SEPARATION WITH REVERSE-MICELLES

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Engineering.

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August 1991

ABSTRACT

Reverse-micelles are surfactant aggregates in an apolar solvent. The surfactants, which must have two hydrophobic tails, cluster around a water core. The effects of the compositions of the solvent and the aqueous phase on water solubilization were studied for two anionic surfactants: sodium di-2-ethylhexylsulfosuccinate (AOT) and dinonylnaphthalene sulfonic acid (DNNSA). Single straight chain alkanes having 6-17 carbons, mixtures of these alkanes and branched alkanes were used as solvents. The aqueous phases consisted of salts and buffers.

For AOT in straight chain alkanes, water uptake increased as the length of the chain approached 9 carbons, the length of the AOT hydrocarbon tail. As the solvent length increased further, a critical carbon number was reached above which there was no water uptake. Different buffers shifted the critical carbon number somewhat. Mixtures of straight chain alkanes behaved similar to single alkanes when compared on the basis of volume-average carbon number. Branched alkanes solubilized more water than their straight chain isomers. For DNNSA the water uptake was the same for all solvents.

RESUMÉ

Les micelles inversées sont des surfactifs qui s'agglomèrent en milieu non-polaire. Les surfactifs, ayant deux queues hydrophobiques, se rassemblent autour d'un noyau d'eau. La fixation de l'eau a été étudiée en fonction de la composition du solvant et de la phase aqueuse sur deux surfactifs anioniques: soient le "sodium di-2-ethylhexylsulfosuccinate" (AOT) et le "dinonylnaphthalene sulfonic acid" (DNNSA). Les alcanes à chaînes linéaires contenant de 6 à 17 carbones, un mélange d'alcanes à chaînes linéaires, ainsi que des alcanes à chaînes ramifiées ont été utilisées comme solvant. Tandis que les phases aqueuses utilisées contenaient des sels et des solutions tampons.

Pour le AOT, en milieu de solvant composés d'alcanes à chaînes linéaires, la fixation d'eau a augmentée pour des longueurs de chaîne s'étendant jusqu'à neuf carbones. Au dessus de ce nombre de carbones, la fixation ne s'éffectue plus. Il est intéressant de noter que la queue d'hydrocarbure du AOT contient 9 carbones.

Ce nombre critique de carbone a été cependant modifié en variant les solutions tampons. Les alcanes ramifiés solubilisent plus éfficacement l'eau que leurs isomères linéaires. Lorsque l'on compare le niveau de fixation d'eau en terme de nombre de carbone volumique, les comportements des mélanges d'alcanes à chaînes linéaires et des alcanes simples donnent des résultats comparables. Pour le DNNSA, la fixation d'eau a été identique pour tous les solvants employés.

ACKNOWLEDGEMENTS

I would like to thank Dr.D.G.Cooper and Dr.M.E.Weber for their supervision, guidance, encouragement and enthusiasm during the course of this research.

I would also like to express my gratitude to the Natural Sciences and Engineering Research Council of Canada and the bequest of Eugenie Ulmer Lamothe for financial support.

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1.0 INTRODUCTION

In the last decade there have been tremendous increases in the production of biochemicals in large quantities by cell culture [Rahaman et al., 1988]. However, the technology for separating biological products from the media in which they are produced has not kept pace with advances in synthesis [Rahaman et al , 1988]. The separation of many biochemicals is still performed by traditional batch type or small scale processes such as electrophoresis, salt and solvent precipitation, and column liquid chromatography. These processes are difficult to scale up to production levels and can become prohibitively expensive unless high value products are being produced [Abbott and Hatton, 1988]. Thus, other efficient, scaleable bioseparation processes with the potential for continuous operation are needed.

An apolar solvent containing reverse-micelles is an attractive solvent for use in the liquid-liquid extraction of biological products because many biochemicals, including amino acids, proteins, and nucleic acids, can be solubilized within, and recovered from, such solutions without loss of native function [Rahaman et al., 1988]. These products are important to the research, pharmaceutical, and industrial communities.

1.1 Definitions

1.1.1 Surfactants

Surface active agents, more commonly known as surfactants, are molecules containing hydrophilic and hydrophobic groups. Such molecules tend to aggregate at the interfaces between an aqueous medium and other phases of the system such as air and oily liquids where they reduce surface or interfacial tension. Effective surfactants reduce the surface tension of water from 72 mN/m to below 30 mN/m.

Surfactants are generally classified by the charge of their hydrophilic groups as anionic, cationic, and nonionic. The hydrophobic group generally is a radical containing from 10 to 20 carbon atoms in a single chain or double chain configuration. The structure of the surfactants, AOT and DNNSA, which were used in the majority of experiments, are shown in Figures 1-1A and 1-1B.

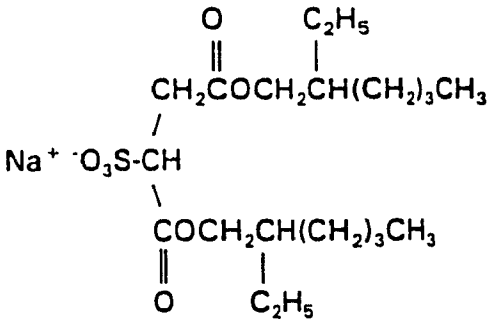


FIGURE 1-1A - Structure of AOT
(sodium di-2-ethylhexylsulfosuccinate)

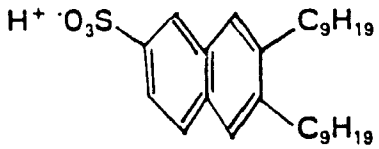


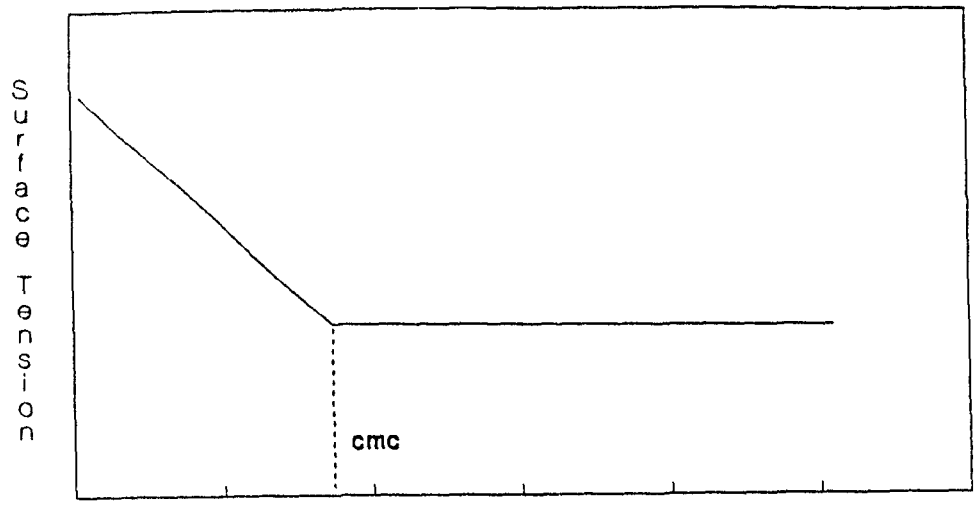
FIGURE 1-1B - Structure of DNNSA
(dinonylnaphthalene sulfonic acid)

These surfactants have anionic hydrophilic groups and double chain hydrophobic groups.

1.1.2 Micelles

In aqueous systems, as the surfactant concentration is increased, the physical, colligative, and spectral properties of the system undergo an abrupt change over a narrow range of concentrations [Ruckenstein and Nagarajan, 1980]. The concentration at which this sharp transition occurs is called the critical micelle concentration (cmc). Below the cmc, the surfactant molecules move to interfaces. At and above the cmc, the molecules associate to form micelles (see Figure 1-2), rather than move to the interface, which means that the surface and interfacial tensions are not further reduced. A micelle is an aggregate composed of hydrophobic tails surrounded by a shell of the hydrophilic head groups. Solubilized lipid may be encapsulated within the micelle's core.

The primary mechanism responsible for micelle formation in aqueous solutions is the hydrophobic effect. For typical amphiphiles, the micelles that persist are those composed of a relatively large number of amphiphiles. Small aggregates (trimers, tetramers, etc.) do not exist in measurable concentrations. The distribution of aggregates arises because a minimum number of amphiphiles must act collectively to shield the lipophiles effectively from the water. Smaller aggregates cannot be arranged to achieve a minimum area of contact between the lipophile and the water. Thus larger aggregates are energetically favoured over smaller aggregates.



(a)

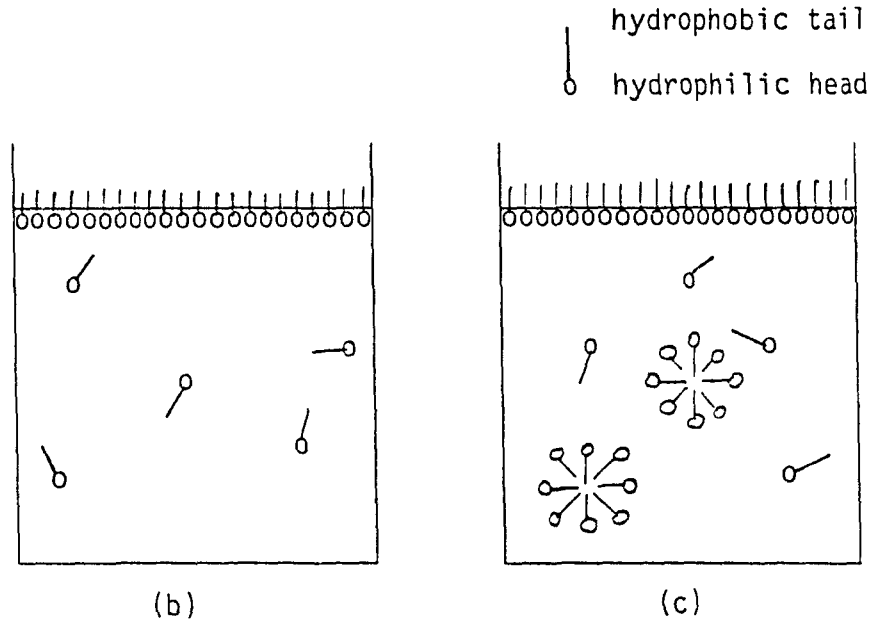


FIGURE 1-2 - Surface tension and micelle formation

(a) surface tension - concentration diagram

(b) surfactant in solution just below cmc

(c) surfactant in solution just above cmc

1.1.3 Reverse-micelles

The physical properties of the nonaqueous surfactant solutions change gradually without any sharp transition as the concentration of the surfactant is increased [Ruckenstein and Nagarajan, 1980], therefore a critical micelle concentration does not exist. Experiments confirm that aggregation into reverse-micelles occurs in nonaqueous solutions even at low concentration. A reverse-micelle is an aggregate composed of hydrophilic head groups surrounded by a shell of hydrophobic tails. Water molecules may be hosted within the reverse-micelle's core. Amphiphile molecules in nonpolar solvents do not experience a hydrophobic effect. The lipophiles remain dispersed among the hydrocarbon molecules, whilst the hydrophiles interact collectively. Thus in nonpolar solvents the environment of the lipophile prior to and after aggregation is not changed in any substantial way. The force responsible for association of amphiphiles is directly attributed to the interaction between hydrophiles. The micelles formed in nonpolar solvents are usually small aggregates due to steric hindrance involved in arranging a large number of amphiphilic compounds with their hydrophiles close together.

Many surfactants do not form reverse-micelles and those which do generally have two hydrophobic tails. One such surfactant is AOT (Figure 1-1A) which forms reverse-micelles when dissolved in nonpolar media, that is the sulfonate head groups reside in the core of the structure and the hydrophobic tailgroups extend outward into the surrounding organic solvent phase as illustrated in Figure 1-3. Polar compounds such as proteins may be encapsulated within the reverse-micelle.

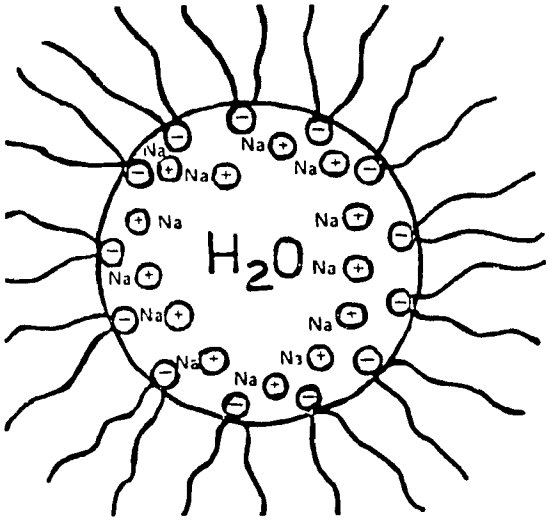


FIGURE 1-3 - Structure of AOT reverse-micelle
(O'Connor, 1987, p.188)

1.1.4 Proteins

Every protein molecule can be considered as a polymer of amino acids. There are 20 amino acids, each with a common backbone combined with one of 20 different side chains (Rgroup)

NH_2 amino end
|
R-CH
|
COOH carboxyl end

Amino acids are joined by a peptide bond between the carboxyl end of one amino acid and the amino end of another. This bond is formed by the loss of a water molecule. Small protein molecules may contain 50 to 100 amino acids while large proteins have up to 300 or more. The molecular weight of an amino acid varies from 75 for glycine to 204 for tryptophan.

1.2 History

The focus of this thesis is the formation of reverse-micelles and the solubilization of water and proteins within reverse-micelles. Accordingly, the historical overview is divided into two parts: (i) formation of reverse-micelles, and (ii) protein uptake.

1.2.1 Nature of organic solvent and surfactant on micelle formation

A list of surfactants which are suitable for forming reverse-micelles can be found in the text of Luisi and Straub (1984). The surfactants which have been most studied are sodium di-2-ethylhexylsulfosuccinate (AOT), Tween, Span, different forms of polyethylene oxide-based surfactants, alkyltrimethylammonium halides (in particular, cetyltrimethylammonium bromide), alkali salts of dinonylnaphthalene sulfonic acid and phospholipids (e.g. phosphatidylcholine, more commonly known as lecithin). Co-surfactants (e.g. benzyl alcohol, hexanol or cholesterol) are sometimes added. An advantage of the AOT system, which helps to explain its popularity, is that no co-surfactant is required [Luisi et al., 1988].

Among the organic solvents used as the dispersion medium, most studies have been made in silicone oil, n-octane, iso-octane, heptane, cyclohexane, benzene, halogenated benzenes, and halogenated alkane solvents such as chloroform [Luisi et al., 1988].

Table 1-1 summarizes the work concerned with the nature of the organic solvent and the surfactant. Many researchers have noted the differences in using aromatic solvents compared to alkanes, but no effort has been made to interpret these results. On the other hand, Aveyard et al.(1986) investigated a wide range of alkanes in systems containing AOT.

TABLE 1-1 - Research into the nature of the organic solvent and surfactant on micelle formation

AUTHORS	YEAR	SIGNIFICANCE OF WORK
Little & Singleterry	1964	solubility of alkali dinonylnaphthalenesulfonates in different organic solvents
Guttman & Kertes	1974	cationic surfactants in organic solvents
Luisi et al.	1979	micellar solubilization of proteins in aprotic solvents
Martin & Magid	1981	carbon-13NMR investigation of AOT R-M with various organic solvents
Luisi et al.	1985 1988	summarized enzymes hosted in various organic solvents and surfactants
Maitra	1985	effect of various solvents
Aveyard et al.	1986	effect of alkane chain length on system containing AOT
Shield	1986	summarized enzymes solubilized in R-M systems
Frieberg	1987	effect of nonionic surfactants with different organic solvents
Abbott et al.	1988	capacity of R-M is increased with higher [surfactant]
Rahaman et al.	1988	CTAB in octane/hexanol and AOT in isooctane, dodecane, and tetradecane
Miletti	1989	effect of mixtures of hexane and hexadecane on water uptake
Woll et al.	1989	R-M are sensitive to polarity and molecular geometry of solvent
Jolivalt et al.	1990	effect of various solvents on AOT
Maitra et al.	1990	lecithin in different organic solvents

1.2.2 Protein uptake

Shield et al. (1986) summarized the early accounts of the solubilization of enzymes within reverse-micelles. They noted that the use of reverse-micelles dates back to 1952 with the research of Hanahan who reported the formation of an enzyme/surfactant complex that retained enzymatic activity in diethyl ether solutions. In this system, phosphatidylcholine was used as both the surfactant and the substrate in a reaction catalyzed by phospholipase. Other biochemists used reverse-micellar systems with natural and synthetic surfactants to study various proteins. It was not until the work of Misiorowski and Wells in 1972 that the influence of conditions within reverse-micelles on enzyme activity was studied. Luisi (1985) and Shield et al. (1986) summarized the directions of research on enzymes in reverse-micelles from 1974 to 1984. Table 1-2 was compiled by combining their tables and adding recent work. Enzymes recovered using reverse-micelles include: α-amylase, α-chymotrypsin, catalase, cytochrome c, dehydrogenase (lipoamide, lactate, hope liver alcohol), glucagon, hydrogenase, lipase, lipoxygenase, lysozyme, pepsin, peroxidase, phosphatase, phospholipase, pyrophosphate, pyruvate kinase, ribonuclease, and trypsin.

TABLE 1-2 - Enzymatic studies in reverse-micelles[a]

AUTHOR	YEAR	SIGNIFICANCE OF WORK
Misorowski	1974	influence of water and cations on activity of phospholipase A2 in phosphatidylcholine/diethyl ether R-M
Martinek	1977	α-chymotrypsin-catalyzed hydrolysis rxn in AOT/isooctane R-M
Luisi	1977	transfer of α-chymotrypsin into TOMAC/cyclohexane R-M
Menger	1979	α-chymotrypsin-catalyzed hydrolysis in AOT/heptane R-M; activity as f(pH,Wo)
Douzou	1979	cryoenzymology: trypsin-catalyzed hydrolysis in AOT/silicone oil R-M
Luisi	1979	transfer of enzymes into organic solvents with retention of activity
Kumar et al.	1980	α-chymotrypsin and bovine serum albumin in various surfactants; activity measurements
Martinek	1981	activity studies of various enzymes in R-M
Pileni	1981	photoreduction of cytochrome c in AOT isooctane R-M
Martinek	1982	peroxidase in AOT/octane; activity of enzyme much higher than in water
Laane	1983	keto steroid conversions by dehydrogenase in CTAB/octane/hexanol R-M
Fletcher	1984	kinetics of α-chymotrypsin hydrolysis in AOT/heptane R-M
Luisi	1984	α-chymotrypsin-catalyzed peptide synthesis in AOT/isooctane R-M
Fletcher et al.	1985	activity of lipase in water-in-oil microemulsions
Goklen et al.	1985	enzyme separation via solubilization in AOT/isooctane R-M

AUTHOR	YEAR	SIGNIFICANCE OF WORK
Luisi	1985	enzymes hosted in R-M in hydrocarbon solution
Dekker et al.	1986-87	continuous enzyme (α-amylase) recovery by liquid-liquid extraction using R-M
Shield et al.	1986	enzymes in R-M as catalysts for organic-phase synthetic reactions
Giovenco et al.	1987	capacity of R-M to lyse bacterial cells instantaneously and accommodate in polar core of surfactant aggregate; CTAB in octane/hexanol
Goklen et al.	1987	liquid-liquid extraction of low MW proteins by selective solubilization in R-M: effect of pH and salt
Abbott et al.	1988	liquid-liquid extraction for protein separation using R-M
Armstrong et al.	1988	selective protein separation using R-M
Luisi et al.	1988	R-M as hosts for proteins and small molecules
Rahaman et al.	1988	recovery of an extracellular alkaline protease from whole fermentation broth using R-M
Leodidis et al.	1989	specific ion effects in electrical double layers: selective solubilization of cations in AOT R-M
Leser et al.	1989	extraction of oil and proteins from vegetable meal using R-M
Woll et al.	1989	bioaffinity separations using R-M
Furusaki et al.	1990	extraction of amino acids by R-M
Jolivalt et al.	1990	extraction of α-chymotrypsin using Aliquat 336 dissolved in esters or in isooctane/isotridecanol R-M
Jolivalt et al.	1990	extraction of proteins using Aliquat 336 in isooctane/isotridecanol R-M

*Abbreviations: AOT, aerosol OT; CTAB, cetyltrimethylammonium bromide; TOMAC, trioctylmethylammonium chloride; Aliquat 336, a mixture of trialkyl (C8-C10) methyl ammonium chlorides); R-M, reverse-micelle; MW, molecular weight.

2.0 PARAMETERS AFFECTING SEPARATION WITH REVERSE-MICELLES

2.1 Factors determining formation of reverse-micelles

Steric factors are important in the formation of reverse-micelles. The relative bulkiness or (more precisely) the ratio of cross-sectional areas of the hydrocarbon to the polar portions of the surfactant affects the size, shape and nature of the aggregates.

Mitchell and Ninham (1981) have described the way in which the structure of surfactant aggregates in an oil-water system changes as phase inversion is approached. Phase inversion occurs when an emulsion changes from an oil-in-water (o/w) to a water-in-oil (w/o) dispersion or vice-versa. They proposed that phase inversion was governed by a packing ratio, P, defined by

$$P = v / a_o l_c \qquad (2\text{-}1)$$

where

a_o = cross-sectional area of surfactant head group

v = volume of the surfactant hydrocarbon tail

l_c = effective length of the surfactant hydrocarbon tail.

The packing ratio provides a measure of the hydrophilic-lipophilic balance (HLB). For $P < 1$ normal micelles are formed, whereas for $P > 1$ reverse-micelles are formed. For $P = 1$ phase inversion occurs and hence the surfactant prefers to be at a plane interface, and the tension of the plane is at a minimum.

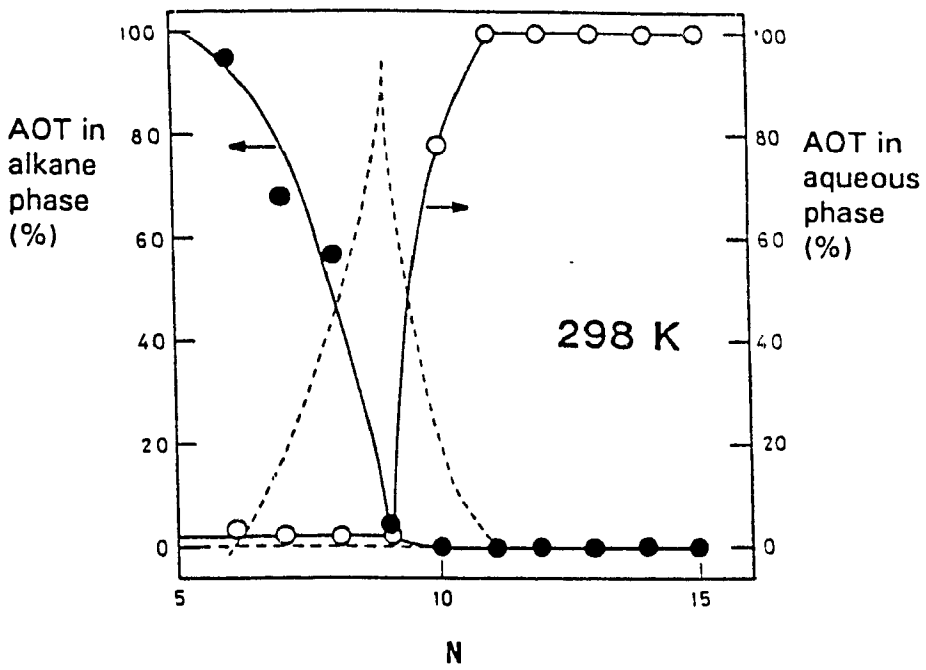
The packing ratio is affected by the hydrophilicity of head group, the ionic strength of solution, the pH, the temperature and the addition of lipophilic compounds such as co-surfactants. Experimentally it has been shown that variation in salt

[Aveyard et al., 1986] or co-surfactant concentration [Tadros et al., 1984 and Aveyard et al., 1986], or temperature [Luisi et al, 1988], or oil chain length [Mitchell and Ninham, 1981 and Aveyard et al., 1986], in two-phase microemulsion systems leads to systematic changes in drop size and in inversion conditions. Changes in a normal micellar system which serve to reduce a_o (e.g. increases in temperature or salt concentration) or to increase v (e.g. increasing penetration of alkane into the surfactant tail region of the aggregate) bring the system closer to phase inversion and lead to a reduction in interfacial tension. These changes have been explained in terms of changes in the relative oil solubility and water solubility of the surfactant.

The head group area, a_o, is assumed to be determined by the competition between head group interactions and the interfacial tension between the hydrocarbon tails and water. The more "hydrophilic" the head group, the stronger the repulsion between adjacent head groups and the larger a_o will be. Large polar head groups and strong electrical repulsion between ionized head groups in dilute electrolyte solutions favor o/w microemulsions where the surfactant film curves in such a way that the head groups are more widely separated than the hydrocarbon tails. The larger the head groups or the stronger the repulsion, the smaller are the drops.

According to Mukhejee et al.(1983), shorter alkanes penetrate into surfactant layers more effectively than do longer chain homologues, as a result of the entropy of mixing. As a consequence, the packing ratio is effectively increased, due to an increase in the interphase volume per surfactant molecule, thus favoring w/o microemulsions. Hence shorter alkanes tend to induce reverse-micelles. Based on this theory, a minimum tension would occur if a series of alkanes were used such that phase inversion occurred.

Many researchers have hypothesized that under most conditions the surfactant remained almost entirely in the organic phase with only a small concentration equal to the cmc present in the aqueous phase. In the instance where no water was solubilized it was stated that the surfactant had transferred entirely to the aqueous phase Consistent with these view but with a crucial addition, Aveyard et al. (1986) measured the distribution of AOT between aqueous NaCl solutions and n-alkanes at room temperature. Their results, which support the above theory are shown in Figure 2-1. For short chain alkanes, say $N \leq 7$, where N is the number of carbons in the alkane, most of the AOT is in the alkane phase and the AOT concentration in the aqueous phase is approximately equal to the cmc. For $N \geq 11$, the AOT is largely in the aqueous phase at concentrations above the cmc. In the region of $N = 9$, much of the AOT is present in a third, surfactant-rich phase. Also consistent with the theory of Mitchell and Ninham (1981) were the data of Rahaman et al.(1988) who measured water uptake and protein solubilization for three different organic solvents. No water uptake occurred when using 50 mM and 200 mM solutions of AOT in the solvents dodecane and tetradecane. On the other hand, water uptake was significant when iso-octane was employed.



The dotted line shows surfactant lost to the surfactant-rich phase.

(●) % AOT in alkane phase
(○) % AOT in aqueous phase

FIGURE 2-1 - Distribution of AOT between aqueous NaCl and n-alkanes (Aveyard et al., 1986, p.1757)

Initial contacting phases
organic: 17 mM AOT in n-alkane, chain length N
aqueous: 0.0684 mol/L NaCl
no mention of pH control

According to Tadros et al. (1984) the co-surfactant plays two major roles in the formation of reverse-micelles: firstly, it effects the packing of the surfactant molecule and secondly, it brings the necessary reduction in interfacial tension, if this could not be produced by the surfactant alone. The addition of a co-surfactant has the effect of increasing the volume without affecting the length (provided the chain length of the co-surfactant does not exceed that of the surfactant) and hence it enables the geometrical packing of the molecules at the interface. Moreover, such molecules act as "padding" separating the head groups.

Aveyard et al. (1986) proved Tadros' theory using various alkanols in heptane at 30°C as shown in Figure 2-2. Starting with a system of 17 mM AOT, dodecanol was added to the heptane. As the mole fraction of dodecanol increased, interfacial tension fell and passed through a minimum as predicted. Hexanol leaves interfacial tension unchanged, and pentanol gives an increase.

Aveyard et al. (1986) investigated the formation of reverse-micelles for a system containing AOT in heptane with NaCl in water. Figure 2-3 (Aveyard et al., 1986) shows the distribution of AOT between an oil and aqueous phase. At very low salt concentration, the surfactant resides in the aqueous phase. Consequently, the formation of reverse-micelles is inhibited due to the surfactant preferring the aqueous phase. At high salt concentration, the surfactant transfers to the oil phase and leaves the aqueous phase devoid of micelles but close to the cmc expected if no excess oil were present. Goklen (1986) found that at very high salt concentrations (>0.2 M), the salt will displace water and reduce water uptake. There will be appreciable water uptake at some intermediate ionic strength.

The quantity of water that can be found inside the micelles is generally expressed as the molar ratio of water to surfactant and referred to as W_o. W_o more than the absolute amount of water or surfactant present in the hydrocarbon solvent, determines most of the structural and physical properties of reverse-micelles [Luisi, 1985]. Luisi (1985) found that at low W_o the physical properties of the water in the reverse-micelles are somewhat different from those of the bulk water. As W_o is increased the water pool properties approach those of pure water through some small differences may remain. In addition W_o is an indication of the size of the reverse-micelle.

Shield et al. (1986) found that up to 60 percent water by weight can be solubilized in an organic solvent containing AOT, by incorporation in these "water pools", but most research involving enzymes in reverse-micelles has been at 1-5 percent water by weight, corresponding to W_o's of 5-40.

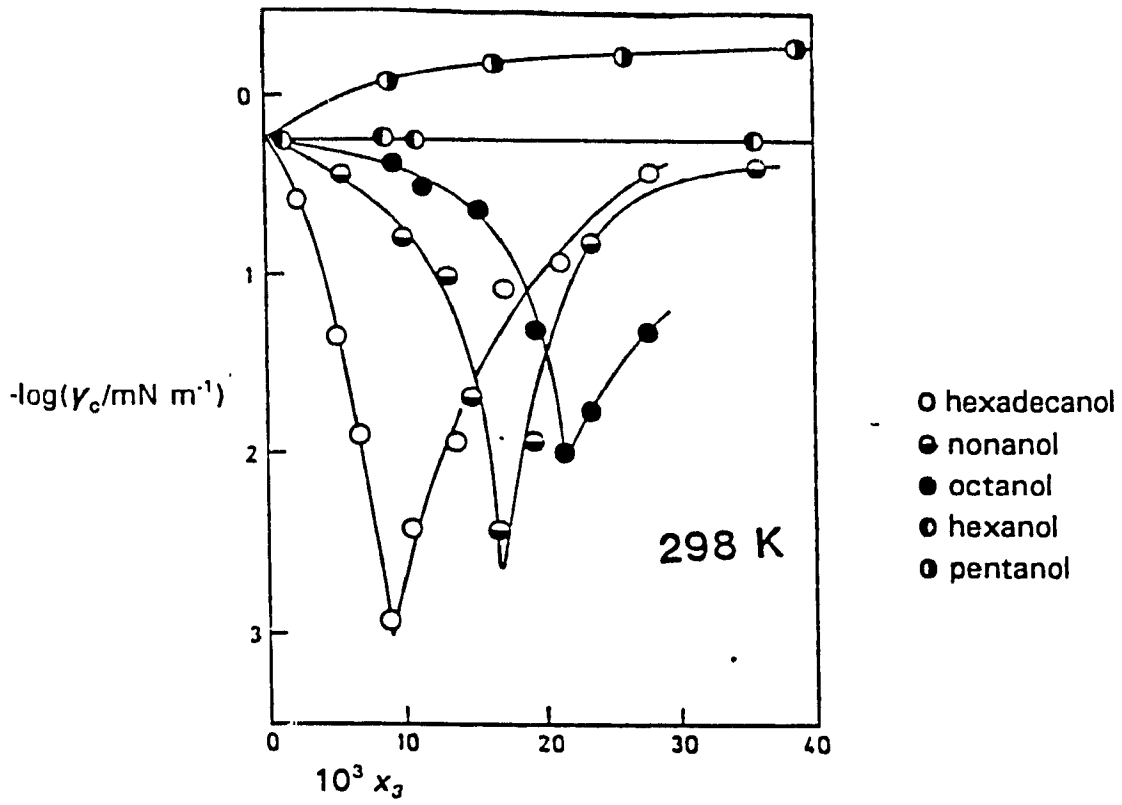
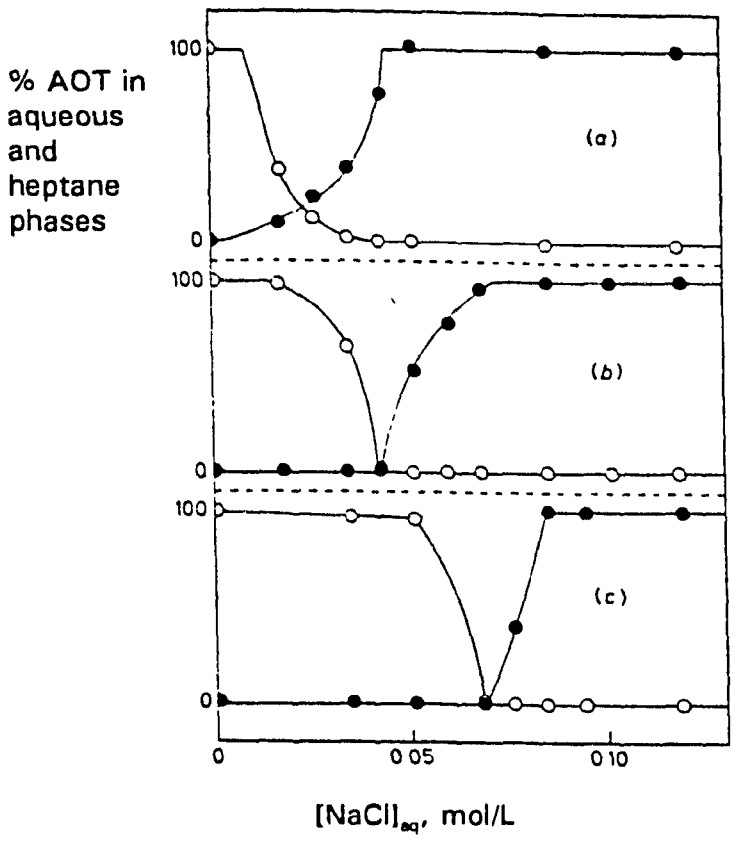


FIGURE 2-2 - Variation of γ_c with mole fraction of various alkanols in heptane (Aveyard et al., 1986, p.1765)

Initial contacting phases
organic: various akanols in heptane
17 mM AOT
aqueous: 17.1 mM NaCl
no mention of pH control



(●) % AOT in heptane phase
(o) % AOT in aqueous phase

FIGURE 2-3 - Distribution of AOT (in aggregated form) between heptane and aqueous NaCl
a. 10°C b. 25°C c. 40°C
(Aveyard et al., 1986, p.129)

Initial contacting phases
organic: unstated concentration of AOT/n-heptane
aqueous: no mention of pH control

2.2 Factors determining protein partition

The primary factors determining protein partition are those discussed in the formation of reverse-micelles. Abbott and Hatton (1988) found that the factors affecting the partitioning of proteins between a bulk aqueous phase and a reverse-micellar solution include pH, ionic strength and salt type, as well as the organic solvents and surfactants used in the extraction. They noted that, in general, the solubilization is controlled by electrostatic interactions between the charged proteins and the surfactant headgroups and by size exclusion phenomena, which depend on the relative sizes of the reverse-micelle and the proteins to be hosted. Thus, any factors which enhance electrostatic attraction or lead to the formation of large reverse-micelles, should facilitate protein extraction and recovery. These factors are discussed briefly below.

2.2.1 pH

The pH affects solubilization of protein in the reverse- micellar phase by determining the protein net charge. Refer to Figure 2-4 below.

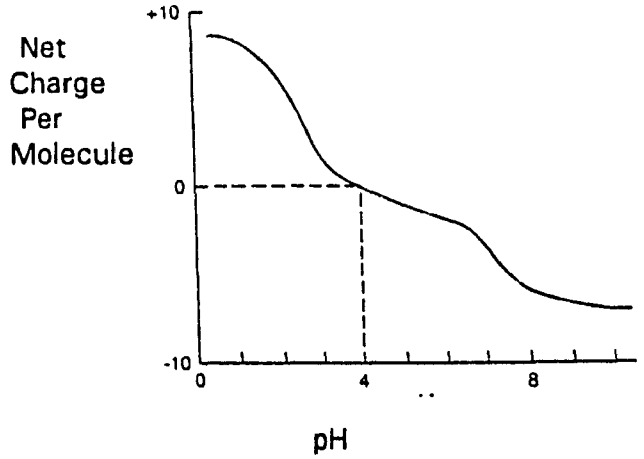


FIGURE 2-4 - Protein charge as a function of pH (Belter et al., 1987)

At low pH, amine ($-NH_2$) side chains are protonated to give a positive charge ($-NH_3^+$); at high pH, carboxylic side chains (-COOH) ionize to give a negative charge ($-COO^-$). The intermediate pH of no net charge is called the isoelectric point (IP). Table 2-1 lists the molecular weights and isoelectric points for various proteins.

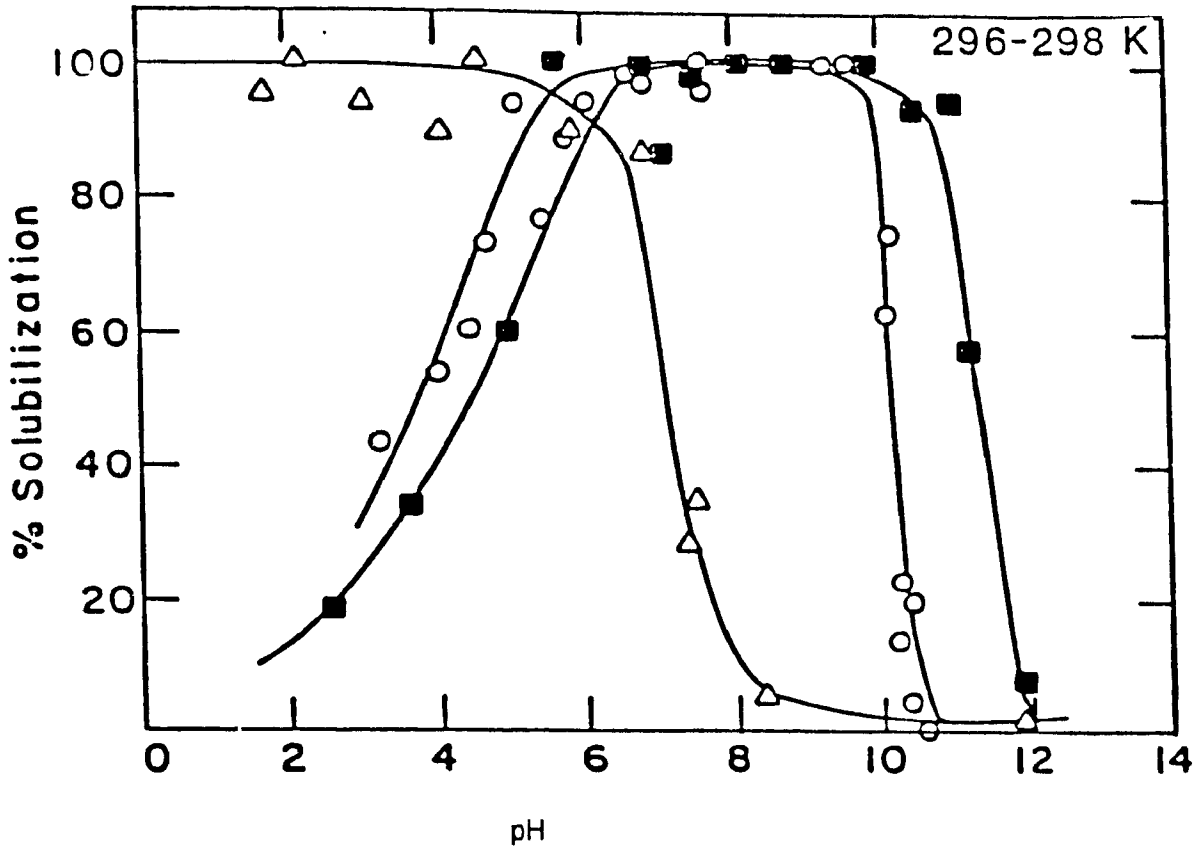
With anionic surfactants, solubilization should be possible only at pHs below the IP of the protein, where electrostatic interactions between the protein and the surfactant headgroups are attractive. At pHs above the IP, partitioning to the reverse-micellar phase should be inhibited because of repulsive interactions. The reverse would be true in the case of cationic surfactants.

Goklen (1986) confirmed the above using AOT (anionic surfactant) with the following low molecular weight proteins: lysozyme, cytochrome-c, and ribonuclease-a which have isoelectric points of 11.0, 10.4, and 7.8, respectively. From Figure 2-5 it is evident that no solubilization was observed for pH > IP for any of these proteins. As the pH of the system was lowered below the IP of each protein, a sudden increase in solubilization was observed, with 100% solubilization occurring for each protein at pHs slightly below their IP values. This 100% solubilization was maintained over several pH units as pH was further reduced. In the case of lysozyme and cytochrome-c, solubilization decreased at very low pH. This was probably caused by the denaturation of the proteins at these low pH values. Rahaman et al.(1988), using the cationic surfactant CTAB, found that protein solubilization was possible only when the enzyme was at a pH above its IP.

TABLE 2-1 - Molecular weight (MW) and isoelectric point (IP) for various proteins[b]

PROTEIN	SOURCE	MW	IP
Cytochrome-c	horse heart	12 300	10.4
Ribonuclease-a	bovine pancreas	13 700	7.8
Lysozyme	chicken egg white	14 300	11.0
ß-Lactoglobulin		18 400	5-10
Trypsin	bovine pancreas	23 000	10.5
Trypsinogen	bovine pancreas	24 000	9.3
Pepsin	porcine stomach mucosa	32 700	< 1
Albumin	chicken egg	44 000	4.5
Albumin	bovine serum	65 000	4.9
Catalase	bovine liver	240 000	?

[b]All proteins were obtained from Sigma except ß-Lactoglobulin which was obtained from Biochem Corp. The source of this information is the catalog from the supplier.



(■) Lysozyme (MW = 14 300, IP = 11.0)
(o) Cytochrome-c (MW = 12 300, IP = 10.4)
(Δ) Ribonuclease (MW = 13 700, IP = 7.8)

FIGURE 2-5 - Effect of pH on protein solubilization
(Goklen, 1986, p.142)

Initial contacting phases
organic: 50 mM AOT/isooctane
aqueous: 1.0 mg protein/mL solution ; 0.1 M KCl
pH adjusted by addition of 0.1 M HCl or NaOH

2.2.2 Ionic strength

Electrostatic interactions can be mediated by ionic strength, as reflected in the expression for the Debye length [Hiemenz, 1986]. The extent to which an electrostatic potential set up by a charged surface can extend into an electrolyte solution varies inversely with the ionic strength of the solution. This is characterized by the inverse Debye length, given by

$$K = \frac{(2000e^2 N_a I)^{0.5}}{(\epsilon kT)^{0.5}} \qquad (2\text{-}2)$$

where K^{-1} is the Debye length in meters, e is the unit of electronic charge (1.60×10^{-19}C), N_a is Avogadro's number (6.022×10^{23}molecules/mole), ϵ is the effective dielectric constant of the medium ($\epsilon = \epsilon_r \epsilon_0$, where ϵ_r is the dielectric constant of the medium and $\epsilon_0 = 8.85 \times 10^{-12} C^2 J^{-1} m^{-1}$), k is the Boltzmann constant (1.3805×10^{-23}J/K), T is the absolute temperature and I is the ionic strength of solution, as defined below:

$$I = \frac{1}{2} \sum z_i^2 M_i \qquad (2\text{-}3)$$

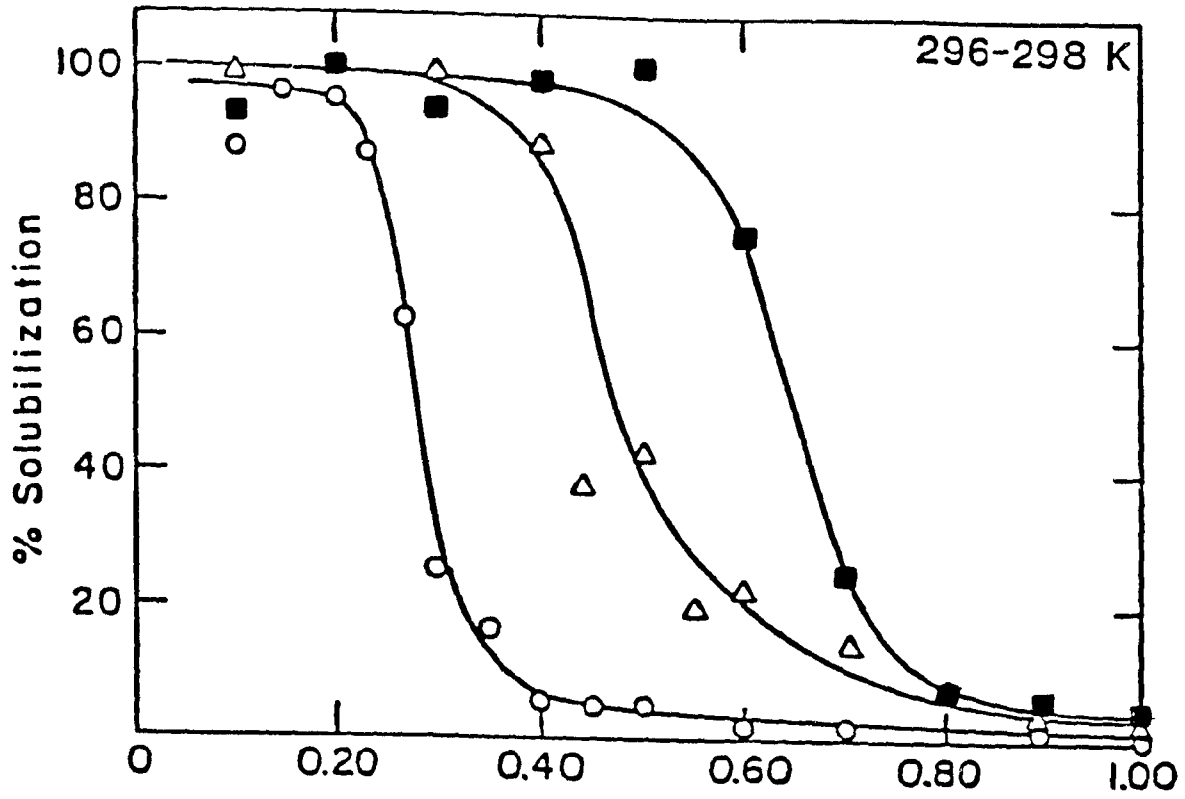
For a symmetrical (z:z) electrolyte, the ionic strength equals $1/2(z^2M + z^2M) = z^2M$. Therefore in a 0.01M solution of 1:1 electrolyte, $I = (1)^2(0.01M) - 0.01M$. For asymmetrical electrolytes (2:1) the ionic strength is equal to 3M and for a (3:1) electrolyte, the ionic strength is equal to 6M.

Abbott and Hatton (1988) found that higher salt concentrations screen the electrostatic interactions between the protein and the surfactant headgroups, thereby reducing the driving force for protein solubilization. They also found that increases in ionic strength may yield smaller reverse-micelles, i.e. the solubilization capacity is diminished because of the size exclusion effect. A third effect of the ionic strength

is to salt out the protein from the micellar phase because of the increased propensity of the ionic species to migrate to the micellar water pools and displace the protein. The work of Goklen (1986) confirms the results of these effects as is evidenced in Figure 2-6 where protein solubilization decreases with an increase in salt concentration.

2.3 Protein extraction from reverse-micelle

Goklen (1986) studied the solubilization behavior of cytochrome-c in 50 mM AOT as controlled by variations in [KCl] between 0.1 and 1.0 M, as shown in Figure 2-6. The recovery of protein was investigated in a complementary experiment, using a 1:1 volume ratio. Cytochrome-c was solubilized in 50 mM AOT in isooctane solution from 0.1 M KCl solution, to a concentration of 1 g/L. This "loaded" micellar phase was then contacted with aqueous solutions of differing KCl concentration, and the de-solubilization, or "back transfer" of the protein to the aqueous phase was measured. Figure 2-7 shows that the protein was almost completely recovered in an aqueous phase of high ionic strength. Similar experiments were conducted with ribonuclease-a but it could not be back transferred to an aqueous phase, even with KCl concentrations as high as 2.0 M at pH=9.7.



(■) Lysozyme (MW = 14 300, IP = 11.0)
(O) Cytochrome-c (MW = 12 300, IP = 10.4)
(Δ) Ribonuclease (MW = 13 700, IP = 7.8)

FIGURE 2-6 - Effect of ionic strength on protein solubilization (Goklen, 1986, p.160)

Initial contacting phases
organic: 50 mM AOT/iso-octane
aqueous: 1.0 mg protein/mL solution
no control over the pH of the system

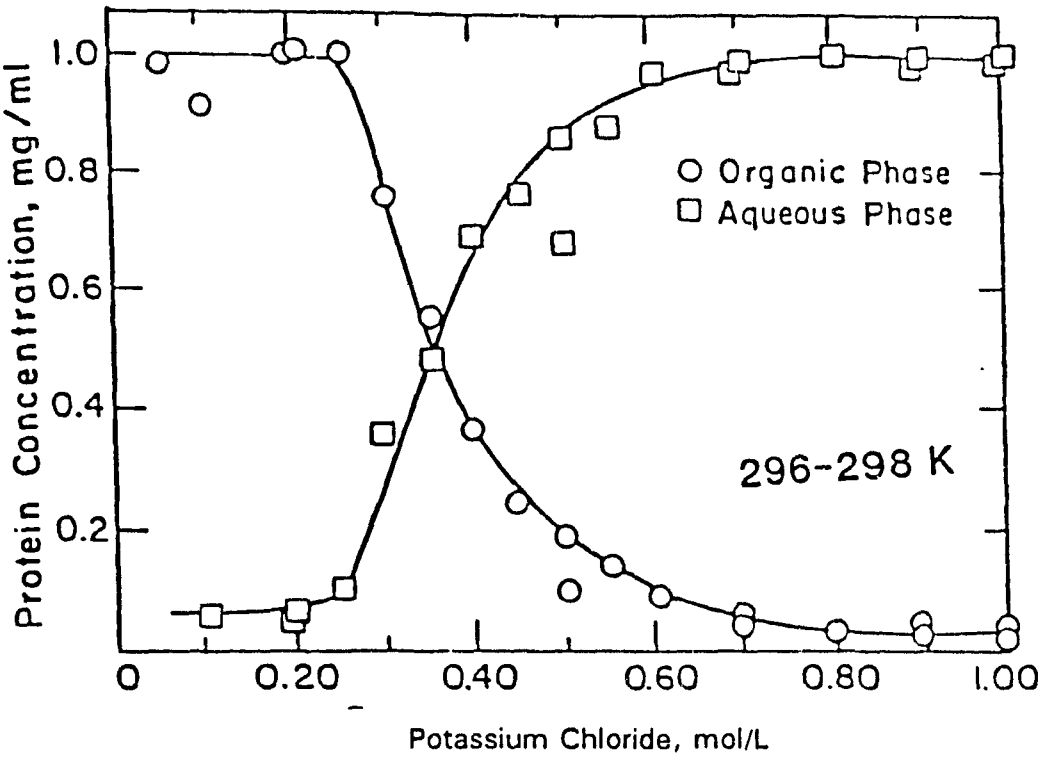


FIGURE 2-7 - Recovery of cytochrome-c from reverse-micelle (Goklen, 1986, p.214)

Initial contacting phases
organic: 50 mM AOT in iso-octane
1 g/L cytochrome-c
aqueous: no control over pH of the system

3.0 PROJECT OBJECTIVES

The objectives of this work were

1. To determine the effect of the following parameters on water solubilization and protein uptake
   a) nature and concentration of surfactant
   b) nature of organic solvent
   c) nature and concentration of salt
   d) nature and concentration of buffer
   e) presence of co-surfactant
2. To determine whether proteins can be recovered from the reverse-micellar phase by varying the organic phase composition.

Two anionic, double hydrocarbon tail surfactants were used: AOT (sodium di-2-ethylhexylsulfosuccinate) and DNNSA (dinonylnaphthalene sulfonic acid).

Organic compounds having different molecular shapes were used as solvents. Straight chain, branched chain and ring molecules were used. Both pure and mixed solvents were tested. The solvents included n-alkanes (where n varied from 6 to 17), cyclohexane, iso-octane, kerosene, and 2,2,4,4,6,8,8-heptamethylnonane.

CTAB (cetyltrimethylammonium bromide) was used as a co-surfactant. CTAB has a cationic hydrophilic group and a single chain hydrophobic group. Other co-surfactants employed included bacitracin and phosphatidylcholine which are both anionic, and palmitin, tripalmitin, pluronic, and heptanol which are neutral.

Aveyard's work suggests that varying the solvent might force release of the protein from the reverse-micelle and that provided the incentive for the second objective.

4.0 MATERIALS AND METHODS

It is important to emphasize that all the solubilization and extraction experiments were performed with synthetic aqueous solutions, i.e. purified proteins solubilized in a suitable buffer. All parameters and components are known, which is not the case with fermentation broths.

4.1 Materials

Two anionic surfactants were used. Aerosol-OT (sodium di-2-ethylhexylsulfosuccinate) was employed in the majority of the experiments. Aerosol-OT (AOT) (99% purity) was obtained from Fisher Scientific (Montreal, Quebec). Dinonylnaphthalene sulfonic acid (DNNSA) dissolved in kerosene (50%DNNSA by weight) was obtained from Pfaltz and Bauer (97% purity).

The following co-surfactants were obtained from Sigma (St.Louis, Missouri): bacitracin, phosphatidylcholine, palmitin, tripalmitin, and cetyltrimethylammoniumbromide. Pluronic was obtained from BASF Corporation (New Jersey, U.S.A.) and heptanol was obtained from Aldrich Chemical Company (Milwaukee, Wisconsin).

The straight chain hydrocarbons, heptane, octane, and hexadecane (99+% purity) were obtained from Aldrich Chemical Company; HPLC grade iso-octane and cyclohexane were obtained from Fisher Scientific; n-hexane and n-pentadecane were obtained from American Chemicals Ltd. (Montreal, Quebec); n-decane, n-undecane, n-dodecane, and n-tetradecane (99+% purity) were obtained from BDH Inc. (Ville St-Laurent, Quebec).

Karl Fischer titrant, AQUASTAR Comp 5 (pyridine free) and 1-propanol were obtained from BDH Inc..

Electrophoresis purity reagent, TRIS (tris(hydroxymethyl) aminomethane) was obtained from Bio-Rad laboratories (Richmond, California). Citric acid (anhydrous) and sodium hydroxide were obtained from Fisher Scientific. Reagent sodium citrate, potassium chloride, and hydrochloric acid were obtained from American Chemicals Ltd..

β-Lactoglobulin (bovine milk) and albumin (chicken egg) were obtained from United States Biochemical Corporation (Cleveland, Ohio). Catalase (bovine liver), lysozyme (egg white), pepsin (porcine stomach mucosa), trypsin (bovine pancreas), and trypsinogen (bovine pancreas) were obtained from SIGMA Chemical Company (St.Louis, Missouri).

Distilled water was used for the preparation of aqueous solutions. All chemicals were used without further purification.

4.2 Methods

Most solubilization experiments were performed by contacting ten (10) mL of aqueous protein solution with ten (10) mL of surfactant-containing organic solution. Buffers and salts were employed in the aqueous solution to stabilize the system when adjusting pH. The phase-contacting experiments were carried out in cork stoppered 50 mL Erlenmeyer flasks. The phases were agitated vigorously for sixty (60) minutes on a vibrating shaker at 250 rpm in a constant temperature room set at 23°C. Phase separation due to gravity was usually complete within two hours. In those instances when the phases did not separate readily, the samples were centrifuged at 5000 rpm

for 20 minutes. Water uptake and protein distribution measurements over time indicated that this equilibration period was more than adequate for equilibrium between the two phase. In most instances the lower phase was an aqueous solution containing ions and a negligible amount of surfactant. The upper phase was a reverse-micellar solution. Pasteur pipettes or syringes were used to collect an aliquot from each phase.

4.2.1 Preparation of solutions

Solutions of salts, buffers and surfactants were prepared on a molar basis, with the preweighed solid or liquid solute (in the case of DNNSA) added to a volumetric flask and filled to the appropriate volume with solvent. Protein solutions were made on a weight per volume basis.

4.2.2 Analytical techniques

Proteins were analyzed by their ultraviolet absorption at 280 nm which (when accounting for background absorbance) should vary linearly with protein concentration, following Beer's Law

$$C_p = \epsilon \, l \, A280 \quad (mg/ml) \qquad (4\text{-}1)$$

where ϵ is Beer's Law proportionality constant or extinction coefficient, l is the path length of the cuvette (usually 1 cm) and A280 is the absorbance at 280 nm.

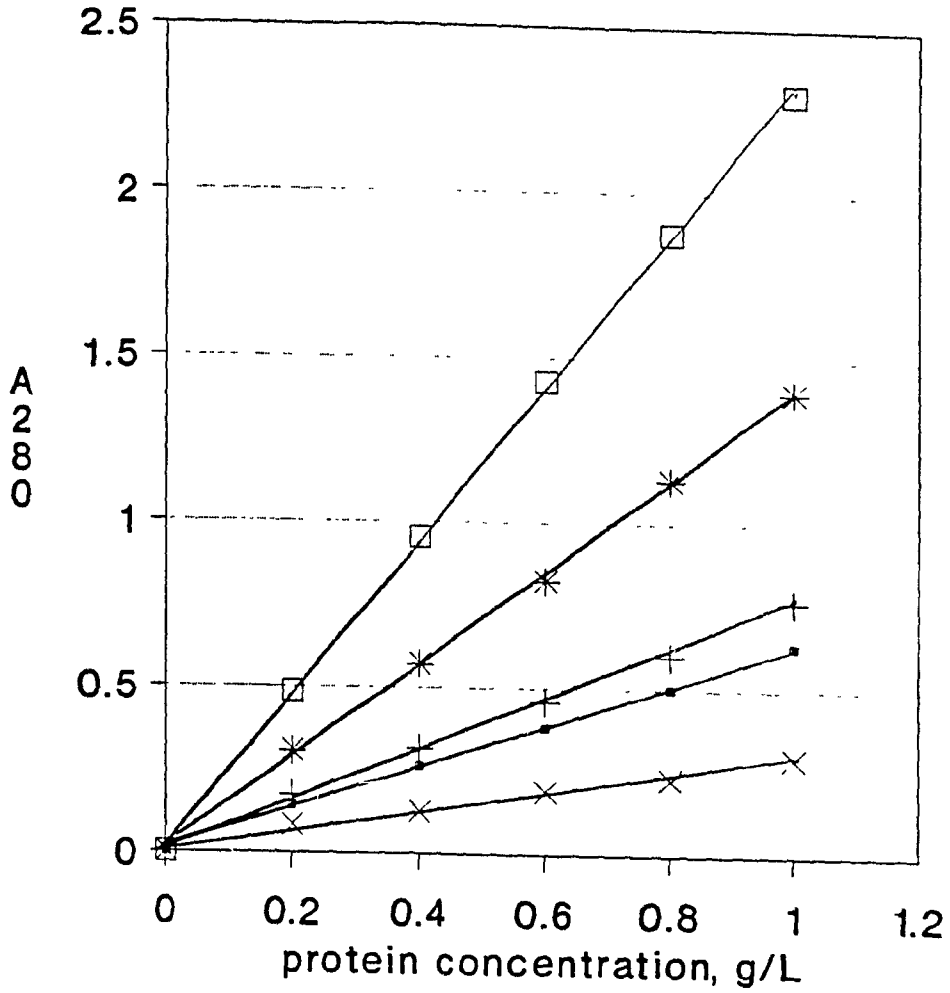
The A280 was usually measured against a solvent standard. Iso-octane was used as a reference for micellar organic solutions, while distilled water was used as a standard for aqueous solutions. Figure 4-1 shows the calibration curves of several proteins ; a good linear proportionality exists in the 0.1 to 1 0 g/L range. All measurements were made with a Varian DMS Spectrophotometer using standard

quartz cuvettes with a 1 cm path length.

This technique was used to measure the protein present both in aqueous and micellar samples. Since the extinction coefficient is essentially the same for proteins in water and in micellar solution [Goklen, 1986, p.108], the extinction coefficient determined for proteins dissolved in aqueous solutions was used for both aqueous and micellar solutions.

Protein measurements in both phases were necessary to determine the percent protein solubilized in the micellar phase. In the case of micellar solutions, Goklen (1986) observed that under certain conditions the microemulsion droplets themselves, though too small to scatter visible light, could scatter enough UV light to inflate the protein assay by 10-15%. Therefore, when protein material balances closed to within 15-20%, they were considered reliable.

Initially the water content of the organic (or top) phase was determined using an FT-IR spectrophotometer manufactured by Bomem-Michelson. Traces of water can be found on the IR spectrum in the following regions: 3450 to 3000cm^{-1}, 1700 to 1500cm^{-1}, and 600cm^{-1} regions due to the high optical density of water (and stretching of the -OH bond). The IR absorption at 3450cm^{-1} for water gave the most consistent results. Figure 4-2 shows the calibration curve of dilute water solutions at 3450cm^{-1}. A relatively good linear proportionality exists up to 3.0 volume percent water. Even without the presence of surfactant, a small amount of water is dissolved in the organic solvent resulting in the non-zero intercept. Higher water contents were determined by Karl Fischer titration using a Brinkmann 701/1 titrator.



(▪) Bovine Serum Albumin
(+) β-Lactoglobulin
(*) Trypsinogen
(□) Lysozyme
(X) Chicken Egg Albumin

FIGURE 4-1 - UV calibration curve for proteins

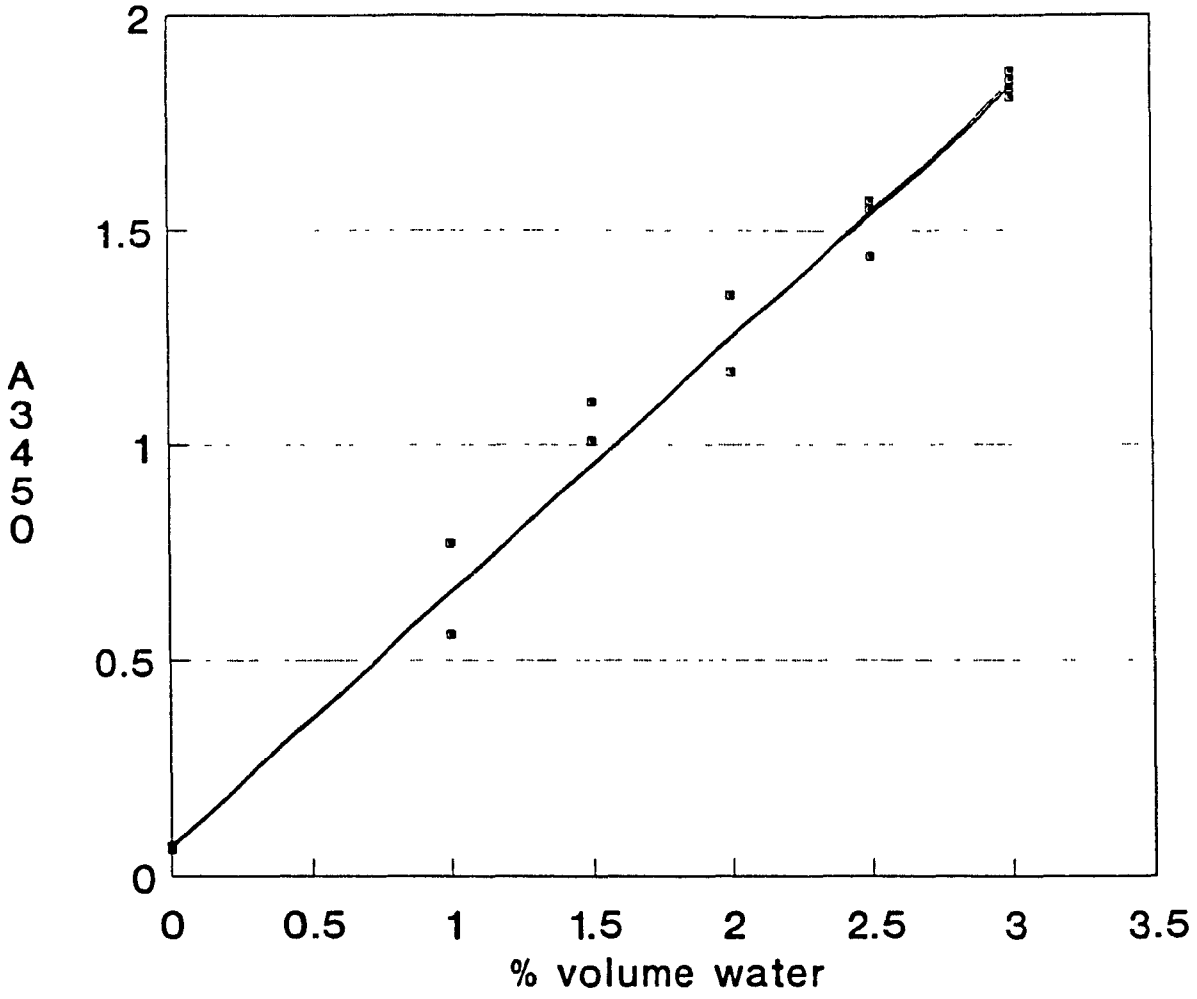


FIGURE 4-2 - IR calibration curve for water

The Karl Fisher (KF) reaction takes place in two steps:

$$CH_3OH + SO_2 + RN \rightarrow [RNH]SO_3CH_3 \qquad (4\text{-}2)$$

$$H_2O + I_2 + [RNH]SO_3CH_3 + 2RN \rightarrow [RNH]SO_4CH_3 + 2[RNH]I \qquad (4\text{-}3)$$

in which RN is a nitrogeneous base. Usually, a water containing sample is added to methanol, and titrated with a solution of the other components. Since long chain hydrocarbons do not dissolve satisfactory in methanol, propanol was employed.

The KF titrant was AQUASTAR Comp 5 from BDH Inc. (Ville St-Laurent, Quebec). It is prepared with an initial water equivalent of about 5 mg H_2O/mL titrant. Atmospheric moisture can penetrate into the titration vessel or into the reagent solutions and cause gradual errors. In practice the titer was checked on a weekly basis and remained constant at 5.35 mg H_2O/mL titrant. Goklen (1986) noted that the water content measured by KF was consistently less than the known value by approximately 10%, probably because the water of hydration of the surfactant headgroup reacted more slowly than unbound core water.

The pH was measured with a Fisher Scientific pH meter. For solubilization experiments, the pH of aqueous protein solutions was measured during preparation.

The density of the organic phase was required for the calculation of the ratio of moles of water in the organic phase to moles of surfactant. A 10 mL pycnometer was employed for density measurement of some organic phases. For the most part, the densities were estimated from the water content and the density of the organic solvent as the estimated and measured densities corresponded to within 4 percent.

The density of the organic phase was determined as shown below.

Let

y = weight water in 100 g sample

X = weight AOT in 100 g sample

C = concentration AOT in organic solvent in moles per liter

grams solvent + AOT = 100 - y **(4-4)**

grams solvent = 100 - y - X **(4-5)**

$$volume\ of\ solvent = \frac{(100-y-X)}{\rho_s} \qquad (4\text{-}6)$$

where ρ_s is the solvent density in g/mL

$$X = (volume\ of\ solvent) \times \frac{C}{1000} \times MW \qquad (4\text{-}7)$$

where MW = molecular weight of surfactant

Hence

$$X = \frac{(100-y-X)}{\rho_s} \times \frac{C \times MW}{1000} \qquad (4\text{-}8)$$

$$X = \frac{(100-y)}{\rho_s}\left(\frac{MW}{1000}\right)C - \frac{X}{\rho_s}\left(\frac{MW}{1000}\right)C \qquad (4\text{-}9)$$

$$X\left[1+\frac{C}{\rho_s}\left(\frac{MW}{1000}\right)\right] = \left(\frac{100-y}{\rho_s}\right)\left(\frac{MW}{1000}\right)C \qquad (4\text{-}10)$$

Finally,

$$X=\frac{(\frac{100-y}{\rho_s})(\frac{MW}{1000})C}{1+\frac{C}{\rho_s}(\frac{MW}{1000})} \qquad (4\text{-}11)$$

Then let

ρ_o = density of organic phase

$$\rho_o=\frac{total\ mass}{(volume\ of\ solvent)+(volume\ of\ water)} \qquad (4\text{-}12)$$

$$\rho_o=\frac{100}{(\frac{100-y-X}{\rho_s})+\frac{y}{\rho_w}} \qquad (4\text{-}13)$$

For an organic phase (50 mM AOT/n-decane) containing 4.6% water by weight, i.e. 4.6 g water in 100 g of organic phase where the organic phase consists of 95.4 g of non-aqueous materials, namely AOT and decane. Assuming that the surfactant does not alter the density of the solvent, the volume of the organic phase is determined by summing the volumes of decane and water as shown below:

y = 4.6 g $\quad \rho_w$ = 0.998 g/ml (@23°C)
ρ_s = 0.728 g/ml
MW = 445.51

$$X=\frac{\frac{95.4}{0.728}(0.445)0.05}{1+\frac{0.05}{0.728}(0.445)}=2.83g$$

$$\rho_o=\frac{100}{\frac{94.5-2.8}{0.728}+\frac{4.6}{0.998}}=0.766g/mL$$

Note that the assumption here is that when AOT is added to a volume of solvent, the volume does not change. The measured density was 0.738 g/mL, 3.8% below the calculated value.

Another plausible assumption is that when the AOT is added, the volume increases as if the AOT behaved like the solvent. Densities were re-calculated and gave similar results to those calculated with the opposite assumption. For simplification, the method described above was used to calculate densities when the density of the organic phase was not measured.

5.0 RESULTS AND DISCUSSION

5.1 Definitions

The data are presented in terms of parameters which are defined below. The amount of water solubilized in the surfactant containing organic phase is usually quoted in weight percent,

$$\%(wt)=\frac{g\ of\ water}{g\ of\ organic\ phase}\times 100\% \qquad (5\text{-}1)$$

and was determined using a Karl Fisher Titrator. Occasionally water results are shown in volume percent,

$$\%(vol)=\frac{mL\ of\ water}{mL\ of\ organic\ phase}\times 100\% \qquad (5\text{-}2)$$

and these were found using the FT-IR. These numbers are proportional to one another but are not numerically the same as they differ by a factor equal to the density of the organic phase. The water content and surfactant concentration in the organic phase are needed for the determination of W_o, the molar ratio of water to surfactant in the organic phase.

$$W_o=\frac{[water]\ moles/L}{[surfactant]\ moles/L} \qquad (5\text{-}3)$$

The protein was monitored in each phase in order to check the protein mass balance. Data are reported only when the protein mass balances closed to within 20%. This degree of closure assured that most of the protein was accounted for and that little had transferred to the organic-aqueous interface.

$$\{protein\ in\ aqu.\ feed\} = \{protein\ in\ org.\ phase\} + \{protein\ in\ aqu.\ phase\} \quad (5\text{-}4)$$

The percent protein solubilized in the organic phase was calculated from the ratio of the concentration of protein in the organic phase to the concentration of protein in the aqueous feed:

$$\%\,protein\ solubilized = \frac{[protein\ in\ organic\ phase]}{[protein\ in\ aqueous\ feed]} \times 100\% \quad (5\text{-}5)$$

To characterize a mixed solvent, the average carbon number (ACN) was determined by multiplying the fraction of each hydrocarbon (on a volume basis) by its carbon number:

$$ACN = \sum x_i N_i \quad (5\text{-}6)$$

where

x_i = fraction of solvent i on a volume basis

and

N_i = carbon number of solvent i.

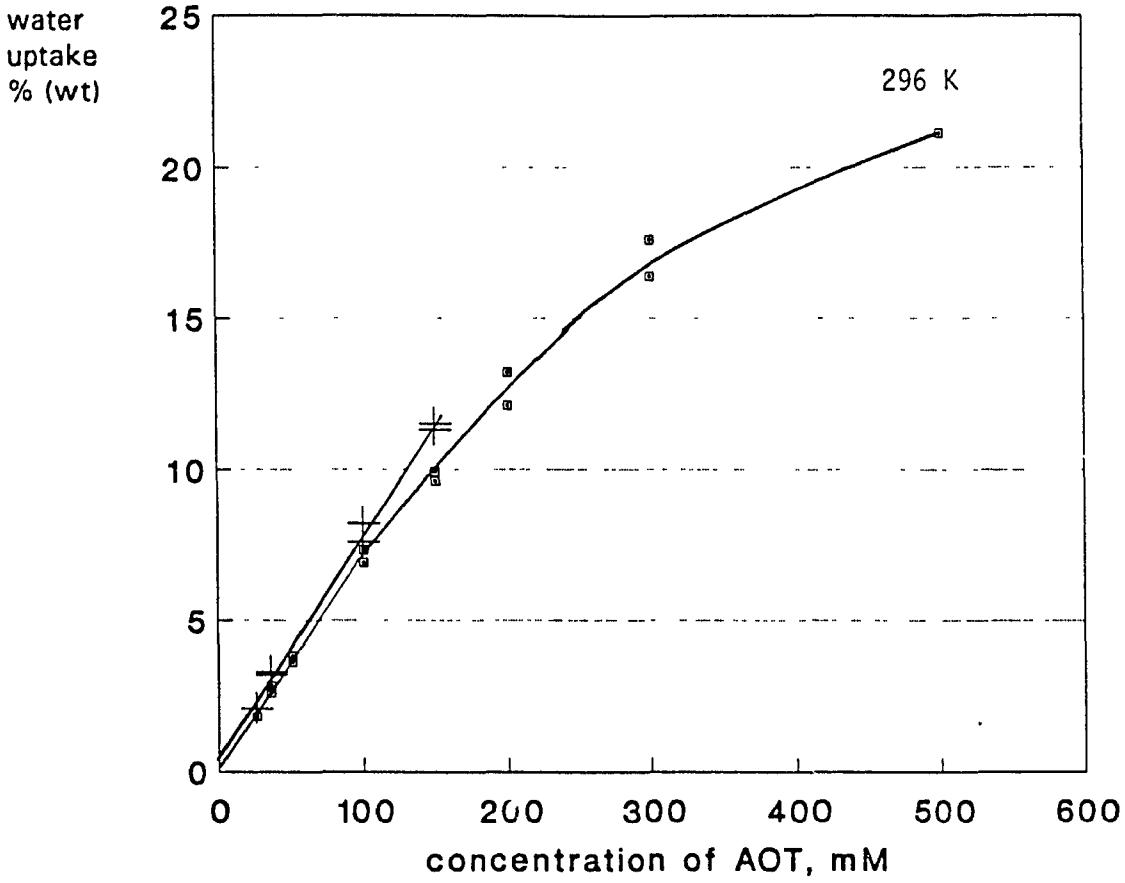
Data for all figures are presented in the Appendices.

5.2 Effect of surfactant concentration on water uptake

Water uptake versus AOT concentration using n-octane and n-decane as the organic solvents is plotted in Figure 5-1. At surfactant concentrations up to approximately 200 mM AOT, the water uptake increased linearly. At higher concentrations, the trend continued except the curve was no longer linear. This increase in water uptake is a result of the corresponding increase in the number of micelles formed. Consequently, the capacity of the reverse-micellar phase for proteins is increased. It is possible that several small micelles regroup to from a large micelle capable of encapsulating a large protein molecule.

5.3 Effect of n-alkane carbon number on water uptake

Figures 5-2A and 5-2B show water uptake versus carbon number (ACN) for nine straight chain alkanes used as solvents. Data were obtained for seven surfactant concentrations. Low surfactant concentrations are shown in Figure 5-2A, while high surfactant concentrations are shown in Figure 5-2B. The shapes of the curves were independent of the surfactant concentration. For a wide range of AOT concentrations, water uptake increased from a carbon number of 6 up to a maximum water uptake at a carbon number of 10. For longer chains, the reverse-micelles were less stable. At a carbon number of 12 and higher, the water uptake was essentially zero. Water uptake increased somewhat as the length of the carbon chain of the solvent approached the length of the carbon chain in the two tails of AOT. The hydrocarbon tails of AOT consist of 9 and 8 carbon atoms respectively (see Figure 1-1A).



(⊡) octane
(+) decane

FIGURE 5-1 - Effect of [AOT] on water uptake

Initial contacting phases
organic: AOT/n-alkane
aqueous: 0.2 M tris ; 0.1 M KCl
pH adjusted to 6.6 by addition of 1.0 M HCl

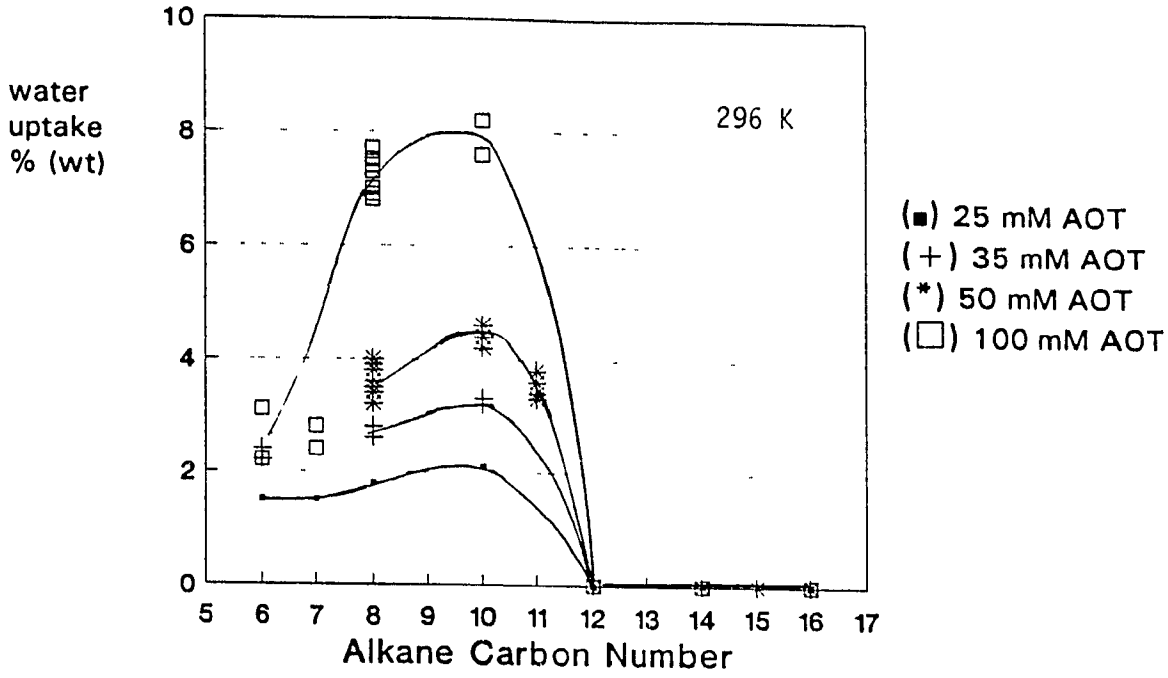


FIGURE 5-2A - Effect of alkane carbon number on water uptake for AOT

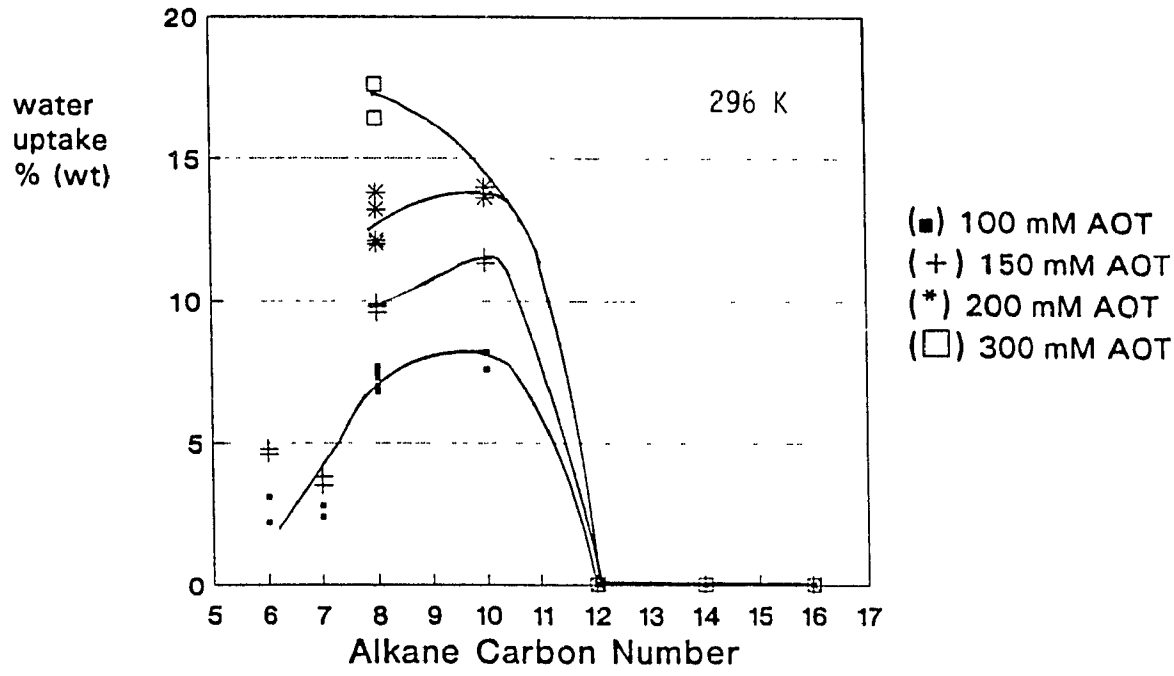


FIGURE 5-2B - Effect of alkane carbon number on water uptake for AOT

Initial contacting phases
organic: AOT/n-alkane
aqueous: 0.2 M tris ; 0.1 M KCl
pH adjusted to 6.6 by addition of 1.0 M HCl

The actual length of the surfactant tail may be determined by using literature values for the various bonds (C-C: 154 pm and C-O: 143 pm). There is very little difference in the length of these two bonds. Accordingly, the longer chain in the AOT molecule (including the focal carbon) is 1364 pm (7 C-C and 2 C-O bonds). The shorter chain has one less C-C bond and thus is only 1210 pm long. The shortest straight chain alkane to exceed the length of the surfactant tail is decane with a length of 1386 pm. Interestingly, water uptake was a maximum with decane employed as a solvent. Water uptake was essentially zero with longer chain alkane solvents than dodecane.

These results are consistent with the work of Aveyard et al.(1986) with alkanes. They found that water uptake dropped drastically at a certain carbon number. These results are also consistent with the theory of Mukhejee et al.(1983) who reasoned that shorter alkanes penetrate into surfactant layers more effectively than do longer alkanes and thus stabilize reverse-micelles.

5.4 Effect of mixtures of n-alkanes on water uptake

Figure 5-3 shows water uptake for solvents consisting of mixed alkanes. The abscissa is the average carbon number (ACN) as defined in eqn 5-8. For the n-alkane mixture range investigated, namely from 9 to 15, the organic micellar phase went through a maximum water uptake at around ACN = 10 and then dropped to zero dramatically between 11 and 12. No water uptake occurred for ACN $\geq$ 12.

Data for mixtures of n-alkanes and pure n-alkanes are plotted on a single curve in Figure 5-4 for an average carbon number of 10. The water uptake was not significantly different for mixtures or pure alkanes. The behavior of mixtures of n-alkanes was similar to that observed for the series of pure n-alkanes. When octane is mixed with decane, dodecane, tetradecane, or hexadecane, a maximum occurs in the water versus ACN curve at some intermediate solvent composition (see Figure 5-3), just as in the case of the n-alkane series (see Figure 5-2). The locus of optima as a function of the volume average ACN of a mixture coincides with that of the corresponding pure n-alkane.

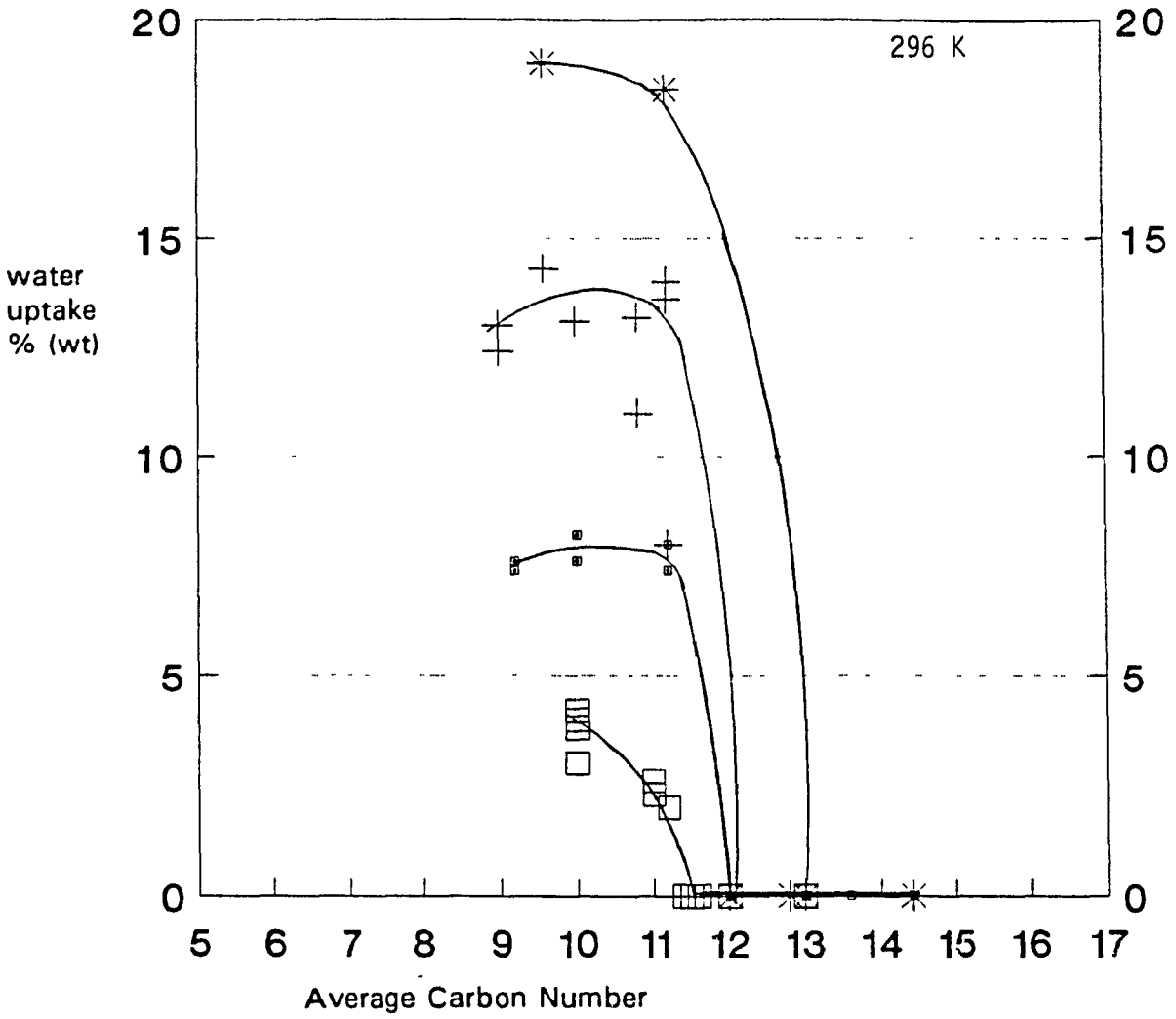


FIGURE 5-3 - Effect of average carbon number on water uptake

Initial contacting phases
organic: AOT/mixture of n-alkanes
aqueous: 0.2 M tris ; 0.1 M KCl
pH adjusted to 6.6 by addition of 0.1 M HCl

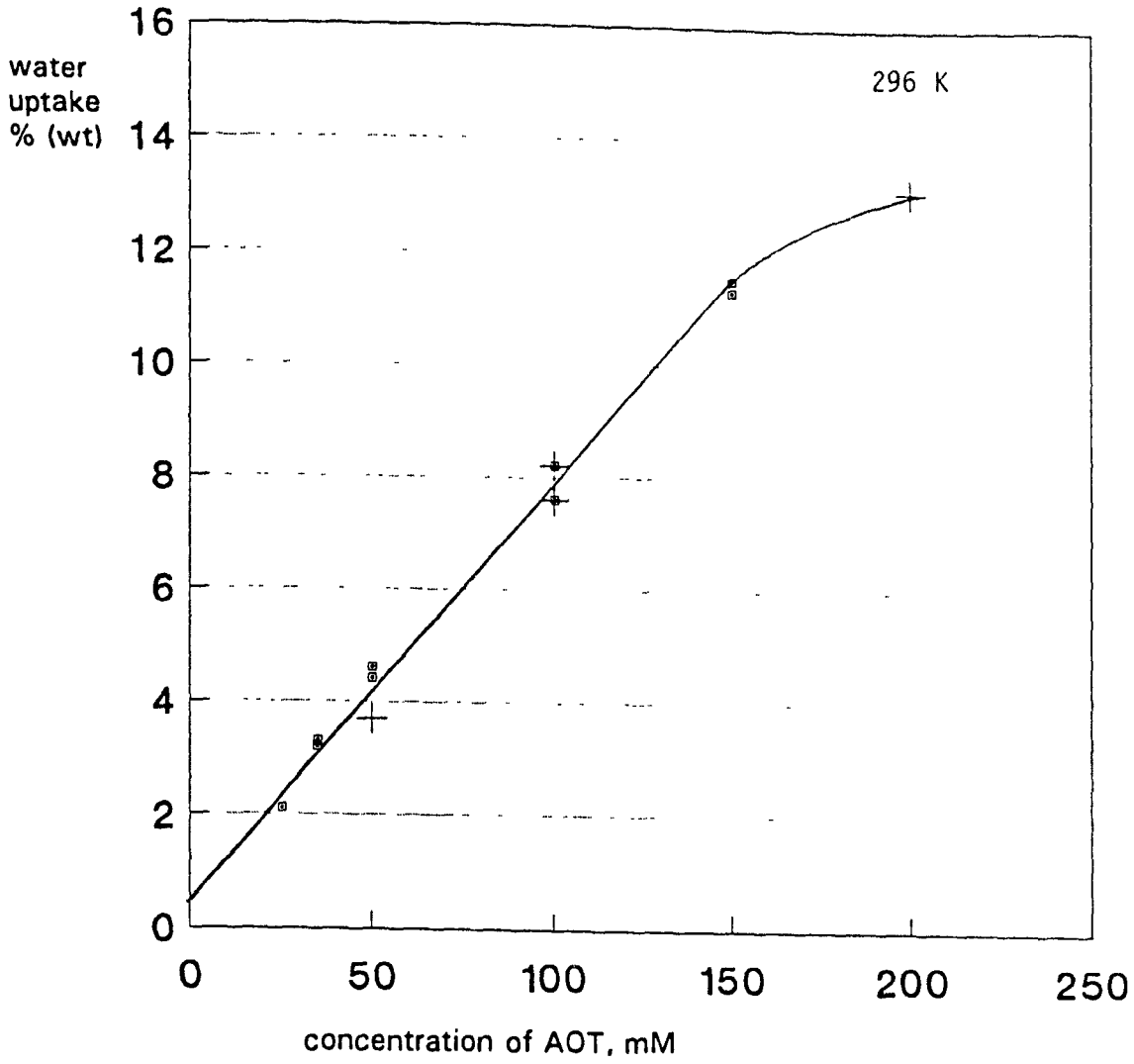


FIGURE 5-4 - Pure alkanes and mixtures of alkanes and their effect on water uptake

Initial contacting phases
organic: AOT/pure n-alkane and mixture
aqueous: 0.2 M tris ; 0.1 M KCl
pH adjusted to 6.6 by addition of 0.1 M HCl

The manner in which the same average carbon number was composed was also investigated. Table 5-1 lists a number of different solvents having an average carbon number of 10 and the corresponding water content with 50 mM AOT as the surfactant. Both binary and ternary mixtures were investigated. There is some variation in water uptake (range of 3-4%) but there is no evident trend.

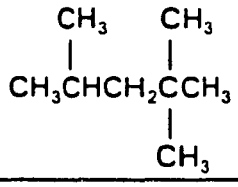
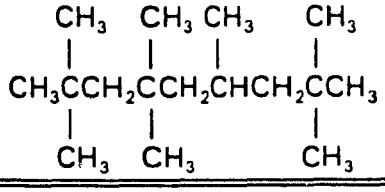
TABLE 5-1 - Effect of composition for ACN of 10

No.	volume percent of alkane						water uptake % (wt)
	C7	C8	C11	C13	C15	C16	
1	25				75		4.0, 3.8
2	50			50			3.7, 3.8
3	62.5				37.5		3.0, 2.9
4	80					20	3.3, 3.1
5		33	67				3.9, 3.8
6		60		40			4.3, 4.2
7		71			29		3.8, 3.8
8		75				25	3.7, 3.7
9	50			35		10	3.5, 3.6
10	60			20		20	3.4
11	65			5		30	3.4, 3.5

5.5 Effect of isomers

When comparing water uptake with different hydrocarbons with the same number of carbons, it is evident that the nature of the structure is important. The structures of various isomers are illustrated in Table 5-2. n-Octane and iso-octane have the same number of carbons but octane is slightly longer. But, as is evident from Table 5-3, they give similar water uptake results. However, their differences in structure are significant; considering that octane is 8 carbons long while iso-octane is only 5 carbons long.

TABLE 5-2 - Structure of some organic solvents

Straight Chain	Branched
n-octane $CH_3(CH_2)_6CH_3$	iso-octane 
n-dodecane $CH_3(CH_2)_{10}CH_3$	kerosene mixture of branched and straight chains
n-hexadecane $CH_3(CH_2)_{14}CH_3$	2,2,4,4,6,8,8-heptamethylnonane 

On the other hand, when one compares the straight chain alkane hexadecane with the very bulky heptamethylnonane, an extreme difference in water uptake occurs. For n-hexadecane there is no water uptake while for heptamethylnonane the water uptake is about 5.2% by weight (see Table 5-3). In this case these structures are significantly different. The n-alkane is 16 carbons long but the length of the branched isomer is only 9 carbons. The straight chain n-dodecane was compared with the highly branched commercially available kerosene whose average carbon number is about twelve. In the case of dodecane no water uptake was evident while kerosene had a water uptake of about 3.3%. It can be concluded that shorter alkanes penetrate more effectively than do longer alkanes even when both alkanes have the same number of carbons. An interesting point here is that heptamethylnonane gives significantly higher water uptake than the octanes.

TABLE 5-3 - Comparing water uptake for long chain alkanes and short bulky hydrocarbons

	SURFACTANT CONCENTRATION	
	50 mM AOT	100 mM AOT
ALKANE	water uptake % (wt)	water uptake % (wt)
n-octane	3.7	7.1
iso-octane	3.6	7.4
n-dodecane	0	
kerosene	3.3	
n-hexadecane	0	
2,2,4,4,6,8,8-heptamethylnonane	5.2	

5.6 Effect of ACN on water uptake for DNNSA

To determine whether a critical ACN existed for a different two-tailed surfactant, dinonylnaphthalenesulfonic acid (DNNSA) was investigated. The only DNNSA which could be purchased was dissolved in kerosene. Thus only mixtures of alkanes were employed with this surfactant. The average carbon number was calculated from equation (5-6) assuming kerosene had an average carbon number of twelve.

Figure 5-5 shows water uptake versus DNNSA concentration. Water uptake increased linearly with surfactant concentration as was the case with AOT at low concentrations. Water uptake with DNNSA was less than half that with AOT as shown in Figure 5-1.

The data presented in Figure 5-6 show that the water uptake was unaffected by the carbon number over the range investigated in contrast to the results with AOT shown in Figures 5-2 and 5-3. The effect of varying hydrocarbon chain length did not occur with DNNSA. Measurements of surfactant concentration showed that it remained essentially completely in the organic phase. No more than 2% of the surfactant was found in the aqueous phase. DNNSA has a much longer and bulkier hydrophobic part than AOT. In addition to having 9 single carbon-carbon bonds, it also has 2 single benzene carbon-carbon bonds and 2 double benzene carbon-carbon bonds. This results in a length of 1942 pm which is about 30% longer than that of AOT. The shortest straight chain alkane to exceed the length of the surfactant tail is tetradecane with a length of 2002 pm. This would appear to mean that the same phenomena with AOT may occur with DNNSA providing the ACN is greater than 14. However, there is only one data point beyond 14 and hence the above statement is only a hypothesis.

Further experimentation is needed to determine this outcome.

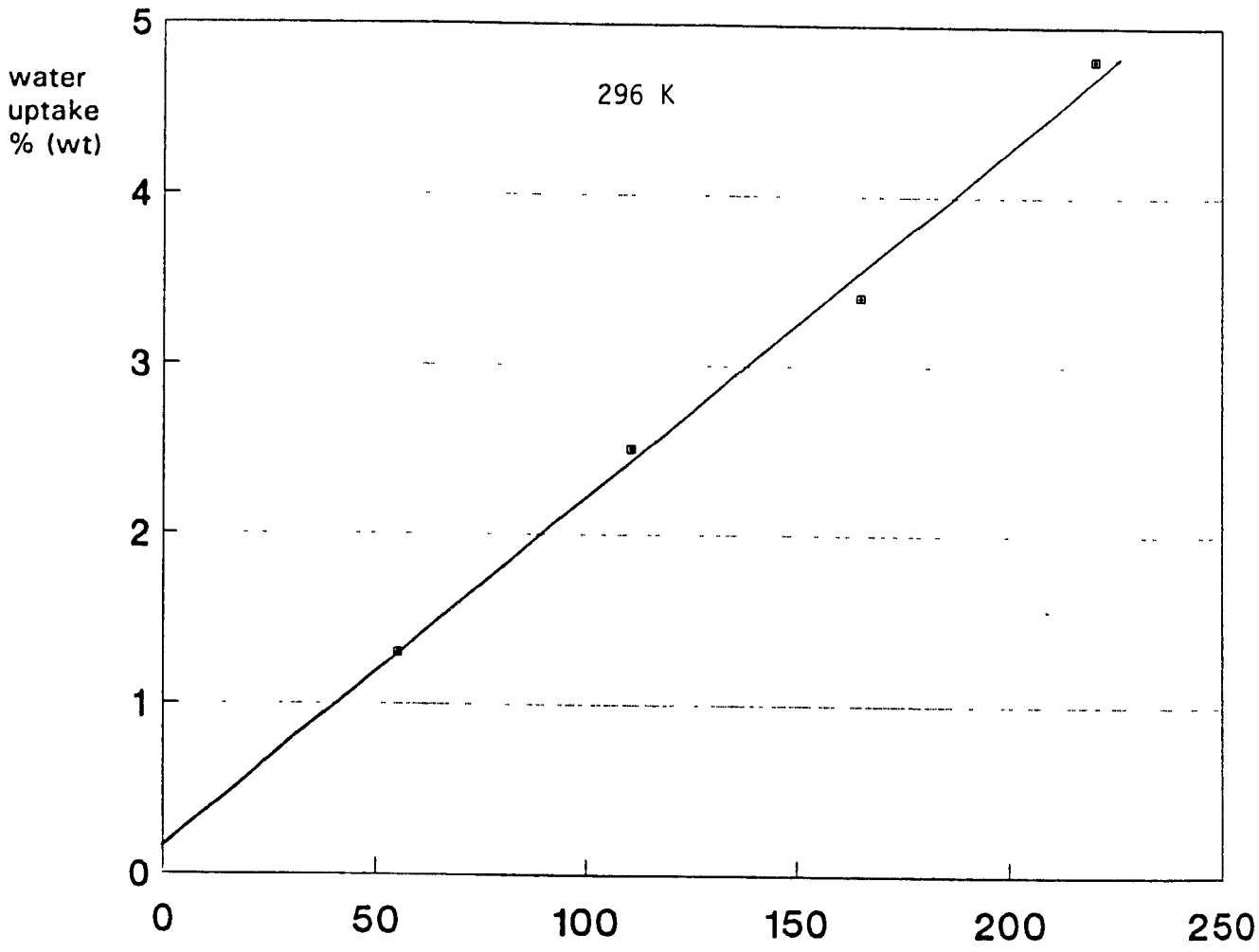
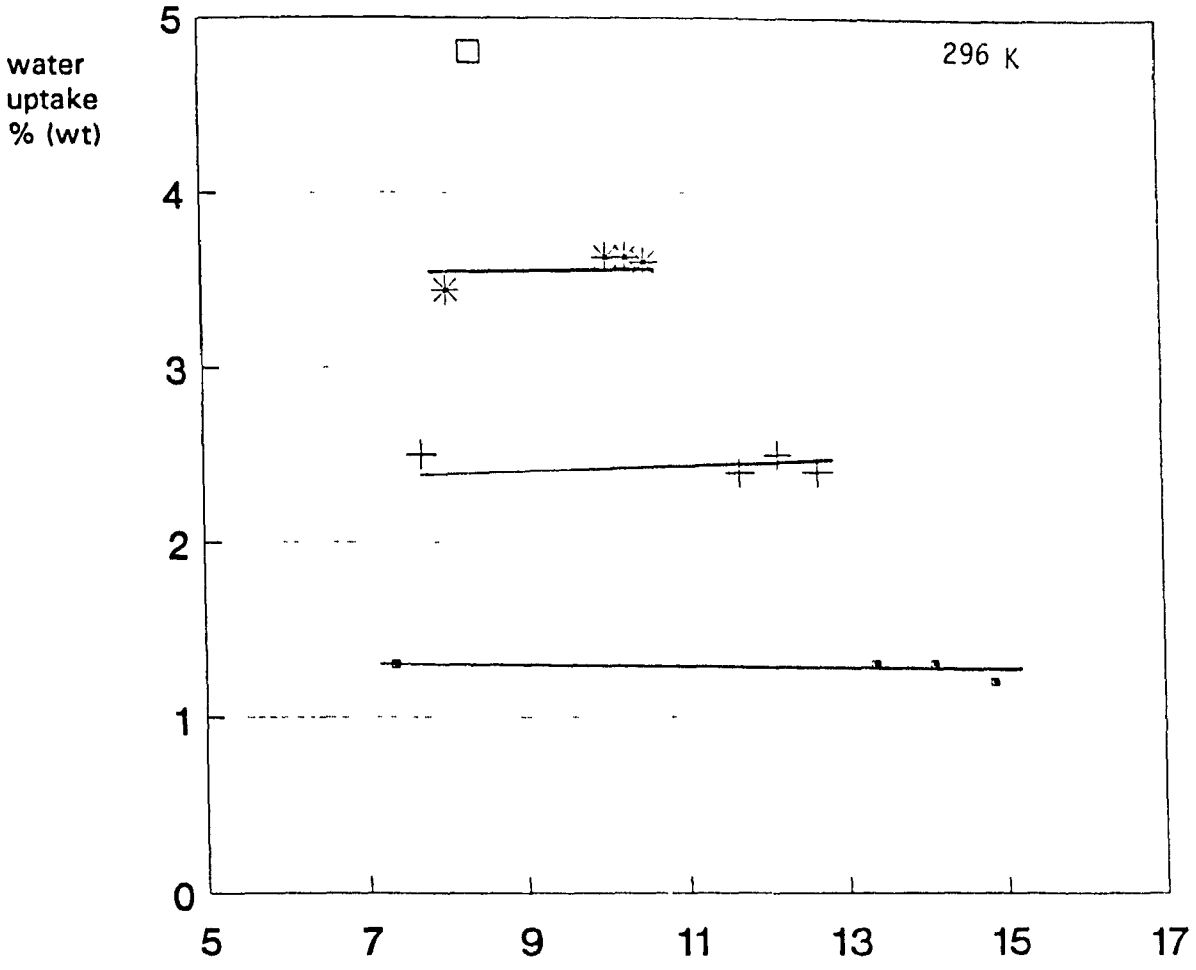


FIGURE 5-5 - Effect of [DNNSA] on water uptake

Initial contacting phases
organic: DNNSA/mixture of kerosene and n-heptane
aqueous: 0.2 M tris ; 0.1 M KCl
pH adjusted to 6.6 by addition of 0.1 M HCl



(▫) 55 mM DNNSA
(+) 110 mM DNNSA
(*) 165 mM DNNSA
(□) 220 mM DNNSA

FIGURE 5.6 - Effect of average carbon number on water uptake for DNNSA

Initial contacting phases
organic: DNNSA/mixture of kerosene and n-heptane
aqueous: 0.2 M tris ; 0.1 M KCl
pH adjusted to 6.6 by addition of 0.1 M HCl

5.7 Effect of buffer and salt types and concentration on the critical n-ACN

Figure 5-7 shows water uptake versus average carbon number for pure n-alkanes with different aqueous buffers. The nature of the buffer played a role in water uptake. Although Goklen and Hatton (1987) have investigated the role of salt and pH on water uptake, their study involved only one organic solvent. From Figure 5-7 it is obvious that the critical alkane carbon number depends on the buffer employed. The critical carbon number range for various buffer systems are shown in Table 5-4.

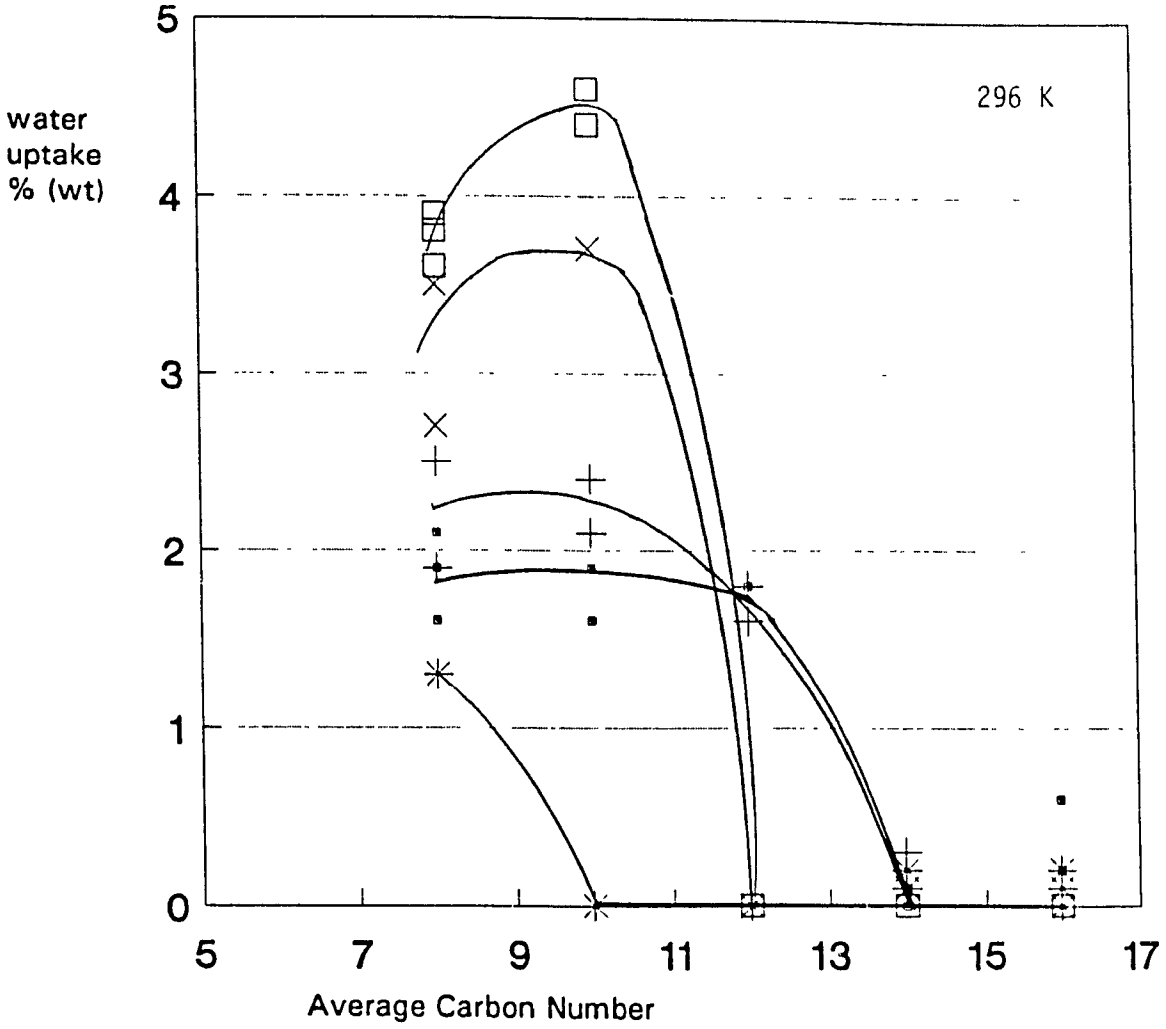
TABLE 5-4 - Critical carbon number range for AOT employing various buffers

Buffer	Critical carbon number
0.1 M KCl & 0.1 M Citric acid (CA)	12-14
0.1 M Sodium citrate	10-12
0.1 M KCl & 0.2 M tris	10-12
0.1 M KCl	12-14
0.1 M NaCl & 0.2 M tris	8-10

It is difficult to make an overall list of different buffers in increasing order of water uptake due to the sensitivity of the system to both the nature and concentration of the buffer. Instead the buffers are compared in pairs. In the case of tris and citric acid (CA), when the potassium ion is replaced by sodium, the critical carbon number decreases. The critical carbon range increases though when tris is replaced by citric acid for potassium chloride and sodium chloride.

The experimental results of this section suggest a method for carrying out the extraction of proteins in AOT systems. Since protein size exclusion from the micelles appears to be an important consideration, these results indicate that for the forward extraction one should choose an accompanying electrolyte that promotes the formation

of large water pools in the absence of protein.



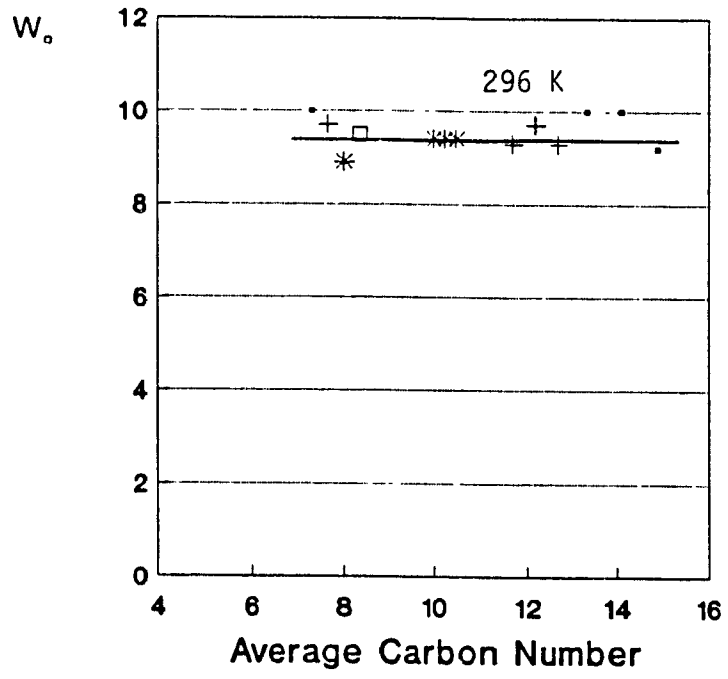
(■) 0.1 M KCl
(+) 0.1 M KCl & 0.1 M CA
(*) 0.1 M NaCl & 0.2 M tris
(□) 0.1 M KCl & 0.2 M tris
(X) 0.1 M Sodium citrate

FIGURE 5-7 - Effect of various buffers on the critical ACN

Initial contacting phases
organic: 50 mM AOT/n-alkane
aqueous: buffer
natural pH of system 1 was 6.6
pH of system 2 was adjusted to 6.6 by add'n of 0.5 N NaOH
pHs of other systems were adjusted to 6.6 by add'n of 1.0 M HCl

5.8 Effect of ACN on W_o for DNNSA

Figure 5-8 shows the water to surfactant ratio, W_o, versus the carbon number at different concentrations of DNNSA. This water-to-surfactant ratio is not affected by either ACN nor the concentration of surfactant. Water content at a fixed surfactant concentration remained constant over the carbon number range investigated, thus W_o was constant.



(■) 55 mM DNNSA
(+) 110 mM DNNSA
(*) 165 mM DNNSA
(□) 210 mM DNNSA

FIGURE 5-8 - Effect of ACN on W_o

Initial contacting phases
organic: DNNSA/mixture of kerosene and n-heptane
aqueous: 0.2 M tris ; 0.1 M KCl
pH adjusted to 6.6 by addition of 0.1 M HCl

5.9 Effect of ACN on water uptake for different proteins

Figure 5-9A shows a plot of water uptake versus ACN for various proteins, namely trypsinogen, lysozyme, and β-Lactoglobulin. The presence of different proteins did not affect water uptake. The percent protein solubilized for the above proteins is shown in Figure 5-9B. For these low molecular weight proteins, water uptake and the percent protein solubilized followed the same trend. This behavior was expected because proteins are not soluble in organics and hence can transfer to the organic phase only if water were present.

5.10 Effect of pH on water uptake and percent protein solubilized using AOT

Figure 5-10 shows a plot of water uptake for different organic solvents versus pH. Results are consistent with the previous discussion that water uptake decreases with an increase in pH for an anionic surfactant. In the case of n-dodecane, water uptake was at a maximum at pH 8 and then dropped off to zero at pH 7. With other solvents the reduction in water uptake was more gradual.

The water uptake and percent protein (trypsinogen) solubilized is plotted versus pH in Figure 5-11. As pH was increased, protein solubilization decreased as was the case with water uptake. Again, this was expected since proteins can only be solubilized in water and denature in organic solvents.

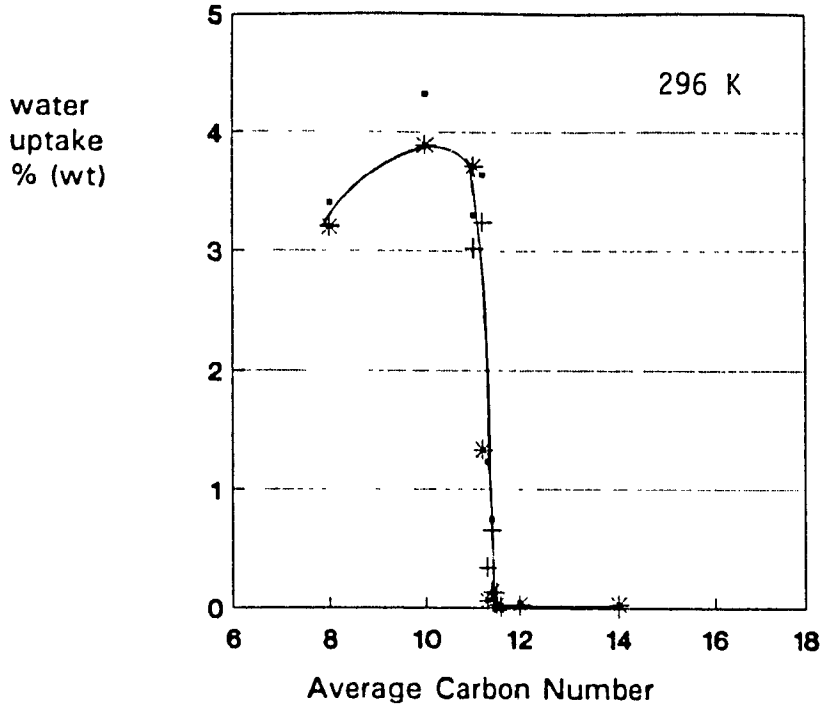


FIGURE 5-9A - Effect of ACN on water uptake for different proteins

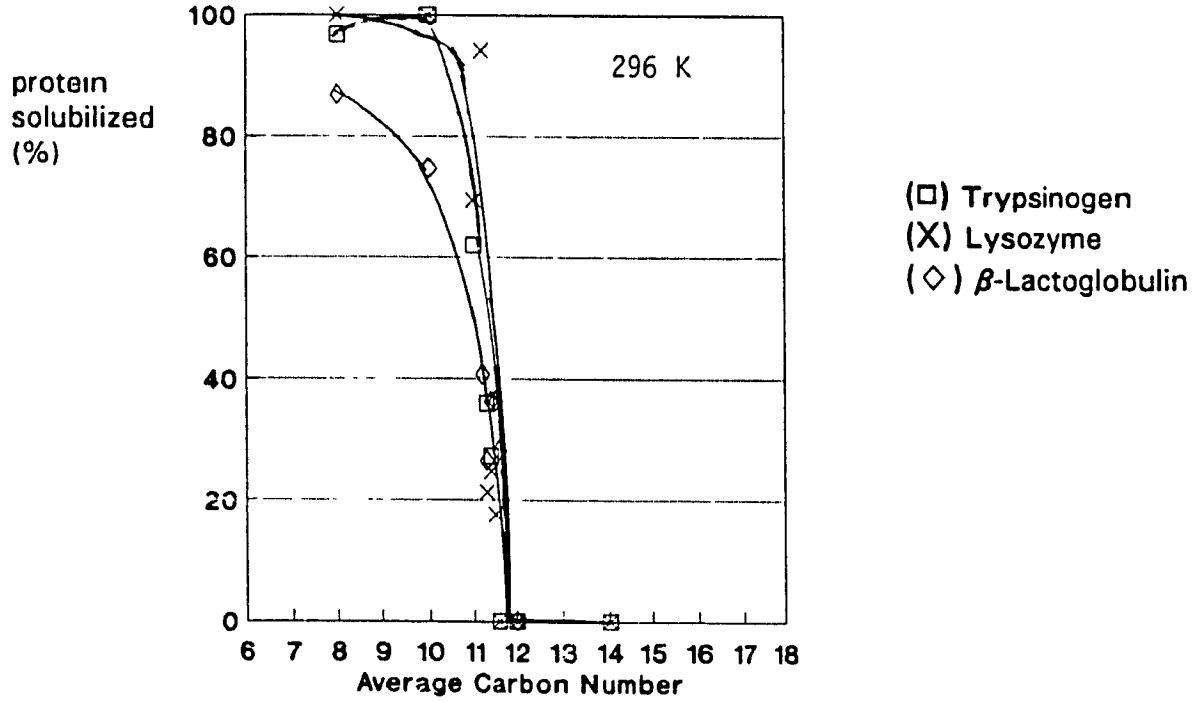
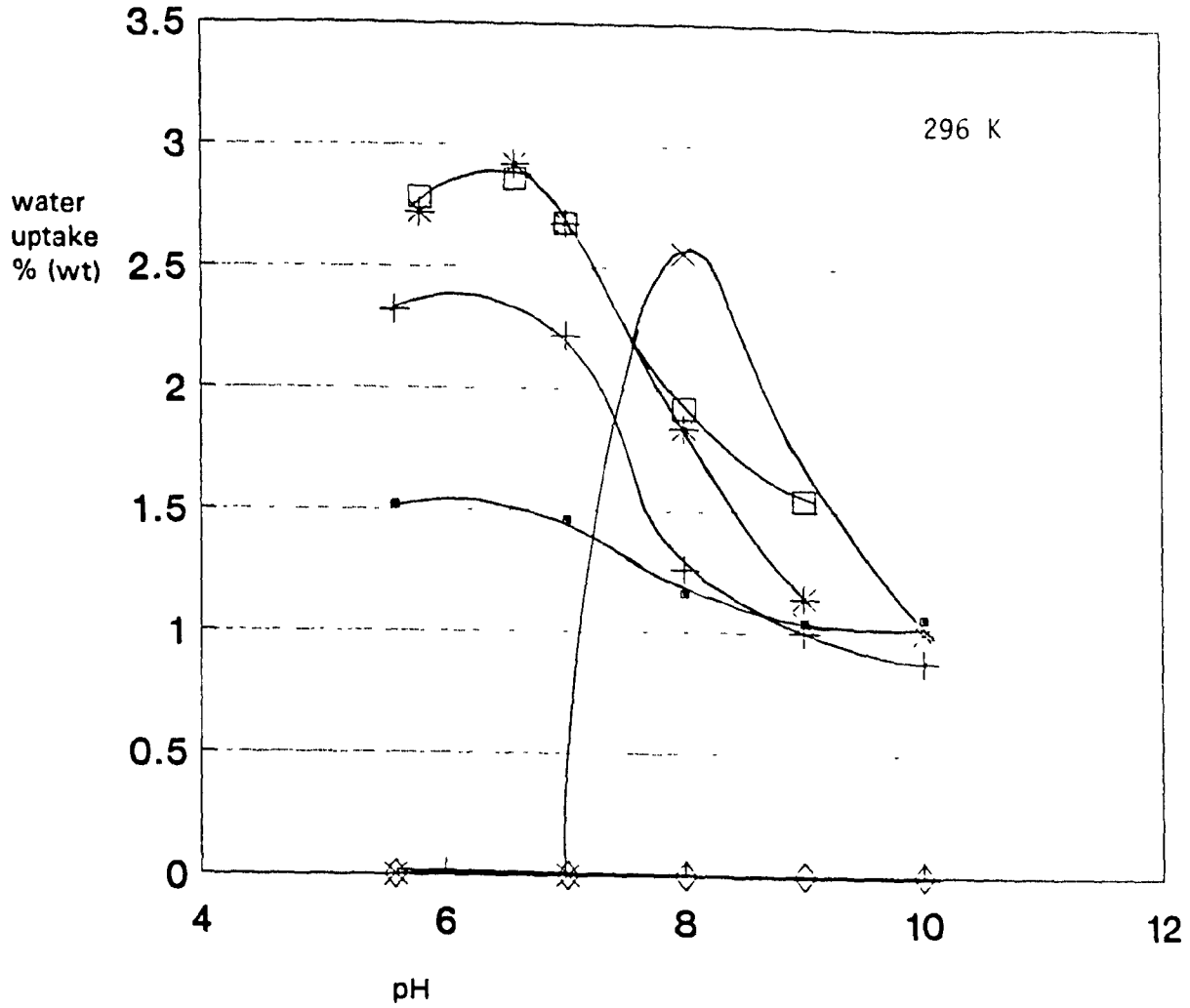


FIGURE 5-9B - Effect of ACN on protein solubilized

Initial contacting phases
organic: 50 mM AOT/n-alkane
aqueous: 0.2 M tris, 0.1 M KCl
pH adjusted to 6.6 by addition of 1.0 M HCl
1 g protein/L solution



(■) Cyclohexane
(+) Heptane
(*) Octane
(□) Iso-octane
(X) Dodecane
(◇) Hexadecane

FIGURE 5-10 - Effect of pH on water uptake

Initial contacting phases
organic: 50 mM AOT/organic solvent
aqueous: 0.2 M tris, 0.1 M KCl
pH adjusted by addition of 0.1 M HCl

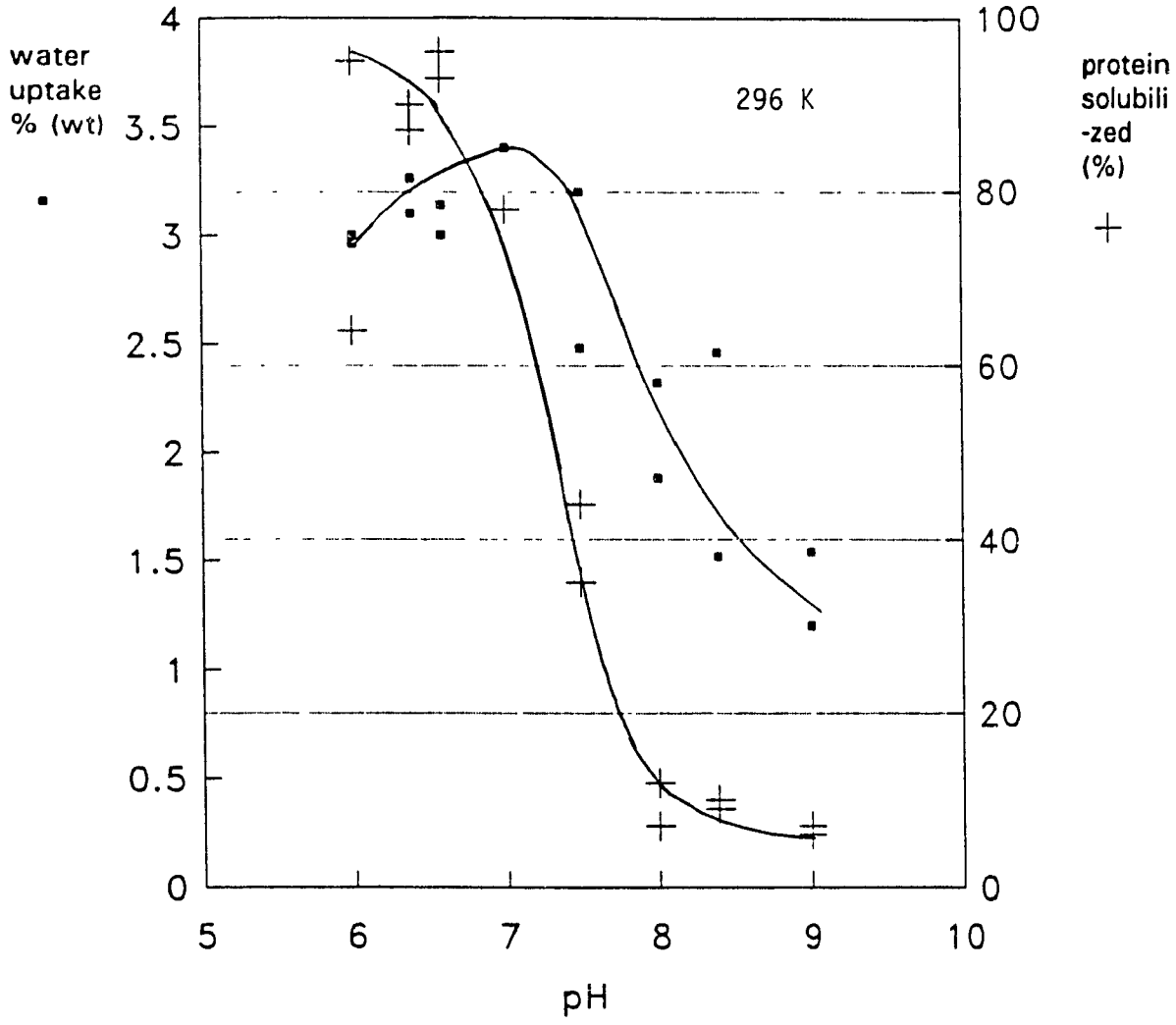


FIGURE 5-11 - Effect of pH on water uptake and percent protein solubilized

Initial contacting phases
organic: 50 mM AOT/iso-octane
aqueous: 0.2 M tris, 0.1 M KCl
1 g/L trypsinogen
pH adjusted by addition of 1 M HCl

5.11 Effect of co-surfactants with AOT

Anionic and neutral surfactants were used because cationic surfactants would form a precipitate with AOT. Experiments were conducted with the anionic surfactants bacitracin and phosphatidyl choline (lecithin) and the neutral surfactants palmitin, tripalmitin, and pluronic. Small amounts of these surfactants were added to 50 mM AOT in n-octane and contacted with an aqueous solution. These added surfactants had low solubilities in the organic phase and had no significant effect on water uptake. As a second choice for co-surfactant it was decided to investigate the use of cetyltrimethylammoniumbromide (CTAB) in an octane/heptanol system. Previous work by Laane (1983) and Rahaman et al. (1988) indicated that CTAB in an octane/hexanol system formed large micelles. CTAB was not that soluble in octane and using this longer chain alcohol seemed to cause some problems with turbidity.

5.12 Water and protein extraction from reverse-micelles

In addition to investigating the effect of water uptake and protein solubilization on average carbon number, the reverse process was also investigated, i.e. forcing the protein and water out of the system. The usual process for transferring the target protein back into an aqueous phase involves adjustment of pH and ionic strength. The research presented here investigated the recovery of the protein from the reverse-micelle through manipulation of the organic phase composition. This was done by first contacting a large volume of 50 mM AOT in iso-octane with an equal volume of the usual aqueous feed These phases were shaken for one hour at 250 rpm and 23°C and then left to settle. The top phase (organic phase) was separated for further use. Varying amounts of this phase were contacted with the appropriate amount of 50 mM AOT in hexadecane to form a total of 10 mL. This gave solvents with different average carbon numbers for the same surfactant concentration. It was hypothesized that if enough hexadecane was added to overcome the critical ACN, water and protein should be forced out of the reverse-micelle. Figure 5-12 shows water uptake versus average carbon number after organic phase manipulation. Data were obtained for three surfactant concentrations. Water uptake increased somewhat as the average length of the carbon chain of the solvent approached the length of the carbon chain in the two tails of AOT. Water was forced out of the organic micellar phase above the critical ACN as was the case in earlier experiments.

Protein extraction by organic solvent manipulation was also investigated. However, mass balances for protein did not close, and these results are not included. Nevertheless, protein analysis showed very little protein in either phase above the critical carbon number. The protein has probably denatured and transferred to the

interphase. Further research is needed to determine whether the protein can somehow be stabilized and transferred back to an aqueous phase without denaturing.

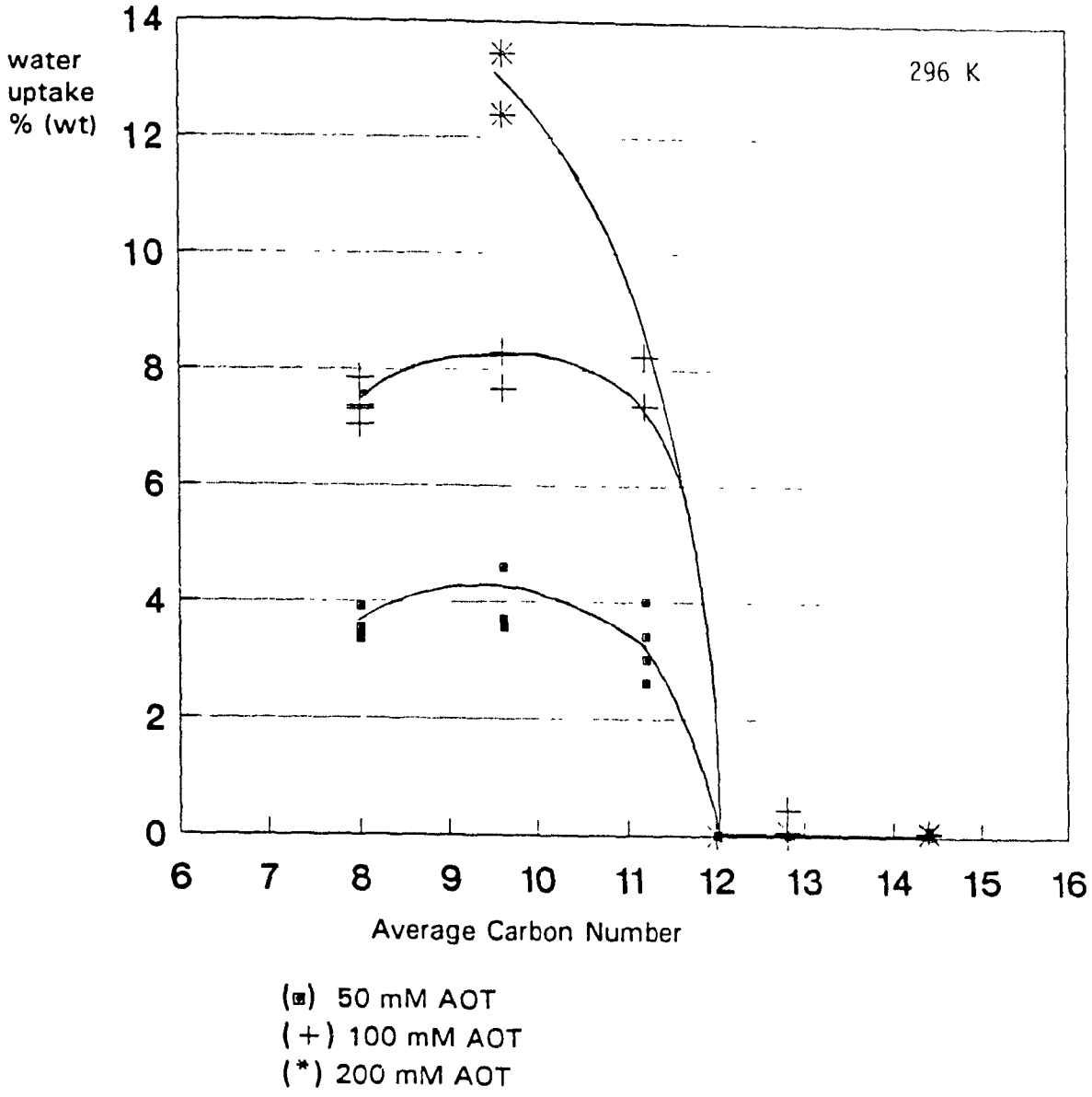


FIGURE 5-12 - Effect of organic phase manipulation on water extraction from reverse-micelles

Initial contacting phases
organic: AOT/n-alkane; solubilized water
aqueous: 0.2 M tris, 0.1 M KCl
pH adjusted to 6.6 by addition of 1 M HCl

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The most significant results of this research are as follows:

(1) In the case of AOT, water uptake increased somewhat as the length of the carbon chain of the solvent approached the length of the carbon chain in the two tails of the AOT. For longer chains, no reverse-micelles were formed and there was no water uptake above a critical ACN range.

(2) The behavior of mixtures of n-alkanes was similar to that observed for the series of pure n-alkanes. The manner in which the average carbon number of the solvent was composed did not affect water uptake significantly.

(3) The branched hydrocarbons give significantly higher water uptake than their straight chain counterparts.

(4) No critical ACN existed for DNNSA over the hydrocarbon range investigated.

(5) The nature of the buffer plays a role in determining the critical ACN for AOT.

(6) Manipulation of the organic solvent after solubilization can be used to extract the water and possibly protein from the reverse-micelle.

6.2 Recommendations

Further research is needed to find surfactants other than AOT. One possibility is the double chain surfactant tetradecyldimethylammonium linoleate used in the work of Schwab and Pryde (1986). Leser et al. (1989) used AOT reverse-micelles for the extraction of proteins from oil-rich vegetable meal, in particular from seed meal of the sunflower and the soybean. Although AOT may be economical, a more biocompatible surfactant would be better because AOT is allowed by the U.S. Food and Drug Administration only to a very limited concentration in food [Leser et al., 1989] Natural vegetable surfactants, i.e. lecithin and other phospholipids, should be investigated. Further experimentation with DNNSA and other surfactants is needed to determine whether the effect which occurred with AOT over varying hydrocarbon length is surfactant specific.

Much effort has been placed on the solubilization of proteins within reverse-micelles but the emphasis should be carried over to the recovery of the protein from reverse-micellar phase. Proteins are most commonly recovered from the micellar phase in a second aqueous stripping phase by simple adjustment of pH and ionic strength. The product could also be isolated batch-wise by transferring the enzymes from the reverse-micellar medium to an aqueous medium allowed by precipitation of the surfactant and evaporation of the organic solvent. Future studies should further investigate the possibility of extracting protein from the reverse-micellar phase by manipulation of the ACN in the organic phase. Finally, a study on the extraction of the same protein with different buffers and its back extraction into different buffers should be pursued.

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APPENDICES

APPENDIX A - CALIBRATION CURVES

(i) For various proteins using ultraviolet (UV) absorption at 280 nm
(data for Figure 4-1)

g/L	Absorbance at 280 nm				
	bovine serum albumin	β-lactoglobulin	trypsinogen	lysozyme	chicken egg albumin
0	0	0	0	0	0
0.20	0.142	0.306	0.483	0.081	0.081
0.40	0.263	0.569	0.954	0.124	0.124
0.60	0.380	0.824	1.427	0.189	0.189
0.80	0.501	1.131	1.879	0.227	0.227
1.00	0.632	1.399	2.303	0.294	0.294

(ii) For water using infrared (IR) absorption at 3450 cm^{-1}
(data for Figure 4-2)

% volume water	A 3450 cm^{-1}
0	0.06, 0.07
1.0	0.77, 0.56
1.5	1.10, 1.01
2.0	1.17, 1.35
2.5	1.57, 1.44, 1.55
3.0	1.85, 1.83, 1.81, 1.87

APPENDIX B - Effect of AOT concentration on water uptake
(data for Figure 5-1)

concentration of AOT, mM	n-octane	n-decane
	water uptake % (wt)	
25	1.8	2 1
35	2.6, 2.8	3.2, 3 3
50	3.8, 3.6	4.6, 4.4
100	7.3, 6.9	8.2, 7 6
150	9.6, 9.9	11 5, 11.3
200	12.1, 13.2	
300	17.6, 16.4	
500	21.1	

APPENDIX C - Effect of n-alkane carbon number on water uptake for AOT (data for Figures 5-2A and 5-2B)

n-alkane	concentration of AOT, mM						
	25	35	50	100	150	200	300
	IR % (wt)		KF % (wt)				
hexane	1.5	2 2,2.4		2.2,3.1	4.8,4.6		
heptane	1.5			2.4,2.8	3.5,3.8		
octane	1.8	2.6,2.8	3.9,3.8 3.6,3.4	7.4,7.3 7.0,6.9	9.6,9.9	12.0,12 .1	
iso-octane			4.0,3.9 3.5,3.5	7.7,7.7 7.7,7.5	9.8	13.8,13 .2	17.6,16 .4
decane	2.1	3 2,3.3	4.6,4.6 4.4,4.2	8.2,7.6	11.5	14.0,13 .6	
undecane			3.8,3.8 3 6,3.4		11.3		
dodecane	0	0	0	0			0
tetradecane			0			0	0
hexadecane	0,0	0,0	0,0	0,0	0	0,0	0,0

APPENDIX D - Effect of mixtures of n-alkanes on water uptake for AOT (data for Figures 5-3 and 5-4)

ACN	mixture of n-alkanes	concentration of AOT, mM			
		50	100	200	300
9.0	0.5C8 & 0.5C10			13 0, 12 4	
9.2	0.85C8 & 0.15C16		7.6, 7.4		
9.6	0.8C8 & 0.2C16			14.3	19
10.0	0.25C7 & 0.75C11	4.0, 3.8			
10 0	0.5C7 & 0.5C13	3.7, 3.8			
10.0	0.8C7 & 0.2C16	3.3, 3.1			
10.0	0.71C8 & 0.29C15	3 8, 3.8			
10.0	0.75C8 & 0.25C16	3 7, 3.7	8.2, 7.6	13.1	
10.8	0.65C8 & 0.35C16			13.2, 11.0	
11.0	0.5C6 & 0.5C16	2.6			
11.2	0.6C8 & 0.4C16	2 0	8.0, 7.4		18 4
11.2	0.8C10 & 0.2C16			14 0, 13.6	
11.5	0.45C6 & 0.55C16	0, 0			
12.0	0.5C8 & 0.5C16	0, 0	0, 0	0, 0	0, 0
12.8	0.4C8 & 0.6C16			0, 0	0, 0
13.0	0.5C10 & 0.5C16	0, 0	0, 0	0, 0	0, 0
13.6	0.4C10 & 0.6C16		0, 0		
14.4	0.2C10 & 0.8C16		0, 0	0, 0	0, 0

APPENDIX E - Effect of average carbon number on water uptake for DNNSA (data for Figures 5-5, 5-6, and 5-8)

ACN	concentration of DNNSA, mM			
	55	110	165	220
	water uptake %(wt)			
7.3	1.3 $W_o = 10.0$			
7.7		2.5 $W_o = 9.7$		
8 0			3.4 $W_o = 8.9$	
8.4				4.8 $W_o = 9.5$
10.0			3.6 $W_o = 9.4$	
10.3			3.6 $W_o = 9.4$	
10.5			3.6 $W_o = 9.4$	
11.7		2.4 $W_o = 9.3$		
12.2		2.5 $W_o = 9.7$		
12.7		2.4 $W_o = 9.3$		
13.3	1.3 $W_o = 10.0$			
14.1	1.3 $W_o = 10.0$			
14.8	1.2 $W_o = 9.2$			

APPENDIX F - Effect of buffer and salt types and concentration on the critical n ACN (data for Figure 5-7)

ACN	buffer				
	0.1 M KCl	0.1 M KCl & 0.1 M CA	0.1 M NaCl & 0.2 M tris	0.1 M KCl & 0.2 M tris	0.1 M sodium citrate
	water uptake % (wt)				
8	2.1, 1.6, 1.9	2.5, 1.9	1.3, 1.3	3.9, 3.8, 3.6	2.7, 3.5
10	1.9, 1.7	2.1, 2.4	0, 0	4.6, 4.4	3.7
12.0	1.8, 1.8	1.8, 1.7	0, 0	0, 0	0
14.0	0.0, 0.1	0.1, 0.3	0.2, 0.1	0, 0	
16	0.2, 0.6	0.1, 0, 0	0.1, 0.2	0, 0, 0	0

APPENDIX G - Effect of ACN on water uptake and percent protein solubilized (data for Figures 5-9A and 5-9B)

ACN	trypsinogen		lysozyme		β-lactoglobulin	
	water uptake % (wt)	protein solubilized (%)	water uptake % (wt)	protein solubilized (%)	water uptake % (wt)	protein solubilized (%)
8.0	3 4	97	3.2	100	3.2	87
10.0	4.3	100	3.9	100	3.9	75
11 0	3.3	62	3.0	69	3.7	
11.2	3.6		3.2	94	1.3	41
11.3	1.2	36	0.3	21	0.1	26
11.4	0.7	27	0.7	25	0.1	36
11.5			0.1	18	0	36
11.6	0	0	0	0	0	28
12.0	0	0	0	0	0	0
14.0	0	0	0	0	0	

APPENDIX H - Effect of pH on water uptake for AOT
(data for Figures 5-10 and 5-11)

pH	water uptake % (vol)	trypsinogen solubilized (%)	organic solvent					
			C7	C8	Ciso-oct	C12	C16	cyclohexane
			water uptake % (vol)					
10.4	0.38, 0.94	7,7						
10.0			0.88			1.00	0	1 06
9.0			1.00	1.14	1.54	1.04	0	1 04
8.6	0.86	11						
8.0			1.26	1.84	1.92	2 56	0	1.16
7.6	1.66, 2.04	66, 59						
7.1	2.62, 2.40	92, 94						
7.0			2.22	2.68	2.68	0	0	1.46
6.6	2.54	95		2.92	2.86			
5.8				2.72	2.78			
5.6			2.32			0	0	1.52

APPENDIX I - Water extraction from reverse-micelles
(data for Figure 5-12)

ACN	concentration of AOT, mM		
	50	100	200
	water uptake % (wt)		
8	3.5, 3 4, 3.4, 3.9	7.0, 7.3, 7.8, 7.4	13.4
9.6	4 6, 3.7, 3.6, 3.6	7.7, 8.3	12.4
11.2	4.0, 3.4, 3.0, 2.6	7.4, 8.2	
12	0, 0	0, 0	0, 0
12.8	0, 0	0, 0.5	0.1, 0.1
14.4	0, 0.1	0.1, 0.1	0.1, 0.1