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ANALYSIS OF SELF-ASSOCIATIONS FROM PARTITION ISOTHERMS

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Self-associations can be studied from the measurements of the partition of the self-associating solute between two immiscible liquids. The apparent partition coefficient, K_{app}, is proportional to the ratio of the apparent weight fraction of monomer, f_a, in each phase. If one assumes that the Adams-Fujita convention for the activity coefficients of the self-associating species applies, then f_a is related to M_{na} and M_{wa}, the apparent values of the number and weight average molecular weights, respectively; and one can use previously developed methods to analyze the self-association. In order to use the method, one must make an independent study at the same temperature of one of the phases by an appropriate thermodynamic method, such as vapor pressure osmometry or sedimentation equilibrium. Then one can test the other phase for the type of self-association present and evaluate the equilibrium constant or constants (k_i) and the nonideal term (BM_1) from the partition data. One can also evaluate the partition coefficient (K_{par}). From these measurements, one can obtain the free energy (ΔG^0) for the association in each phase and for the transfer between phases. Temperature-dependence studies will provide the enthalpy (ΔH^0) or entropy (ΔS^0) of self-association or transfer. This method should be quite useful for studying small molecules of biological importance.

1. Introduction

There is much interest in the partition of a solute or solutes between two liquid phases. Indeed, this formed the basis of Craig's [1,2] elegant countercurrent distribution method, which was used in the isolation of some small peptide hormones [3–6] and antibiotics [7,8]; this technique is also used in the fractionation of peptide mixtures [9]. Countercurrent distribution can be used to study self-associations and mixed associations, but most studies in this area have been restricted to simulations [10–13]. More recently, partition between two aqueous polymer phases has been used in the analysis and separation of biological macromolecules and cell particles [14,15]. Partition between an immobilized gel and a mobile, liquid phase has been used very elegantly in protein chemistry [16–18]; this method, known as analytical gel or molecular sieve chromatography, has been applied to the study of self-associations or mixed associations in protein solutions. Here we will discuss a different situation, namely, the partition of a self-associating solute between two immiscible liquids. In particular, we will discuss the analysis of self-associations from partition isotherm data; this is an area that has not been widely explored. This technique should be quite useful in studying small molecules of biological importance.

Partition isotherms have been used together with conductivity measurements to determine the distribution of hexadecylpyridinium chloride be-

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tween water and nitrobenzene at 25 °C [19]; these studies were conducted above and below the critical micelle concentration to determine the degree of dissociation and to measure the activity coefficients of the solute in both solvents. More recently, Czapkiewicz and Czapkiewicz-Tutaj [20–23] have studied partition isotherms of quaternary salts of the R'_3 NRBr type, above and below the critical micelle concentrations. The salt is completely dissociated in the aqueous phase and partially associated (in the form of ion pairs) in the organic phase. They used the Debye-Hückel theory to calculate mean ionic activity coefficients. At the critical micelle concentration, a sharp change in the slope of the partition isotherm occurs. Very recently, Vadnere and Lindenbaum [24] have used partition isotherms of various bile salts to study the distribution of these bile salts between 1-octanol and aqueous buffer solutions. From their partition data, they were able to study the self-association of the bile salts in aqueous solution and also to evaluate the partition (or distribution) coefficient. Their analysis was done assuming ideal solution conditions.

The purpose of this communication is to provide a general treatment for the analysis of self-associations under ideal or nonideal conditions from partition isotherms. It will be shown that one can obtain direct measurement of the weight fraction of monomer, f_1, in ideal solutions, or its apparent value, f_a, under nonideal conditions. Having f_a available at various concentrations, one can use previously developed relations [25–31] to obtain M_{wa}, the apparent weight average molecular weight, and M_{na}, the apparent number average molecular weight. For ideal self-associations, M_{na} and M_{wa} assume their true values of M_{nc} and M_{wc}, respectively. One can use f_a, M_{na} and M_{wa} to test for the presence or absence of a variety of self-associations, and once a model has been found, it is possible to evaluate the equilibrium constant or constants (k_i) and the nonideal term (BM_1). It will be shown that one can obtain the same information from partition isotherms of self-associating solutes that one does from membrane osmometry, vapor pressure osmometry, sedimentation equilibrium or elastic light scattering; in addition, one can obtain the partition coefficient for the system being studied.

2. Evaluation of the apparent weight fraction of monomer (f_a)

We follow the assumption by Nernst [32–34] that only monomer is partitioned between the two phases. There is no restriction on the self-association of the solute in either or both phases. This situation, illustrated in fig. 1, was also one of the schemes used by Vadnere and Lindenbaum [24]. The condition for phase equilibrium is [33–35]:

$$u_1^\alpha = u_1^\beta \tag{1}$$

$$T^\alpha = T^\beta$$

here u_1^i is the molar chemical potential of the monomer in phase i ($i = \alpha$ or β), and

$$\mu_1^i = (\mu_1^0)^i + RT \ln a_1^i$$

$$= (\mu_1^0)^i + RT \ln y_1^i c_1^i \tag{2}$$

in phase i. For the solute in phase i ($i = \alpha$ or β), $(\mu_1^0)^i$ represents the standard state chemical potential of the monomer, a_1^i is the activity, y_1^i the activity coefficient, and c_1^i the monomer concentration in g/l. It should be noted that

$$\lim_{c \to 0} y_1^i = 1 \tag{3}$$

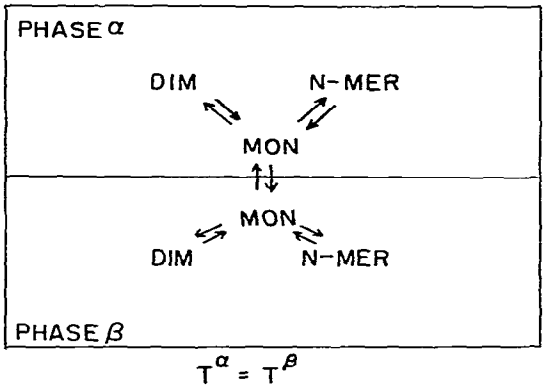

Fig. 1. Schematic representation of the partition of a self-associating solute between two phases α and β. The solute may or may not undergo self-association in both phases at the same time. It is assumed that the partition between phases occurs through the monomer only. The two liquids α and β are immiscible. The system is at constant pressure.

One can use eqs. 1 and 2 to obtain the partition coefficients, K_{par}, which for

$$(MON)^{\alpha} \leftrightarrows (MON)^{\beta} \tag{4}$$

is defined by

$$K_{par} = \frac{a_1^{\beta}}{a_1^{\alpha}} = \frac{c_1^{\beta}}{c_1^{\alpha}} \frac{y_1^{\beta}}{y_1^{\alpha}} = K_c K_y \tag{5}$$

K_c is simply the ratio of monomer concentrations and is not a true partition coefficient unless the solution is ideal, similarly, $K_y = y_1^{\beta}/y_1^{\alpha}$ is simply a convenient symbol for the ratio of activity coefficients. K_{par} is related to the standard state chemical potentials by

$$K_{par} = \exp\left[\left\{(\mu_1^0)^{\alpha} - (\mu_1^0)^{\beta}\right\}/RT\right] \tag{6}$$

Now note that

$$K_c = \frac{c_1^{\beta}}{c_1^{\alpha}} = \frac{K_{par}}{K_y} \tag{7}$$

and

$$\lim_{c \to 0} K_c = K_{par} \tag{8}$$

since

$$\lim_{c \to 0} K_y = \lim_{c \to 0} \frac{y_1^{\beta}}{y_1^{\alpha}} = 1 \tag{9}$$

While one can define K_{par} by eq. 5 one cannot measure K_{par} directly since it is impossible to isolate physically the monomer from a self-associating system without destroying the chemical equilibrium. Instead, one defines K_{app}, the apparent partition coefficient, where

$$K_{app} = c^{\beta}/c^{\alpha} \tag{10}$$

If self-association occurs in both phases, then the total solute concentration in phase i, c', becomes

$$c^i = (c_1 + c_2 + c_3 + \ldots)^i = \left(c_1 + k_2 c_1^2\left(y_1^2/y_2\right) + k_3 c_1^3\left(y_1^3/y_3\right) + \ldots\right)^i \tag{11}$$

$i = \alpha$ or β

$c_j = k_j c_1^j\left(y_1^j/y_j\right), j = 2, 3, \ldots$

One can write K_{app} as

$$K_{app} = \frac{c_1^{\beta}\left[1 + k_2 c_1\left(y_1^2/y_2\right) + k_3 c_1^2\left(y_1^3/y_3\right) + \ldots\right]^{\beta}}{c_1^{\alpha}\left[1 + k_2 c_1\left(y_1^2/y_2\right) + k_3 c_1^2\left(y_1^3/y^3\right) + \ldots\right]^{\alpha}} = \frac{c_1^{\beta}}{c_1^{\alpha}} \frac{f_1^{\alpha}}{f_1^{\beta}} = \frac{K_{par}}{K_y} \frac{f_1^{\alpha}}{f_1^{\beta}} \tag{12}$$

here f_1^i is the weight fraction of monomer in phase i.

$$f_1^i = (c_1/c)^i \; (i = \alpha \text{ or } \beta) \tag{13}$$

and

$$1/f_1^i = (c/c_1)^i = \left(1 + k_2 c_1\left(y_1^2/y_2\right) + k_3 c_1^2\left(y_1^3/y_3\right) + \ldots\right)^i \tag{14}$$

It is now possible to evaluate K_{par} from careful measurements of K_{app} at various concentrations, since

$$\lim_{c \to 0} K_{app} = K_{par} \tag{15}$$

Note that as the total concentration, $c \to 0$, $f_1^i = 1$ because of the law of mass action; it is also evident from eq. 14 that $\lim f_1^i = 1$. In addition, $\lim_{c \to 0} y_1^i = 1$ [33–35] and $\lim_{c \to 0} K_y = 1$. At this point, let us define a function Q, where

$$Q = \frac{K_{app}}{K_{par}} = \frac{1}{K_y} \frac{f_1^{\alpha}}{f_1^{\beta}} = \frac{y_1^{\alpha} f_1^{\alpha}}{y_1^{\beta} f_1^{\beta}} \tag{16}$$

We now consider how to use Q in the analysis of self-associations under various situations. Note that Q contains the weight fraction of monomer in both phases.

2.1. Case I

Suppose that there is no association of solute in phase α, then

$f_1^{\alpha} = 1$ (no self-association)

Now if the amount of solute in phase α is very small so that one might have to use radiolabelled solutes in order to detect the solute in phase α,

then one might assume $y_1^\alpha = 1$ and for this case

$$\frac{1}{Q_I} = f_1^\beta y_1^\beta \tag{17}$$

$(f_1^\alpha = 1$ and $y_1^\alpha = 1)$

One can also obtain the activity of monomer in the β-phase, since $c^\beta/Q = c_1^\beta y_1^\beta = a_1^\beta$. If one makes appropriate assumptions about the activity coefficient, y_1^β, then one can proceed with the analysis of self-association in phase β. This will be discussed later.

2.2. Case II

Suppose that there is no association of solute in phase α so that $f_1^\alpha = 1$, but we will assume that the solute is quite soluble in both phases so that y_1^α is not necessarily one. If the solution α were ideal then $y_1^\alpha = 1$ and one would use case 1. If $y_1^\alpha \neq 1$ then it is necessary to find values of y_1^α as a function of c^α at the desired temperature, or else one will have to determine y_1^α experimentally, which could be done by vapor pressure osmometry [28], for example. If it is assumed in phase α that

$$\ln y_1^\alpha = (BMc)^\alpha \tag{18}$$

where B is a constant whose value depends on the temperature T and the solvent α, M is the true molecular weight of the solute and c^α is the solute concentration, then one can obtain the apparent molecular weight M_{app} from [25]:

$$\Delta E = K_{vp}(c/M_{app})^\alpha \tag{19}$$

where ΔE is the imbalance (in μV) of the Wheatstone bridge in the vapor pressure osmometer. K_{vp} is the calibration constant for the vapor pressure osmometer; it is determined from a separate calibration experiment in the same solvent and at the same T on the substance of known molecular weight. M_{app} is related to M by

$$\frac{1}{M_{app}} = \frac{1}{M_1} + \frac{Bc}{2} \tag{20}$$

The activity coefficient can be determined from the osmotic coefficient, g, since [28]

$$g = \frac{M}{M_{app}} = 1 + \frac{BMc}{2} \tag{21}$$

and

$$\ln y_1 = 2(g-1) \tag{22}$$

Having a value of y_1, one then obtains

$$\frac{1}{Q_{II}} = \frac{y_1^\alpha}{Q} = f_1^\beta y_1^\beta \tag{23}$$

The analysis of the self-association in phase β will then be done in the same way as it is with the other cases.

2.3. Case III

There is self-association in both phases, and the solute is readily soluble in both phases. For this case, one would have to study the self-association of the solute in either solutions of α or β at the same temperature T. Generally one should choose the solution whose solvent has the lower latent heat of vaporization (ΔH_v), since ΔE is inversely proportional to ΔH_v. The self-association of the desired solution (α or β) would be studied by various thermodynamic techniques, such as vapor pressure osmometry or sedimentation equilibrium. For an organic solvent vapor pressure osmometry would be the method of choice [26,28,30,31], and assuming that this technique is used, one uses the following relations. Assume that in the desired solution [25–31]

$$\ln y_i = iBM_1c \tag{24}$$

for the associating solute. Then one obtains M_{na} from ΔE measurements, since [26,28,30,31]

$$\Delta E = K_{vp}(c/M_{na}) \tag{25}$$

where M_{na} is the apparent number average molecular weight and K_{vp} the vapor pressure osmometer calibration constant. M_{na} is related to M_{nc} by

$$\frac{M_1}{M_{na}} = \frac{M_1}{M_{nc}} + \frac{BM_1c}{2} = g \tag{26}$$

where g is the osmotic coefficient. One can also obtain M_{wa} and $\ln f_a$ and use them to test for the presence or absence of a variety of self-associations using previously described methods. Once the self-association in one of the phases has been

characterized, then eq. 16 becomes

$$\frac{y_1^{\alpha} f_1^{\alpha}}{Q} = \frac{1}{Q_{III}} = y_1^{\beta} f_1^{\beta} \tag{27}$$

The problem at hand is how to analyze the $y_1^i f_1^i$ ($i = \alpha$ or β) data when self-association occurs in either or both phases. For simplicity, we will use $1/Q$ in the discussion that follows, although one must bear in mind which one of the three cases one is encountering.

3. Analysis of self-associations from partition isotherms

In order to use partition isotherm data, it is necessary to assume some descriptive function for the activity coefficient y_1 ($i = \alpha$ or β). A very useful description of the activity coefficient has been to assume that eq. 24 is obeyed. When this is true, then [25–31]

$$y_n / y_1^n = 1 \tag{28}$$

$$y_1 = e^{BM_1 c} \tag{29}$$

and

$$f_1 y_1 = f_1 e^{BM_1 c} = f_a = 1/Q \tag{30}$$

Note that

$$\lim_{c \to 0} f_a = \lim_{c \to 0} 1/Q = 1 \tag{31}$$

This f_a is the same f_a obtained by other thermodynamic methods (vapor pressure osmometry, sedimentation equilibrium, membrane osmometry and elastic light scattering). We can use f_a to evaluate M_{wa} and M_{na}. First one notes that [25–31]

$$\ln f_a = \ln f_1 + BM_1 c = -\ln Q \tag{32}$$

and that

$$\frac{M_1}{M_{wa}} = 1 + \frac{\mathrm{d} \ln f_a}{\mathrm{d} \ln c} = 1 - \frac{\mathrm{d} \ln Q}{\mathrm{d} \ln c} \tag{33}$$

where

$$\frac{M_1}{M_{wa}} = \frac{M_1}{M_{wc}} + BM_1 c \tag{34}$$

Also one notes that

$$\frac{cM_1}{M_{na}} = \int_0^c \frac{M_1}{M_{wa}} \mathrm{d}c \tag{35}$$

becomes

$$\frac{cM_1}{M_{na}} = c - \int_1^{(1/Q)} c \mathrm{d} \ln Q = c + c \ln(1/Q) - \int_0^c \ln(1/Q) \mathrm{d}c \tag{36}$$

and

$$\frac{M_1}{M_{na}} = 1 - \frac{1}{c} \int_1^{(1/Q)} c \mathrm{d} \ln Q = 1 - \ln Q + (1/c) \int_0^c \ln Q \mathrm{d}c \tag{37}$$

The relationship between M_{na} and M_{nc} is given by eq. 26. Now one can use the same relationship used in the other methods to analyze self-associations. Two useful relations, in which the nonideal term BM_1 has been eliminated, are [27,28]

$$\xi = \frac{2M_1}{M_{na}} - \frac{M_1}{M_{wa}} = \frac{2M_1}{M_{nc}} - \frac{M_1}{M_{wc}} = 1 + \frac{2}{c} \int_0^c \ln Q \mathrm{d}c + \frac{\mathrm{d} \ln Q}{c \ln c} - 2 \ln Q \tag{38}$$

or

$$\nu = \frac{2M_1}{M_{na}} - \ln f_a = \frac{2M_1}{M_{nc}} - \ln f_1 = 2 + \frac{2}{c} \int_0^c \ln Q \mathrm{d}c - \ln Q \tag{39}$$

These values of the quantities ξ and ν are independent of the model for the association, yet one can use them and also the quantity, $\eta = \frac{M_1}{M_{wa}} - \ln f_a$, to test for the presence or absence of a variety of self-associations and also evaluate the equilibrium constant or constants (k_i) and the nonideal term (BM_1).

For example, assume that a monomer-n-mer association is present. This is described by

$$nP_1 \leftrightarrows P_n \ (n = 2, 3, \ldots) \tag{40}$$

where P represents the self-association solute.

Whenever eq. 24 applies, the total solute concentration becomes [22–28]

$$c = c_1 + k_n c_1^n \tag{41}$$

Furthermore, one notes that

$$f_1 = c_1/c \tag{13}$$

$$f_n = \frac{k_n c_1^n}{n} = 1 - f_1 \tag{42}$$

and since $c_1 = f_1 c$ it follows from eq. 42 that

$$\frac{1 - f_1}{f_1^n} = k_n c^{n-1} \tag{43}$$

One can evaluate f_1 from ν; recall that ν is obtained from eq. 39. For a monomer-n-mer association, ν is given by

$$\nu = \frac{2 + 2(n-1)f_1}{n} - \ln f_1 \tag{44}$$

since

$$\frac{M_1}{M_{n\alpha}} = f_1 + \frac{f_n}{n} = \frac{nf_1 + f_n}{n} = \frac{1 + (n-1)f_1}{n} \tag{45}$$

For each choice of n ($n = 2, 3, \ldots$), one can solve for f_1 by successive approximations since $0 < f_1 \leq 1$. These values of f_1 are used in plots based on eq. 43. For the correct choice of n, one will obtain a linear plot, going through the origin or close to it, with a slope of k_n for the correct choice of n. If the plot curves, deviates significantly from an intercept at or near the origin, or gives negative values for k_n, then the model fails and other models have to be tried. Once a model has been found, one can evaluate BM_1, the nonideal term from eq. 26 or eq. 32.

Once values of k_n are obtained, they can be converted to molar association constants K_n, since

$$K_n = k_n M_1^{(n-1)}/n, \qquad n = 2, 3, \ldots \tag{46}$$

Since the molecular weight of monomer is the same in both phases K_{par} can be used as it stands to calculate the free energy of transfer. Thus the free energy of self-association or transfer is obtained from

$$\Delta G_t^0 = -RT \ln K_t \quad (K_t = K_n \text{ or } K_{par}) \tag{47}$$

The enthalpy of self-association or transfer, ΔH_t^0, is obtained from a Van't Hoff plot of $\ln K_t$ vs. $1/T$, since

$$[\partial \ln K_t/\partial(1/T)]_p = -\Delta H^0/R \tag{48}$$

Finally, the entropy of self-association or transfer is available from

$$\Delta S_t^0 = (\Delta H_t^0 - \Delta G_t^0)/T \tag{49}$$

4. Discussion

What is exciting about this treatment is that it shows how one can obtain f_n, M_{na} and M_{wa} (or their true values under ideal conditions) from partition isotherms. Thus, this method can provide the same information that other thermodynamic methods (vapor pressure osmometry, membrane osmometry, elastic light scattering, sedimentation equilibrium) used for studying self-associations provide [25–31]. This treatment establishes a link between these methods. From partition isotherms one can also obtain values of the partition coefficients. In order to analyze self-associations, it is necessary to establish the degree of aggregation for one of the phases (probably the organic phase) by an independent measurement, such as vapor pressure osmometry or other appropriate thermodynamic techniques. This way one of the $y_1' f_1'$ ($i = \alpha$ or β) values in eq. 16 can be determined. If vapor pressure osmometry is used, one must have a nonvolatile solute and a one-component solvent. With mixed solvents, the vapor composition is not the same as that of the solvent mixture, and the interpretation of the experimental data could be difficult. Although the example discussed here was a monomer-n-mer association, the partition isotherm method can be applied to a wide variety of indefinite and discrete self-associations using previously described methods [25–31].

Besides characterizing a self-association, this method provides a measurement of the partition coefficient. When these studies are performed at different temperatures, then one can obtain the thermodynamic functions (ΔH^0, ΔG^0 and ΔS^0) for the self-association and also for the transfer

between phases (see eqs. 47–49), which could mimic transfer from an aqueous phase to a membrane. It should be noted that Vadnere and Lindenbaum [24] did do temperature-dependent studies in their work on the distribution of bile salts between aqueous buffer solutions and 1-octanol.

In order to determine K_{par}, it is necessary to measure K_{app} (see eq. 10) carefully. If the material is slightly soluble in one of the solvents, then one could use radiolabelled compounds and measure the radioactivity in both phases using a scintillation counter or some other apparatus to detect the radioactivity. Vadnere and Lindenbaum [24] used this procedure in their studies on bile salt self-associations. When the solute absorbs visible or ultraviolet light, one can use a spectrophotometer to monitor the concentration in each phase. If this does not apply, there may be a sensitive colorimetric test for the solute that could be used. Some procedures that others have used to study association phenomena from partition isotherm measurements are described in refs. 33 and 34. The procedure to the followed depends on the material being studied.

The concentration dependence of the partition isotherm as well as a plot of c^{α} vs. c^{β} can provide some insights into the solution behavior of the system. For an ideal system with no self-association, a plot of K_{app} vs. c should be constant. On the other hand, if self-association occurred, then one would obtain curved plots of K_{app} vs. c; this was encountered by Vadnere and Lindenbaum [24] in their studies on bile salt self-association. At constant T, one can make plots of c^{α} vs. c^{β} to see if there is an abrupt change in the slope, which indicates a very strong self-association and the formation of micelles; some refer to the concentration at which the abrupt slope change occurs as the critical micelle concentration. This type of behavior was encountered by Czapkiewicz and Czapkiewicz-Tutaj [22] in their studies with quarternary ammonium bromides. When Vadnere and Lindenbaum [24] made similar plots at constant T of $c_{organic}$ vs. $c_{aqueous}$ for their bile salt studies, they obtained smooth plots without abrupt slope changes, and this was attributed to a reversible self-association in which several species are present. This is in accord with other studies on bile salt self-association [36,37].

One feature of this technique is that it allows one to study a stronger self-association from measurements of partition isotherms and independent characterization of the weaker self-associating system. Suppose that a nonionic detergent were partitioned between water and an organic solvent. Further, suppose the detergent is readily soluble in both solvents, but that it associates more strongly in water. If the size of the aggregates (micelles) in the aqueous solution were such that M_{nc} or M_{na} were greater that 10000 (this is about the upper limit of detection for vapor pressure osmometry), then one could not do vapor pressure osmometry experiments on the aqueous solutions. In order to study independently the self-association in the aqueous phase, one would have to resort to sedimentation equilibrium or elastic light scattering experiments. This would require determination of the refractive index increment for both techniques, and also determination of the solute's partial specific volume for sedimentation equilibrium experiments. With light scattering experiments one must strive to remove all dust from the solution, otherwise the experimental results would have a larger error. Sedimentation equilibrium experiments take time, and it is usually difficult to perform experiments above 30 °C because of drive oil fogging the schlieren lenses. So, if one characterizes the behavior of the solute in an organic phase by vapor pressure osmometry, then knowing this information plus having knowledge of the partition coefficient allows one to analyze the self-association in the aqueous phase, as Vadnere and Lindenbaum [24] have demonstrated in their studies with bile salts.

The partition isotherm method should be a very powerful method for studying self-associations of small molecules of biological importance. It can be applied to ionizable or non-ionizable solutes. When the solute ionizes in aqueous solutions one may have to pay attention to the various factors affecting the self-association pH, buffer concentration, and ionic strength [24]. In principle, the partition isotherm method should be applicable to macromolecules, but here one has the problems of surface denaturation of proteins at the interface of aqueous and organic solvents, as well as problems of

solubility in the nonaqueous solvent. This was a limitation of Craig's elegant countercurrent distribution method [9,38].

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