Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Rabie, Hamid R. and Vera, Juan H.(1998) 'A Simple Model for Reverse Micellar Extraction of Proteins', Separation Science and Technology, 33: 8, 1181 — 1193
To link to this Article: DOI: 10.1080/01496399808545248
URL: http://dx.doi.org/10.1080/01496399808545248

SEPARATION SCIENCE AND TECHNOLOGY, 33(8), pp. 1181–1193, 1998

A Simple Model for Reverse Micellar Extraction of Proteins

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ABSTRACT

A simple thermodynamic model is developed for the extraction of proteins with reverse micelles formed with the contact method. The model is based on the ion-exchange reaction of the protein and the surfactant counterion at the reverse micellar interface. Using the equilibrium constant for this reaction and the equilibrium constants of the protein reactions in the aqueous phase, a simple expression is derived for the effects of salt type and concentration, pH, surfactant concentration, and volume ratio of two phases on the extraction. Results on the extraction of α-chymotrypsin into dioctyldimethyl ammonium chloride (DODMAC) reverse micelles are well predicted by the model. The negatively charged proteins are extracted from the aqueous phase by exchanging with the Cl^- counterion of DODMAC at the reverse micellar interface. The presence of counterions different from chloride in the system, which are introduced through addition of a salt, has a significant effect on the extraction. The added counterions are exchanged with the chloride of the surfactant at the reverse micellar interface, therefore changing the nature of the surfactant. This change in the nature of the surfactant, in turn, alters the extraction of negatively charged proteins.

INTRODUCTION

Batch-type processes such as column chromatography, salt or solvent precipitation, and electrophoresis are still used for separation and purification of many proteins on a large scale. There is a clear need for efficient, scalable bioseparation processes that can be operated on a continuous basis. Liq-

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uid–liquid extraction technology has been recognized as potentially useful for this purpose (1, 2). Two classes of two-phase extraction systems are suitable for protein recovery: 1) biphasic aqueous polymer systems and 2) systems in which an organic reverse micellar phase is in equilibrium with a conjugated aqueous phase. After proteins are extracted to the reverse micellar phase, they can be backextracted by contacting the organic phase containing the proteins with a fresh aqueous phase at a proper pH or at high ionic strength. The present study deals only with the initial extraction of proteins using the contact method.

There have been several experimental studies on the extraction of proteins with reverse micelles. Reverse micelles have been employed for the extraction and purification of several industrially relevant proteins, including amylases (3), proteases (4), lipases (5), and food proteins (6). Using reverse micelles, proteins have been successfully extracted from complex feed mixtures including whole and distributed cells (7), fermentation broths (8), and dried solids (9).

Two important experimental findings have been reported by various groups: 1) positively charged proteins are preferentially extracted into anionic surfactant systems, whereas negatively charged proteins are preferentially extracted into cationic surfactant systems; and 2) extraction decreases with ionic strength. Based on these results, it has been hypothesized that the main factors affecting the partitioning of proteins are electrostatic interactions and size exclusion phenomena (6, 10, 11). The addition of salt, and consequently the increase of the ionic strength, has been proposed to have two effects: 1) electrostatic interactions between charged protein molecules and charged surfactant head groups are decreased due to Debye screening, thus reducing extraction; and 2) due to shrinkage of the reverse micelles with the addition of salt, proteins are excluded from the reverse micelles (6, 12).

As discussed by Rabie et al. (13), there are some discrepancies in the above hypotheses, and they indicate that the precise relations among different parameters affecting extraction are not yet clear. For example, it was found that size exclusion is more pronounced with sodium than with potassium or cesium in a sodium bis-2-(ethylhexyl) sulfosuccinate, AOT, reverse micellar system (14). It is known, however, that AOT reverse micelles are significantly larger in the presence of sodium than in the presence of potassium or cesium (15).

There have been relatively few studies on the modeling of reverse micellar extraction of proteins. Bonner et al. (16) were among the first to consider this problem. They hypothesized that in order to encapsulate a protein inside a reverse micelle, small reverse micelles must combine to form a bigger reverse micelle, thus providing an extra volume equal to that of the protein. Woll and Hatton (17) used a similar idea with the extension of relating the

size of the filled reverse micelles to the protein size and charge, and developed a phenomenological model for the effect of pH and surfactant concentration at a fixed ionic strength. The model, however, was found to be correlational but not predictive (18).

Bratko et al. (19), in their shell and core model, assumed that electrostatic interactions and the ideal mixing of the proteins into the micellar solution are the important model constituents. The predictions of this model appear to agree with the experimentally observed protein solubilization as a function of salt concentration. However, this agreement is a direct consequence of their use of a Langmuir-type isotherm for the solubilization and does not reflect any inherent quantitative capability of the model (18). Later on, other groups proposed other solubilization models (12, 20). However, because of the complexity of the protein molecules and of the system, the partitioning behavior is still not predictable.

Some authors recently demonstrated that the extraction of a protein molecule into an organic phase containing ionic surfactants is due to direct interaction between the protein and individual surfactant molecules. Carlson and Nagarajan (21) speculated that the first step in extraction of a protein into an organic phase is the formation of a transferable complex between protein and surfactant molecules. Adachi and Harada (22) showed that extraction of cytochrome c by the contact method involves complexation between AOT and cytochrome c. Matsuura et al. (23) showed that insulin could be solubilized in 1-octanol by complexing the protein with the ionic surfactant SDS (sodium dodecyl sulfate).

Much of the previous work on protein extraction has focused on the use of AOT and triocylmethyl ammonium chloride (TOMAC) (24), and little attention has been paid to develop new reverse micellar systems for this emerging technology. An important limitation for these two surfactant systems is that for most proteins solubilization occurs in a narrow pH range (about 1–2 pH units) (12).

Recently, a new reverse micellar system using dioctyldimethyl ammonium chloride (DODMAC) has been employed to extract proteins. This system does not have the limitations of AOT and TOMAC for the pH range of extraction (13). The mechanism by which proteins are extracted into the reverse micelles was found to be the ion-exchange reaction of negatively charged proteins and the positively charged surfactant head groups in the reverse micellar phase. It was also observed that the nature of the solvent, the nature of the cation of a salt, and the concentration of the cosurfactant had no significant effect on the extraction of proteins with DODMAC. In the present study a simple model based on ion-exchange reactions is developed to predict the extraction of proteins into the reverse micellar phase. The pre-

dictions of the model are compared with experimental results obtained for extraction of α-chymotrypsin with DODMAC.

MATERIALS AND METHODS

The commercial surfactant Bardac LF-80 was obtained from Lonza Inc. (Fair Lawn, NJ). This surfactant contains 80 wt% DODMAC in an ethanol water solution. It was concentrated by vacuum evaporation as explained by Rabie and Vera (25). Reagent grade isooctane from Fisher Scientific (Montreal, QC), and Karl Fischer solvent from BDH Inc. (Toronto, ON) were used. α-Chymotrypsin (bovine pancreas) was obtained from Sigma (Saint Louis, MO) and used as received. The molecular weight (MW) and the isoelectric point (pI) of this protein are 25,000 and 8.6, respectively. All other chemicals were obtained from A&C American Chemicals Ltd. (Montreal, QC). Deionized water with an electrical conductivity lower than 0.8 μS/cm was used for all experiments.

The experimental procedure is shown schematically in Fig. 1. The initial organic phase was prepared by adding purified surfactant to decanol to obtain the desired molar ratio of decanol to DODMAC (2.5/1). Organic solvent was then added to make up the required volume. An aqueous electrolyte solution containing protein and salt was then contacted with the organic solution. The pH of the initial aqueous phase was adjusted by adding HCl or NaOH. The volume ratio of the two phases was set at unity. The phases were vigorously shaken for 2 hours at 23°C and then left to stand for 1 week at the same temperature. The phases were then separated for analysis. Sampling at regular periods ensured that the settling time used here was adequate to obtain equilibrium.

The water content in the organic phase was measured by a Karl Fischer titrator Model 701 (Metrohm Ltd., Herisau, Switzerland). The pH of the aqueous phase was measured by a Model 691 pH meter (Metrohm, Ltd.). The concentrations of proteins in the aqueous phase were measured by a Cary 1/3 UV spectrophotometer (Varian Techtron Pty. Ltd., Victoria, Australia) at 280 nm (A_{280}). The concentration of protein solubilized in the organic phase was also measured by UV spectrophotometry as explained by Rabie et al. (13).

SOLUBILIZATION REGIONS

As shown in Fig. 1, there are essentially four distinct regions in a reverse micellar system where a solute can be solubilized. They are: (i) the bulk of the organic phase, (ii) the interface of the surfactant hydrophilic groups and the water pool in the reverse micelles, (iii) the water pool inside the reverse

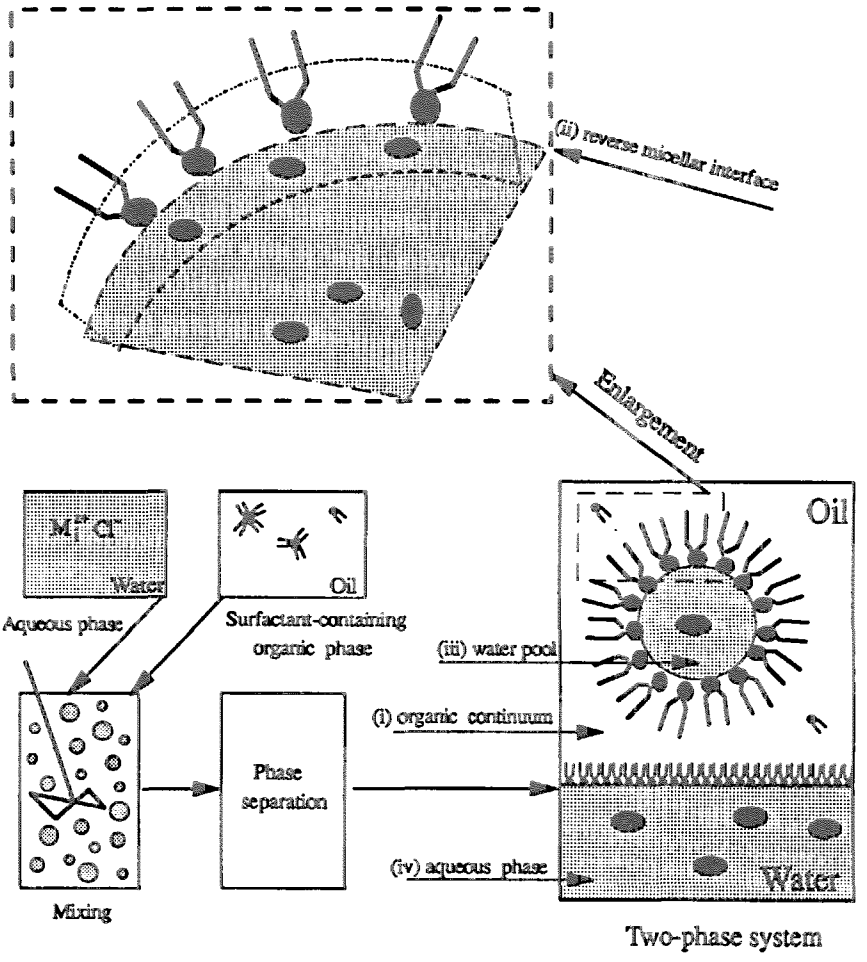

FIG. 1 Diagram of the experimental procedure and the solubilization sites (solid dot with double legs), DODMAC surfactant (cross-hatched oval), solute.

micelles, and (iv) the excess aqueous phase. Salts and proteins are essentially insoluble in organic solvents. Therefore, only the latter three regions are considered in this study.

The reverse micellar interface is where the heads of the surfactant molecules are placed shielding the water pool from contact with the organic solvent. The interface is considered here to be a uniform solubilization environment. As shown by Rabie and Vera (26–28), the interface is chemically active

due to strong electrostatic effects of the surfactant head groups. Different solutes are able to react with the surfactant head groups and form different complexes. Thus, the nature of the solute at the interface is different from that in the water pool, where the solute is more likely to be in a similar state as in the excess water. The concentrations of different solutes in the water pool can be assumed to be the same as those in the excess aqueous phase (27–29).

MODELING

The initial and final conditions of the system are described as follows. In the initial state, an aqueous phase of volume V^0 containing H_2O, chloride anion, hydroxide anion, and protein with concentrations C^0_{Cl} and C^0_{OH} and C^0_P, respectively, is contacted with an organic phase of volume $\overline{V}^0$ containing organic solvent and the surfactant with concentration $\overline{C}^0_S$. In the final state (at equilibrium), the aqueous phase has a volume V and it contains surfactant, chloride anion, hydroxide anion, and proteins with different charge numbers at concentrations C_S, C_{Cl}, C_{OH}, and C_{P^z}, respectively. The organic phase of volume $\overline{V}$ contains organic solvent, surfactant ($\overline{C}_S$), bound anions to the surfactant head groups at the reverse micellar interface ($\overline{C}_{i,b}$), chloride anion ($\overline{C}_{Cl^-}$), ions in the water pool, and H_2O in reverse micelles.

The concentrations of the surfactant and the bound ions in the organic phase are defined as moles of the surfactant or bound ion per unit volume of water-free organic phase. This volume is the same as the initial organic phase volume since the solubility of organic solvent in water is very small. It has been found that the fraction of DODMAC in the bulk water at equilibrium is negligible in the presence of salts (25), so the concentration of surfactant in the organic phase at equilibrium can be assumed to be the same as the initial value.

The model proposed here for extraction of proteins with reverse micelles in a Winsor type II system is based on the following assumptions:

(1) The concentration of any solute in the water pool is assumed to be the same as that in the excess aqueous phase at equilibrium.
(2) At equilibrium, all the surfactant is in the organic phase participating at the reverse micellar interface.
(3) The equilibrium constant of ion-exchange reactions can be expressed in terms of concentrations.
(4) The solubility of solutes in the organic solvent is negligible.
(5) The presence of protein has no significant effect on the distribution of other ions.

(6) Although a protein molecule can have different charge numbers, for simplicity it is assumed that only two forms of protein exist. These are a protein at its isoelectric point having no net charge and a protein carrying z negative charges.

Assumptions (1) to (3) have already been discussed in detail elsewhere (28). Assumption (4) holds for proteins and salts. Assumption (5) is valid under the experimental conditions of this study since the concentration of protein used here is extremely small. A concentration of 1 g/L of α-chymotrypsin is equivalent to 40 μM. Assumption (6) is based on the fact that due to the steric effects (considering the geometry of reverse micelles and the conformation of protein), a protein molecule with several charges cannot bind all of the charges to the surfactant head groups. Therefore, there should be a maximum number of binding sites for a specific protein, even though this number cannot be measured experimentally.

The positively charged proteins do not play any major role in extraction of proteins with DODMAC; therefore, there is no need to consider them in this study. The value of z was determined by trial and error. Different values were assumed in order to obtain the best fit of the model presented below and the experimental data of protein extraction presented in Fig. 2 (discussed in the following section). When increasing the value of z did not enhance the

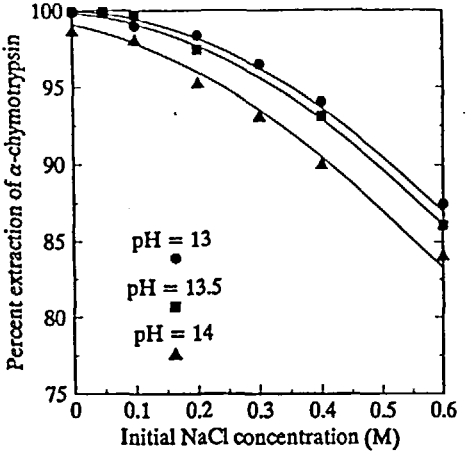

FIG. 2 Extraction of α-chymotrypsin as a function of NaCl concentration for different pHs: initial organic phase, 100 mM DODMAC, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein. The data are from the present study. The solid curves show the results obtained with the model using $z = 2$.

correlation of experimental results, the lowest value was chosen. In this way the value of z was obtained as 2. The present model is developed for a cationic surfactant like DODMAC in the presence of one protein and hydroxide and chloride anions. It can be applied to other cationic or anionic surfactants with ad-hoc modifications, and it can be extended to systems containing more than one protein and two anions.

Proteins in the aqueous phase at their isoelectric points have no net charge. However, in the presence of acids or bases they undergo a series of reactions which results in a net charge on the protein molecule. Proteins can gain one or several charges, positive or negative, depending on the pH. For example, α-chymotrypsin can have charges from -16 to $+14$ in a pH range varying from 12 to 3 (30). The reaction of a protein molecule at its isoelectric point (P^i) with hydroxide to produce a protein with z negative charges (P^{z-}) can be represented by

$$P^i + zOH^- \rightleftarrows P^{z-} \tag{1}$$

The equilibrium constant of Reaction (1) in terms of concentrations is

$$K_{P^z} = \frac{C_{P^{z-}}}{C_{P^i}(C_{OH})^z} \tag{2}$$

The negatively charged protein in the water pool undergoes an ion-exchange reaction with the surfactant counterion, and this is written as

$$zSCl + P^{z-} \rightleftarrows S_zP + zCl^- \tag{3}$$

where the protein anion P^{z-} in the water pool replaces the surfactant (SCl) bound counterion, and releases chloride anions into the water pool and into the excess aqueous phase. The equilibrium constant of Reaction (3) in terms of concentrations is

$$K_S^{P^{z-}-Cl} = \left(\frac{\overline{C}_{P^{z-},b}}{C_{P^{z-}}}\right)\left(\frac{C_{Cl}}{\overline{C}_{Cl,b}}\right)^z \tag{4}$$

In Eq. (4) the concentrations of protein anion and chloride anion in the excess aqueous phase are used instead of their concentrations in the water pool based on Assumption (1). For simplicity, the symbol $K_S^{P^z}$ is used instead of $K_S^{P^{z-}-Cl}$ in the following equations.

The mass balance of protein is then formulated as

$$C_{P^i} + C_{P^{z-}} + r\overline{C}_{P^{z-},b} = C_P^0 \tag{5}$$

where r is the initial volume ratio of organic to aqueous phase:

$$r = \overline{V}^0/V^0 \tag{6}$$

As shown previously (27), hydroxide undergoes an ion-exchange reaction with the chloride of the surfactant:

$$SCl + OH^- \rightleftarrows SOH + Cl^- \tag{7}$$

with the equilibrium constant of K_S^{OH} defined in terms of concentrations as

$$K_S^{OH} = \frac{\overline{C}_{OH,b} C_{Cl}}{\overline{C}_{Cl,b} C_{OH}} \tag{8}$$

The equilibrium constant of Reaction (7) has been reported as 0.096 (25, 27). This reaction results in a new form of surfactant (SOH) at the reverse micellar interface. The negatively charged protein can be extracted with this form of surfactant as well. However, the corresponding reactions are not independent from the other reactions presented above.

The concentrations of chloride and hydroxide anions in different regions of the reverse micellar system can be obtained independent of proteins, based on Assumption (5). Equation (8) with the mass balances on surfactant, hydroxide, and chloride can be used to obtain the distribution of these two anions. A detailed derivation of this model is available elsewhere (27). The final equations for the calculation of the equilibrium concentrations of chloride and hydroxide in the different regions are presented in the Appendix.

The data are reported as the overall percent extraction of protein to the organic phase, which is calculated from

$$\xi_P = 100 \times (C_P^0 - C_P)/C_P^0 \tag{9}$$

Combining Eqs. (2), (4), and (5) with $z = 2$ results in

$$\xi_P = \frac{100 \times r}{\frac{1}{K_S^{Pz} K_{P^z}} \left(\frac{C_{Cl}}{\overline{C}_{Cl,b} C_{OH}} \right)^z + \frac{1}{K_S^{Pz}} \left(\frac{C_{Cl}}{\overline{C}_{Cl,b}} \right)^z + r} \tag{10}$$

In Eq. (10) the concentrations of chloride and hydroxide in the excess aqueous phase and the concentration of bound chloride at the reverse micellar interface are obtained from the model presented in the Appendix. In Eq. (10) there are two parameters (K_S^{Pz} and K_{P^z}) to be evaluated. The evaluation of these parameters is discussed in the following section.

RESULTS OF THE MODEL

All the data reported here were obtained in Winsor type II systems. The model is compared with the experimental data for the system DODMAC–decanol–isooctane–NaCl–α-chymotrypsin–water. Figure 2 shows the variation of α-chymotrypsin extraction with initial NaCl concentration for different initial pH values in the aqueous phase. The parameters in Eq. (2) were found as explained below.

Using the value of $z = 2$, the two parameters in Eq. (10), $K_S^{P^2}$ and K_{P^2}, were evaluated by a least-squares fit of Eq. (10) to the data in Figure 2. They are $K_S^{P^2} = 250$ and $K_{P^2} = 3.2 \times 10^{24}$. The curves in Figure 2 are the least-squares fits with these parameters.

As shown in Fig. 2, the extraction decreased with the addition of salt as well as with an increase in pH. The addition of either NaCl or NaOH (used for pH adjustment) provides chloride or hydroxide, respectively, which competes with the protein molecules for extraction with the surfactant head groups.

Figure 3 shows the effect of surfactant concentration on extraction of α-chymotrypsin for different initial NaCl concentrations. The curves are the predictions of Eq. (10) using the parameters given above; no additional fitting was performed. The effect of surfactant concentration on extraction does not appear directly in Eq. (10). However, it affects the extraction through changes in the distribution of hydroxide and chloride as shown previously (27), and as it can be seen from the equations presented in the Appendix.

As shown in Fig. 3, the extraction increases with addition of surfactant, and it reaches a plateau at higher surfactant concentrations. The minimum amount of surfactant required to extract α-chymotrypsin quantitatively from the aqueous phase (for example, 90% extraction) increased with the initial salt concentration. Similar results were obtained by Fletcher and Parrott (31)

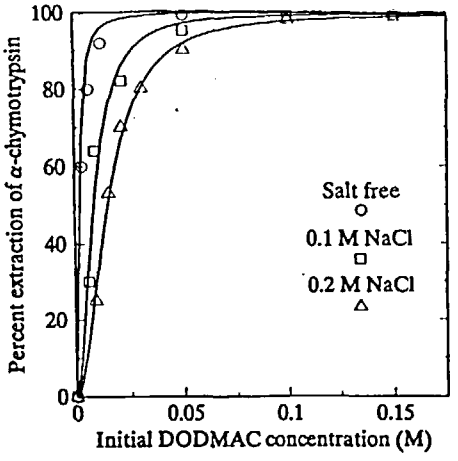

FIG. 3 Extraction of α-chymotrypsin as a function of DODMAC concentration for different initial NaCl concentrations: initial organic phase, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein, pH 13.5. The data are from the present study. The solid curves show the prediction of the model.

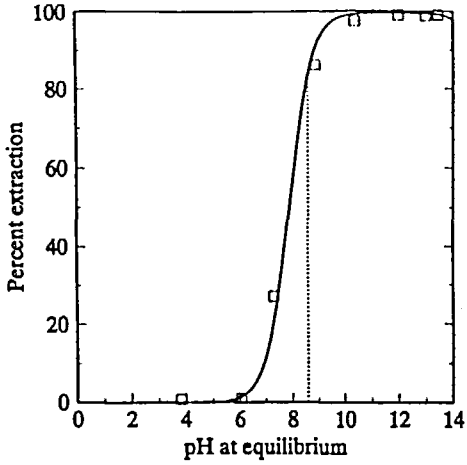

FIG. 4 Effect of pH on the extraction of α-chymotrypsin: initial organic phase, 100 mM DODMAC, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein, 100 mM NaCl. The experimental data are from Ref. 13. The solid curve shows the prediction of the model.

for extraction of α-chymotrypsin with AOT in heptane, and by Ichikawa et al. (32) for extraction of cytochrome c with AOT in isooctane.

Figure 4 shows the extraction of α-chymotrypsin as a function of pH. The curve in Fig. 4 shows the prediction of extraction by the model without any additional fitting. The predictions of the model shown in Figs. 3 and 4 are in good agreement with the experimental results. The dashed line in Fig. 4 indicates the approximate isoelectric point of the protein. Over a wide range of pH above the isoelectric point, where the protein molecules are negatively charged, almost 100% extraction was obtained.

Previous work on TOMAC and AOT showed (12) that the maximum extraction of different proteins was 40–70% for TOMAC and about 80–100% for AOT, both in a narrow pH range. The dramatic drop in the extraction of proteins at favorable pH values, which is a characteristic of AOT and TOMAC, was not observed with DODMAC. This dramatic drop was found (12, 32) to be due to the formation of a surfactant–protein complex which usually precipitates at the interface between the aqueous phase and the organic phase. No precipitate was observed for the results obtained with DODMAC.

SUMMARY

A simple mathematical model was developed to predict the extraction of proteins for the effect of pH, surfactant concentration, salt concentration, and volume ratio. This model, which is based on the ion exchange of negatively

charged protein with the surfactant counterion at the reverse micellar interface, has predictive properties. The extraction increases with addition of surfactant and reaches a plateau at high surfactant concentrations. However, it decreases with addition of salt.

APPENDIX

The molar ratio of hydroxide to chloride at equilibrium in the excess aqueous phase (R_{OH}) can be obtained from the following equation:

$$2R_{OH} = R^0_{OH} + K_S(\delta - 1) + \sqrt{(R^0_{OH} + K_S(\delta - 1))^2 + 4K_S(R^0_{OH} + \delta)} \quad \text{(A-1)}$$

where R^0_{OH} is the initial molar ratio of hydroxide to chloride in the aqueous phase and δ is defined as

$$\delta = r\bar{C}^0_S/C^0_{OH} \quad \text{(A-2)}$$

The equilibrium concentration of chloride in the excess aqueous phase is then calculated using the following equation:

$$C_{Cl} = \frac{C^0_{Cl} + C^0_{OH}}{1 + 1/R_{OH}} \quad \text{(A-3)}$$

and the equilibrium concentration of hydroxide in the excess aqueous phase is calculated from

$$C_{OH} = \frac{C^0_{Cl} + C^0_{OH}}{1 + R_{OH}} \quad \text{(A-4)}$$

The concentration of bound chloride to the surfactant head groups at the reverse micellar interface is obtained from the mass balance on chloride:

$$\bar{C}_{Cl,b} = \bar{C}^0_S + (C^0_{Cl} - C_{Cl})/r \quad \text{(A-5)}$$

and the concentration of bound hydroxide at the reverse micellar interface is then calculated using Eq. (8).

ACKNOWLEDGMENT

The authors are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support.

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Received by editor March 6, 1997
Revision received September 1997