with phthalic acid to Equation 5 and with anthranilic acid to Equation 6. The limiting capacity factors obtained by the least squares method are as follows: phthalic acid: $k_0 = 6.3$, $k_{-1} = 2.72$, and $k_{-2} = 0.47$. Anthranilic acid: $k_0 = 5.06$, $k_1 = 2.14$, and $k_{-1} = 1.71$. The pK_{a_m} values are listed in Table III

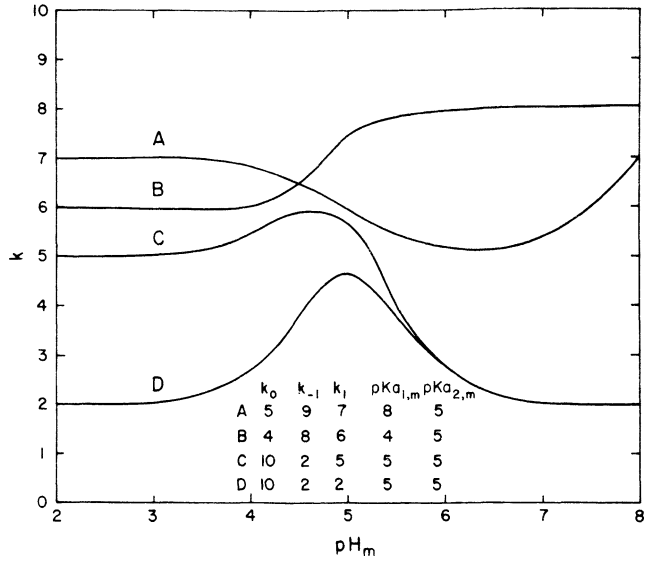

Figure 10. Plots of the capacity factor of ampholytes vs. the pH of the eluent. The curves were calculated from Equation 6 with the parameters shown on the graph

parameters, provided the third is known, from capacity factors measured in a sufficiently wide pH range. Figure 7 illustrates plots of k vs. $(k - k_{-1})/[H^+]_m$ for homovanillic, benzoic, and salicylic acids. It is seen that according to Equation 23 straight lines are obtained and the slopes, $-K_{a_{m,1}}$, are in good agreement with the experimental values. This graphical method can give a reasonable parameter estimation even if all three parameters are unknown and one of them can be guessed. A similar graphical approach can be used to evaluate data for monoprotic bases when Equation 4c is recast in a linearized form similar to Equation 23.

In principle, the theory makes it possible to calculate the capacity factor ratio for the un-ionized and fully ionized solutes according to Equation 19a. Unfortunately such calculations are hampered by the lack of sufficient data on the dielectric constant of the stationary phase and the dipole moment of the solutes. Nevertheless, with the simplifying assumptions discussed in the previous section, Equation 19a could be reduced to Equation 22 which enabled us to calculate approximate k_0/k_{-1} values. For instance, by substituting the literature values, $\mu = 0.76$ Debye (*31*) and $V_S = 96.5$ cm^3, for benzoic acid we found that $k_0/k_1 = 5.0$, which is in fair agreement with the experimental value of 6.0. The theoretical k_0/k_{-1} value for salicylic acid was calculated with $\mu = 2.65$ Debye (*31*) and $V_S = 95.7$ cm^3 as 3.8, which compares well with the observed ratio of 4.1. The calculation for homovanillic acid with $\mu = 3.1$ Debye and $V_S = 117$ cm^3 gave a theoretical k_0/k_{-1} value of 3.29, which is identical to the experimentally determined ratio.

For lack of literature data for the other acids investigated, we calculated the average dipole moment of the monoprotic acids listed in Table II by using Equation 22 and the average values $V_S = 110$ cm^3 and $k_0/k_{-1} = 3.5$. The average dipole moment of 2.7 Debye thus obtained is commensurable with that of similar compounds whose dipole moment is given in the literature (*31, 32*) and this finding lends further support for the theory.

Diprotic Acids. The pH dependence of the capacity factor for diprotic acids is expressed by Equation 5 which is illustrated for certain typical cases in Figure 8. As suggested by Equation 5 and seen on the graph the shape of the curves is largely determined by the difference between the two pK_a values. When $pK_{a_{m1}}$ and $pK_{a_{m2}}$ are very close, sigmoidal curves are obtained and the behavior of monoprotic acids and the diprotic acid is indistinguishable. When the pK_a values are well separated, the curve can be viewed as a composite of two sigmoidal curves.

Figure 9 shows the measured pH dependence of the capacity factor of phthalic acid, and the least-squares fit of the data to Equation 5 by the solid line. The limiting capacity factors and the pK_{a_m} values obtained from computer analysis are given in the caption and in Table III, respectively. The chromatographic, titrimetric, and literature values of both pK_a's are in reasonable agreement as seen in Table III.

Following the procedure outlined in the previous section for the calculation of the capacity factor ratio by using Equation 22, we calculated k_0/k_{-1} for phthalic acid. The dipole moment, $\mu = 2.6$ Debye, was taken from the literature (*31*) and the molar volume was calculated as 104.2 ml. The result (3.5) does not agree well with the experimental value (2.3). We suspect that the dipole moment given in the litera-

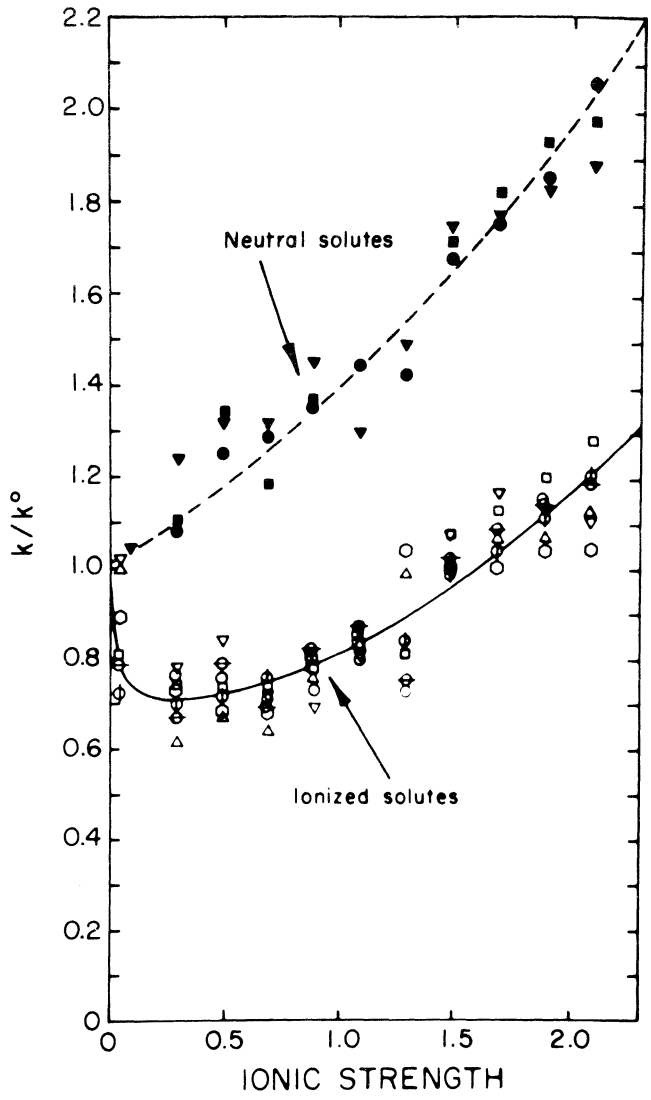

Figure 11. The dependence of the normalized capacity factor on the ionic strength of the eluent for a variety of un-ionized (- - -) and ionized (—) solutes

$k°$ denotes the capacity factor at zero ionic strength. The broken line was calculated by using Equations 20e and 20f for un-ionized acids. The solid line was calculated from Equations 20g and 20h. The chromatographic conditions are: Column, Partisil 1025 ODS; eluent, 0.01 M phosphate buffer, pH 4.0 with increasing concentration of KCl; temperature, 25 °C. The symbols represent the following solutes: (▼) 3,4-dihydroxyphenylacetic acid; (●) homovanillic acid; (■) vanillmandelic acid; (▽) normetanephrine; (○) metanephrine; (□) 3-*O*-methyldopamine; (△) dopamine; (○) adrenaline; (⊖) tyramine; (Φ) paranephrine

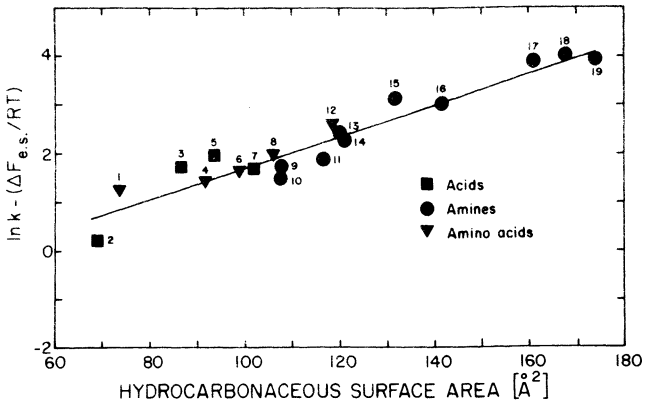

Figure 12. Graph illustrating the relationship between the logarithm of the capacity factor corrected for electrostatic effects and the hydrocarbonaceous surface area of various solute molecules

Column, Partisil 1025 ODS; eluent, 1.0 M Na_2SO_4 in 0.1 M phosphate buffer, pH 2.05; temperature, 25 °C. Symbols: (■) acids, (▼) amino acids, and (●) amines. The numbers represent the following solutes: (1) anthranilic acid, (2) 3,4-dihydroxymandelic acid, (3) 3,4-dihydroxyphenylacetic acid, (4) 3,4-dihydroxyphenylalanine, (5) mandelic acid, (6) 4-aminophenylacetic acid, (7) vanillmandelic acid, (8) tyrosine, (9) norphenylephrine, (10) octopamine, (11) normetanephrine, (12) phenylethylamine, (16) 3-*O*-methyldopamine, (17) paranephrine, (18) *N*-methylphenylethylamine, (19) ephedrine

ture is in error and the actual value is higher. When μ = 4.4 Debye is used in the above calculation, the theoretical and experimental capacity factor ratios are the same.

Ampholytes. The pH dependence of the capacity factor for ampholytes is expressed by Equation 6 and illustrated in Figure 10 with certain arbitrarily chosen parameters. Since it is somewhat more difficult to assess a priori the relative magnitude of the capacity factors of the zwitterionic and monopolar forms of the ampholyte, for the sake of illustration the k_0 values were chosen to be higher than, lower than, or intermediate to the two k_2 values. It is seen that the k vs. pH_m plots have widely different shapes even if the parameters are similar.

The operational upper limit of the eluent pH (pH_m = 7) with the Partisil ODS columns precluded the investigation of the naturally occurring amino acids within a sufficiently wide pH range to test Equation 6. Therefore anthranilic acid was chosen as a model substance for such a test. Figure 9 shows the capacity factors measured at different pH_m values; the solid line was calculated from Equation 10e with the parameters given in the legend. As shown in Table III, the chromatographically measured pK_{a_m} values are in accord with the corresponding pK_a values determined by other methods.

The capacity factors of the two anthranilic acid monopoles, k_{-1} and k_1 are 1.71 and 2.14, respectively. The observed smaller capacity factor for the anion could be explained by its smaller ionic radius compared to that of the cationic monopole. Yet, the k_1/k_{-1} ratio for anthranilic acid appears to be greater than expected from this consideration. By using Equation 19a with the geometric mean of the two capacity factors, we obtain a dipole moment of 4.6 Debye whereas the literature value is 1.52 Debye (*31*). If anthranilic acid behaves as a zwitterion under our experimental conditions, we obtain following Kirkwood's approach (*17*) a dipole moment of 13.6 Debye which is similar to that of the naturally occurring amino acids. The intermediate value of 4.6 Debye calculated from our data can be readily explained by the observation (*33*) that the neutral anthranilic acid is present in aqueous solutions mainly as a simple dipole and, to a much lesser extent, as a zwitterion.

Effect of Ionic Strength. We have shown elsewhere (*3*) that increasing salt concentration in the eluent augments the capacity factors of neutral solutes in hydrophobic chromatography. The effect has been ascribed to the increasing surface tension of the eluent and the concomitant increase in the energy required for cavity formation (*35*).

With ionized solutes, electrostatic effects must also be considered in present theory. In chromatography the ionic strength of the eluent is usually high so that the Debye–Hückel theory is not applicable. In order to make our approach suitable for practical conditions we adapted the semi-empirical treatment of Lietzke et al. (*16*). It describes the behavior of salt solutions as a combination of the dilute solution behavior described by the Debye–Hückel model and the properties of randomized fused salts.

Equation 20g describes the effect of changing ionic strength on the capacity factor of a monopole. Since all the parameters of this expression are known with the exception of $(\epsilon - \epsilon^*)/\epsilon^*$, $\Delta\Phi$ and the leading constant, it reduces to the form of Equa-

tion 20h at high ionic strength. As described in the previous section, we evaluated α, β, and k_i^z for seven amines by using Equation 20h. Then a normalized theoretical curve, which is shown in Figure 11, was constructed for these ionized solutes with the mean values α and β and with $k_i^z = 1$. The most important feature of this curve is the minimum in the normalized capacity factor at $I = 0.3$. For comparison, the experimental capacity factors normalized to their individual k_i^z values are also plotted vs. the ionic strength in Figure 11. Although the experimental points scatter about the theoretical curve, the fit is fairly good because the deviations are random and seldom exceed 10%.

In order to demonstrate the significance of the electrostatic term in Equation 20g, the effect of the ionic strength on the capacity factor of three acids which are un-ionized under the experimental conditions is also shown in Figure 11. The ionic strength dependence given by Equations 20e and 20f can be expressed by Equation 20h with $\alpha = 0$. By using this approach, we obtained a theoretical curve in a procedure similar to that described above and plotted together with the normalized capacity factors of the un-ionized acids on Figure 11. The curve differs markedly from the corresponding curve for the ionized solutes as it shows no minimum in the normalized capacity factor, which monotonously increases with the ionic strength. Again, the deviation of the experimental points from the theoretical values is small (less than 10%) and random.

For certain families of substances, such as carboxylic acids, amines and amino acids, we have shown (*3*) a linear relationship between ln k and the hydrocarbonaceous surface area of the solute molecules. According to the solvophobic theory, such a linear relationship should be generally observed with solutes of similar molecular geometry and relatively large nonpolar ligands when the cavity term dominates the energetics of the solute–solvent interaction. The different intercepts obtained for the individual families on a plot of ln k vs. the hydrocarbonaceous surface area has largely been attributed to the fact that the acids were neutral whereas the amines and amino acids were ionized under the conditions of the experiment.

The theoretical approach presented in this study can be used to correct the logarithm of the capacity factor by the appropriate electrostatic term according to Equations 21a and 21b. The electrostatic contributions were calculated from Equations 14a and 16d for un-ionized dipoles and monopoles, respectively. For zwitterions, Equation 13 of Reference *17* was used. The dielectric constant of the stationary phase was assumed to be 35, vide supra. When the dipole moments were unknown their values were taken as 3 and 17 (*34*) for neutral and zwitterionic species, respectively. Substituting the ΔF_{es} and ΔF_{es}^z values thus obtained into Equations 21a and 21b, we evaluated the LHS's of these equations for the individual solutes and plotted the resulting values vs. the corresponding hydrocarbonaceous surface areas.

Figure 12 shows the results for 19 solutes. As seen on the graph and as manifested by a correlation coefficient of 0.96, the fit of the data points to a straight line falls short of the expectations. Several of the assumptions made in the calculations can be responsible for the relatively poor fit. For instance, we used the same correction for all amines which constitute the largest group of compounds on the graph, assuming that they all have the same ionic radius. Since it cannot be true, this simplification explains in part the poor correlation of the amino group and this strongly affects the correlation of the entire set of solutes. In addition, we assumed that the electrostatic component of the gas phase association term (Equation 17c) is the same for the different solutes. This crude approximation may also be responsible for the scattering of the data points.

Nevertheless, the results give ample evidence that our treatment of the interaction between the solvent and an un-ionized dipole, simple ion, and an ionic dipole as manifested by Equations 14a, 16d, and the adapted Kirkwood approach, respectively, is essentially correct. Without evaluating the interaction energies for the three individual cases, the plot in Figure 12 would consist of three parallel lines as shown elsewhere (*3*). It is noted that the calculation of the interaction energy for zwitterions by Equation 14a would yield capacity factor values with an error of 2 to 3 orders of magitude. Therefore, the treatment of zwitterionic substances requires the special consideration advanced by Kirkwood (*17*).

Evidently the lack of adequate data on the physical properties even for such relatively simple biological substances greatly impedes a rigorous testing of the theory. On the other hand these compounds are far more complex than the model substances such as hydrocarbons or alcohols which have been used almost exclusively in more exact thermodynamic studies of the chromatographic process.

In fact, the solvophobic theory appears to be the only approach to the analysis of hydrophobic chromatography with biological substances in which coulombic, hydrogen bonding, and hydrophobic interactions are amalgamated. Even with the limitations stated above, Figure 12 eminently conveys the fundamental feature of hydrophobic interactions that the magnitude of the nonpolar contact area plays a paramount role in determining the interaction energy between the solute and the ligand of the stationary phase.

As we have shown, however, solute–solvent interactions can also have a great effect on the overall chromatographic behavior and can be theoretically evaluated for ionic substances as well.

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