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Mark A. Eiteman[a]
[a] DEPARTMENT OF BIOLOGICAL AND AGRICULTURAL ENGINEERING DRIFTMIER ENGINEERING CENTER, UNIVERSITY OF GEORGIA ATHENS, GEORGIA

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Partitioning of Charged Solutes in Poly(Ethylene Glycol)/Potassium Phosphate Aqueous Two-Phase Systems

MARK A. EITEMAN
DEPARTMENT OF BIOLOGICAL AND AGRICULTURAL ENGINEERING
DRIFTMIER ENGINEERING CENTER
UNIVERSITY OF GEORGIA
ATHENS, GEORGIA 30602

ABSTRACT

A mathematical model based on the dissociation of charged compounds and the pH of each phase is developed to describe the partitioning of charged compounds in aqueous two-phase systems. Observed partition coefficients of several charged and uncharged compounds, including three pairs of oppositely charged analogs (tryptamine/indole 3-acetic acid, 5-methoxytryptamine/5-methoxyindole 3-acetic acid and 2-(*p*-tolyl) ethylamine/*p*-tolyl acetic acid), are compared in identical poly(ethylene glycol)/potassium phosphate aqueous two-phase systems over the pH range of 5.5 to 9.2. Among these pairs, the partition coefficients of the acids increased with increasing pH, from 8.4 to 33.3 for indole 3-acetic acid, from 11.4 to 53.9 for 5-methoxyindole 3-acetic acid, and from 4.2 to 17.7 for *p*-tolyl acetic acid. The amine partition coefficients also increased with increasing pH, from 4.0 to 7.8 for tryptamine, from 5.8 to 12.2 for 5-methoxytryptamine, and from 1.6 to 3.0 for 2-(*p*-tolyl) ethylamine, respectively. Consistent with the derived model, the greatest rate of increase in the partition coefficients of the acids occurs at low pH, while the greatest rate of increase in amine partition coefficients occurs at high pH. The ratio of partition coefficients for these pairs predicted by the model agrees with the observed partition ratio. The results indicate that charge, in addition to hydrophobic effects previously described, plays a major role in the partitioning of biological compounds.

INTRODUCTION

Solutions of two water soluble but mutually incompatible components, such as poly(ethylene glycol) (PEG) and dextran, or PEG and certain

salts, can form aqueous two-phase systems. Albertsson (1) showed that two liquid phases form when a threshold concentration of either component is exceeded. A solute added to such a system often partitions unequally between the phases, and its partition coefficient, K, is defined as this solute's upper phase concentration divided by its lower phase concentration. Many types of solutes, including small organic molecules (2–4), salts (5, 6), peptides (7) and proteins (1, 8, 9), have been shown to partition in aqueous two-phase systems. Since such systems are composed primarily of water, they provide a gentle environment for the fractionation of biomaterials (10–15). Aqueous two-phase systems are also environmentally benign, which is an extremely important consideration for large-scale processes having low impact on natural environments and for maintaining low toxicity of purified biological compounds which humans may ultimately ingest or inject.

In order to provide some rationale for the selection of a particular aqueous two-phase system for a desired separation, models must be developed to predict partition coefficients. Numerous studies have focused on the general prediction of partition coefficients in aqueous two-phase systems. Since partition coefficients are thought to depend on several factors including solute hydrophobicity (16, 17), molecular weight (18), temperature (10), pH (19–21), solute charge (22) and the presence of additional salts (23, 24), models and correlations which incorporate these effects have been developed. These correlations include recent derivations based on the Flory–Huggins lattice model (25, 26), a modified lattice model (27), UNIQUAC (28, 29), an extension of an osmotic pressure virial expansion (22), and Hill solution theory (30, 31). This present study focuses on the effect that the charge of a solute has on its partition coefficient.

That solute charge has some effect on the partition coefficient has been recognized for over 20 years. Reitherman et al. (32) measured an electric potential between phases and correlated the partitioning of negatively charged human erythrocytes with this difference in potential. Johansson (6) showed that the partitioning of proteins could be correlated with salt partitioning. Johansson (33) and Albertsson (34) developed equations for protein partition coefficients as a function of the protein's net charge and the difference in potential between the phases. Since the mechanism and magnitude of charge effects have been poorly understood, researchers merely refer to "charge-sensitive" and "noncharge-sensitive" phase systems when describing charge-related behavior (35, 36). One phenomenon observed has been "cross-partitioning" (7, 19, 20): in two PEG/dextran/phosphate systems, one having an addition of sodium chloride and the other sodium sulfate, the partition coefficients of a protein "crossed" at its isoelectric point. Clearly, several factors contribute to the observed

partition coefficients. Unfortunately, there has been limited effort to isolate the charge effect from other potentially confounding effects such as a hydrophobic effect. In order to optimize biological separations, the effect of charge alone must be understood and quantified.

Ideally, to study the effect that solely the charge of a solute has on its partition coefficient, the partition coefficient of a charged solute should be compared to the partition coefficient of the *same* solute in an uncharged state in the same phase system. Unfortunately, this idealized experiment is not possible because the charge of a compound may not be altered without altering either the compound or the phase system. However, this condition may be approximated by comparing the partition coefficients of two compounds having analogous structures: one carrying a charge over a certain pH range while the other does not. An example of such an analogous pair of compounds is benzoic acid and benzyl alcohol. One might also compare the partition coefficient of a compound having a negative charge with an analogous compound having a positive charge, such as indole 3-acetic acid and tryptamine, respectively. In this case the hydrophobicity of the methylene amino group on the amine is approximately the same as that of the carboxylic acid group on the acid (37). Therefore, any difference between the partitioning of these two compounds should be due primarily to the difference in charge.

Eiteman and Gainer (38) showed that a measured pH difference between the phases of an aqueous two-phase system has a predictable effect on the partition coefficients of charged solutes. Zaslavsky et al. (39) confirmed the existence of a pH difference between the phases through the use of solvatochromatic dyes. Using a mass balance equation for all species (charged and uncharged) in a phase system, models were derived (38) to predict the partition coefficient of a charged solute relative to the partition coefficient of an analogous uncharged solute. Specifically, the partition coefficient of a charged solute depends upon the partition coefficient of an uncharged analog of that solute, the dissociation of the solute, and the pH in each phase. A goal of this present study is to derive useful expressions for the partition coefficient of other singly-charged solutes and compare the predicted behavior with results obtained from partitioning several solutes of biological interest.

MATHEMATICAL MODEL

In any aqueous two-phase system, a singly-charged solute (e.g., a monocarboxylic acid or an amine) may exist as an uncharged or charged species, and these species coexist in each phase. Therefore, one must consider the partition coefficient of both the charged species and the uncharged

species when calculating the observed partition coefficient. In order to derive a model describing the partitioning of a positively charged solute (such as an organic amine), the partitioning of the uncharged and charged species must be considered individually.

The partitioning of an uncharged species is first considered. The uncharged species has a partition coefficient, K_0, due entirely to nonelectrostatic forces, such as hydrophobic, size, or excluded volume effects:

$$K_0 = \frac{c_0'}{c_0''} = k\frac{x_0'}{x_0''} \tag{1}$$

In Eq. (1), c is the concentration and x is the mole fraction of a species in the upper (′) and lower phase (″). k is the particular proportionality constant needed to convert units of concentration to mole fraction for dilute solutions.

The partition coefficient *actually* measured for a solute which can have a single positive charge depends on both the uncharged (0) and charged (+) species:

$$K = k\frac{x_+' + x_0'}{x_+'' + x_0''} = K_0\frac{\frac{x_+'}{x_0'} + 1}{\frac{x_+''}{x_0''} + 1} \tag{2}$$

The measured partition coefficient, K, has no subscript to emphasize that its value includes both the positively charged and neutral species.

The charge of those organic compounds which can carry a charge will depend on the pH of the solution. For example, the relationship between the solution pH and the charge of a solute such as an amine (A^0 in the neutral form, AH^+ when it has a single positive charge) may be expressed in terms of an equilibrium:

$$AH^+ \rightleftharpoons A^0 + H^+ \tag{3}$$

An equilibrium constant, K_{b1}, is defined by the equilibrium described by Eq. (3):

$$K_{b1} = \frac{a_0 a_H}{a_+} \tag{4}$$

where the subscript "b" is reserved for equilibria of positively charged compounds, and the subscript "1" emphasizes that an uncharged and singly-charged species are in equilibrium. At equilibrium, Eq. (4) must be satisfied for both phases. For the upper phase, Eq. (4) may be rewritten as

$$K_{b1}\gamma_+' x_+' = \gamma_0' x_0' a_H' \tag{5}$$

In Eq. (5), species activities (a) are expressed as the product of the activity coefficient (γ) and mole fraction (x). For convenience, an activity ratio, Λ_+, is defined as the solute activity coefficient for the neutral species divided by the activity coefficient of the positively charged species (i.e., γ_0/γ_-). Substituting the expressions for the solute mole fractions in each phase (e.g., Eq. 5) into Eq. (2) results in the following expression:

$$\frac{K}{K_0} = \frac{1 + \Lambda'_+ \, 10^{(pK_{b1} - pH')}}{1 + \Lambda''_+ \, 10^{(pK_{b1} - pH'')}} \tag{6}$$

This equation does not predict the measured partition coefficient of the positively charged solute, but rather predicts the *ratio* of such a solute's observed partition coefficient (charged and uncharged species) to the partition coefficient of the uncharged species alone. This partition ratio depends on the pH in each phase, the activity ratio, and the equilibrium constant. Equation (6) is analogous to a previously (38) derived expression for the partition ratio of a negatively charged solute such as an organic acid:

$$\frac{K}{K_0} = \frac{1 + \Lambda'_- \, 10^{(pH' - pK_{c1})}}{1 + \Lambda''_- \, 10^{(pH'' - pK_{c1})}} \tag{7}$$

In Eq. (7), Λ_- is the activity ratio defined as the solute activity coefficient for the neutral species divided by the activity coefficient of the negatively charged species. The equilibrium constant, K_{c1}, of the organic acid is given a subscript "1" to emphasize that the equilibrium is between an uncharged and singly-charged species. In this case the equilibrium constant for the neutral species and the charged species is

$$K_{c1} = \frac{a_- a_H}{a_0} \tag{8}$$

Equations (6) and (7) indicate that if the activity ratios in the two phases are equal and $\Delta pH = pH'' - pH' = 0$, then $K = K_0$. However, the presence of a small pH difference between the phases indicates that K is not equal to K_0 for some range of pH, even if both activity ratios are equal to 1. Since the partition coefficients of individual species are often not measurable, K_0 in practice must be estimated by one of two means: an uncharged analog of the charged solute is partitioned to approximate the neutral species, or a model which considers all noncharged effects is used to predict the partition coefficient of the neutral species. With a value for K_0, Eq. (6) or (7) may be used to predict the partition coefficient of the charged solute, K, which includes the contributions of the charged and uncharged species.

Another useful expression is obtained by dividing Eq. (6) by Eq. (7). This expression predicts the ratio (R) of the partition coefficient of a negatively charged solute (A) by the partition coefficient of an analogous positively charged solute (B). If the four activity *ratios* (Λ) are each assumed to be 1 for the case of dilute solutes, then a comparison of the negative and positive solutes may be simplified:

$$R = \frac{K_N}{K_P} = \frac{10^{-pH''}(10^{-pH'} + 10^{-pK_{c1}})(10^{-pH''} + 10^{-pK_{b1}})}{10^{-pH'}(10^{-pH''} + 10^{-pK_{c1}})(10^{-pH'} + 10^{-pK_{b1}})} \quad (9)$$

K_N is the observed partition coefficient of the solute which is negatively charged at some pH, and K_P is the observed partition coefficient of the analogous solute which can be positively charged. K_N and K_P are not equal to partition coefficients of the charged species alone (and therefore the notation K_- and K_+ is *not* used), but rather to the observed sum of the charged and uncharged species as indicated by Eq. (2). Moreover, because oppositely charged solutes may interact with each other, the two partition coefficients (K_N and K_P) should be compared in two separate but identical phase systems. Equation (9) predicts how a positively charged solute can partition differently from a negatively charged solute.

Several important complications limit the practical utility of Equations (6), (7), and (9). The values of the activity ratios are unknown, and must either be assumed to be 1, as they were in Eq. (9), be assumed equal, or be considered adjustable parameters. Also, since equilibrium constants generally depend upon numerous solution properties (temperature, ionic strength, dielectric constant, etc.) (40), their values must be estimated, perhaps by using equilibrium constants in pure water as an approximation. Since the addition of PEG decreases the dielectric constant of an aqueous solution, the equilibrium constant of an organic acid presumably is greater in a two-phase system than in pure water. In fact, the equilibrium constant of a given solute may actually be different in each phase of a two-phase system. The effect this "equilibrium constant difference" has on the partition ratio depends on its magnitude relative to ΔpH. The pH in each phase must be measured, and because the pH is measured with an electrode or with dyes, the measurement is subject to the same interpretation as equilibrium constants in nonaqueous environments. Finally, the mere addition of a charged solute to the two-phase system may alter the pH difference, the equilibrium constants, and the activity ratios. The partition coefficient may therefore change as more solute is added. This phenomenon would likely be more pronounced when numerous solutes are concentrated in the two-phase environment.

Despite the limitations of Eqs. (6), (7), and (9), these models are suitable for answering some basic questions concerning the partitioning of charged

compounds in aqueous two-phase systems. Useful information would include the comparison of partition coefficients for neutral, positive, or negative solutes in a given aqueous two-phase system and the selection of optimal two-phase systems and operating pH for a particular desired separation.

The partitioning of any charged solute between phases does not imply unequal distribution of charges across the phases. The partitioning of one charged species is presumably accompanied by the partitioning of any one of the numerous co-ions present in the complex two-phase systems, thereby maintaining charge neutrality in each phase.

MATERIALS AND METHODS

A series of 1.00 M potassium phosphate solutions was prepared as described elsewhere (38, 41). The phase-forming polymer used in these solutions is poly(ethylene glycol) (PEG) with a molecular weight of 8000. In the absence of any solute, these phase systems at 25.0°C have been previously shown (38) to result in a positive pH difference between the phases, that is, the measured pH of the upper phase is greater than the pH of the lower phase.

The following solutes were used for partitioning studies: indole 3-acetic acid, tryptamine (Sigma Chemical Co., St. Louis, Missouri), 5-methoxyindole 3-acetic acid, 5-methoxytryptamine, 3-hydroxybenzoic acid, 3-hydroxyanthranilic acid, 3-hydroxybenzyl alcohol, *p*-tolyl acetic acid, and 2-(*p*-tolyl) ethylamine (Aldrich Chemical Co., Milwaukee, Wisconsin).

The partition coefficients of these solutes at 25.0°C in the phase systems were determined by HPLC. Approximately 2–6 mg of a single solute was added to 10.0 mL of two-phase solutions, well below the solubility limits. The phases were placed at 25.0°C (±0.5°C) under nitrogen, thoroughly mixed for 2 days, allowed to equilibrate for 3 days, then carefully separated with Pasteur pipets immediately before analysis. The HPLC system comprised Gilson model 306 pumps, 231 autosampler, and an Applied Biosystems 759A UV/vis detector. The column was a Waters Radial-Pak C_8, with eluant and detector settings appropriate to separate the pure solute of interest from impurities arising from the PEG and solute sample. Partition coefficients could be determined by this method consistently within 8% error.

RESULTS AND DISCUSSION

Equations (6), (7), and (9) do not provide a value for the partition coefficient of a charged compound. Rather, these equations indicate how the

partition coefficient of a charged compound compares to the partition coefficient of a neutral compound or to an oppositely charged compound. Therefore, one method to examine the applicability of these models is to partition at least two compounds over a range of pH.

Equation (7), for example, predicts the ratio of partition coefficients of negatively charged and neutral compounds. Figure 1 shows the observed partition coefficients of an organic acid, 3-hydroxybenzoic acid, and an organic alcohol, 3-hydroxybenzyl alcohol. The partition coefficient of the neutral alcohol increases with increasing pH, an observation attributable to the change in the tie line length rather than the pH itself (41). The observed partition coefficient of the acid also increases with increasing pH. However, the two sets of partition coefficients do not increase at the same rate. Since these two compounds are roughly identical in size and hydrophobicity, any difference in the observed partition coefficients is attributed to the difference in charge according to Eq. (7). At low pH the organic acid is uncharged, and would therefore be expected to have approximately the same partition coefficient as the alcohol. As the pH increases, however, a greater portion of the acid in solution becomes charged. As Eq. (7) predicts, for systems which have a positive pH difference between the phases, the partition coefficient of an acid should be-

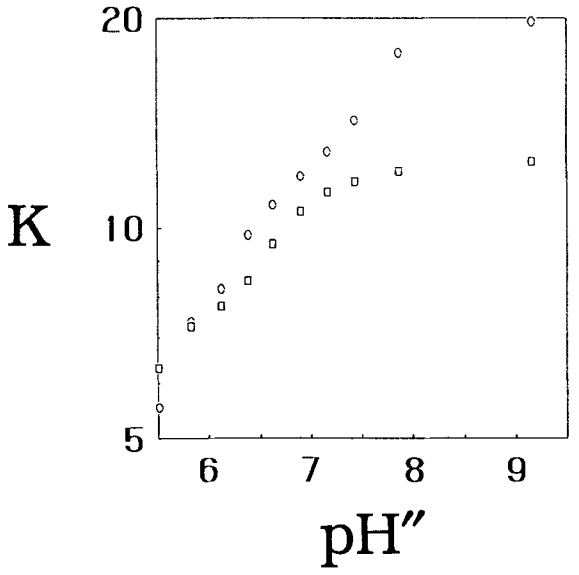

FIG. 1 Observed partition coefficients of 3-hydroxybenzyl alcohol (□) and 3-hydroxybenzoic acid (○) in a series of PEG/potassium phosphate aqueous two-phase systems.

come increasingly greater than the partition coefficient of the neutral alcohol. The results shown in Fig. 1 support these general predictions.

Figure 2 shows the partition coefficients of three organic acids in the series of PEG/potassium phosphate systems. The partition coefficients again increase with increasing pH. The greatest rate of increase in the partition coefficients occurs at the lowest pH. The difference in partition coefficients between these three solutes is most likely due to their difference in hydrophobicity (41). For example, the partition coefficient increase observed by adding a methoxy functional group to indole 3-acetic acid is 0.15–0.20 log units in each of the 10 systems. The partition coefficients of *p*-tolyl acetic acid are similarly consistently lower than the partition coefficients of indole 3-acetic acid.

Figure 3 shows the partition coefficients of three organic amines, analogous in structure to the acids, in the series of PEG/potassium phosphate systems. Like the acids, the greatest partition coefficient for each of these solutes occurs at the highest pH. Unlike the acids, however, the partition coefficients of the amines do not change significantly in the low pH phase systems. Again, the difference in partition coefficients between these solutes themselves may be attributed to differences in hydrophobicity. The order of partition coefficients in any one phase system is 5-methoxyindole

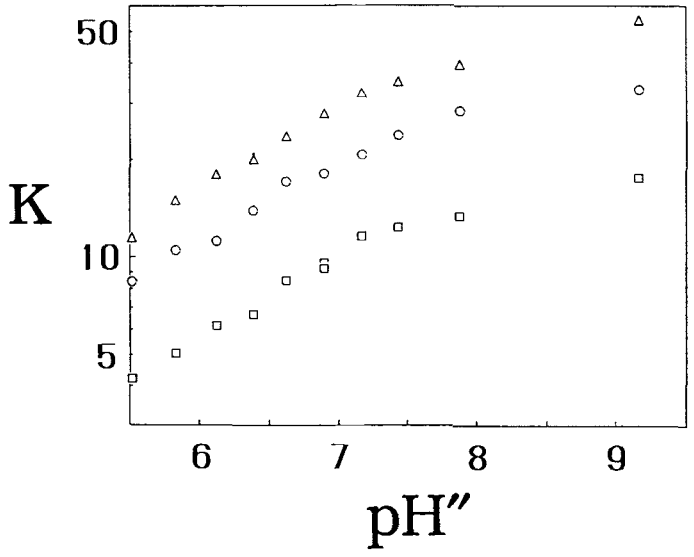

FIG. 2 Observed partition coefficients of three organic acids in a series of PEG/potassium phosphate aqueous two-phase systems: 5-methoxyindole 3-acetic acid (△), indole 3-acetic acid (○), and *p*-tolyl acetic acid (□).

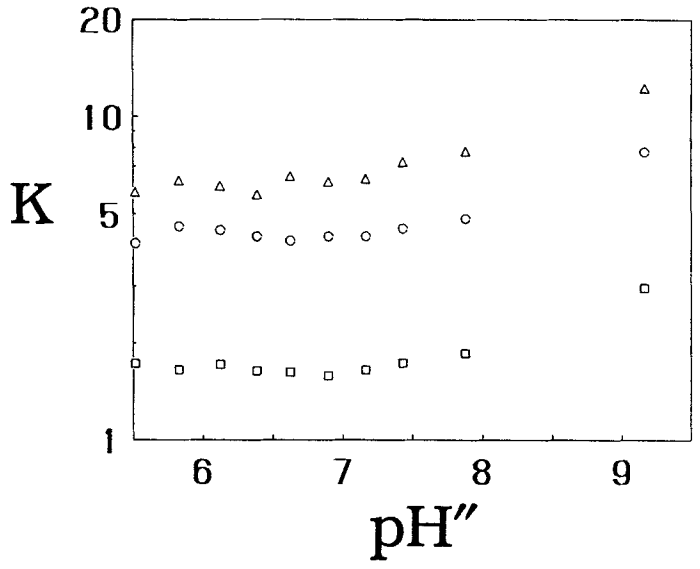

FIG. 3 Observed partition coefficients of three organic amines in a series of PEG/potassium phosphate aqueous two-phase systems: 5-methoxytryptamine (△), tryptamine (○), and 2-(*p*-tolyl) ethylamine (□).

tryptamine > tryptamine > 2-(*p*-tolyl) ethylamine, the same observed for the analogous acids. The difference in partition coefficients between 5-methoxyindole tryptamine and tryptamine in any one phase system is similarly 0.15–0.20 log units.

Equation (9) may be used to predict the ratio (R) of the partition coefficient of an acid to the partition coefficient of an analogous amine. Figure 4 shows the partition ratio for the three acid and amine pairs whose partition coefficients are shown in Figs. 2 and 3, respectively. The solid line is the ratio predicted by Eq. (9) using the discrete pH values measured in the 10 phase systems and a pK_{c1} of 4.75 and a pK_{b1} of 9.25 (the approximate values of each of the three pairs in water) (42). The observed partition ratio for each of the three solute pairs increases slowly with increasing pH to a maximum ratio at a pH of 7.5–8.0. For systems of positive pH difference between the phases, the model correctly predicts that the partition coefficients of each acid are greater than the partition coefficients of their analogous amine. Equation (9) predicts the observed partition ratio behavior particularly well at low pH, and also supports the observed maximum in the partition ratio at intermediate pH values. For each of the three pairs, the observed maximum is less than the maximum value predicted by Eq. (9).

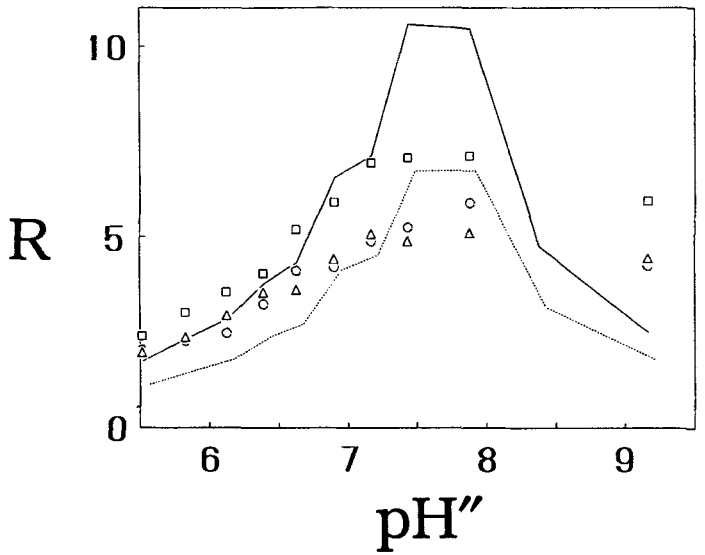

FIG. 4 Observed partition ratio ($R = K_N/K_P$) of pairs of analogous compounds in a series of PEG/potassium phosphate aqueous two-phase systems: 5-methoxyindole 3-acetic acid/5-methoxytryptamine (△), indole 3-acetic acid/tryptamine (○), and *p*-tolyl acetic acid/2-(*p*-tolyl) ethylamine (□). Solid line: Model prediction by Eq. (9). Dotted line: Model prediction by Eq. (9) with ΔpH set equal to 0.10 units below observation.

Several comments should be made concerning the use of Eq. (9) and the observed ratios. Identical values for the two dissociation constants, pK_{c1} and pK_{b1}, were used for all three acid and amine pairs in the calculation of the predicted ratio. As noted in the Introduction, an accurate value for a dissociation constant of a solute in each of these two-phase systems cannot be readily obtained. Fortunately, Eq. (9) is *not* sensitive to values of pK_{c1} and pK_{b1}, even when a value is close to the pH of one of the phases. A change of 0.50 pH units in the value of either constant does not alter the shape of the predicted ratio in Fig.4, and alters the predicted partition ratio by just over 5% only near the maximum.

However, the ratio predicted by Eq. (9) is rather sensitive to the pH of each phase. The dotted line in Fig. 4 shows the predicted partition ratio when the pH difference between the phases is assumed to be 0.10 pH units less than the value actually measured in the phase systems without any solute. The partition ratio predicted by Eq. (9) decreases at all values of pH with a decreasing pH difference between the phases. One possible explanation for the overprediction is small inaccuracies in the pH measurement, particularly at a pH of 7.0–8.0. Another possible explanation

for the differences between observed and predicted partition ratios is that the four activity ratios are not equal to 1 as they were assumed to be in the derivation of Eq. (9). Of course, another phenomenon not incorporated in the model may account for the observed behavior.

The model predictions and observations indicate and quantify some important effects that solute charge has on the partition coefficient. In systems of positive pH difference, like PEG/potassium phosphate systems, the partition coefficient of a negatively charged compound will be greater than a neutral compound of identical hydrophobicity. Similarly, the partition coefficient of a positively charged compound will be less than that of an otherwise identical neutral compound. A positively charged solute will not necessarily partition into the opposite phase as a negatively charged solute. Rather, the partition coefficient of a positively charged solute will be shifted from the partition coefficient of a negatively charged solute. Complicating this picture is the variation in the charge of solutes with pH. Also, the pH difference between the phases, an important term in Eq. (9), changes with the tie line length and by the addition of a solute (Eiteman, unpublished data).

If a single compound can be both negatively charged and positively charged (e.g., an amino acid), then it will have a relatively low partition

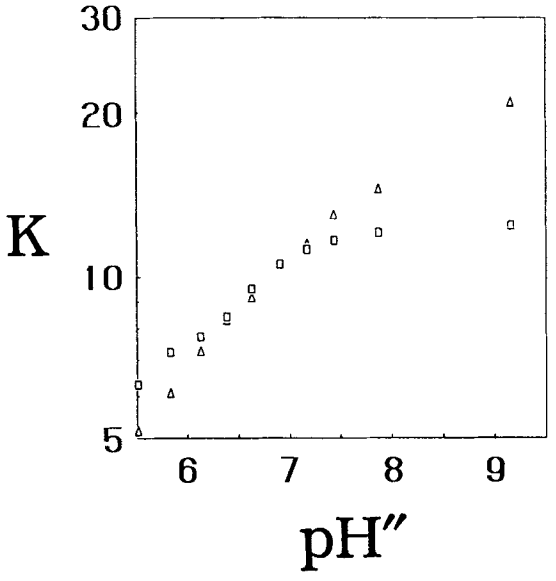

FIG. 5 Observed partition coefficients of 3-hydroxyanthranilic acid (△) and 3-hydroxybenzyl alcohol (□) in a series of PEG/potassium phosphate aqueous two-phase systems.

coefficient when it is positively charged and a relatively high partition coefficient when negatively charged. The partition coefficients are relative to a neutral compound of identical hydrophobicity. In order to examine this prediction, 3-hydroxyanthranilic acid was partitioned in the series of PEG/phosphate systems. Figure 5 shows the partition coefficients of this compound in addition to 3-hydroxybenzyl alcohol, assumed to approximate a neutral analog. These two solutes are assumed to have approximately the same hydrophobicity. When positively charged at low pH, 3-hydroxyanthranilic acid has partition coefficients below those of the analogous alcohol, as predicted. In contrast, at higher pH, where the acid is negatively charged, the acid has partition coefficients greater than the neutral compound. At intermediate values of pH, this compound has a net charge of 0 and partitions like the alcohol, as predicted.

CONCLUSIONS

As Eq. (9) predicts for a system having a positive pH difference between the phases, a negatively charged solute has a greater partition coefficient than an otherwise identical positively charged solute. The difference between the partitioning of oppositely charged solutes may be correlated to the pH difference between the phases. Solutes of different charge do not partition preferentially into different phases. Rather, changing the charge of a solute shifts the partition coefficient of the compound. The size of this shift depends on the pH difference between the phases and the dissociation constants for the two charged compounds. These principles may be useful to optimize extraction of biological molecules using aqueous two-phase systems.

All solutes studied were relatively hydrophobic, that is, they had partition coefficients greater than 1. No assumptions concerning solute hydrophobicity were made in the derivation of Eqs. (6), (7), and (9). Additional solutes must be partitioned to show the applicability of these equations to more hydrophilic solutes. Furthermore, the phase system used has a positive pH difference between the phases. Additional studies of partitioning of charged compounds will be necessary if a system of negative pH difference is identified.

NOMENCLATURE

a_i	activity of species i
c_i	concentration of species i
K	(measured) partition coefficient

K_{bi} equilibrium constant for dissociation between species of i positive charge and i − 1 positive charge
K_{ci} equilibrium constant for dissociation between species of i negative charge and i − 1 negative charge
R partition ratio ($= K_N/K_P$)
x_i mole fraction of species i
γ_i activity coefficient of species i
Λ_i activity ratio for species i ($= \gamma_{i-1}/\gamma_i$)

Subscripts

H hydrogen ion
0 neutral species
− negatively charged species
+ positively charged species
N solute which may be negatively charged
P solute which may be positively charged

Superscripts

′ upper phase
″ lower phase

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