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Gas Hydrate Formation in Reversed Micelles

Applications to Bioseparations and Biocatalysis

HUYEN NGUYEN,[1] A. MADHUSUDHAN RAO,[1] J. B. PHILLIPS,[1] VIJAY T. JOHN,*[,1] AND WAYNE F. REED[2]

Departments of [1]Chemical Engineering; and [2]Physics, Tulane University, New Orleans, LA 70118

ABSTRACT

We describe a technique to modify protein solubility and optimize enzyme activity in reversed micellar solutions. The technique is based on the ability of hydrates of natural gas to form in the microaqueous phase. Clathrate hydrates are crystalline inclusions of water and gas, and their formation in bulk water has traditionally been studied with relevance to natural gas recovery. We have found that hydrates can form in the environment of the microaqueous pools of reversed micelles, and that their extent of formation can be well controlled through the thermodynamic variables of temperature and pressure. Additionally, formation of hydrates affects the size and aggregation number of the micelles, and thus influences the solubility and conformation of encapsulated proteins. We demonstrate how the concept can be used in two applications: (i) protein extraction into reversed micelles and subsequent recovery, and (ii) optimization of enzyme activity in reversed micelles.

Index Entries: Natural gas; reversed micelles; hydrates; protein extraction; biocatalysis.

INTRODUCTION

Reversed micelles are water-in-oil microemulsions that are capable of encapsulating a variety of molecular species, particularly macromole-

*Author to whom all correspondence and reprint requests should be addressed.

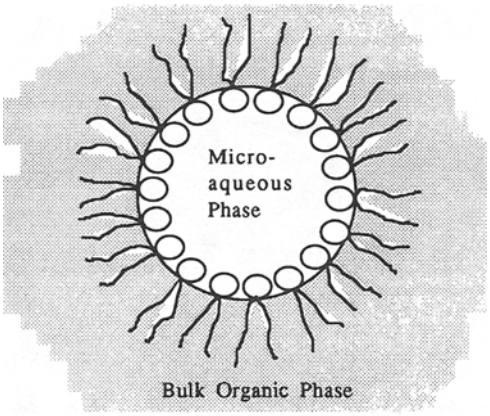

Fig. 1. Schematic of a reversed micelle.

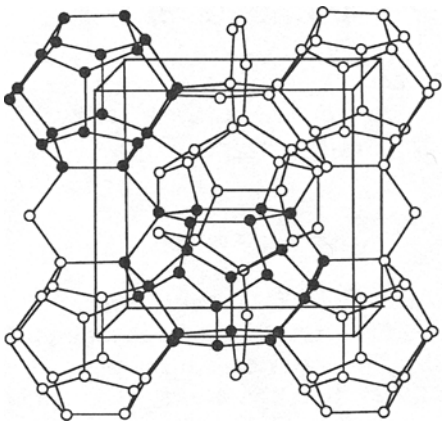

Fig. 2. Unit cell of gas hydrate structure I.

cules. The schematic of Fig. 1 illustrates a reversed micelle formed with an anionic surfactant (dioctyl sodium sulfosuccinate, commonly referred to as AOT); encapsulation typically involves electrostatic interactions between the solute and the anionic surfactant head groups. From an applications point of view, protein-containing reversed micelles have been extensively studied for their relevance to biomembrane mimetics (*1*), protein extraction processes (*2,3*), and biocatalysis (*4–6*). More novel uses of reversed micelles are emerging; an example is recovery (or removal) of heavy metal ions from aqueous solution through ion-exchange with the Na^+ cations in the microaqueous phase (*7*).

In this paper, we review some of our recent research on the formation of clathrate hydrates in reversed micelles, and the applications of the phenomenon to biomolecule separation and enzyme catalysis. Clathrate hydrates are crystalline inclusions of gas and water, formed when water is contacted with gas species at appropriate thermodynamic conditions of temperature and pressure. Figure 2 illustrates the cage-like crystal structure of the unit cell of Structure I hydrate; gas molecules (the guest species)

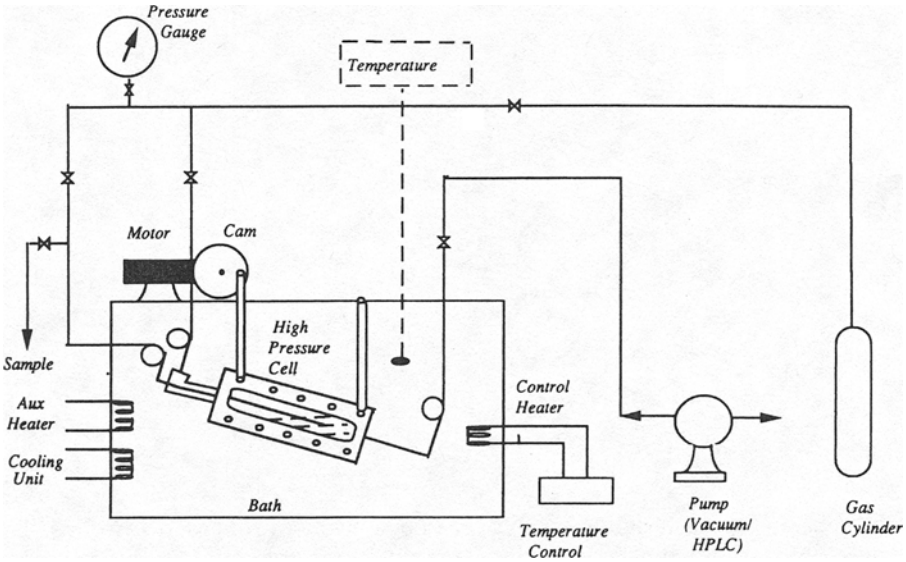

Fig. 3. Schematic of the experimental set-up.

trapped within these cavities stabilize the crystal structure through dispersion interactions with the host water molecules (*8*). It is thought that vast hydrate deposits exist in permafrost regions and under the sea floor, and hydrates have traditionally been studied for their relevance to natural gas recovery and transmission (*9*). Thus, hydrate research has, up to the present, dealt with formation in bulk water systems; formation in the microaqueous phase of reversed micelles is a rather new and interesting phenomenon. In a recent paper, we have shown that hydrates can form in reversed micelles (*10*). This paper discusses some aspects of hydrate formation, and potential applications of the phenomenon to biotechnology.

RESULTS AND DISCUSSION

Figure 3 is a schematic of our experimental apparatus for hydrate formation in reversed micelles. The set-up is very similar to that used by Holder and Hand (*11*) for hydrate studies in bulk water systems, and consists of a high pressure, glass-windowed view cell, suspended in a temperature-controlled water bath; the contents of the cell are kept rocked by attachment of the cell to a rotating cam.

Figure 4 illustrates the characteristics of hydrate formation in protein-free reversed micelles; the actual data is listed in ref. 8. The quantity w_o is the water-to-surfactant molar ratio, which has a strong influence on the size and aggregation number of the micelles, and the nature of the microaqueous phase. For example, micelle size decreases with w_o; in addition, at lower w_o values, much of the intramicellar water is considered as bound

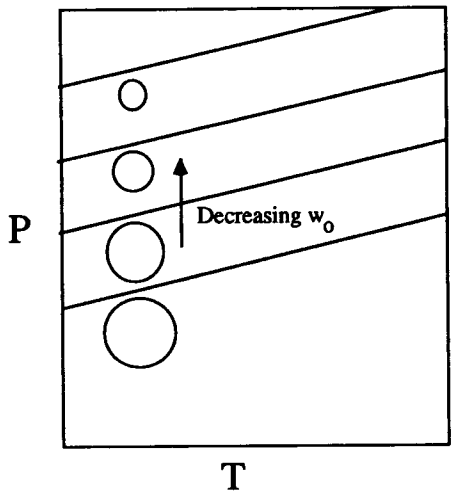

Fig. 4. Characteristics of Hydrate Formation in reversed micelles. Each line represents the univariant formation conditions for reversed micellar solutions of a specified w_o.

water, with a reduced thermodynamic activity (*12,13*). It is noted from Fig. 4, that hydrate formation is dependent on w_o. At each w_o, hydrate formation in reversed micelles follows univariant equilibrium, i.e., at a given temperature, hydrates form at a specific pressure. Furthermore, the smaller the w_o of a reversed micellar solution, the higher the pressure for hydrate formation at a given temperature. The reasoning is intuitive, and is related to the fact that the activity of water decreases with w_o (*12,13*). The free energy based driving force required to reorient the water molecules to the crystalline hydrate form increases as the water activity decreases, and this is manifested by the increase in the pressure for hydrate formation (*8*).

A noteworthy result on our thermodynamic studies on empty reversed micelles is the fact that by changing the pressure, the w_0 value of a reversed micellar solution can be adjusted, with consequent adjustment of micelle size. This is seen by simply examining Fig. 4 from another perspective; increasing the pressure (adding gas to the system) simply leads to the formation of more hydrates from the microaqueous phase. The nucleated hydrate crystals drop out of the reversed micelles and to the bottom of the cell (hydrates being more dense than the bulk organic); the supernatant then consists of reversed micelles containing less water, and thus, of a smaller size and, hence, a lower w_o. The process is completely reversible, and micelle size can be increased again simply by releasing gas; the hydrates dissociate, and the water released is spontaneously back-incorporated into reversed micelles. Furthermore, once the size (or w_0), pressure, and temperature correlations are established, it is possible to adjust the size to any desired value simply by adjusting the pressure and temperature of the system. As will be seen shortly, the ability to adjust w_0 and

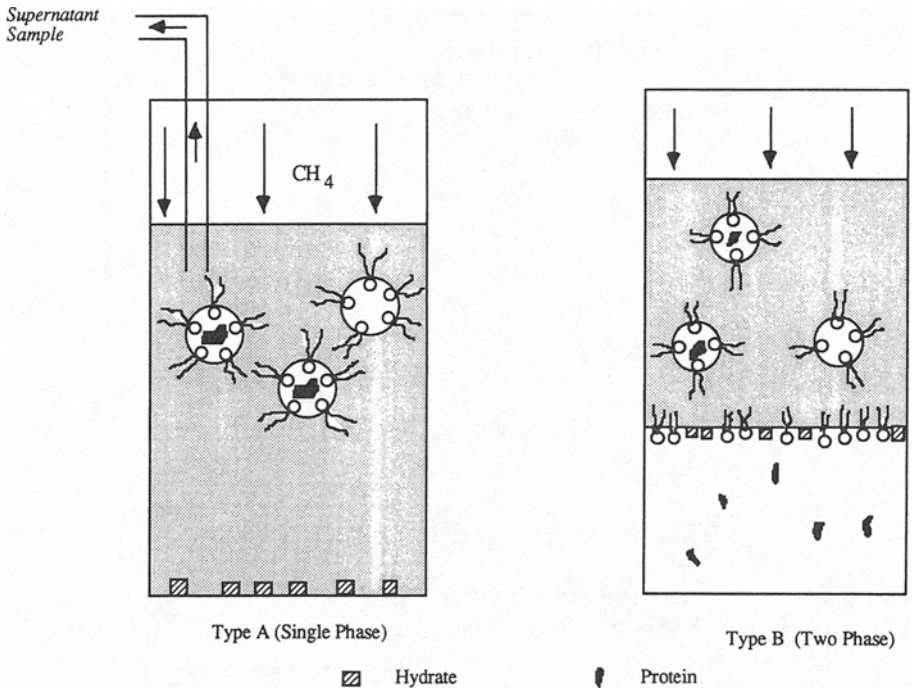

Fig. 5. Type A (single phase) and Type B (two-phase) systems.

micelle size (at constant surfactant concentrations) has implications to the solubility and behavior of proteins in reversed micelles. As a quantitative illustration, pressurization to about 2.9 MPa is necessary to initiate methane hydrate formation in reversed micelles of w_o 15, at a temperature of 273.15 K. Further pressurization results in additional hydrate formation, water drop-out, and reduction in w_o; a w_o value of 4 is achieved at about 4.8 MPa. An experimental temperature of 273.65 K was used in most of our work.

We next discuss the implications of the thermodynamics of hydrate formation to cases in which proteins are encapsulated in the micelles. Two kinds of systems are considered, a macroscopically single phase system (Type A system), and a two-phase system (Type B system), as shown schematically in Fig. 5. In type A systems, the hydrates are found at the bottom of the cell; in type B systems, at the interface between the two bulk phases. Sampling tubes inserted into the high-pressure cell allow withdrawal of fluid at elevated pressure for subsequent analysis.

We next turn to results for protein-containing systems. Some interesting observations have been made to date. The first relates to hydrate formation in a macroscopically single-phase reversed micellar solution containing the proteins cytochrome-*c* or α-chymotrypsin. We have found that hydrate formation does not desolubilize proteins in reversed micelles, as part of the microaqueous phase is removed. Samples of the supernatant were withdrawn at different pressures (different degrees of hydrate for-

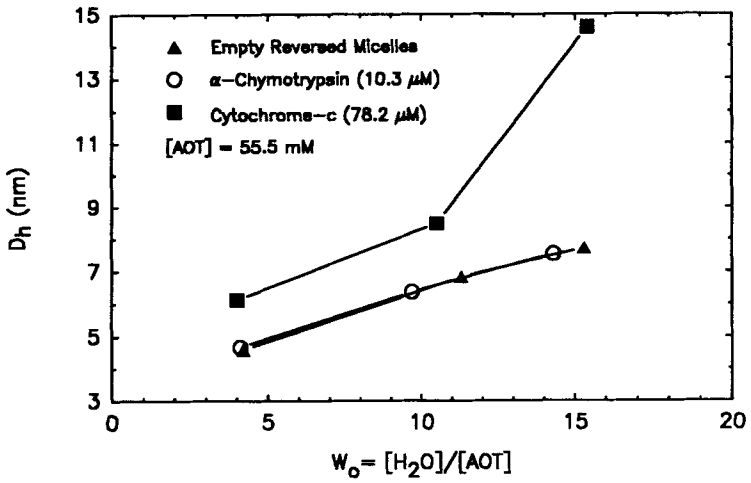

Fig. 6. Size characteristics of protein-containing reversed micelles as modified by hydrate formation. D_h is the hydrodynamic diameter.

mation) and analyzed for protein content (uv spectroscopy at 280 nm, and a modified Lowry method), water content (Karl-Fischer titration), and particle size (dynamic light scattering). The results are shown in Fig. 6; for both cytochrome-*c* and α-chymotrypsin, it was found that hydrate formation did not decrease protein solubility, and it is clearly seen that particle size decreases as increased hydrate formation lowers the water content of the micelles. The stability of proteins in reversed micelles as water content is decreased (at constant surfactant concentration) is a rather new observation.

The observation that proteins, once encapsulated in single-phase reversed micelles, stay encapsulated, brings about a conjecture about the maximum amount of protein a micellar solution can hold (the maximum fractional occupancy possible). Protein encapsulation into reversed micelles is usually done in one of three ways (*14*)

1. The phase transfer method of contact of a reversed micellar solution with a protein-containing aqueous phase;
2. The injection method of forming reversed micelles by adding the required quantity of protein-containing water to AOT and isooctane; and
3. The solid extraction method wherein a reversed micellar phase is contacted with solid protein.

The injection method is the most rapid method of protein transfer, and usually allows encapsulation of more protein than the other methods. With azocasein as the model protein, we used the injection technique to saturate the reversed micelles with protein and found that a maximum of about 8.9 mg/mL azoacasein could be incorporated into micelles at w_0 15 (Fig. 7). On the other hand, only about 1.1 mg/mL azocasein can be solu-

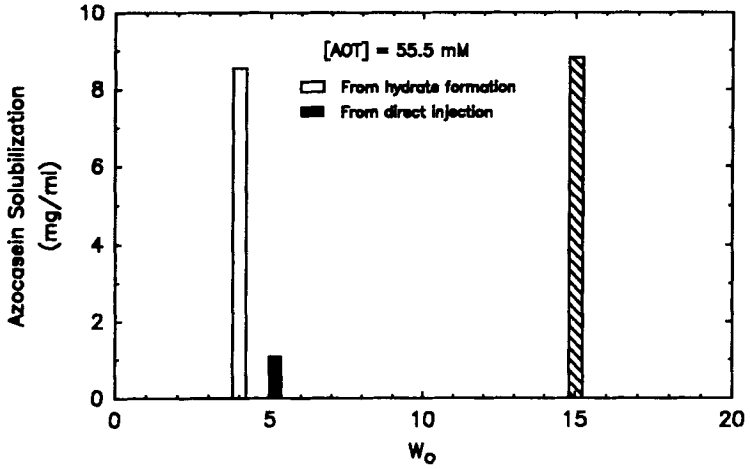

Fig. 7. High protein loading in low water content reversed micelles through hydrate formation. The cross-hatched and filled bars refer to individually prepared solutions. The unfilled bar refers to the solution of w_o 15 (cross-hatched) reduced to w_o 4 through hydrate formation.

bilized at w_0 5 through the injection technique, implying a very low fractional occupancy. We have found that by forming hydrates in reversed micelles of w_0 15 containing 8.9 mg/mL protein, all the protein is retained in the micelles even when the amount of water present is decreased to yield a w_0 of 4. Thus, hydrate formation appears to be a way to pack high protein concentrations into reversed micelles under minimal water conditions. The observation may have applications to drug delivery systems with hardened micelles (*15*), where high drug component concentrations are required in minimal volume reversed micelles.

Another interesting observation relates to the activity of enzymes in reversed micelles, as affected by hydrate formation. It has been very clearly shown in the literature (*16*) that several enzymes exhibit maximum activity at an optimal w_0, usually between 9 and 15. The exact reason is not clear, although Kabanov et al. (*16*) have presented a rationale that may have some validity. According to these authors, at high w_0, natural conformational fluctuations could prevent enzyme catalytic sites from being in the correct position to accept substrates at the particular instant that the substrate approaches the site. At low w_0, too severe restrictions on conformation destroy activity. At an optimal w_0, it is postulated that the enzyme is locked into its optimal conformation. Whatever the true reason (or reasons), it should be realized that reversed micelles in contact with a bulk aqueous phase (Type B systems) solubilize a maximum of water, and their w_0 values are usually greater than 20. It therefore seems reasonable to assume that enzymes extracted by the phase transfer method would exist with nonoptimal activity. Decreasing w_0 by adding surfactant often has a deleterious effect, again, a fact not clearly understood but

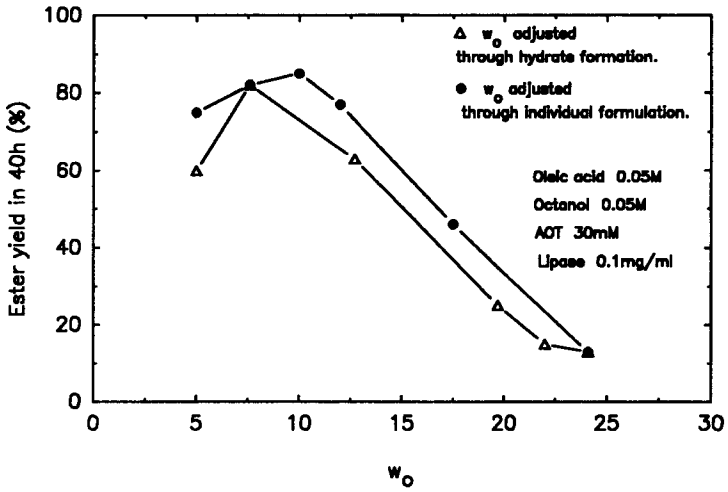

Fig. 8. Lipase activity as modified by hydrate formation.

superficially explained away by citing adverse protein-surfactant interactions, or the increased number of micelles created.

Since hydrate formation is a method to control w_0 and micelle size, we have tried to apply the approach to examine enzyme activity, using lipase-catalyzed ester synthesis (of octanoyl-oleate) as the model reaction. Introducing an initial lipase-containing micellar solution of w_0 25, we have formed hydrates and removed samples at progressively higher pressures (and therefore, lower w_0), and then conducted activity tests. As Fig. 8 indicates, we do see a maximum in activity, which we have confirmed by doing the activity test after making up separate samples of different w_0 values. A model reaction for α-chymotrypsin (hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide) also shows that the hydrate method of adjusting w_0 can lead to optimal activity (Fig. 9). It is important to note that whereas lipase is an interfacial enzyme and hence resides at the oil-water interface of the micelle, α-chymotrypsin is a core-resident enzyme (*17*).

The third observation directly relates to protein extraction from an aqueous phase (Type B systems). At moderately low ionic strengths, protein can be transferred into reversed micelles through phase transfer, whereas at high ionic strengths, protein transfer does not take place (*18*); in fact, high ionic strengths are used to back-transfer proteins from reversed micelles to an aqueous phase (*18*). At very low ionic strengths, a cloudy emulsion phase is seen, rather than an optically clear microemulsion phase. This is because at such low ionic strengths, the lack of charge screening implies repulsive interactions between the anionic head groups, and leads to the formation of large emulsion droplets and high water solubilizations. Under these conditions, protein solubilization into the emulsion phase is negligible. For cytochrome-*c*, an ionic strength of 0.1 *M* KCl is necessary for transfer into the micellar phase. When this two-phase

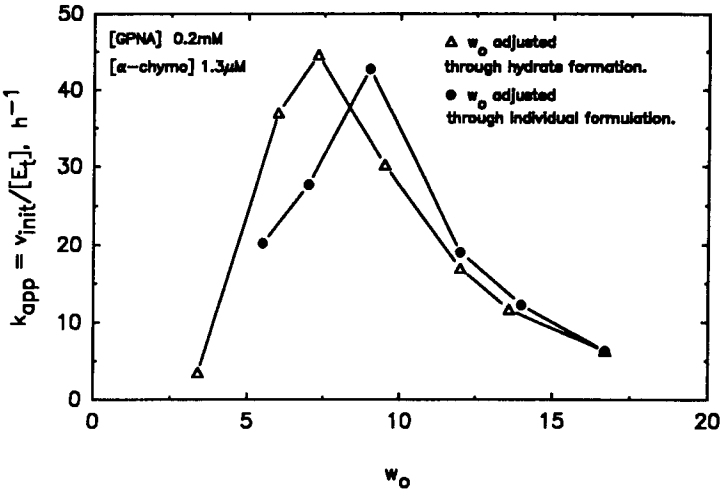

Fig. 9. α-Chymotrypsin activity as modified by hydrate formation. k_{app} is the apparent reaction rate constant in the Michaelis-Menten equation.

system (System B) is introduced into the hydrate cell, and hydrate formation initiated, the aqueous phase becomes converted to hydrates and decreases in volume. We have found that when a substantial amount of the water has been converted to hydrates, there comes a stage when the emulsion phase becomes optically clear, and the cytochrome-*c* is quantitatively extracted into the resulting reversed micellar phase. We believe that removal of water through hydrate formation eventually leads to collapse of the large emulsion droplets to the microemulsion state. Furthermore, the increase in ionic strength caused by conversion of part of the aqueous phase to pure water (crystalline hydrates) leads to some charge screening, and a consequent transformation of the supernatant phase from emulsion to microemulsion character. Proteins, driven from the aqueous phase to the microemulsion, are stabilized through coulombic attraction. Recovery of the protein back into an aqueous phase simply involves contacting the microemulsion phase with an aqueous phase of very low ionic strength; the microemulsion solubilizes water to transform back into an emulsion, and the protein is rejected into the aqueous phase. The process is shown schematically in Fig. 10. It is interesting to note that the entire procedure takes place without any external adjustment of pH or ionic strength.

The above results apply to hydrate formation, with methane as the hydrate-forming gas species. When ethylene is used, we have recently found that pressurization beyond a critical pressure results in destabilization and precipitation of solubilized protein. We attribute the effect to the fact that the higher solubility of ethylene in isooctane (compared to methane) leads to an expansion of the bulk solvent phase that perturbs micellar integrity. Further work is in progress to understand the phenomenon

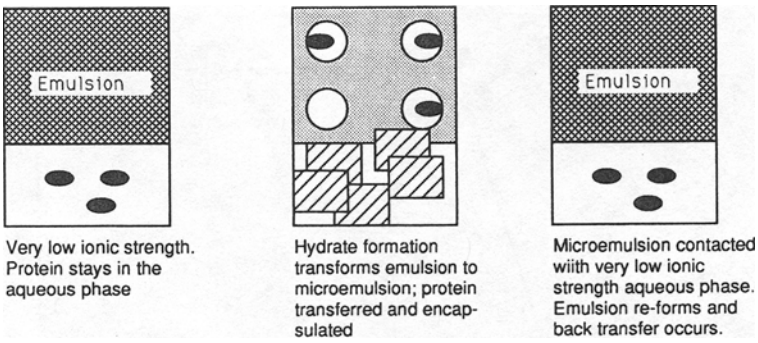

Fig. 10. Protein extraction and recovery at low ionic strength.

more thoroughly. The fact that proteins can be released easily from reversed micelles through contact with pressurized ethylene implies the possibility of a methodology for protein recovery after extraction into reversed micelles.

In this regard, it is important to cite the recent interesting work by Smith and coworkers at Battelle Pacific Northwest Labs. (*19*). These authors have used supercritical ethane, propane, Xe, and so on, in contact with an aqueous phase to form reversed micelles in the dense gas phase. The objective here is to extract material from the aqueous phase into the dense gas phase. This work of ours, with ethylene in contact with an isooctane reversed micellar solution, is different; we are attempting to destabilize proteins in liquid phase reversed micelles, for subsequent recovery.

SUMMARY

We have been able to demonstrate that clathrate hydrates can form from the microaqueous pools of reversed micelles, when a micellar solution is contacted with hydrate forming gas at appropriate thermodynamic conditions of temperature and pressure. Clathrate hydrates are crystalline inclusions of gas and water; while their formation in bulk water has been well studied, this research is among the earliest to study the physical phenomena associated with formation in the minimal water environment of reversed micelles. We have found that the pressure–temperature conditions for hydrate formation are dependent on the characteristic water to surfactant ratio, w_o, that influences the size of the micelles. The conditions for hydrate formation can be used as a probe to understand the nature of the microaqueous phase. Furthermore, by adjusting the extent of hydrate formation simply by changing the pressure, the micelle size can be altered reversibly. Extension of the work to protein-containing micelles has shown that enzyme activity can be altered through hydrate formation-induced size modifications. Finally, we have shown that hydrate

formation can, under specific conditions, alter protein solubilities and packing densities in reversed micelles. The potential relevance of the phenomenon to the biotechnological applications of protein purification and biocatalysis are discussed. From a fundamental viewpoint, the research addresses a hitherto unstudied physical phenomenon that may have wide applications.

ACKNOWLEDGMENT

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