SOLUBILIZATION OF ENZYMES AND NUCLEIC ACIDS IN HYDROCARBON MICELLAR SOLUTIONS

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I. GENERAL CONSIDERATIONS ABOUT REVERSE MICELLES

A. Introduction

Surfactant molecules are amphiphilic, possessing nonpolar regions (frequently *n*-alkyl group[s]) as well as polar regions (generally referred to as the head groups) which may or may not bear charges. If the head group is negatively charged, one refers to the surfactant as anionic; cationic surfactants have positively charged head groups and nonionic surfactants have polar, but neutral, head groups. The term surfactant is a contraction of surface active agent, which emphasizes the tendency of the molecules to concentrate themselves at surfaces and interfaces (air/water, water/oil, etc.). As a result of solvophobic association, these molecules form a structurally rich variety of organized assemblies, including micelles, microemulsions, and lyotropic mesophases (the lamellar structure, or planar bilayer, being a simple model for the cell membrane). Thus, lipids are naturally occurring surfactants.

The phenomenology of self-assembly has been reviewed extensively by numerous authors,[1-7] but it can be said that many features of it are incompletely understood. For nonspecialists (in the area of surfactant science) who wish to design a micellar system in which to solubilize biopolymers, we feel it is useful to briefly review what is known of the parameters, especially those of surfactant molecular structure, which control the morphology of the organized assemblies. We shall have a great deal to say about a particular anionic surfactant, Aerosol OT (AOT or sodium bis[2-ethylhexyl]sulfosuccinate), which forms reverse micelles in hydrocarbon solvents. These micelles take up (solubilize) water, forming water pools which may have radii exceeding 100 Å; they make it possible to study biopolymers in the presence of limited amounts of water, whose structure is often quite different from that of normal bulk water. This water is considered to be similar to that found at polar/apolar interfaces in vivo.

Two important principles must be kept in mind when using reverse micelles as hosts for biopolymers (or indeed small molecules), whether one's aim is structural or catalytic studies. First, the reverse micelles are dynamic entities which exchange the contents of their water pools and surfactant interfaces with one another and with bulk solvent (in the case of the hydrophobic components). Second, solubilizates may alter the gross structural features of

the micelles (for example, their size and shape or their water content) and cause changes at the molecular level (for example, in conformational) equilibria in the surfactant interface and water pools. Usually, these interactions of the host micelles with the guest biopolymers must be determined each time a new system is studied. One cannot assume that a hotel room (the micelle) is immune to guest-induced modifications or personalizations.

B. The Surfactant Packing Parameter

In this review, we shall deal extensively with ternary systems (or pseudoternary ones, wherein the additional components provide a minor perturbation) composed of water, hydrocarbon, and surfactant. Self-assembly may be described in terms of the curvature which exists at the hydrocarbon-water interfaces in these systems. Thus, Ninham and coworkers[8-11] have defined a surfactant packing parameter, $v/a\ell$: v is the volume of the hydrocarbon chain of the surfactant, ℓ is an optimal length close to that of the fully extended chain, and a is the head group area, which is determined by a balance of opposing surface forces, chiefly a minimization of hydrocarbon-water contact vs. head group repulsions. Figure 1 shows the aggregates expected for various parameter values and one sees an evolution of structures from those having positive curvature (normal spherical, followed by cylindrical, micelles) to those with zero net interfacial curvature (lamellar liquid crystals or the disordered bicontinuous microemulsions) and finally to structures possessing negative curvature (reverse micelles and water-in-oil microemulsions). It has been suggested that phospholipids having small head groups form reverse micelles in membranes;[12] reverse micelles may also be transient intermediates in cell fusion.

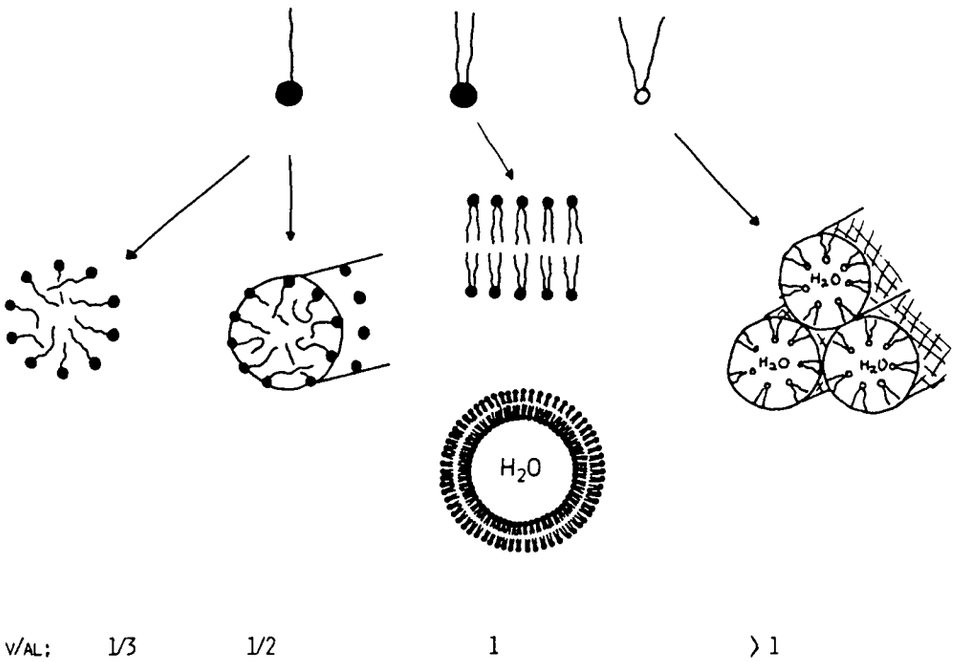
The zero-order theory for the numerical values of the packing parameter presented in Figure 1 ignores curvature corrections: that is, the recognition of the fact that head-group repulsion is different at curved than at planar interfaces. These values come simply from geometrical considerations:[10] the use of surface-to-volume relationships for the various aggregate shapes. Thus, consider a normal spherical micelle containing $\bar{n}$ surfactant monomers and hence having a hydrocarbon core volume (in the absence of oil) of $\bar{n}v = (4\pi/3)\ell^3$ and a core surface of $\bar{n}a = 4\pi\ell^2$. Rearrangement gives $v/(4\pi/3)\ell^3 = a/4\pi\ell^2$, from which one obtains $v/a\ell = 1/3$.

Intermicellar interactions also affect the structural changes observed. Thus solutions containing cylindrical micelles, may evolve into cylindrical lyotropic mesophases as the surfactant concentration increases. These contain normal or reverse cylindrical aggregates packed in hexagonal arrays.

When compared to single-tailed surfactants, double-tailed surfactants such as AOT show an increased tendency to negative curvature. This can be seen in the ternary phase diagrams of Figure 2. Pure AOT is itself not a crystalline solid, existing instead as a reversed hexagonal (F) mesophase. This tendency is easily understood: adding a second alkyl chain of length equal to or shorter than the first increases v without affecting a or ℓ. One can understand also the changes in structure which occur when a fourth component such as electrolyte (which decreases a) or a cosurfactant, often a medium chain length alcohol (which increases v more than it increases a) is added.

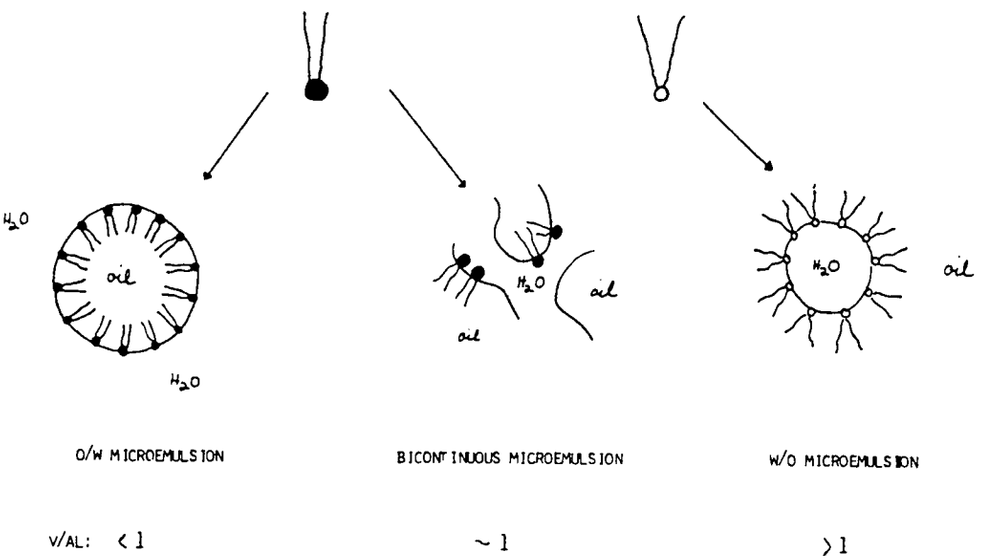
The $L_1 \rightarrow E$ transition of Figure 2a is in part due to the electrolyte effect, while the $E \rightarrow D$ transition is an example of a cosurfactant effect. The surfactant hexadecyltrimethylammonium bromide (C_{16} ≡ cetyl also, thus CTAB) does not form micelles in linear or branched alkanes. However, when cosurfactants such as $CH_3(CH_2)_nOH$ (n at least three) are used, reverse micelles form. If the alcohol has a long enough alkyl tail, it can function as the oil as well (Figure 2a). One can even understand observations of oil specificity (as a subclass of cosurfactant effects), and we shall return to this point for the case of AOT in later sections.

It is advantageous to be able to work whenever possible with reverse micellar systems



SURFACTANT PACKING PARAMETER, BINARY SYSTEMS

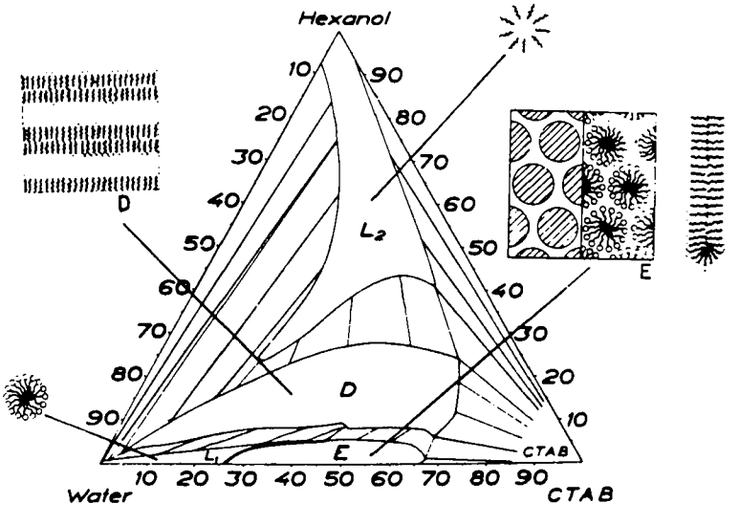
A



SURFACTANT PACKING PARAMETERS, TERNARY SYSTEMS:
MICROEMULSIONS WITHOUT COSURFACTANTS

B

FIGURE 1. Predictions of aggregate morphology using surfactant packing parameters.



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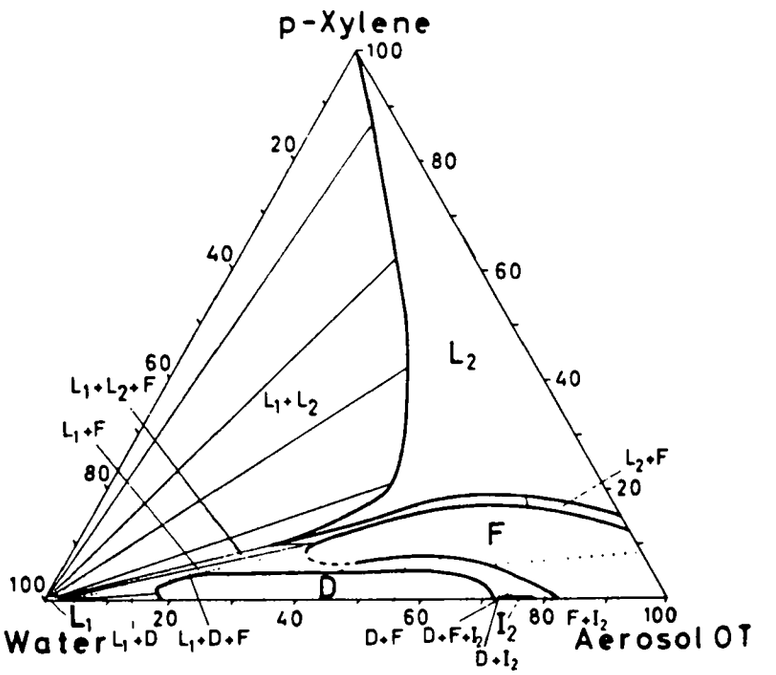


B

FIGURE 2. (A) Phase diagram (at 25°C) for the ternary system hexadecyltrimethylammonium bromide (CTAB)-1-hexanol-water. Concentrations are expressed in weight percent. (B) Phase diagram (at 20°C) for the ternary system sodium *bis*-(2-ethylhexyl)sulfosuccinate (AOT)-*p*-xylene-water. (From Ekwall, P., *Adv. Liq. Cryst.*, *1*, 1, 1975. With permission.)

which contain no cosurfactant, because the cosurfactant partitions among the continuous phase, the surfactant monolayer, and the water pool. This partitioning will depend upon water and surfactant concentrations, so that it must be determined for each new micellar preparation. When solubilizing biopolymers, one also wishes to avoid molecules as biologically aggressive as the medium chain length alcohols.

C. Aggregation of Surfactants in Apolar Solvents

Excellent comparisons of micelle formation in water vs. apolar solvents are available; for more information, see References 1, 2, and 4. We shall concentrate here on the micelles formed in apolar solvents.

We consider the aggregation which occurs in apolar solvents in the absence of all but adventitious water. It is impossible to obtain either completely dry surfactants or solvents, but generally somewhat less than one mol of water per mol of surfactant will be available. The point is that aggregates will occur even when a well-defined hydrocarbon/water interface is not present. We may then describe the appearance of that interface from a molecular perspective, by speaking of the solubilization of water into the reverse micelles.

In aromatic, aliphatic, and halogenated hydrocarbons, surfactant aggregation is dominated by solvophobic interactions involving the head groups. These groups are sequestered in the interior of the aggregates, largely shielded from interactions with bulk solvent. Important contributions to micelle stability in apolar solvents come from hydrogen bonding, dipole-dipole, dipole-induced dipole, and dispersion interactions. Several classes of surfactants which aggregate in apolar solvents are shown in Table 1. The formation and characterization of reverse micelles have been reviewed recently.[1,6] Unlike surfactant aggregation in aqueous solutions, which is often characterized by a well-defined critical micelle concentration (CMC) and monomer $\leftrightarrow$ $\bar{n}$-mer association ($\bar{n}$ frequently taking values of ca. 50 to 100), surfactants in nonpolar solvents often display indefinite self association.[5,6,13-21] In several cases the stepwise equilibrium constants for the aggregation monomer $\leftrightarrow$ dimer $\leftrightarrow$ trimer $\leftrightarrow$ . . . $\bar{n}$-mer have been found to be equal; average aggregation numbers are frequently as low as three to seven at moderate total surfactant concentrations. Mono-, di-, and trialkylammonium salts and many nonionic surfactants display this aggregation behavior.

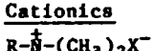
There are also surfactants which display monomer $\leftrightarrow$ $\bar{n}$-mer association in nonpolar solvents and they often contain two hydrocarbon tails. Dialkyl sulfosuccinates,[22-30] of which AOT is an example, dinonylnaphthalene-sulfonates,[31,32] and phosphatidylcholines[33-39] are perhaps the most extensively studied. Aggregation numbers typically range from 12 to about 30, but dodecylammonium benzoate forms a trimer in a monomer $\leftrightarrow$ $\bar{n}$-mer association.[40] Even with these surfactants, the definition of a CMC is not without ambiguity. Thus in the case of AOT,[41-44] there is evidence for a premicellar trimer. In certain solvents the aggregation of AOT to 6-mers and 14-mers has also been inferred.[45]

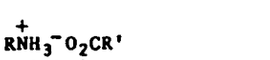
In cases where CMCs have been measured for surfactant aggregation in apolar solvents, they are frequently in the millimolar range. For the case of AOT, the residual water present in surfactant and solvent participates in a highly structured network involving the head groups of the surfactant. The smallest AOT aggregates[29,46] observed in isooctane (2,2,4-trimethylpentane) have a hydrodynamic radius of 15 Å, which is longer than the extended length of the surfactant molecule (ca. 11 to 12 Å). Eicke assigns the 15 Å dimension to an AOT — H_2O pair, while Maitra[47] notes that at low water content, the positions of the AOT molecules are staggered with respect to the micellar center (see Figure 3). As the micellar cores are enlarged by water, this staggering decreases, and the area available per AOT head group at the oil/water interface[48] reaches a plateau value (Figure 4, ca. 55 Å^2) which is close to that found for AOT monolayers.

For the "dry" AOT micelles, the aggregation number shows a large dependence on the hydrocarbon solvent used.[1,49] Weight-average molecular weights, determined by static light

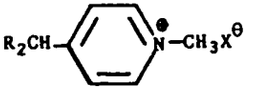
Table 1
SURFACTANTS WHICH AGGREGATE IN APOLAR SOLVENTS

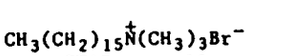
Cationics





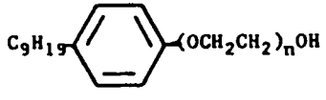
RNH$_3^+$ $^-$O$_2$CR'
R>>R'
or vice versa
R=nC$_{12}$H$_{25}$, R'=CH$_3$; DAP



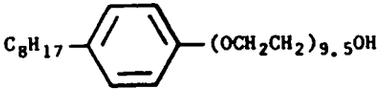


CH$_3$(CH$_2$)$_{15}$N$^+$(CH$_3$)$_3$Br$^-$
CTAB; usually
requires a cosurfactant

Nonionics

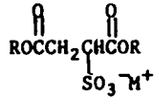


ethoxylated nonylphenols;
added H$_2$O is required

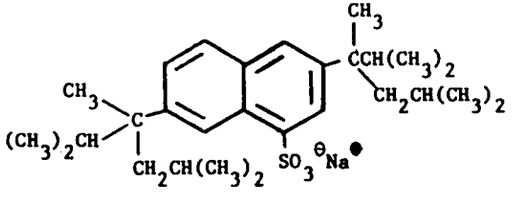


Triton X-100

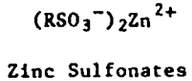
Anionics



sulfosuccinates
R = 2-ethylhexyl
M$^+$ = Na$^+$:AOT

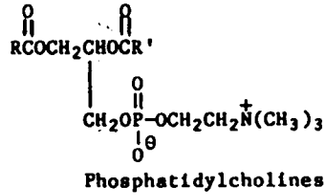


Sodium Dinonylnaphthalenesulfonates



Zinc Sulfonates

Zwitterionics



Phosphatidylcholines

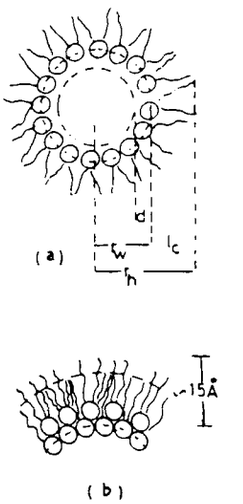


FIGURE 3. (a) AOT reverse micelle with an aqueous core. (b) Staggered association of AOT molecules at the interface. (From Maitra, A., *J. Phys. Chem.*, 88, 5122, 1984. With permission.)

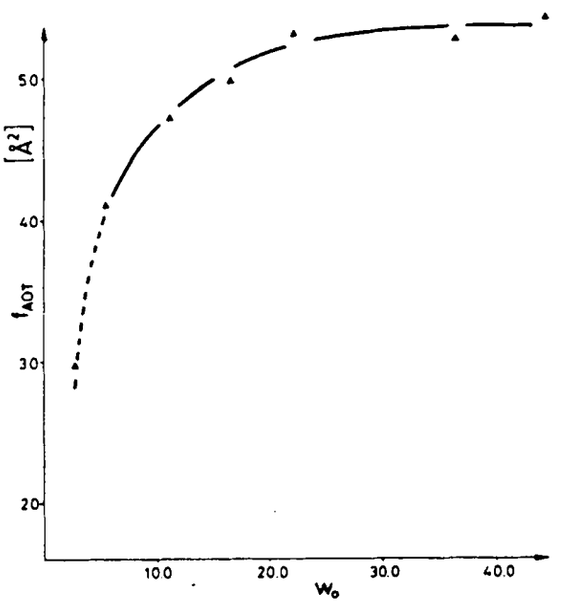


FIGURE 4. Average area of the hydrocarbon-water interface covered by one AOT molecule plotted against aliquots of added water (25°C). (From Eicke, H.-F. and Kvita, P., *Reverse Micelles*, Luisi, P. L. and Straub, B. E., Eds., Plenum Press, New York, 1984, 21. With permission.)

scattering measurements, for the micelles in *n*-octane, *n*-decane, and *n*-dodecane are 30, 37, and 44, respectively. The micellar size increases as the ability of the hydrocarbon solvent to penetrate into the curved AOT monolayer decreases. Extensive penetration of the solvent increases v and hence the surfactant packing parameter; reverse micelle size decreases as the value of $v/a\ell$ increases. Stated in another way, large values of $v/a\ell$ mean highly curved surfactant monolayers will be possible.[10]

D. Water Solubilization by Reverse Micelles

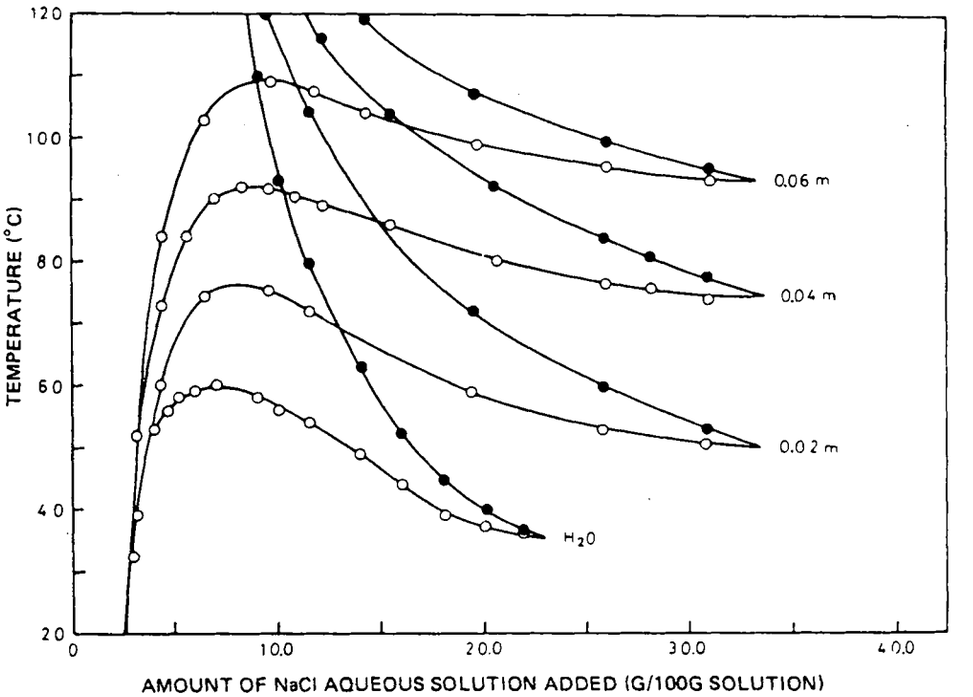
The ability of reverse micelles to solubilize water in apolar solvents at ambient temperature varies widely. We will express the molar ratio of water to surfactant as w_o. In solvents such as toluene and CCl_4,[50] AOT solubilizes an amount of water only slightly in excess of the hydration requirements of the sulfonate head groups and their sodium counterions (ca. 4 and 6, respectively), while solubilizing amounts substantially in excess of hydration requirements in aliphatic hydrocarbon solvents (in some cases w_o exceeds 70). The solubilizing properties of AOT arise from the operation of several effects. We defer discussion of the ability of AOT to change its effective $v/a\ell$ by conformational change until we discuss the physical characteristics of the surfactant interface.

Mitchell and Ninham[10] have computed the dependence of the maximum allowable water pool size upon $v/a\ell$, using simple geometry and the assumption that none of the continuous phase (oil) penetrates into the micelles. The closer $v/a\ell$ is to one, the larger the water pool can be. In fact, the maximum water pool size depends upon the oil used, because the oil can penetrate into the surfactant interface, increasing v. In the absence of any specific oil-head group interactions, the oils best at penetration will be those which are molecularly most similar to the surfactant tails. Thus, $w_{o,max}$ for AOT solutions decreases as the carbon number of an *n*-alkane increases.[51]

Micellar size of course increases as w_o increases; indeed, for some surfactants, one may say that water pool formation serves as a nucleating site for proper micelle formation. Thus, an aggregation pattern involving indefinite self-association may shift so that micelles having many tens of surfactant monomers appear in the solution. The appearance of free water ($w_o \geq 10$ to 15), which coincides with the aggregates acquiring a water pool encased by a curved surfactant monolayer having a constant area per head group (Figure 4), marks the transition to the water-in-oil (w/o) microemulsion domain.[1] However, in this review we shall use the terms reverse micelle for the aggregates present at all w_os.

Eicke and co-workers[52] have found that adding benzene to AOT micellar solutions in isooctane first increases $w_{o,max}$, then decreases it as the benzene-to-AOT molar ratio exceeds ten. The initial increase was rationalized on the basis that benzene is a very efficient penetrator, interacting with the ester carbonyls and desolvating (removing isooctane from) the surfactant interface. It is not clear whether these differential penetration effects lead to micellar size changes or not. However, one must be aware of this possibility when one alters the composition of a reverse micellar preparation.

When solubilizing biopolymers, one often employs water pools which are buffered. The presence of electrolytes in the water pools will change $w_{o,max}$ (usually decreasing it), so that it may be necessary to determine the phase diagram of the system anew when a new buffer is introduced. Below a w_o of 1.6, solubilization of NaCl from the solid state does not occur.[53] Figure 5 shows the situation at a single AOT concentration in cyclohexane for solubilization of a simple electrolyte solution.[54] If one considers only the lower phase boundary (often called the coexistence or solubilization curve, open circles in Figure 5), $w_{o,max}$ decreases as the NaCl concentration increases. For an ionic surfactant, a decreases when electrolyte is added, because the increase in ionic strength decreases the head-group repulsions. Per unit charge, divalent cations (Figure 5b) lower $w_{o,max}$ for AOT in cyclohexane a bit more than the monovalent cations. The resulting increase in $v/a\ell$ is then responsible for the decrease



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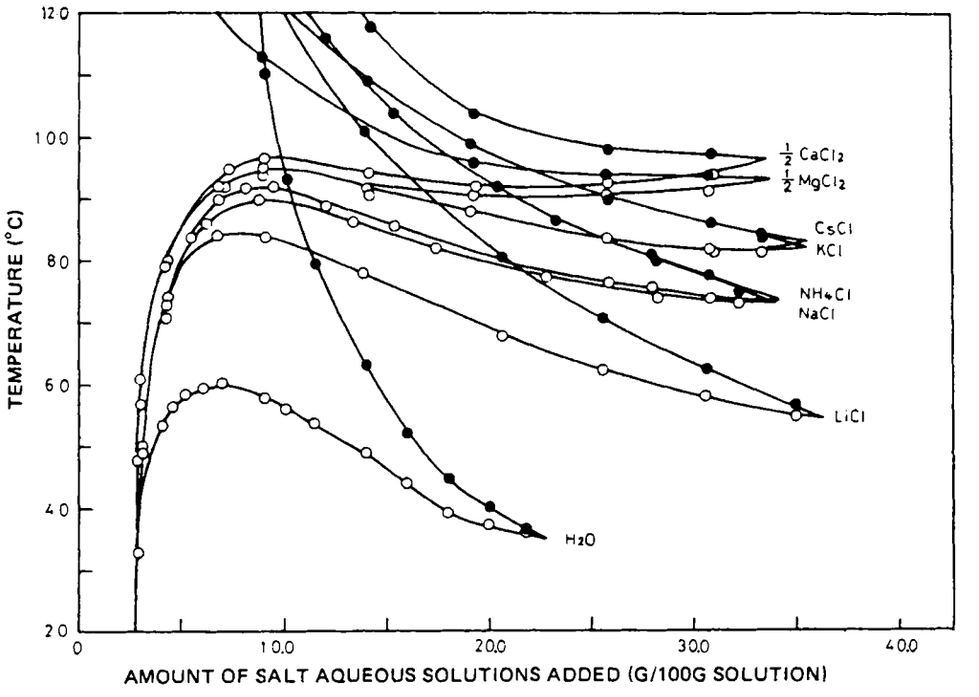


B

FIGURE 5. (A) The effect of NaCl concentration on the solubilization of water by AOT in cyclohexane. (B) The effect of variations in the cation of the salt on the solubilization of water by AOT in cyclohexane. (Both diagrams from Kon-no, K. and Kitihara, A., *J. Colloid Interface Sci.*, 41, 47, 1972. With permission.)

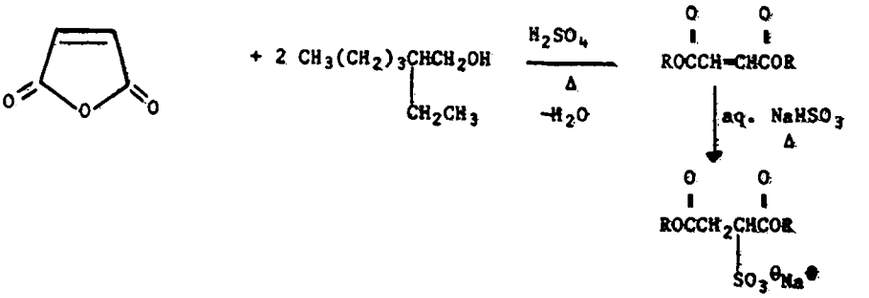
in solubilization. This rationalization does not, however, explain the effect of electrolytes on the solubility curve (closed circles in Figure 5).

E. Effect of Surfactant Purity on the Micellar Properties: The Particular Case of AOT

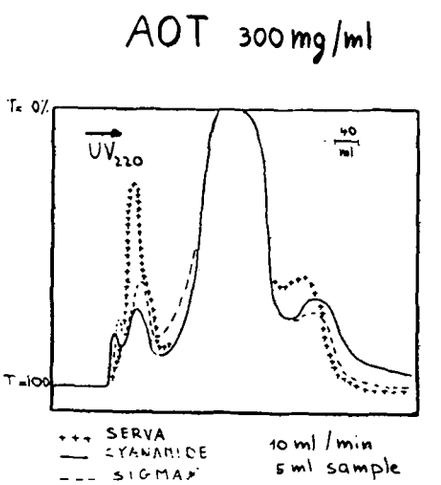
The check of the chemical purity of AOT or of any other surfactant for that matter, is an important prerequisite for the reliability of chemical studies on biopolymers solubilized in reverse micelles. Unfortunately, none of the commercially available preparations seem to meet the required chemical purity. Figure 6 shows HPLC profiles for three commonly used AOT sources (Serva, Sigma, and Cyanamid). In all cases, three distinct components are evident, and the relative intensity of these three peaks often varies from preparation to preparation of a given commercial source. Figure 6c shows the UV-absorption spectra of the three preparations, before HPLC purification. The impurities absorbing in the 250 to 300 nm region are certainly a considerable disturbance in carrying out spectroscopic studies of proteins or nucleic acids in reverse micellar solutions. Of course, the contribution of these impurities to spectroscopic properties can in principle be eliminated by using the appropriate reference cell. The problem is, however, the effect of such impurities on the behavior of the guest biopolymers and all the corresponding problems of reproducibility.

Figure 6c shows titration curves for the three commercial preparations, before HPLC treatment. Notice in some of the curves the clear evidence for acid impurities. In a recent paper,[55] we have shown the comparison in UV-absorption and titration properties of AOT samples from a single commercial source, but purified according to different purification procedures described in the literature. All these data together indicate that the HPLC method is the most efficient for preparing AOT samples with a minimal absorbance and acidity content.

The nature of these impurities is not completely clarified. It is, however, helpful, in this respect, to consider two chemical reactions. One is the synthesis of AOT, which is usually prepared according to the following scheme:



The other reaction, whose importance has been emphasized by Fletcher and co-workers[56] is a hydrolysis reaction of AOT which is either acid- or hydroxide-promoted, once water solutions are solubilized in AOT hydrocarbon micellar solutions. In other words, even if one prepares reverse micellar solutions using carefully purified AOT, hydroxide-promoted, or acid-catalyzed hydrolysis of the ester groups occurs when an aqueous solution has been solubilized. AOT has a half-life of ca. 3 days at room temperature and a pH of 12, but at neutral pH reverse micelles containing solubilized water show only ca. 0.1% decomposition of the AOT. The hydrolysis generates 2-ethyl-1-hexanol, which is highly surface active, as well as various ionic forms of sulfosuccinic acid. Whether present initially or generated by hydrolysis, the alcohol can function as a cosurfactant which may affect micellar size and the rates of solubilizate (and surfactant) exchange between micelles. The hydrolysis of course also changes the pH of the water pools, so that it is advisable to use a well-buffered pool



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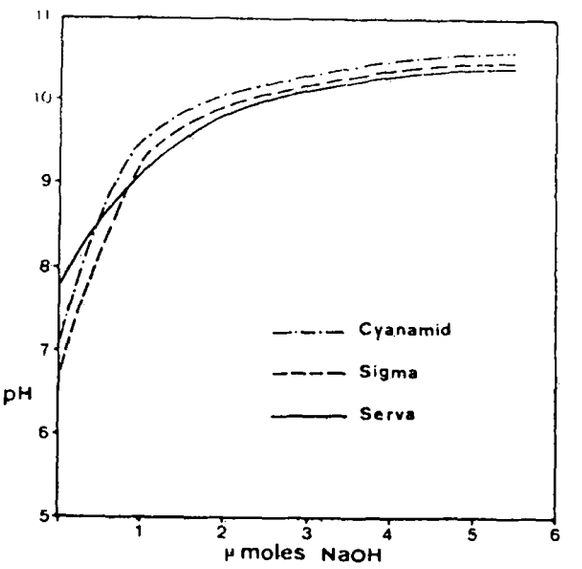
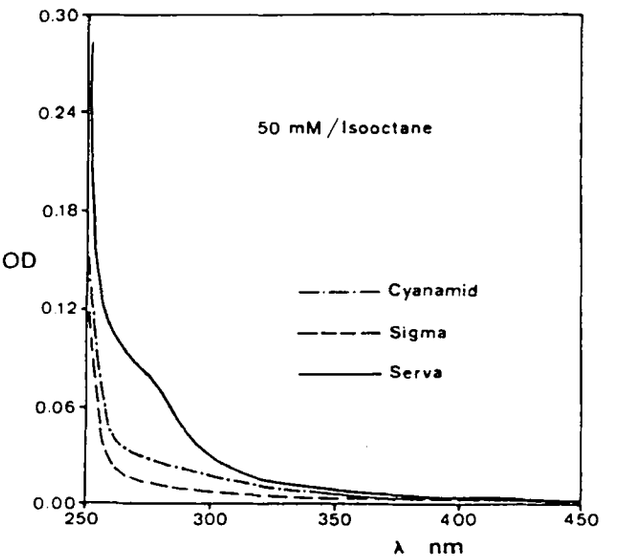


B

FIGURE 6. Characterization of different commercial AOT samples. (a) HPLC trace. (b) Titration of a 220 m*M* AOT solution in 1:1 CH_3OH-H_2O. (c) UV absorption of methanolic 50 m*M* AOT.

when constancy of the pH is important. The issues of what one means by water pool pH and of buffer selection are discussed in Sections I. and L.

Sodium bisulfite is a strong electrolyte, in addition to being an acidic impurity in virtually all commercial AOT samples. A facile procedure for evaluating how well electrolytic im-



C

purities have been removed is the determination of $w_{o,max}$ for AOT in cyclohexane;[57] an increase from 20 to 70 can be obtained. AOT reverse micelles prepared with commercial AOT have radii (at various w_o values) which are about 10% larger than micelles prepared with AOT purified by HPLC.[58,59] The presence of water-soluble acidic impurities also makes the starting pH of the water pools uncertain.

It would be advisable for authors to report the characterization of each AOT sample with at least three parameters: the absorption spectrum in the 250 to 320 nm region, the titration curve, and the determination of $w_{o,max}$ under established conditions (e.g., for a 100 m*M* AOT solution at room temperature using bidistilled water in the absence of any added salt). A qualitative idea of the presence of salt in the AOT preparations can be also obtained by measuring the conductivity of standard water/methanol solutions of AOT.

F. Dynamics of Solubilizate Exchange Between Micelles

A number of studies have indicated that while exchange of small solubilizate molecules among reverse micelles is generally much more rapid than any chemical reactions in which the molecules may participate (this may not be true for enzymes as solubilizates), exchange is a factor of ca. 10^3 below the diffusion-controlled rate.[60,61] Expressed in another way, only 1 in each 1000 micellar collisions results in solubilizate transfer. Exchange processes on time scales ranging from 100 μs to 10 msec have been observed for the Ni^{2+}/murexide ion pairs[62] and for the $TbCl_3$/phenylacetic acid pair [63] (monitored by enhancement of Tb^{3+} luminescence intensity) in AOT reverse micelles. Schelly and co-workers[45,64] have investigated the kinetics of picric acid and 7,7,8,8-tetracyanoquinodimethane solubilization by AOT and other reverse micelles in benzene (using concentration-jump experiments with absorption detection), identifying a rapid rate process (submillisecond) associated with adsorption at the AOT carbonyl groups and additional processes corresponding to further penetration of the picric acid to the level of the sulfonate of the surfactant head groups. The presence of a cosurfactant in the interfacial layer loosens the interface and modestly increases the exchange rates observed.[65]

Figure 7 depicts two possibilities for the exchange of water pool contents. In part A, the two water pools remain intact and the transfer of water and other solubilizates occurs through

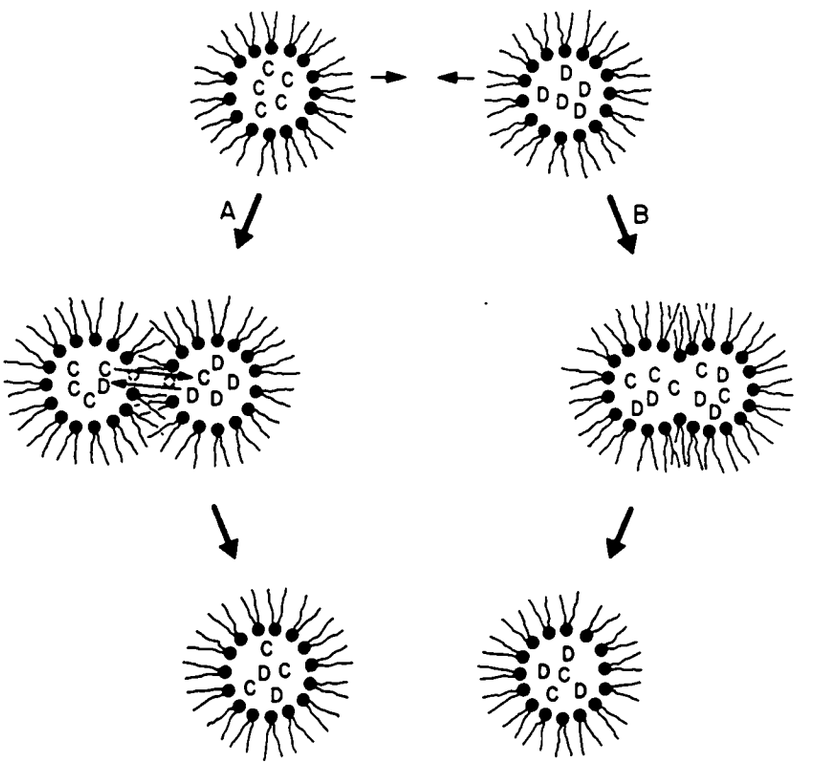


FIGURE 7. Possibilities for the exchange of water pool contents: (A) with maintenance of water pool integrity; and (B) with formation of a transient dimer.

the interfacial layers. In this model the transfer rate should depend upon solubilizate size, which is not found experimentally for small molecules.[66] Part B proposes instead that the water pools fuse, so that a single larger transient aggregate is produced. This model predicts that the exchange rate should be independent of solubilizate size. This process will involve the release of some surfactant into the continuous phase (with possible transient formation of "dry" reverse micelles). To understand this, assume that AOT is the surfactant and w_o = 25, which means a water core radius[47] of 43 Å and 420 AOT molecules per micelle. Fusion of two such water cores gives R_c = 54 Å and requires only 670 AOT molecules per micelle (not 840, assuming the area per AOT at the water core surface is 55 Å^2). The surfactant release will make a positive contribution to $\Delta S^{\ddagger}$ for the overall process, which makes it an attractive proposal.

G. Molecular Characterization of the Surfactant Interface

The w_o-dependent conformational properties of AOT molecules in the surfactant interface of reverse micelles have been investigated using ^{1}H and ^{13}C NMR.[25,47,57,67-69] The two hydrocarbon tails are conformationally distinct, making AOT in some respects a rather good model for phosphatidylcholines, which are important constituents of cell membranes. The staggered conformations about the C_1C_1' bond are shown in Figure 8. As w_o increases in isooctane, the conformational equilibrium I ↔ II shifts towards II. This is detected by monitoring by ^{1}H NMR the w_o-dependent coupling constants of the ABX pattern for the protons attached to C_1 and C_1', see Figure 9a. The interfacial environment of the carbonyl carbon C-2′ is substantially less polar in II than in I, and this is evident in the w_o-dependence

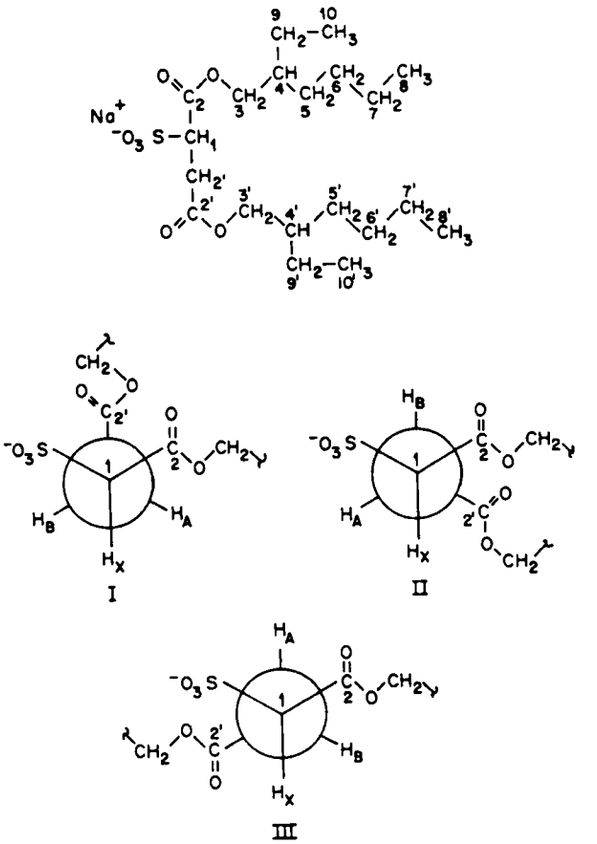


FIGURE 8. Staggered conformations for the AOT molecule about the C_1-C_1' bond. Rotamer III is of low amphiphilicity and is not appreciably populated in the micelles.

of the ^{13}C chemical shift (Figure 9b) of the C-2's. In contrast, C-2, which sits deeper in the interface, is essentially unaffected by the increase in solubilized water. C-1 (Figure 9b) and its attached proton (Figure 9c) also undergo changes in chemical shift as w_o is increased; all the w_o-sensitive chemical shifts reach relatively constant values in the regime of w_o = 10 to 20. We shall see in Section I. that the properties of the solubilized water approach those of pure, bulk water in the same w_o region.

Rotamer I has a large effective volume because of the manner in which the hydrocarbon tail commencing at C-2' is splayed out with respect to the one commencing at C-2. It is disfavored as w_o increases because the ratio of A_{HC}/A_{WP} (area per surfactant tail to area per surfactant head in the aggregate) must decrease on geometrical grounds. Thus, rotamer I (which has a smaller $v/a\ell$) dominates at higher w_o. Other surfactants (see Table 1) which form reverse micelles lack this sort of conformational feature (didodecyldimethylammonium halides, for example, see Reference 70), and this is one factor in their lower capacities for solubilizing water. AOTs ethyl side chains also play an important role in this swelling by water, since sodium *bis-n*-alkylsulfosuccinates[23] in which the alkyl tail-alkyl tail interactions are stronger than in AOT (these *n*-alkyl derivatives are crystalline solids) usually have a low maximum w_o.

The w_o dependence of the rotational correlation times (evaluated from ^{13}C T_1 values) for carbon atoms in the hydrocarbon tails indicates increased overall mobility of the AOT

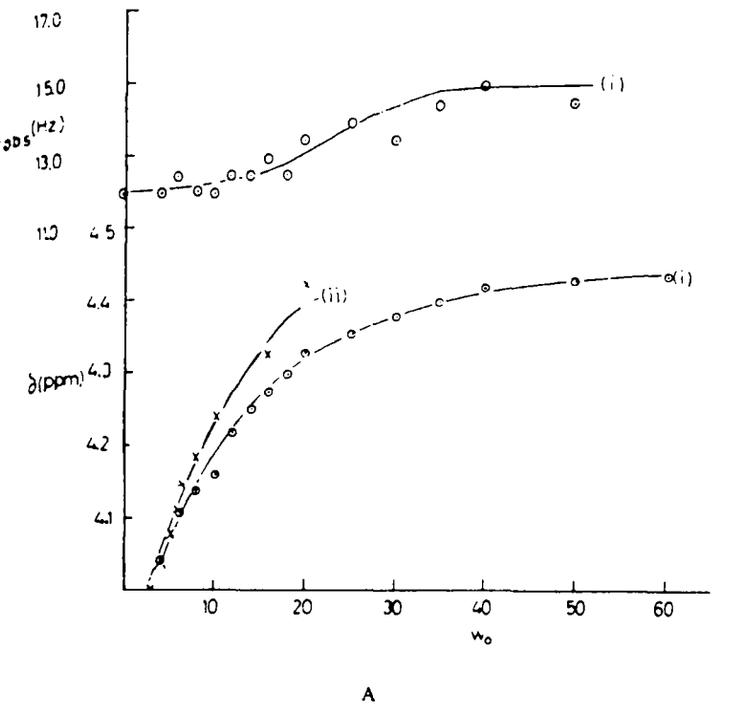


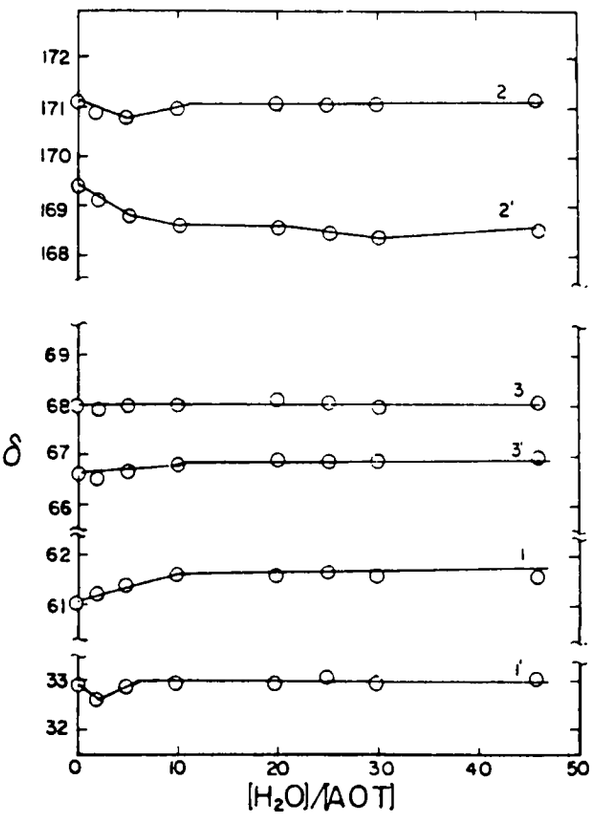
FIGURE 9. NMR data for AOT reverse micelles. (A) Variation of water proton chemical shifts and observed coupling constant values, J_{obs}, with w_o in H_2O-AOT-oil reverse micelles: (1) H_2O-AOT-isooctane and (2) H_2O-AOT-cyclohexane. (From Maitra, A., *J. Phys. Chem.*, 88, 5122, 1984. With permission.) (B) Dependence of ^{13}C chemical shifts for carbons 1-3 of AOT upon water content in H_2O-AOT-cyclohexane. (From Magid, L. J. and Martin, C. A., *Reverse Micelles*, Luisi, P. L. and Straub, B. E., Eds., Plenum Press, New York, 1984, 81. With permission.) (C) Dependence of proton chemical shifts upon water content in H_2O-AOT-isooctane. (From Eicke, H.-F. and Kvita, P., *Reverse Micelles*, Luisi, P. L. and Straub, B. E., Eds., Plenum Press, New York, 1984, 21. With permission.)

molecule, but a decrease in the extent of segmental mobility as w_o increases[57,69] and the continuous phase is squeezed out of the micelle. This decrease in segmental mobility parallels the decrease in area per hydrocarbon tail which occurs at the surfactant/continuous phase boundary as w_o increases. The extent of solvent penetration into the surfactant layer may also be inferred from the NMR data: there is a more marked preference for gauche conformers in AOTs hydrocarbon tails at low w_o when cyclohexane (a weak penetrator) is the solvent.

Aromatic and chlorinated hydrocarbons (stronger penetrators) interact with AOTs ester groups,[52] causing a shift towards rotamer II, even at low w_o; they are also not easily displaced from the surfactant interface, which keeps $w_{o,max}$ lower than for the case of aliphatic hydrocarbons.

H. w_o-Dependent Properties of the Water Pool Water and Sites of Solubilization for Small Molecules

We have already mentioned that in the case of AOT, the properties of the solubilized water depend upon w_o. Below a w_o of ca. 10 (the numerical value cited depends on the hydration requirements of the surfactant), the water pool water has properties which are substantially different from those of normal bulk water. For many solubilized biopolymers,



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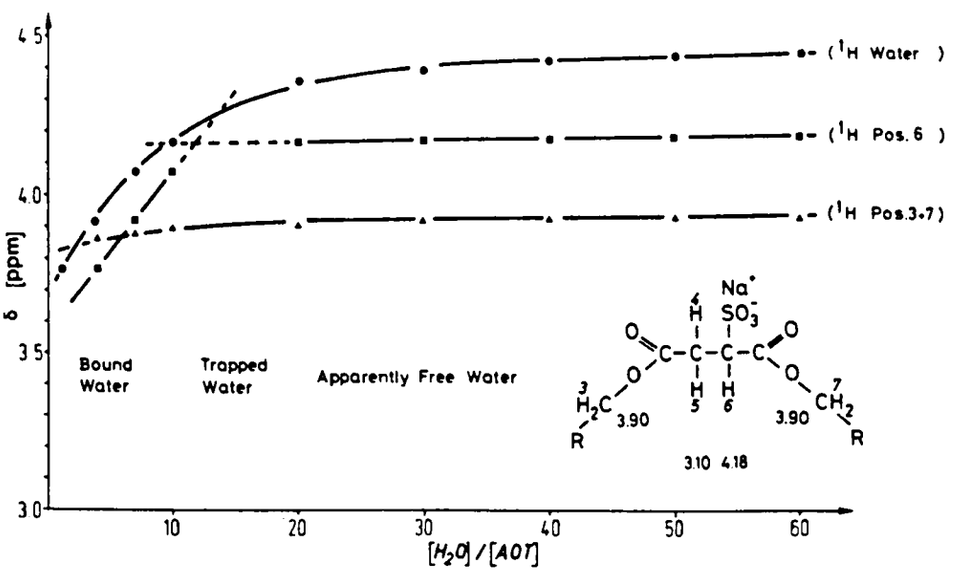


C

the greatest catalytic activity and/or changes in conformation are found at w_os below 10 to 12.

The properties of this solubilized water have been studied using a number of techniques; some investigators have looked at the water directly, while others have drawn inferences about its properties from its effect on the spectra (absorption, fluorescence, NMR, etc.) of cosolubilized species. The use of a probe molecule to monitor the nature of the water requires that one knows the location of the probe: how does it sit within the micelle (entirely in the water or at an interfacial site), and is the average position of solubilization independent of w_o? We shall discuss the two classes of data separately.

There is an enormous literature on the hydration of biopolymers and lipids. Because in reverse micellar systems facile control of the water-to-surfactant ratio is possible, one has accessible systems where the water population varies from completely "bound" to (mostly) free bulk water. One can infer (vide infra) that the bound water in these systems is rather similar to the interfacial water associated with biological membranes in vivo. This feature has stimulated extensive study and use of reverse micellar systems in chemical and biological applications.

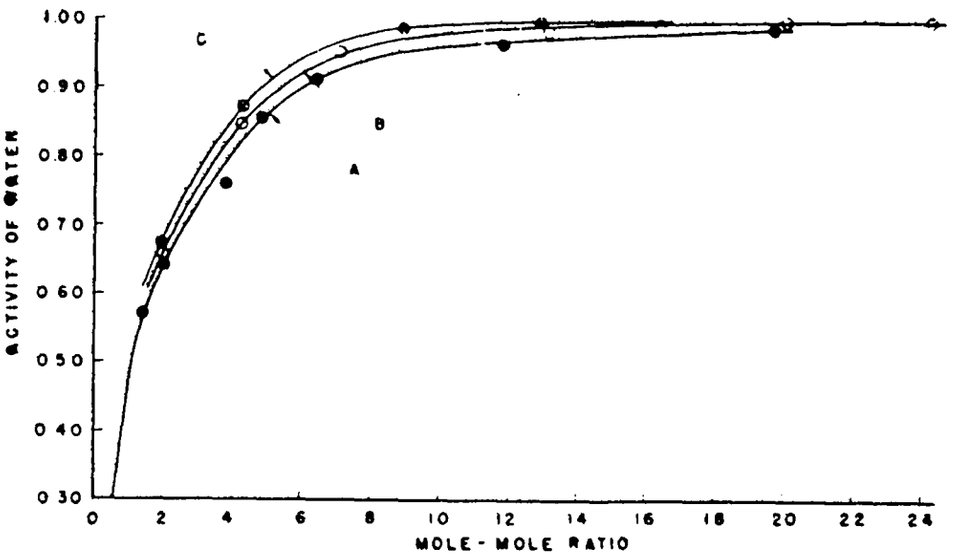
What is meant by "bound" water? The operational definition specifies that it is water possessing properties which are detectably different from those of pure bulk liquid water:[71] these properties include extent of hydrogen bonding, effective dielectric constant and viscosity, mobility (translational and rotational), etc. One should recognize that for a given population of water molecules, different techniques will give differing assessments of the fraction of molecules which are bound. Thus, one quotes a w_o range for the appearance of free (bulk) water (also referred to as the formation of a proper water pool) in reverse micelles. For biopolymers,[72] one can identify at least two types of bound water: (1) irrotationally bound water, which is site bound to the molecule for ca. 10^{-6} sec; and (2) more loosely bound water (reorientation or exchange time ca. 10^{-9} sec) whose rotational motion is significantly hindered and whose freezing point is significantly depressed. The water of hydration for a biopolymer will typically include a few molecules of type (1), but one to two monolayers of water of type (2).

I. Characterization of the Water Pools in the Absence of Probe Molecules

For AOT reverse micelles in various hydrocarbons, an increase in w_o has the following consequences for the solubilized water: (1) an increase in activity, Figure 10a;[73] (2) a decrease in the apparent molar heat capacity, Figure 10b;[74] and (3) an increase[74,75] in the apparent molar volume (i.e., a decrease in the density). Note that the limiting value (in all cases that of pure bulk water) for these properties is reached for a w_o = 10 to 15.

Differential scanning calorimetric (DSC) measurements[76] for the AOT/water/isooctane system detect no phase change (solid ↔ liquid) for the solubilized water until w_o = 7.7. At higher w_os, water melting at 0°C is observed. Since the DSC scans are begun at a temperature of −50°C, one must worry, at least at $w_o > 20$, about separation of the micellar solution into two phases,[29] one of which is almost pure water. However, below a w_o of 7, simple visual observation (of large samples outside the DSC) detects no clouding of the solution. It is possible that at high w_os, one simply observes the melting of (almost) pure ice crystals arising from this phase separation. At low w_os this criticism cannot be made.[29] Proton NMR linewidth measurements for AOT-solubilized water also indicate it possesses a normal melting point.[77] In contrast, for the cationic surfactant CTAB,[77] which forms reverse micelles in chloroform, large depressions of the freezing point were observed (to −20°C at w_o = 0.8; −6°C at w_o = 2.2). Douzou and co-workers[78] have used AOT reverse micelles, w_o = 5.5, to solubilize cytochrome c and trypsin; absorption spectra for cytochrome c could be obtained down to a temperature of −38°C.

Proton (^{1}H) NMR chemical shifts (δ) for AOT-solubilized water have been determined



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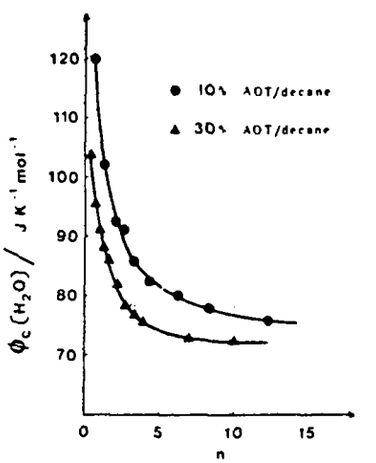


B

FIGURE 10. Properties of water in AOT reverse micelles. (A) The activity of water in AOT-hydrocarbon-water systems as a function of w_o (mol-to-mol ratio) solubilized in a 10% AOT-hydrocarbon solution. A, hexadecane; B, dodecane; and C, octane. (From Higuchi, W. I. and Misra, J., *J. Pharm. Sci.*, 51, 455, 1962. With permission.) (B) Apparent molar heat capacities of water as a function of w_o (≡n) in the AOT-decane system. (From Morel, J. P. and Morel-Desrosiers, N., *J. Chim. Phys.*, 81, 109, 1984. With permission.)

in a number of solvents by several investigators.[47,48,79-83] Figure 11a[81] shows that δ approaches the value (4.8 ppm) for pure bulk water at high w_o. Smaller δ values (i.e., resonance at higher field strength) suggest weaker hydrogen bonding. Since proton exchange between water molecules is rapid on the NMR time scale, one observes a single average chemical shift (δ_{obs}). Maitra[47] assumes that the bound water is of a single type, so that $\delta_{obs} = X_F\delta_F + X_B\delta_B$ (the Xs are mole fractions of free [i.e., proper water pool] and bound water, respectively). From X_B and the water pool radii, he computes the thickness of the bound water layer to be 3 to 4 Å (it decreases slightly as w_o increases).

The w_o-dependence of the rotational correlation times (evaluated from ^1H spin-lattice relaxation times) for the water molecule solubilized by AOT is represented in Figure 11b.[79,80] The motional restriction is large at low w_o; once again, the value for pure bulk water (3×10^{-12} sec) is approached at high w_o. Similar results were obtained for reverse micelles comprised of Triton X-100® (a nonionic surfactant, see Table 1) and 1-hexanol in cyclohexane.[84,85]

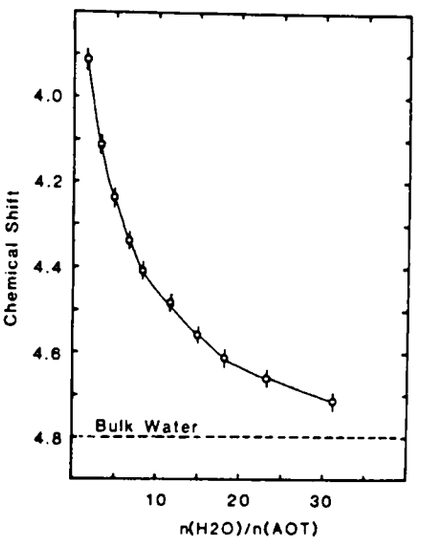
Infrared data have also been obtained by a number of groups[81,86-89] for water in reverse micelles. In the 1800 to 2000 nm region, water absorbs strongly due to a combination mode of stretching and bending vibrations. Bulk water exhibits a broad band at 1930 nm, while water in the gas phase or in an organic solvent (i.e., nonhydrogen bonded water) has a sharp band at 1875 or 1900 to 1910 nm, respectively. In contrast to NMR, separate IR bands are observed for each water population.

For CTAB reverse micelles in $CHCl_3$, λ_{bound} ($w_o = 0$) is 1996 nm, and the authors[88] infer that the water-CTAB interaction (H_2O with the N $(CH_3)_3$ head group) is of the coordination type. The value of λ_{bound} decreases sharply until $w_o = 1$, then more slowly.[88,89] The decrease has been used to infer that as w_o increases, the water is less strongly hydrogen bonded,[89] but this is undoubtedly incorrect, since λ begins at 1996 nm and remains above 1930 nm (the value for bulk water). The break in λ_{bound} at $w_o = 1$ is more interesting; it has been interpreted[88] to mean that the first water molecule interacts much more strongly with the CTAB head group than subsequent ones. Perhaps this first water molecule is irrotationally bound. In contrast, IR data (1800 to 2000 nm region) for AOT reverse micelles in $CHCl_3$[88] show no evidence for a special first water molecule. This is in disagreement with the proposal[1] that at $w_o < 1$ AOT reverse micelles have water molecules forming hydrogen-bonded bridges between two sulfonate head groups.

Thompson and Gierash[81] have collected IR data in the overtone region for the OH stretch of solubilized 11 M HOD (to simplify the analysis) in AOT reverse micelles in *n*-heptane. Bulk water exhibits a band at 1670 nm, while water not involved in a tetrahedral array of hydrogen bonds absorbs at ca. 1400 nm. At $w_o = 1.5$, only the 1400 nm band is seen; it shifts to longer wavelengths as w_o increases, and the 1670 nm band is first clearly present at $w_o = 8.3$ (a shoulder is apparent at lower w_os). The red shift in the shorter wavelength band is thought to reflect changes in the nature of the hydration: the first added waters hydrate an $-SO_3^-Na^+$ intimate ion pair complex; upon further addition of water, insertion of water between the anion/cation pair occurs with a concomitant shift in wavelength.

What can one conclude from thermodynamic and spectroscopic studies about the w_o-dependent properties of water solubilized in reverse micelles? First, that the first several water molecules per surfactant molecule are bound to the interface as water of hydration; the hydration number is low for cationic surfactants (CTAB, for example, will have one water of hydration per head group, perhaps two per bromide ion), higher for anionic surfactants with sodium counterions (AOT evidently has six per Na^+, two to four per sulfonate head group and perhaps one of the ester carbonyl groups is also hydrated). Eicke[48] finds that the bound-to-free water transition for alkali *bis*-(2-ethylhexyl)sulfosuccinates follows the hydration requirements of the cation, occurring at $w_o = 4$ for Cs^+, which is weakly hydrated.

Compared to normal bulk water, the bound water is motionally restricted, may have a



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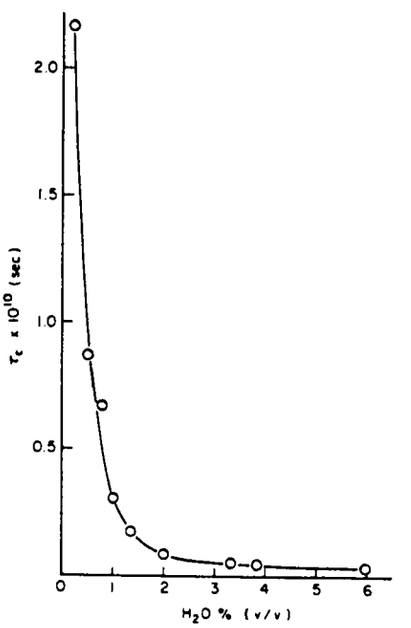


B

FIGURE 11. More NMR data for the water in AOT reverse micelles. (A) Proton chemical shift of the water resonance as a function of w_o in 0.067 *M* AOT (octane-d_{18}). (From Thompson, K. F. and Gierasch, L. M., *J. Am. Chem. Soc.*, 106, 3648, 1984. With permission.) (B) The effect of water content on the rotational correlation times (τ_c) in 0.067 *M* AOT (heptane-d_{16}). Correspondence between percent H_2O and w_o here is 1% (8.2); 6% (49.3). (From Wong, M., Thomas, J. K., and Nowak, T., *J. Am. Chem. Soc.*, 99, 4730, 1977. With permission.)

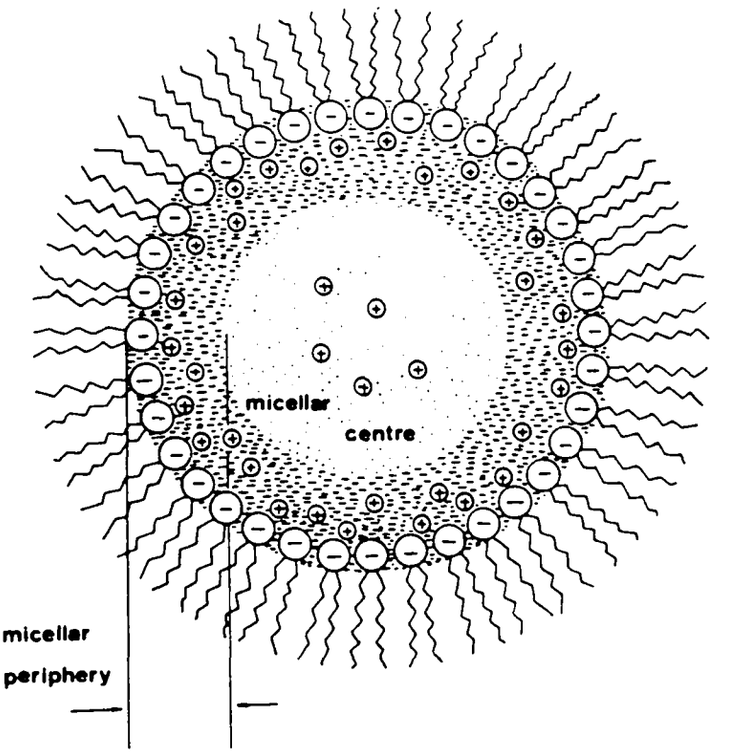


FIGURE 12. The AOT reverse micelle. (From El Seoud, O. A., *Reverse Micelles*, Luisi, P. L. and Straub, B. E., Eds., Plenum Press, New York, 1984, 81. With *permission.*)

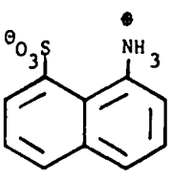
depressed freezing point, and lacks the normal hydrogen-bonded structure. When sulfonate head groups are present, a portion of the hydrating water is involved in hydrogen bonding, and this is undoubtedly the reason why less perturbation than with cationics is observed in the 1800 to 2000 nm region of the IR spectra. Very little of this water is irrotationally bound; however, water molecule exchange, even at the lowest w_o values, is occurring on the nanosecond time scale. Figure 12 shows, for AOT, a visualization of the reverse micelle when w_o exceeds the hydration requirement. One can see that there are at least three distinct solubilizate environments available: interfacial, in the bound water layer, and in the water pool. Exchange among these sites may be rapid for small molecules. Probe molecules with a dimension exceeding 4 Å cannot experience a pure bound water environment; the thickness of the bound water layer remains roughly constant as w_o increases, so that water pool water predominates at the higher w_os (for example, at w_o = 40, only 17% of the total water is in the bound layer). The effective sodium ion concentration, $[Na^+]_{eff}$, may be several molar in the bound water layer.

J. Inferences about the Water Pools Based on the Behavior of Solubilized Probe Molecules

The w_o-dependence of water pool polarity (often designated micropolarity) has been investigated using solubilizates which have solvent-sensitive UV-VIS absorption spectra[90-94] and/or solvent-sensitive fluorescent lifetimes and quantum yields.[95-98] In such studies, it is important to determine by an independent technique the location of the solubilizate within the reverse micelle, so that one is certain that the probe molecule is indeed located within the water pool rather than adsorbed at the interface (i.e., at the micelle boundary).

At low w_o, contact between probe and interface is, of course, unavoidable. Even molecules which are quite soluble in water, such as phenols, are found to bind at the interface,[90-94] with the hydrocarbon portion of the molecule intercalated among the surfactant tails. Such interactions can be detected by monitoring the effect of the solubilizate on the chemical shifts for surfactant protons close to the head group.

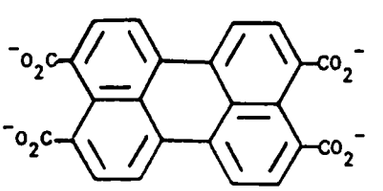
We provide a specific example of the difficulties in interpretation which may arise when one uses probe molecules. Wong et al.[96,97] determined the w_o dependence of the quantum yields and λ_{max} values for ANS



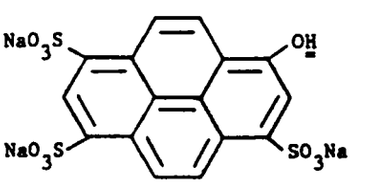
ANS

fluorescence in AOT reverse micelles. Since ANS fluorescence has been investigated in many organic and mixed/organic aqueous solvents for which Kosower Z values are known (this is an empirical solvent polarity scale), Figure 13 could be constructed. One sees that the apparent polarity (and hence dielectric constant) of the solubilized water increases rapidly until $w_o = 12$ and more slowly thereafter. However, it attains at high w_o a maximum value characteristic of an aqueous alcoholic solution, rather than the value (dotted line) for bulk water. This disagrees with the more direct measurements of the properties of water at high w_o; it is likely that part or all of the solubilized ANS sits at or in the AOT interfacial layer, so that even at high w_o, an average Z for bound plus free water is being determined. When pyridine 1-oxide is used as the probe, the bulk water Z is reached[95] at $w_o = 32$, lending further support to the notion of differences in probe partitioning.

Fluorescence polarization decay[99-103] and ^{13}C T_1 values[104] have been used to assess microviscosities (η) of the water pools. As expected, the apparent ηs are high at low w_os, decreasing to values close to that of bulk water once the hydration requirements of the surfactant are met. One also has in these measurements the problem of solubilizate location. Electrostatic repulsion between surfactant head groups and the solubilizate may be used to ensure a free water environment (once the size of the free water core exceeds the dimensions of the probe molecule). Pyrene tetracarboxylate (PTC) and pyranine have been used to good advantage in studying AOT systems. On the other hand,



PTC



Pyranine

(= designates an acidic proton)

if one wants to ensure almost exclusive interfacial solubilization, an amphilic probe may be used, for example,[101] 2-(*N*-tetradecyl)amino-naphthalene-6-sulfonic acid.

The measured decay may arise from rotational diffusion of the micelle-probe complex as a rigid unit as well as from rotational diffusion of the probe within the micelle. Each

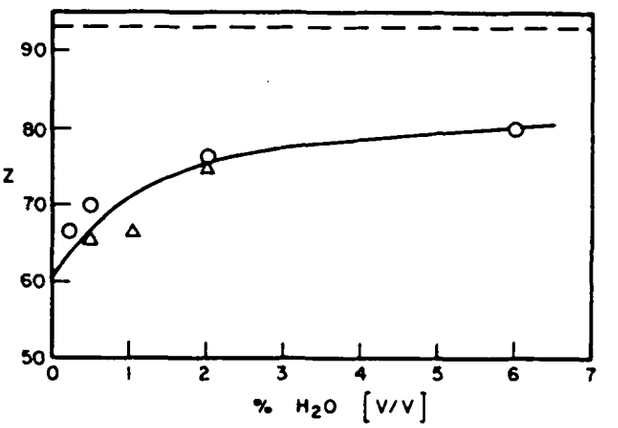


FIGURE 13. Kosower's Z value evaluated from ANS fluorescence results for 0.067 *M* AOT in (Δ) dodecane and (o) heptane. (From Wong, M., Thomas, J. K., and Gratzel, M., *J. Am. Chem. Soc.*, 98, 2391, 1976. With permission.)

solubilization site will in principle have a different D_r; associated (via a Stokes-Einstein relationship) with each will be an apparent microviscosity. Zinsli[101] has fit his observed multiexponential decay curves for 1-aminonaphthalene-4-sulfonic acid (inferred to reside only in the solubilized water) in AOT reverse micelles at several w_os using two microviscosities, with η (bound water layer) $>\eta$ (water pool). He estimates that the bound water layer (see Figure 12) is 4 to 8 Å thick. The ESR measurements of Yoshioka[105] on a nitroxide spin probe solubilized in AOT reverse micelles also permit one to differentiate between bound and free water in the micellar interiors.

Pyranine is a much stronger acid in its excited than in its ground state. Recently, Bordez and co-workers[103] have determined the w_o-dependence for the rate constant of proton dissociation from excited state pyranine. The rate constant may be expressed as $k_1 = k_1^\circ(a_{H_2O})^n$, with $n = 6.9$ (the same value it has in concentrated aqueous electrolyte solutions) and a_{H_2O} values taken from Reference 106. This means that k_1 reaches a plateau value at $w_o = 12$. At low w_o proton transfer is slowed because few water clusters, $(H_2O)_n$, which can accept a proton, are available. Escabi-Perez and Fendler[107] found ultrafast excited-state proton transfer for pyrene-1-carboxylic acid solubilized in reverse micelles of dodecylammonium propionate.

Perhaps the least ambiguous probe species for the properties of solubilized water is the hydrated electron,[108,109] whose capture probability and spectroscopic properties have been found to depend upon w_o for AOT reverse micelles. Below a w_o of five, no hydrated electrons can be produced; Pileni and co-workers[108] ascribe this to immobilization of the water molecules. Above a w_o of 20, the absorption spectra observed for the hydrated electrons is the same as found for bulk water. At intermediate w_os, the effects observed can be rationalized in terms of variations in $[Na^+]_{eff}$. Similarly, the w_o dependence of the quenching rate constant (quenchers are azide ion or tryptophan) for singlet oxygen in AOT reverse micelles[110] provides evidence for increases in water mobility as w_o increases.

The information obtained in the probe studies discussed here corroborates the microscopic picture of the solubilized water developed in the previous section and summarized in Figure 12. It is obviously preferable to study the properties of the water directly, rather than relying on spectroscopic or reactivity probes.

K. Binding Constants of Solubilizates to Reverse Micelles

Solubilizate binding constants have been determined for a few reverse micelle — small

molecule (phenols, imidazole, and simple alcohols) combinations.[91,111,112] In the case of imidazole binding to dodecylammonium propionate (DAP) reverse micelles, the use of the chemical shift dependence on DAP concentration for imidazole protons led to nonunique (i.e., proton-dependent) binding constants, a physically unreasonable situation.[112] When one is studying chemical reactions involving reactants which partition among the water pools, surfactant interface or the continuous phase, one must know the effective concentration of each reactant at the reaction locus. This question of local vs. overall reactant concentration is discussed further in a subsequent section.

Competition between solubilizates for interfacial binding sites may be used to obtain qualitative information on binding constants. Imidazole is capable of displacing *p*-nitrophenol[90] (detected by a shift in pK_a for the phenol) from the AOT interfacial layer in AOT reverse micelles. Generally, the more polar (or the more interfacially active) a solubilizate, the better it binds to reverse micelles. Rates of aminolysis[95] for a series of *p*-nitrophenyl alkanoates in AOT reverse micelles support this: the longer the alkyl tail (i.e., the more hydrophobic the reactant), the lower the binding constant and consequently the slower the reaction is.

L. Empirical Determination of pH for the Water Cores of Reverse Micelles; pK_a Values for Solubilizates in Reverse Micelles

The determination of pH of the water pool (pH_{wp}) of reverse micelles is difficult both from the conceptual and the experimental point of view. If one accepts the view that water inside reverse micelles is a solvent different from bulk water, one is left with the conclusion that the common procedures used to measure the pH in aqueous solutions (a pH electrode, or indicators) do not apply, simply because the electrode, or the indicators, are not calibrated for this novel solvent. An additional problem lies in the fact that the physical properties of the water in the water pool of reverse micelles depend upon w_o — so that one would need, in principle, a calibration of each w_o; a difficulty at yet a different level lies in the relation between activity and concentration of hydrogen ions.

As hopeless as it sounds in principle, the problem of the evaluation of pH can be approached in practice in a number of ways. First of all, when w_o is rather large (say above 20), one can make the approximation that we are dealing with "normal" water. In this case, the usual indicators and other probes can be used as in water. The only important precaution here is to make sure that the probe is located in the middle of the water pool. If the indicator is bound on the micellar wall, or sticks out in the apolar phase, then of course the number one reads is deprived of validity. This observation is particularly relevant if one considers that most indicators are large aromatic molecules with a hydrophobic or amphiphilic nature. Indeed, it has been shown that under certain conditions different indicators give different answers.[113-116]

Conceptual and experimental difficulties are of course larger in the w_o region below 20, where the water of the water pool is "anomalous". Actually, difficulties are dramatic at w_o values below eight or so, where most of the water is supposed to be bound to the micellar wall (at least in the case of AOT). Conceptually, the fact that most of the water is bound does not inhibit the definition and the evaluation of the pH, since experimentally we are generally concerned with activity more than with concentration measurements. However, then, of course, the interpretation of data is extremely difficult.

What then can one do in this case, when one needs some indication of the hydrogen concentration in the w_o range, say, three to ten? This is, by the way, the w_o range which is most interesting for enzymes solubilized in reverse micellar solutions, namely, where both activity and conformational properties deviate most strongly from those found in aqueous solutions. The only reasonable and simple way is to use an arbitrary acidity scale. One can, for example, utilize an indicator — chosen to be as much as possible water soluble — and

use it to monitor whether the enzyme experiences the same "acidity" as in another experiment. In this way, one can, for example, study the dependence of certain enzymatic properties (e.g., activity or percent of secondary structure) on pH. This is allowed — one has only to remember that the "pH" evaluated under such condition, say, at $w_o = 5$ with AOT, cannot be taken as an absolute measurement of $[H^+]$.

After this introduction, let us see what has been done in the field in order to cope with the question of pH in reverse micelles. Smith and Luisi[117] assume that solubilization in AOT reverse micelles in fact does not change the pK_a; thus for a given pH, the $H_2PO_4^-/HPO_4^{2-}$ ratio is the same in bulk water as in the water cores. Since ^{31}P NMR chemical shifts are sensitive to pH, the water core pH is thus determined for various AOT reverse micellar solutions by locating the observed chemical shift on a calibration plot produced by measurements of bulk water phosphate buffer solutions. By this method, pH_{wp} was found to be generally within 0.4 pH units of pH_{st} (which is the pH of the solution to be solubilized). However, does this method actually measure pH_{wp} — that is an average of some sort for the bound water layer plus the water pool? In fact, it probably measures the apparent pH value for the center of the water core — because the anions involved are so hydrophilic and are repelled from the like-charged surfactant interface.

Let us assume for the moment that in fact $pH_{wp} \approx pH_{st}$. pK_as determined using this assumption ($pK_a = pH_{st} - \log([A]/[HA])$ for several indicators are given in Table 2. Menger and Saito[90] found a pK_a of 11.5 for *p*-nitrophenol in AOT reverse micelles containing solubilized phosphate buffer. When an imidazole buffer was used instead, a portion of the phenol had a pK_a of 7.6 to 7.9, and the amount of it present increased with increasing imidazole concentration. From these data, the authors inferred that imidazole is displacing *p*-nitrophenol from interfacial binding sites into the water pools. Indicators which have one or more hydrophobic forms generally show large apparent pK_a shifts in the acid-weakening direction in AOT reverse micelles. In contrast, for indicators whose ionization is of the ($- \leftrightarrow =$) charge type, (so that it is likely that both species reside mainly in the water pool), the pK_a increase is less than one unit. The observed shift in pK_a is also independent of w_o. Examples are sodium hydrogen maleate[116] and 4-nitrophenol-2-sulfonate.[116,118]

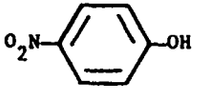
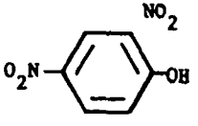
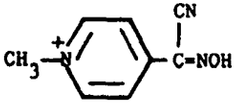
When pH_{st} is used, one obtains the result[92] that pK_as for hydrophobic indicators depend upon w_o in AOT and in nonionic reverse micellar systems. Since these indicators are interfacially bound, the proper water core pH is actually that of the bound water layer, pH_b. El Seoud has computed pH_b and pH_f (the value in the interior, i.e., the proper water pool portion of the micellar cores of the AOTs) for various pH_{wp} and w_o values, and the results are displayed in Figure 14. The source of the difference in the two pHs ($pH_f > pH_b$ except below a pH_{wp} of one) is the ion-exchange equilibrium occurring between Na^+ and "H^+":

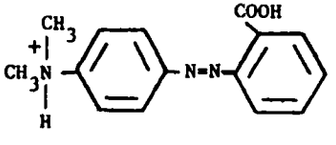
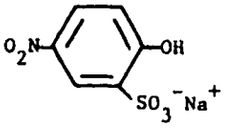
$$Na^+_b + H^+_f \rightleftarrows Na^+_f + H^+_b$$

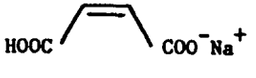
This has the effect of accumulating hydrogen ions in the bound water layer. The gap between pH_f and pH_b is larger at the larger w_o because the water pool volume (where H_f resides) is so much larger than at low w_o. When pH_b is used in the Henderson equation for hydrophobic indicators, the increase in pK_a observed upon transfer from bulk water to the AOT reverse micelles is less than one unit, and it is independent of w_o.

Provided one uses the proper pH in the Henderson equation, the pK_a shifts one computes for solubilizates in AOT reverse micelles are small. One always observes an acid-weakening effect (pK_a increases), and this may be attributed to a solvent effect (a decrease in solvent polarity suppresses ionization) and/or to retardation of ionization (in the case of interfacially solubilized indicators) because of hydrogen bonding to the sulfonate head groups (or ether oxygens in the case of nonionic surfactants).

Table 2
pK_a VALUES FOR INDICATORS IN AOT REVERSE MICELLES

			
in water	7.14	4.1	4.6
in AOT (interfacial)	11.5	5.8	5.15
(water pool)	7.6-7.9		

		
in water	4.95	6.0
in AOT (interfacial)	8.2	-
(water pool)		6.2

	
in water	6.24
in AOT (water pool)	6.5

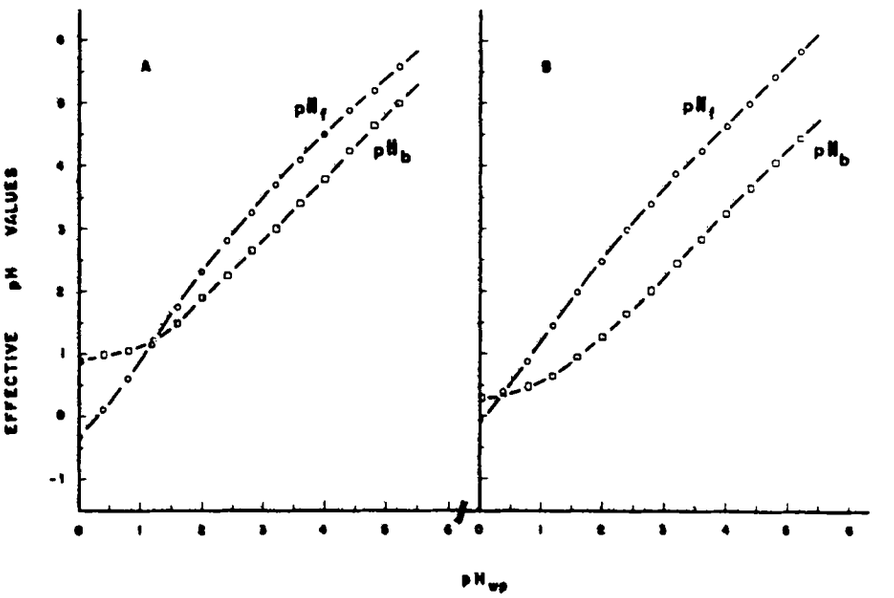


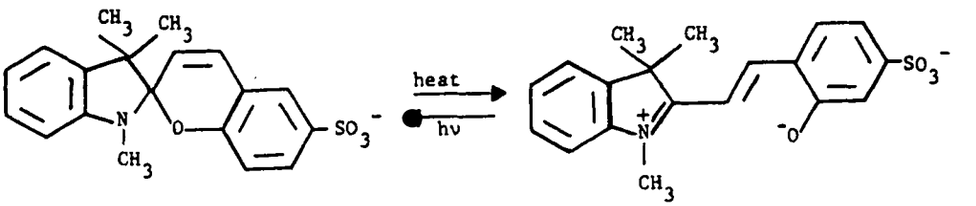
FIGURE 14. Variations of the pH values at the micellar periphery (pH_b) and at the center part of the pool (pH_f) as a function of pH_{wp}. Part A is for a small pool (w_o = 10), whereas part B is for a large one (w_o = 40). These effective pH values are for AOT = 0.1 *M*. (From El Seoud, O. A., *Reverse Micelles*, Luisi, P. L. and Straub, B. E., Eds., Plenum Press, New York, 1984, 81. With permission.)

M. Reverse Micelles as Novel Microenvironments for Chemical Reactions

The effects of reverse micelles on a wide variety of chemical reactions (metal ion-ligand exchange, ester hydrolysis and aminolysis, proton transfers, etc.) have been investigated and the topic has been reviewed a number of times in recent years.[4,5,111,119-121] Chemical reactions have also been studied in normal micellar systems, and it is legitimate to ask in what respects these complementary milieu differ. For bimolecular reactions involving small molecules, the increases in velocity (over the values in bulk water for the same overall concentration), found in normal micellar systems are dominated by a concentration effect: solubilization of the reactants by micelles increases their effective concentrations. Of course, for the evaluation of a real "micellar catalysis", this concentration effect must be taken care of.[122] Unfortunately, this useful and trivial correction of the rate enhancement is seldom done. For some reactions, such as unimolecular decarboxylations,[123] it has been verified that generalized medium effects (the dielectric constant of the normal micelles surface being lower than that of bulk water) also affect the rate constant. One would expect reverse micelles to be analogous to normal micelles in these two respects, and for many reactions this is the case.

The dependence of the reactivity in aminolysis of a series of aliphatic *p*-nitrophenyl esters on alkyl tail length in AOT reverse micelles (acetate fastest, dodecanoate slowest) is the reverse (as expected based on partitioning considerations) of what one finds in normal micellar systems.[95] Increasing the amount of water (and therefore the pool size) increased the aminolysis velocity because the effective concentration of the ester in the reverse micelles increases. No unusual effects due to the properties of the water pool of the reverse micelles could be discerned.

Sunamoto and co-workers[124] have investigated in AOT reverse micelles the reaction given in Equation 1. They have found a substantial rate decrease for the ring opening and subsequent *cis-trans* isomerization. This



decrease is interpreted to arise from the particular microenvironment of the reverse micelles. In several cases where water pool water is a reactant, such large rate enhancements have been observed [125] (for example, the acquation of Cr^{3+} complexes), that one can speak of real micellar catalysis, i.e., of a marked increase of the bimolecular rate constant. In certain other cases, for example, the hydration of 1,3-dichloroacetone,[126] there is a mechanistic change (revealed in this case by a proton inventory study) upon going from bulk water to water pools in AOT reverse micelles, i.e., two different transition states for $w_o = 1.3$ and for $w_o = 11.1$ have been inferred.

There have been attempts made to exploit the idea that micelle-reactant interactions should be more specific (in the sense of one reactant orientation being markedly favored over others and/or chiral discrimination by surfactants of optically active reactants) in reverse than in normal micelles. Thus, Jaeger and Ippoliti[127] attempted to carry out lactonization of long chain ω-hydroxyacids in reverse micelles; however, the production of polyesters was not significantly hindered by the micellar environment. Similarly, only modest chiral discrimination has been observed in reactions carried out in reverse micelles: Kon-no and coworkers[128,129] observed an enantioselectivity of 2.5 in the aminolysis reaction of (+) or (−)-α-phenethylamine with (+) or (−)-*p*-nitrophenyl-α-methoxyphenyl acetates in cationic reverse micelles. From the examples of stereoselective catalysis in normal micellar and vesicular systems, it is likely that providing for multiple hydrogen bonding and/or hydrophobic interaction sites for surfactant-reactant interactions will be necessary to produce significant stereoselectivity.[120]

N. Sizes of Reverse Micelles with and without Added Biopolymers

Reverse micellar sizes have been determined for a number of surfactants other[130,131] than AOT. However, we shall discuss in detail only the data available for AOT, since it has been studied by a number of methods (in several solvents and at many w_o values), including scattering techniques (dynamic light scattering,[29,132,133] small-angle X-ray scattering,[134] and small-angle neutron scattering[60,135-139]), ultracentrifugation,[28,132] viscometry,[132] and fluorescence polarization[99,100] measurements. As w_o increases, the diameters of the central water pools increase, and the number of water and AOT molecules per aggregate also increase. Below a w_o of 10 to 15, the apparent average micellar size[29] is independent of temperature and pressure (see, however, the paragraphs on interactions). There is a modest distribution of micellar radii in the systems investigated; this polydispersity has been evaluated by both SANS[136,137] and LS.[133]

Figure 15 gives the average dimensions for AOT reverse micelles in isooctane.[29] If the stoichiometric concentration of AOT is 0.10 *M*, then the average surface-to-surface separation between two micelles is 82 Å ($w_o = 10$) and 165 Å ($w_o = 50$). At the lower w_o, the distance of separation is larger than the micellar diameter; at the higher one it is less.

Dynamic light scattering, which allows one to obtain an apparent translational diffusion coefficient (and thus an apparent hydrodynamic radius for the micelles, see Reference 29 and Table 3), and static SANS measurements, which allow one to obtain apparent water pool and hard sphere radii directly (see References 60, 135-139, and Table 4), are complementary techniques for structural studies of reverse micelles. In the case of LS, the scattering

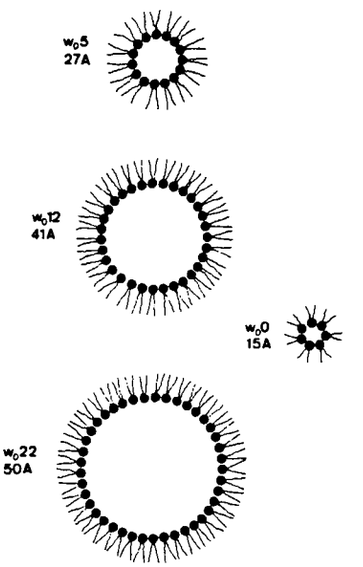


FIGURE 15. Sizes of AOT reverse micelles in isooctane. Radii given are $R_{wp} + \ell_{AOT}$.

Table 3
THE DEPENDENCE OF HYDRODYNAMIC RADIUS ON w_o FOR AOT REVERSE MICELLES IN ISOOCTANE AT 30°C (LIGHT SCATTERING)

w_o	R_H,Å	R_w,Å*
0	15	—
5	31	19
9	35	23
15	40	28
27	61	49
38	105	93
50	145	133

Note: w_o is the molar ratio of water to AOT.

* $R_w = R_H - 12$ (i.e., assuming no solvent penetration).

From Eicke, H.-F. and Zulauf, M., *J. Phys. Chem.*, 83, 480, 1979. With permission.

is observed provided the refractive index of the particles and the solvent differ. Since n_{part} depends on water content, i.e., on w_o, optical matching phenomena[140] have been observed (the nature of the oil also affects Δn). For a polydisperse sample, not all micelles will be invisible at a given overall w_o. Therefore, LS measurements made close to an optical match

Table 4
THE DEPENDENCE OF THE HARD SPHERE AND WATER POOL RADII ON w_o FOR AOT REVERSE MICELLES IN HEPTANE AT 25°C (SANS)

w_o	R_{HS}, Å	R_w, Å
10	33	26
15	36	29
20	42	35
30	—	48
40	—	60
50	—	90
60	—	120

From Fletcher, P. D. I. and Robinson, B. H., *Ber. Bungenges Phys. Chem.*, 85, 863, 1981. With permission.

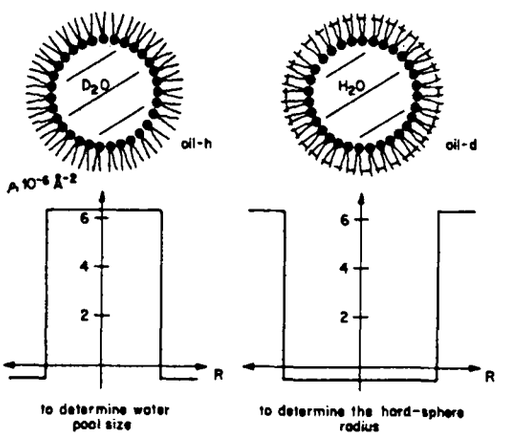


FIGURE 16. Representation of the two contrasts commonly used in SANS measurements on reverse micelles.

point may yield apparent mean micellar radii which represent only a portion of the actual micellar population.

In SANS measurements on AOT micellar systems the coherent scattering (i.e., the portion which contains structural information) observed is due to differences in nuclear scattering cross section between deuterium and hydrogen. Thus if one wishes to do size determinations on the water cores alone, one can solubilize D_2O. On the other hand, to determine the hard-sphere radius of the micelles (and limit of penetration for the continuous phase), one can use H_2O and deuterated hydrocarbon. Figure 16 depicts these two types of contrast in scattering cross-sections. As the data in Table 4 demonstrate, the hard-sphere radius for an AOT micelle at large w_o is about 7 Å larger than the water core radius. Thus, the outer portion of the surfactant layer is penetrated by the continuous phase.

We have used the adjective apparent to describe the micellar radii determined by dynamic LS or static (total intensity) SANS measurements at finite particle concentrations. Similarly,

one determines apparent molecular weights by static SAXS or LS or by ultracentrifugation. The point is that the particles interact with each other, and these interparticle correlations (as opposed to intraparticle correlations, which give rise to what one calls the form factor) produce a contribution to the scattering which is referred to as a structure factor.

The interaction potential for AOT reverse micelles is comprised of a hard-sphere repulsive term plus a term coming from a short-ranged attractive potential,[141,142] whose molecular origin is overlapping of the tail of the surfactant; many other reverse micellar systems behave similarly.[143] The depth of the attractive potential well increases with increasing particle size and temperature; for some w_os and particle concentrations, this potential can be sufficient to produce a critical transition, which involves separation of the micellar solution into two: one solution dilute in micelles, the other concentrated. This phase separation does not involve a change in particle size for AOT micelles[135-139] or for many other micellar systems.[144-148] Critical concentration fluctuations, characterized by a correlation length whic . diverges, occur in solutions approaching phase separation, and dynamic LS measurements determine it rather than the micellar sizes. In contrast, because SANS measurements cover a range of momentum transfers (Q) where the micellar form factor shows Q-dependence, one can determine both correlation lengths and micelle sizes.[135-139]

The approach to phase separation also produces more facile droplet communication, and this gives rise to a phenomenon known as percolation, in which a microscopic path for, for example, ion transport appears in the solution. This is detected by a sudden increase (often by several orders of magnitude) in the specific conductivity of the solution. For AOT micelles these paths are transient, but in other micellar solutions the water cores may be destroyed and permanent water conduits appear (for an example in another ternary system, see Reference 149).

How does one know if one's size determinations are being seriously compromised by the presence of interactions? The best way is to make measurements at several particle concentrations and then extrapolate the apparent radii or molecular weights to infinite dilution, where interactions are absent. However, for reverse micellar solutions, simple dilution with pure hydrocarbon may decrease the particle size. The presence of some water and surfactant in the diluting solvent prevents this, but determining the amount to use is not trivial.

Alternatively, one can accept the fact that one is determining apparent sizes and use them for comparative purposes. If one's samples are well within the one phase region of the AOT/water/hydrocarbon phase diagram, only hard-sphere repulsions operate and the dependence of apparent particle size on volume fraction is modest. This constitutes a strong argument for always determining what changes, if any, occur in the phase behavior when a biopolymer is added. It is absurd to compare the apparent size of an empty micelle to a solution of the correlation length of filled micelles (which is measured if the addition of the biopolymer moves the solution close to phase separation).

II. BIOPOLYMERS IN REVERSE MICELLAR SOLUTIONS

On the basis of the background information summarized until now, let us discuss the behavior of biopolymers, in particular enzymes and DNA, solubilized in hydrocarbon solvents via reverse micelles. We will consider first the solubilization procedure and the little which is known about the solubilization mechanism. The question of the size and structure of biopolymer-containing reverse micelles will be considered next. Then will follow a closer inspection of the composition of the reverse micelle which hosts the biopolymer, including questions about the changes in conformation experienced by the macromolecule with respect to the normal aqueous solution. This issue is connected closely with the problem of the structure of water inside the reverse micelle, and in turn these two factors (structure of the biopolymer and of water) are important determinants for the biological activity. After this

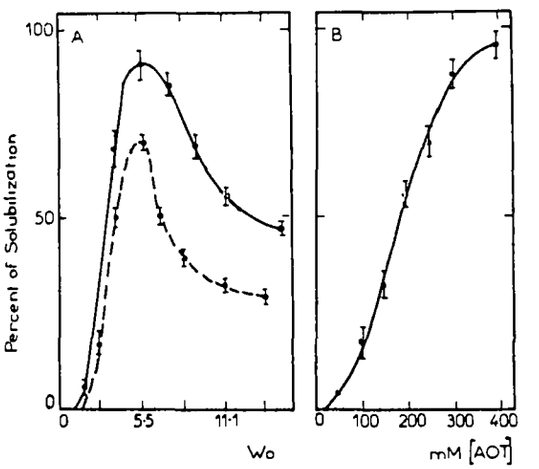


FIGURE 17. Solubilization of the Folch-Pi proteolipid in AOT-water-isooctane reverse micelles. The overall concentration of the proteolipid in the assay was 20 μM. A micellar solution of this concentration corresponds to a 100% solubilization. (A) The percent of solubilization at 300 (——) and 200 (---) mM AOT. (B) The percent of solubilization against AOT concentration, at w_o = 5.56. (From Delahodde, A., Vacher, M., Nicot, C., and Waks, M., *FEBS Lett.*, 172, 343, 1984. With permission.)

section on activity, we will consider the biotechnological implications of enzymes, nucleic acids, and other biomaterials in reverse micelles. We will terminate this presentation with a short discussion about the most urgent questions which are still open in the field.

A. Solubilization of Biopolymers in Reverse Micelles

Currently, there are three methods which are most commonly employed to solubilize macromolecules in reverse micellar solutions:[150-152] (1) starting from the biopolymer in the solid state, by stirring the biopolymer powder with the micellar solution at a given w_o; and (2) with a phase transfer process, in which the protein is initially present in an aqueous solution, and is extracted by a supernatant hydrocarbon micellar solution. These two first methods appear of quite general validity for proteins and all sorts of low molecular weight biomolecules, but they are not suitable for high molecular weight nucleic acids, in particular DNA. Method (3) is by injecting in the hydrocarbon micellar solution a few microliters of a stock aqueous solution of the biopolymer. This is a quite general method for water-soluble macromolecules, but it is not suitable obviously for certain water-insoluble proteins (we will discuss this case later on).

Let us consider some experimental data, beginning with the solid phase extraction method. Preliminary data have been published a few years ago[151] which are now under further scrutiny.[156] Figure 17 reports new data concerning the solubilization of a protein which constitutes the myelin structure, i.e., lipophilin (or Folch.-Pi protein), which is water insoluble. For the first time lipophilin could be studied in solution, and, more interesting, one has now a means to study the interaction with the other protein (myelin basic protein, MBP) comprising the myelin structure, under conditions which can be considered similar to those in the membrane environment. For the biological implications, the reader is referred to the work of Delahodde et al.[154,155] Here, we want to comment briefly about the solubilization data (Figure 17 is taken from the cited work of Delahodde and co-workers).

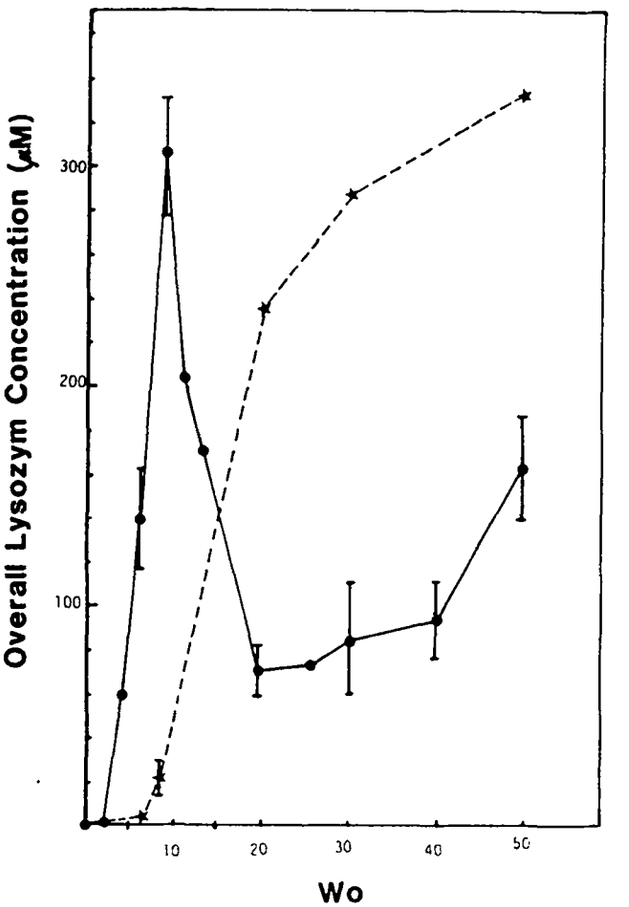


FIGURE 18. Lysozyme extracted from the solid state as a function of the initial w_o in 50 m*M* AOT (isooctane). A 100 μ*M* solution in water of the "acid" (——) powder from Fluka has a pH of 3.5 to 4. A 100 μ*M* solution of the "basic" (---) powder from Sigma has a pH of 9.0. The solution of the basic powder can be acidified and the protein precipitated and lyophilized. Extraction of this solid preparation affords the extraction profile of the "acid" powder.

The maximal solubilization of both proteins in AOT/hydrocarbon takes place at a very small w_o value corresponding to less than 0.5% water in v:v. This is surprising, as one would have expected that, at least for water-soluble proteins, the solubilization extent would increase by increasing the concentration of water in the apolar phase. The case of lysozyme, which was studied by us earlier[153] is not much different. Quite recently, upon repeating these experiments with lysozyme, we have learned something new about this kind of solubilization process. In fact, the extent of solubilization and the form of the curve was found to be dependent on the commercial source of the protein. When this phenomenon was studied more deeply, it was found that the difference was due to the ionization state of the protein in the powder form: when lysozyme was precipitated from a very acidic solution, the curve in Figure 18 was obtained, whereas when the powder was obtained from an alkaline solution, the curve was much shifted towards larger w_o values, with no sharp maximum at low w_o. Details will be given later, in a paper now in preparation. These findings indicate already a very important clue for the solubilization of the protein from the solid state: that, at least

in the case of the anionic surfactant AOT, the protein has to be charged positively. When this is so, the maximum extraction power of the micellar hydrocarbon solution is around w_o 6 to 8, at least in the case of very small proteins. Notice that, considering the arguments presented in the Introduction, water under those conditions is supposed to be bound to the polar internal walls of the micelles — in other words there should be no "free water" at w_o values below ca. 7 to 8. On the other hand, one should mention that w_o given in the abscissa of Figure 18 is a phenomenological (overall) value, which does not necessarily represent the intrinsic w_o value of the protein-containing micelles. We will come back to this point later when we discuss the size of protein-containing micelles. On that occasion, we will see that the most likely w_o value for lysozyme-containing micelles, in a solution having a nominal w_o of 6 to 7, is around 14. However, even when this is taken into account, the fact remains, that the maximal extraction of the protein takes place at a tiny concentration of free water in the reverse micelle. In turn, this indicates that novel solubilization capacity is one of the novel physical properties possessed by the water of the water pool with respect to bulk water. Why this solubilization power is maximal at such a low content of free water is not clear as yet. We will see later on that for such conditions the enzymatic activity is higher also — a finding which certainly adds to the general fascination of the field and to the mystery of water structure.

Notice that the concentration expressed in the ordinates of Figures 17 and 18 is an overall concentration, i.e., referred to the total volume of the solution. If one were to express the concentration of the solubilized biopolymer in terms of water pool concentration, one would obtain at $w_o = 5$ a concentration factor of ca. 200 larger. For substances soluble only in the water microphase, the two types of concentrations are related by the relation $C_{ov} = F_w \cdot C_{wp}$, where F_w is the volume fraction v/v of water. This local concentration is larger than the maximal solubility of lysozyme in an aqueous solution. We can take this opportunity to present a rough numerical representation of the concentration parameter characterizing the micellar solution. In the case of lysozyme in Figure 18 at an AOT concentration of 50 m*M*, the concentration of micelles is 0.43 m*M*, and the overall concentration of the protein after extraction at $w_o = 5$ is 0.10 m*M*. The average degree of occupancy of the micelles (if no change in micellar number occurs) is then 23%, and the concentration of lysozyme in the water pools is 0.022 *M*.

Let us consider now the phase transfer, another method to solubilize proteins in apolar solvents via reverse micelles. A few representative experiments, which are also a reappraisal of our earlier findings,[150,151] are presented in Figure 19. We see how trypsin, present in an aqueous solution at various salt concentrations, is extracted by the AOT of the supernatant isooctane solution. The micellar solution contains initially no water, so it is also extracted from the aqueous solution. From Figure 19b one can see the amount of water extracted into the micellar solution as a function of the salt concentration of the aqueous solution and the relationship between protein and water uptake.[156]

A few observations are immediately apparent from inspection of Figure 19: (1) at the optimal salt concentration, the transfer of protein is almost quantitative; (2) the maximal transfer of protein corresponds to the maximal transfer of water into the organic phase; and (3) there is a certain degree of specificity in the transfer process, for example, NaCl and $CaCl_2$ influence differently the extent of transfer of a given protein, and different proteins behave somewhat differently at a given salt concentration.

The experiments whose data are partly summarized in Figure 19 were aimed at studying the relative rate of transfer of different proteins from a water-to-a-lipidic phase under conditions which are as close as possible to physiological ones. For these reasons the two phases were stirred only very gently, taking care to avoid disturbing the interface mechanically. However, it should be noted that all the data points represented in Figure 19 are at equilibrium. This has been checked by the time dependence of the protein concentration (no change in

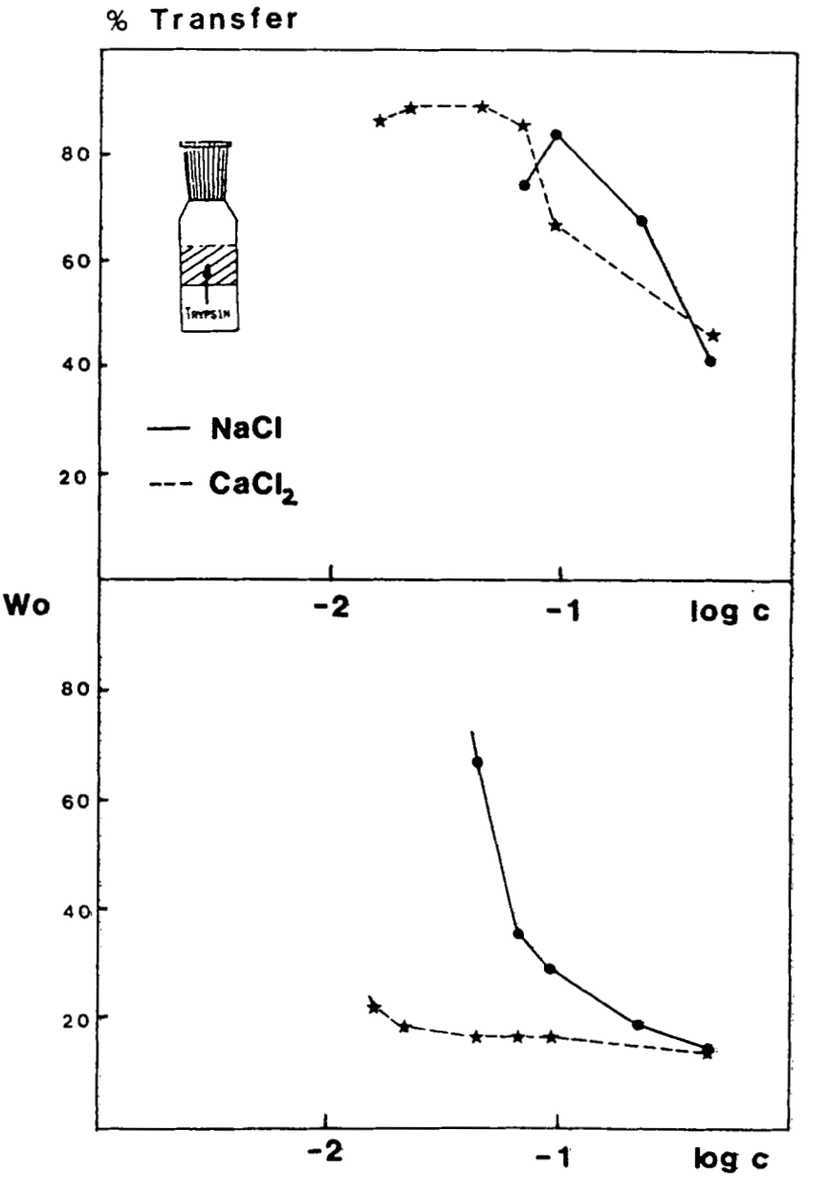


FIGURE 19. Percent transfer of trypsin (initial concentration of 15 μ*M* in water) into 50 m*M* AOT (isooctane) as a function of supporting electrolyte concentration (c) in the aqueous subphase. Final water content in the organic phase as a function of (c).

the plateau value for over a period of 20 hr); furthermore, the same concentration values have been also obtained in the hydrocarbon solution after a long period of violent stirring and mixing the two phases.

These experimental findings suggest a series of observations and questions. A first question is what are the driving forces which are responsible for the transfer of the protein from the aqueous environment (which one would expect to be much more compatible with the nature of a hydrophilic protein) to the micellar environment? In other words, why is the free energy balance in favor of the protein in the hydrocarbon micellar solutions?

No thermodynamic study of protein-containing reverse micelles has been attempted with the aim of answering such a question. One can suggest, as a first working hypothesis, that it is the peculiar structure of water which plays the most important role. In particular, one can envision that in the system there are three limiting types of water: the water of the bulk aqueous solution, the water at the interphase between water and hydrocarbon, and the water inside the water pool. The first type of water is the least ordered and the water in the water pool is the most structured of the three. If this picture is accepted, then a physicochemical rationalization of the transfer could be the following: that the protein is brought first to the interphase, then into the water pool so that the uptake corresponds to an increase of entropy — a process of disorganization of the structure of water. This idea, which in its simplest form would ignore enthalpic contributions, is now under close scrutiny in our group (in particular by Dr. Maestro) from a theoretical point of view.

Let us consider now the parallel behavior of water and protein uptake. A first question in this regard is whether the transfer of the protein into the micellar solution is a passive process. In other words, is water the element which is primarily taken up by the already formed micelles in the hydrocarbon solution, and the protein is transferred simply because it is statistically distributed in the water?

That this is not so is suggested by various considerations. Firstly, the concentration of the protein to be found in the micellar solution would be proportional to the volume fraction of the transferred water — in the best case, it would be 3% of the protein initially present in the aqueous solution (at ca. w_o = 30). Instead, we are dealing with a transfer efficiency which is larger by at least one order of magnitude. (Note in this regard, that if one were to measure the protein concentration in terms of water pool volume, one would obtain a value which is larger by yet a couple of orders of magnitude.) Furthermore, as already observed, the process of protein transfer is somewhat specific — which also speaks against the theory of a simple passive transfer.

The influence of the salt concentration is also not clear. Control experiments without biopolymers in the water phase gave the same results as in Figure 17 (see Figure legend) — namely, no stable and transparent hydrocarbon micellar solution in contact with a water phase could be obtained. This is due perhaps to separation of the excess water being transferred at low ionic strengths. The reason for the maximum in the salt molarity of 100 to 200 m*M* is also not apparent at the present stage. Let us speculate on the events which might occur at the water/micellar solution interface when the solubilization protocol is followed. The interface will possess a monolayer of surfactant, which is depicted in Figure 20. Surfactant micelles located in the bulk hydrocarbon could collide with the interface, opening a channel through which transfer of water and other solubilizates occurs (Figure 20a). Subsequent detachment of the modified micelle completes the process. Alternatively, the original micelle may lose its identity completely (Figure 20b), with the new micelle being ejected from the planar interfacial layer. Entropic considerations should favor process A. For the case of the previous solubilization protocol, it is useful to consider the work of Shaeiwitz and co-workers[157] on the mechanism of solid solubilizate incorporation into normal micelles. Monomeric surfactant adsorbs on the surface of the solid (in the case of a reverse micellar solution, an intervening layer of water is likely to form). Solubilizate and surfactant molecules organize to form a sort of hemimicelle while still on the surface, and this aggregate then desorbs from the surface, forming a solubilizate — swollen full micelle which diffuses away into the solution.

It is clear that a limiting factor for progress in this area is due to our lack of understanding of the basic thermodynamics of the hydrocarbon micellar solutions, with or without solubilized biopolymer. It should perhaps be added, as a kind of positive counterpart, that the practical application of the phase transfer of proteins from water into reverse micelles does not have to wait for the complete elucidation of all the thermodynamic questions. In fact,

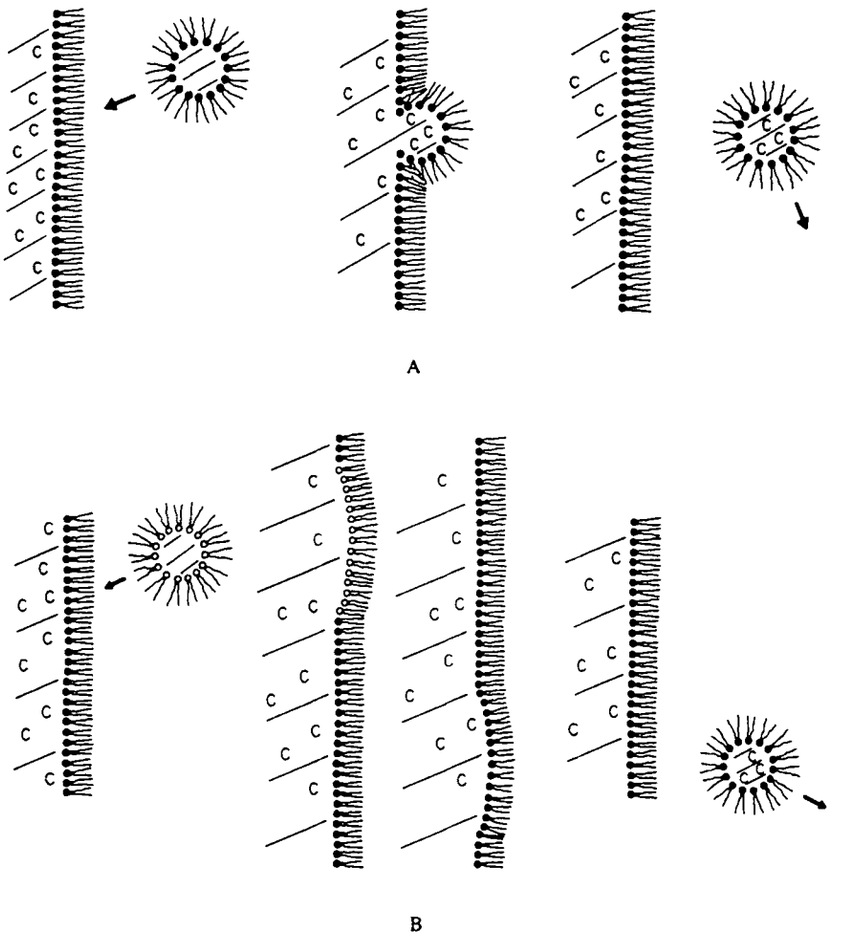


FIGURE 20. Models for solubilizate transfer through a water/micellar solution interface. (A) With maintenance of micellar integrity. (B) With loss of micellar identity.

the exploitation of the extraction principles illustrated above to protein purification and isolation has already begun, as we will discuss later. For this, one needs eventually to reextract the protein from the hydrocarbon solution into a water solution again. We have called this process, which is quite possible and with high yields, "backwards transfer"[150,151] — which will not be discussed here. It should be mentioned however that the two phase transfer processes, forward and back transfer, can be joined together in what has been termed "double transfer", an experiment illustrated in Figure 21.[150,151] In this case, a macromolecule dissolved in the first water phase A can be vectorially transferred into the second water phase B, the process being actualized by the micelles contained in the hydrocarbon bridge. The driving force in this case is the salt concentration gradient between the two aqueous phases. There is transfer of water accompanying the transfer of the protein — a case of osmosis finally — but again the process is not a passive one. This phenomenon of double transfer has not as yet been studied with attention. The interest, aside from practical application in the field of protein chromatography, lies in the fact that the experiment illustrated in Figure 21 simulates the transfer of a biological macromolecule between two water phases through a lipidic barrier — a situation for which there are, to the best of our knowledge, no better synthetic models.

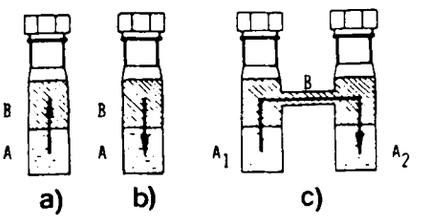


FIGURE 21. Phase transfer processes. In the "forward-transfer": (a) The protein is transferred from the water phase A into B; in the "backward transfer". (b) The protein is recovered from the hydrocarbon micellar solution into an aqueous solution; in the "double transfer" method. (c) The protein is vectorially transported from a first water phase A_1 to a second water phase A_2 via reverse micelles present in the hydrocarbon bridge. The vials are gently shaken during the experiment. The shaking is interrupted periodically for the removal of small aliquots to monitor the concentration of the transported biopolymer.

Finally, let us briefly consider the third solubilization method, the so-called injection method. Operationally, this is the simplest and fastest method. Following the injection of a few microliters of stock aqueous solution of the biopolymer into the hydrocarbon micellar solution, the solution remains cloudy for a short time (1 min up to a few minutes), and then it clears up with gentle hand-shaking. No sonification is generally used for the preparation of biopolymer-containing micelles. Of course, only in a limited range of conditions are the biopolymer-containing micellar solutions thermodynamically stable. A simple representation of these stability regions is illustrated in Figure 22: the space beneath the curves represents the region where clear micellar solutions are formed, whereas the region outside the bell-shaped curve gives rise to cloudy solutions, which we discard for further studies.

The injection method is the simplest, and the one which is most commonly applied for the preparation of biopolymer-containing micelles. It has, however, some drawbacks. Lacking information on phase diagrams which include the concentration of the guest molecule as an independent variable, one does not know *a priori* whether the injected amount of material will give rise to a thermodynamically stable micellar solution. Indeed, one often observes that the optical density of the biopolymer solutions prepared in this way decreases with time, due to material separating out of the hydrocarbon solution. We often noticed this phenomenon in the case of high molecular weight DNA, although the loss of the absorbance at 260 nm can generally be contained within 5% in several hours, till a plateau is reached.[151,158,159] We have mentioned at the beginning of this section that for high molecular weight DNA only the injection method works. Extraction of plasmid PBR322 from the powder state does not work. The injection method works even starting from solutions at relatively high pHs (9 to 10) (see References 158 and 159), where the macromolecule is negatively charged. This is surprising, in view of the fact that AOT is a negatively charged surfactant. This may suggest even that the micellar solutions of DNA prepared with the injection method are not thermodynamically stable, i.e., do not correspond to a stable equilibrium situation, even if the optical density remains constant for days once the plateau value is reached. Conversely, it is possible that the extraction from the solid state of the biopolymer does not take place because of kinetic effects. We need to know something more about the thermodynamics and the dynamics of the solubilization processes in order to clarify this point.

Let us give now a numerical example of typical concentrations obtained with the injection method. The limiting factor is the solubility of the biopolymer in the stock aqueous solution which is going to be injected into the hydrocarbon solution. For example, in the case of

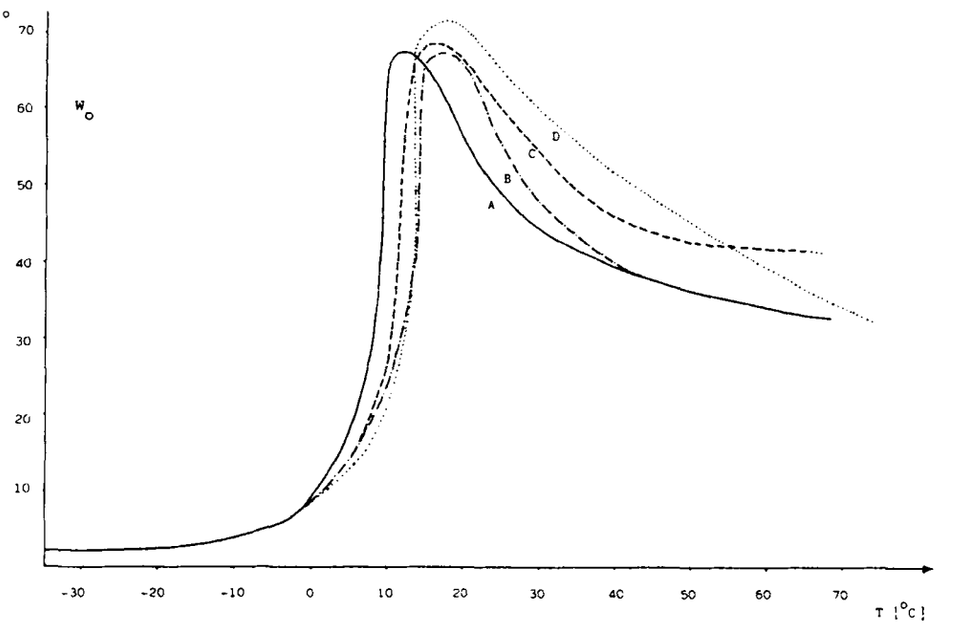


FIGURE 22. Stability curves for 50 m*M* AOT − 50 m*M* borate buffer (pH 8.5)-isooctane system (A) containing enzymes; lipoxygenase (B), C_{ov} = 0.16 μ*M*; ribonuclease (C), 175 μ*M*; and (D) 17.5 μ*M*.

lysozyme we can typically prepare stock solutions of about 20 mg/mℓ, corresponding to 0.0014 *M*. The phenomenological concentration in the micellar solution, or overall concentration, is smaller by the dilution factor, e.g., for 50 m*M* AOT at w_o = 10 it will be 0.0127 m*M*. Lysozyme, in the reverse micellar solution, will be confined in the water microphase, and the water pool concentration will obviously be equal to the initial aqueous stock solution, 0.0014 *M*. In this case, only 4% of the reverse micelles will be filled with protein.

As a final consideration in this section, let us point out something about the overall physical picture of a reverse micellar solution which is usually not generally realized. This concerns the average distance between the micelles, first discussed in Section I.N. In a 100 m*M* AOT solution at w_o = 20, for example, the micelles occupy only a small volume fraction of the total (ca. 8% assuming the accepted dimensions for reverse micelles[29] with hydrocarbon chains included, namely, a radius of ca. 47 Å). However, the average surface-to-surface intermicellar distance is close to the diameter of a micelle, as discussed in Section I.N. The picture one should keep in mind, and which can be very useful for further considerations (e.g., for enzyme kinetics), is one in which the micelles in solution are close to each other.

B. The Structure of Protein-Containing Reverse Micelles: General Considerations

What is the structure of these biopolymer-containing micelles? Where on average in the water core does the biopolymer reside? How does this depend upon its hydrophilicity and net charge? Are these micelles spheres? Do the biopolymers cluster in a few large micelles or are they distributed throughout the available water cores? Does a redistribution of water occur in the system: expressed as w_o, are enzyme-containing micelles water-rich, empty micelles water-poor?

The evidence on the solubilized conformations of the biopolymers and activity, at least for hydrophilic biopolymers, favors the one-enzyme-per-micelle proposal and the so-called water-shell model.[56,160,161] That is to say, the biopolymer resides in the water pool, surrounded

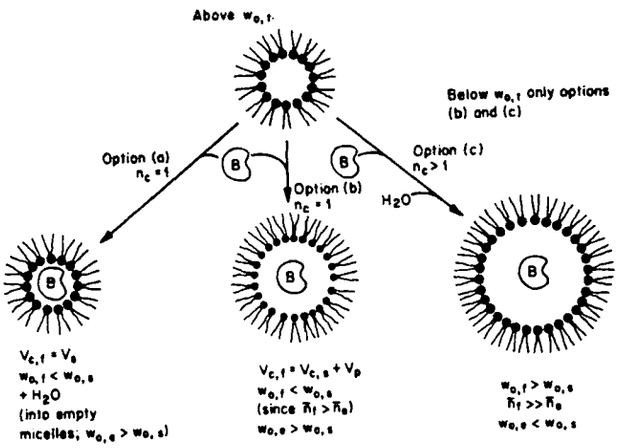


FIGURE 23. Possible consequences of biopolymer incorporation into reverse micelles. The number of empty micellar cores needed to make a single filled core is denoted by n_c. See the text for explanation of other symbols.

by a shell of water which protects it from the surfactant wall and from the hostile hydrocarbon solvent. Let us assume for the moment that biopolymer solubilization does not affect the micellar shape. Figure 23 itemizes the possible consequences of biopolymer incorporation into reverse micelles. We consider separately the low w_o and high w_o regions, where the dividing line depends upon the size of the biopolymer; it occurs when the core volume (V_c) of the starting micelles just exceeds the volume of the biopolymer (V_p). We shall call this transitional w_o, $w_{o,t}$. For small biopolymers (< 40,000 mol wt), this transitional w_o is below the region where the area per AOT head group reaches its limiting value of 55 Å^2. Note that if a biopolymer sticks to the surfactant interface at the periphery of the water core then this value of 55 Å^2 of water surface per AOT head group no longer holds.

The various structural possibilities are fully characterized if we can specify for each filled micelle: R_c, $\bar{n}_{AOT}$, w_o, and the number of empty micelles needed to produce one filled micelle. The best unit here is perhaps the empty micellar core. Above $w_{o,t}$, the biopolymer may enter the water pool and

1. Displace water such that $R_{c,s} = R_{c,f}$, allowing $\bar{n}_{AOT}$ to remain the same, while $w_{o,f} < w_{o,s}$. This requires that $w_{o,e} > w_{o,s}$.
2. Swell the core by its own volume so that

$$R_{c,f} = \left(\frac{V_{c,s} + V_p}{(4/3)\pi}\right)^{1/3}$$

   $\bar{n}_{AOT}$ will increase, because $R_{c,f} > R_{c,s}$; $w_{o,f}$ will again be less than $w_{o,s}$ and $w_{o,e} > w_{o,s}$.
3. Attract additional water, so that $w_{o,f} \geq w_{o,s}$ and $w_{o,e} < w_{o,s}$. This possibility will require both the water and AOT from more than one starting, empty micelle. If two empty micellar cores were to coalesce to form one filled micelle, we would have the permanent version of the transient dimer discussed earlier in the section on the kinetics of solubilizate exchange.

Below $w_{o,t}$, the biopolymer cannot fit into the existing micellar core by simple displacement of water, so it must organize a new micelle around itself. Options (2) and (3) are available, with (3) more likely than (2) if a large protein is to ensure that it retains a water shell.

C. Experimental Studies for the Characterization of Protein-Containing Reverse Micelles

It would be desirable to have a thermodynamic theory which predicts the dimensions and structure of reverse micelles as a function of the chemical structure of the surfactant and of the overall micellar parameters, such as water content and concentration of guest molecules as discussed above. This is still well out of reach. For the time being we have to rely entirely on experimental methods in order to gather information about size/structure of biopolymer-containing micelles in hydrocarbon solvents. This is also not easy. A main difficulty is the heterogeneity of the micelle population, since it consists generally of filled and unfilled micelles. In fact, it is operationally difficult to solubilize biopolymers in hydrocarbon micellar solution up to a (theoretical) 1:1 stochiometric relation with the number of starting, empty micelles, and even when this happens, the actual distribution of guest molecules is not known — it must be determined experimentally. When the concentration of unfilled micelles is much larger than the concentration of guest macromolecules, then the assumption of "one single biopolymer molecule per single micelle" is probably correct, but then the experimental determination of the parameters of the filled micelle must be sought out of a large background of unfilled ones, which sets very high demands upon the sensitivity of the experimental methods.

The first approach to the characterization of protein-containing micelles in the system isooctane/AOT/water was undertaken with analytical ultracentrifugation.[160] Indeed, this appears the most proper way to operate since, by monitoring the protein at 280 nm, the unfilled micelles are "invisible" and even a small signal originating from the filled ones can be used to determine sedimentation and diffusion coefficients and molecular weights.

These first experiments gave molecular weights for the filled micelles which were, not surprisingly, higher than those of the unfilled ones. RNase, lysozyme, and liver alcohol dehydrogenase were the enzymes used. Both sedimentation and diffusion coefficients were determined, so that molecular weights could be calculated according to the Svedberg equation:

$$M = \frac{RT}{1 - \bar{v}\rho} \cdot \frac{S}{D}$$

To decide upon the components making up the micelle, some assumptions were required. It was assumed that there was one enzyme molecule per micelle, and that $w_{o,f} = w_{e,s}$ (this is a particular subset of Option (3)). Let i equal the number of water molecules per empty starting micelle and j the same for the enzyme-filled micelle. Then the w_o assumption gives $i/\bar{n}_e = j/\bar{n}_f$ and the molecular weight is expressed as

$$M = M_P + jM_w + \bar{n}_f M_{AOT} = M_P + jM_w + (j/w_o)M_{AOT}$$

This equation can be solved for j and the relationship $V_c = jV_w + V_P$ used to calculate the radius of the micellar core.

Figure 24[160] depicts the w_o-dependent enlargement of the water cores which this treatment predicts. Figure 25 shows that RNase is protected by only a thin sheath of water when the overall w_o is small. At this small w_o, two water cores have merged to make the RNase-containing micellar core. Note the crucial role of the $w_{o,f} = w_{e,s}$ assumption: it controls the core size which is derived.

Levashov, Martinek, and co-workers[163-165] have also done velocity ultracentrifugations, in order to determine sedimentation coefficients, for enzyme-filled AOT reverse micelles.

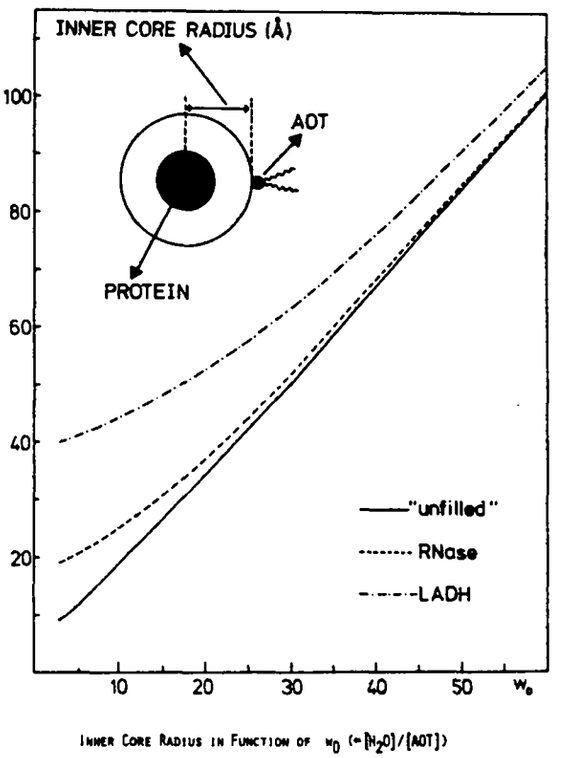


FIGURE 24. Dimensions of AOT reverse micelles as a function of w_o. The full line corresponds to the data of Eicke and co-workers. (From Bonner, F. J., Wolf, R., and Luisi, P. L., *J. Solid Phase Biochem.*, 5, 255, 1980. With permission.)

α-Chymotrypsin (α-CT) is by far the most extensively studied enzyme in their investigations. They "visualize" the micelles by staining the water cores with 2,4-dinitrophenol (which may in fact be interfacially solubilized), so that they observe both the filled and empty micelles sedimenting: this is a double-boundary experiment, and the filled micelles sediment more rapidly. Levashov, Martinek et al. observed that the empty micelles in the α-CT containing solution have the same sedimentation coefficient as the empty starting micelles, which should mean that the $w_{o,f}$ assumption of Luisi and co-workers is correct.

There are problems with the interpretation of the sedimentation coefficients observed for the α-CT-containing micelles at various starting w_o values. Since Martinek and co-workers did not measure any diffusion coefficients, they are unable to calculate molecular weights. They therefore proceed as follows, writing the expression:

$$s_f = s_e \cdot \frac{R_e}{R_f} \cdot \frac{M_f}{M_e} \cdot \frac{1 - \bar{v}_f\rho}{1 - \bar{v}_e\rho}$$

where the ratio R_e/R_f replaces the diffusion coefficient ratio D_f/D_e (recall that diffusion coefficients and radii are related through the Stokes-Einstein relationship).

Above $w_{o,f}$ (which is 11.6 for α-CT), they assume that α-CT inserts itself into an empty micelle with no change in AOT or water content. Thus $M_f = M_e + M_p$. As we have discussed earlier, this is physically impossible. Once this assumption is made, R_e/R_f is fixed, since s_f, s_e, $\bar{v}_f$, and $\bar{v}_e$ are all determined experimentally. These radii are equal to R_{core} +

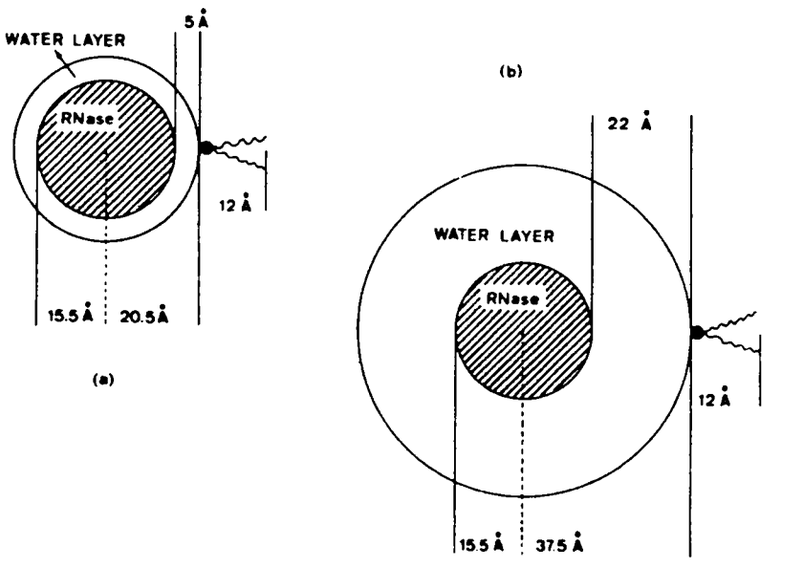


FIGURE 25. Model (cross-section) and dimensions for an AOT micelle containing one RNase molecule. (a) $w_o = 5$; (b) $w_o = 20$. (From Bonner, F. J., Wolf, R., and Luisi, P. L., *J. Solid Phase Biochem.*, 5, 255, 1980. With permission.)

AOT length. However, in addition, $V_{e,f} = V_{H_2O} + xV_P$, and x (which one might expect to be one), must be only about 0.1 for $V_{c,f}$ and R_f to be self-consistent. In other words, it appears that α-CT "disappears" when it is solubilized. R_f will always demand this sort of physically unreasonable result if the assumption about M_f sets it at too low a value.

Below $w_{o,t}$, the solubilized α-CT must create a new micelle about itself, so that the assumption of constant AOT and water content must be abandoned. How much enlargement does one choose? In order to predict the dependence of s_f upon s_o, option (3) is employed, and it is assumed that $\bar{n}_f$ (whatever the w_o) $= n_{e,s}^-$ ($w_o = 11.6$). This fixes the surface area of the micellar core; and gives $R_f = 21.9$ Å ($V_{c,f} = 44{,}000$ Å^3). Furthermore, it is assumed that $w_{o,f} = w_{e,s}$. Once again, the inference is that the α-CT in part "disappears" when it is solubilized: 61,000 Å^3 $= V_{H_2O} + V_{\alpha\text{-CT}}$ using this collection of assumptions.

The notion that α-CT has an extremely high apparent density in AOT reverse micelles cannot be correct. We shall see in our discussion of light scattering data that α-CT does not increase the hydrodynamic radius of the micelles and suggest what inferences can be drawn from the observed sedimentation coefficients. It should be clear from our discussion of Martinek's work, why it is so important to determine apparent molecular weights directly.

Recently, Zampieri and co-workers[166] have made a novel modification of Martinek's double boundary technique. In separate runs at constant (AOT), w_o and enzyme concentration they use in turn $Cr_2O_7^{2-}$ to monitor the water cores and 2-naphthoate, a cosurfactant dye, to monitor the AOT interfacial layer. The optical densities (Figure 26) associated with the two populations of sedimenting micelles are then directly proportional to the four stoichiometric concentrations: AOT and water in filled and empty micelles, respectively. Thus, $w_{o,f}$ and $w_{o,e}$ may be determined.

Further numerical elaboration of these data is possible. Under the assumption of one protein molecule per micelle — which is reasonable when the concentration of protein is much smaller than the concentration of initial micellar aggregates — the concentration of filled micelles can be set equal to the protein concentration. From this, one can estimate the aggregation number of AOT, as well as the number of water molecules in the filled

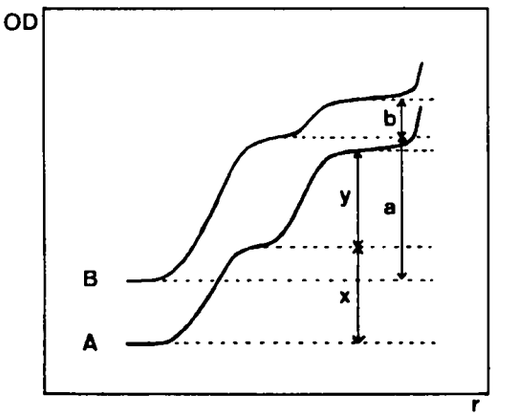


FIGURE 26. Scanner traces of sedimentation profiles (optical density vs. radial position) of reverse micelles with w_o = 5.5 at 48,000 rpm and 25°C approximately 40 min after the start of sedimentation. (a) Cell 1, micelles containing MBP and bichromate, monitoring the H_2O distribution at 336 nm, where x and y are the absorption of unfilled and filled micelles, respectively. (b) Cell 2, micelles containing MBP and naphthoic acid, monitoring the AOT distribution at 313 nm, where a and b are the absorption of unfilled and filled micelles, respectively.

Table 5
CHANGE OF THE STRUCTURE OF AOT REVERSE MICELLES UPON UPTAKE OF PROTEINS[a]

	Myelin basic protein[b]	Lysozyme[b]	α-Chymotrypsin[b]
w_o	5.5 → 11.3	7.9 → 12.1	13.7 → 25.8
n_{H_2O}[c]	960 → 1700	1350 → 1540	4130 → 2900
N_{AOT}[d]	175 → 152	172 → 128	302 → 94
r_{wp}[e]	175 → 25.9	. . . → 24.8	. . . → 28.9
$\overline{M}_w$	. . . → 116,700	. . . → 99,300	. . . → 110,200

[a] As determined by double probe ultracentrifugation. The molar concentration of the proteins in these experiments is in the range of 40—60 μ*M*, the initial molar concentration of reverse micelle is 290 μ*M* for lysozyme and myelin basic protein, and 166 μ*M* in the case of α-chymotrypsin.

[b] The first figure refers to the situation before, the second after uptake of protein. The initial values depend obviously from the starting conditions (w_o, protein concentration, etc.)

[c] Number of molecules of water in the water core.

[d] Aggregation number of AOT.

[e] Inner core radius.

micelles. Also, the size of the filled micelles can be roughly evaluated; under the assumption of spherical micelles, taking the size of the enzyme, as known from the literature and the size of water molecule (30 Å^3), one can calculate the volume of the water core of the micelle. The area per AOT head group is given by $4\pi R_{c,f}^2/\overline{n}_{AOT}$.

Whereas detailed data are given elsewhere,[166] we would like to examine here some basic results, as shown in Table 5. In the three cases investigated until now, when the micelles

take up protein there is an increase of w_o with respect to the value of unfilled micelles. The enlargement depends both on the size of the protein, and on the starting w_o value; in other words there is no such thing as a single w_o value for protein-containing micelles (even for a given protein). Notice that in the case of lysozyme and myelin basic protein (MBP), which have about the same size, the number of water molecules inside the water core is ca. 1500 to 1700: this corresponds, roughly speaking, to 3 layers of water, and one can picture that one of these is needed to solvate the AOT head groups (indeed, this would correspond to ca. 7 water molecules per sulfonate group), with the other 2 layers solvating the protein. Notice that for all the 3 cases investigated, the inner core radius is within the range 25 to 29 Å, and also the molecular weights of the filled micelles are rather close to each other. Notice, finally, that the aggregation number of AOT tends to decrease upon uptake of a protein. However, the protein "needs" more water, which brings about an increase of the w_o value, as mentioned above.

It is clear that this technique is very powerful, and also very simple. Its limits should also, however, be mentioned: the method does not work (the sensitivity is not enough) when the occupancy degree is low; or when the difference in size (actually, in the sedimentation coefficients) of the filled and unfilled micelles is too small. Also, some assumptions are needed in order to evaluate the inner core radius (e.g., about the volume of both water and protein). It should be added that from such techniques, one can also determine the w_o.

It is important to add that the w_o value of the empty micelles which coexist in solution with the filled ones is smaller than the initial w_o, so that mass balance is retained. The amount of "shrinking" is proportional to the degree of occupancy of the protein.

Chatenay and co-workers[167] have made dynamic LS measurements on the MBP system at 50 m*M* AOT and $w_{o,s} = 5.6$. They use several BMP concentrations and find that the apparent hydrodynamic radius (evaluated from the intensity autocorrelation function, C[t]) increases from 29 Å (no MBP) to 43 Å when the molar ratio of MBP to starting empty micelles (designated f) is 0.3. Recall that 12 Å should be subtracted from these radii to obtain the apparent water core radii.

The function C(t) can be fit from $f = 0$ to $f = 0.3$ by assuming that a mixture of empty micelles ($R_H = 29$ Å) and filled micelles (one MBP each) are present, with the filled micelles having been constructed on average from 3.3 empty ones. Note that it is more proper to say 3.3 water cores coalesce, since we know that some AOT will be expelled into the solution because of surface-to-volume considerations. At a w_o of 22, the limiting f is 0.7; on average each filled micelle is constructed from 1.4 empty ones. Above these limiting fs, there must be multiple occupancy of the filled micelles.

The interpretation of the LS data disagree with the ultracentrifugation data about the sizes of the empty micelles in the presence of enzyme, but it is possible that a smaller R_e than 29 Å would work as well in the curve fitting. Remember that when $w_o = 0$, the hydrodynamic radius of an AOT micelle is ca. 15 Å. The LS and ultracentrifugation data do agree that the MBP-filled micelles at $w_o = 5.6$ have water core radii of 25 to 30 Å.

The MBP data discussed up to now are consistent with single occupancy of the filled micelles before multiple occupancy occurs. However, the question of protein clustering in a few filled micelles is still an open one. Fletcher and Robinson[168] have made preliminary SANS measurements (thus sizing directly the water cores) on a cyclic pentapeptide solubilized in AOT micelles; they see a dimension which might be ascribed to empty micelles, even though they are working above 500% theoretical occupancy. Deviation of the filled micelles from sphericity is also still an open question. In a SAXS study on rhodopsin (a hydrophobic protein) solubilized in phospholipid reverse micelles in hexane, Ramakrishnan and co-workers[169] identified two characteristic dimensions. They believe it likely that a protein molecule bridges two reverse micelles, producing prolate ellipsoidal complexes in solution whose semimajor and semiminor axes are detected.

SANS, because of the possibility of sizing the water cores directly, is a particularly attractive technique for obtaining structural information in these systems. Magid and co-workers[170] have made measurements on MBP-containing AOT micelles at w_o = 7, 12, and 20 (0.10 *M* AOT, D_2O water pools), at 50% theoretical occupancy. As we have seen from the dynamic LS data, this means there is certainly multiple occupancy at w_o = 7. Two characteristic dimensions may be obtained (the data analysis assumed that two populations of spherical particles were present): 87 and 27 Å (the empty starting micelles had 21 Å water cores). The 27 Å dimension could arise from the singly occupied micelles (it is consistent with the LS and ultracentrifugation data) if they are spheres, with the large dimension being some convolution of multiply-occupied micelles (the scattering is proportional to R^6, so that a few large particles would scatter strongly). The other possibility is of course that the filled micelles are not spherical.

SANS data[170] for solubilized lysozyme at low theoretical occupancy (10 to 20%) also gives a large second dimension. If the filled micelles remain spherical, this means that lysozyme clusters: that is, a significant fraction of the filled micelles are multiply occupied in the presence of many empty ones.

Let us return to the structural data for α-CT in AOT reverse micelles. Fletcher and Robinson[171] have made dynamic LS measurements from w_o = 5.6 to 55.6 and they find no increase in R_H, even when the theoretical percent occupancy is above 100% (possible when w_o exceeds 25). SANS[170] and SAXS[134] measurements agree that with α-CT, enlargement of the water core is minimal: in other words, option (1.) appears to operate with α-CT. There are other data which support α-CTs following the water-shell model: quasielastic neutron scattering[172] indicates that α-CT does not retard the diffusion of AOT molecules along the AOT/water interface.

Fletcher and Robinson[173] have repeated Martinek's ultracentrifugation experiments for α-CT, finding sedimentation coefficients which agree well. He points out, however, that the micelles sediment too quickly to be the spheres seen by light scattering. He believes in fact that the α-CT containing water pools are shaped like butternut squashes, with the α-CT in the small end of the pool. Such a shape would sediment more quickly than the equivalent sphere. A recent attempt to determine the dimensions of reverse micelles with cytochrome-c has been proposed by Pileni and co-workers.[134] They found that the small protein (with a molecular weight of 11,000) does not bring about any appreciable increase of size in the w_o range. The techniques used in this case were SAXS and hydrated electron generation/capture.

One may wonder why electron microscopy is not widely employed for tackling the problems of dimensions and structure of reverse micelles. The problem is purely technical, whereas in the case of aqueous solutions the various experimental difficulties connected with the fast elimination of water from the sample have been painstakingly overcome, the case of hydrocarbon solutions has never been systematically taken into examination until now, and a long series of control experiments must still be carried out before reliable data are obtained from this technique. Preliminary experiments carried out in our group for plasmid-containing AOT reverse micelles give a diameter of ca. 300 Å, which is in agreement with expectations derived on the basis of geometrical considerations. However, these data must wait confirmation.

D. The Structure of Biopolymers in Reverse Micelles

As we have seen in the preceding section, the knowledge of the overall size of protein-containing reverse micelles is only now beginning to accumulate. Knowledge of the internal structure of reverse micelles which host biopolymers is even more scarce. The questions here are where are the biopolymers located in the reverse micelle? Is their conformation altered and how? What about the location/structure of the other components of the interior

of the reverse micelles, for example, water and the counterions of the surfactant? All these questions are of course interdependent.

As already mentioned, the first model which was proposed and somewhat supported by experimental evidence is the so called "water-shell model", according to which the hydrophilic protein is hosted in the water pool and is protected by water layers from the surfactant molecules and from the organic solvent.[160] The evidence for this model is only indirect, and based on the following experimental observations: (1) circular dichroism and fluorescence spectra, as well as enzymatic activity data, show a clear dependence on w_o. In other words, the protein is sensitive to the concentration of water in the water pool, so the most likely picture is one in which the protein is in direct contact with water; (2) enzymes are still active, with a pH profile and kinetic patterns which closely resemble and, in some cases, are superimposable with those obtained in aqueous solutions; (3) under conditions of nonwater starvation ($w_o \geq$ ca. 10) both CD and fluorescence spectra closely resemble those in aqueous solutions, which indicates that both the overall protein conformation and its environment are largely aqueous; and (4) finally, the ultracentrifugation data discussed in the previous section indicate there is a minimal w_o requirement for a protein hosted in the reverse micelle.

Although none of the above evidence is compelling, the weight of all the indirect experimental evidence is really considerable in favor of the water-shell model. Thus, any alternative proposal has to be made consistent with the above body of information.

In principle, other modes of hosting a biopolymer inside reverse micelles can be envisaged, particularly if the protein is not very hydrophilic. A few possible situations are represented in Figure 27. It is quite conceivable, for example, that a highly hydrophobic protein may tend to interact with the hydrocarbon chains of the surfactant, or even with the organic solvent. A model in which the protein adheres to the charged internal wall of the micelle is very attractive in view of the analogy with enzymes bound to insoluble matrices. As a possible candidate for a model different from the water-shell, one could cite lysozyme, which is denatured in AOT reverse micelles in the absence of substrate.[174] However, the situation is complex, as the CD spectra of lysozyme bound to NAG-inhibitors are very similar in water and in reverse micelles, again indicating essentially the same, or a very similar, conformation. It has been suggested that the interaction of the surfactant with the active site (rather hydrophobic in nature) might be the cause of the denaturation (rather than an interaction of the enzyme surface with the charged micelle wall).

Of particular interest is the case of rhodopsin studied by Ramakrishnan and co-workers[169] who found evidence of a small amount of a very high molecular weight component as well. They interpreted the data on the basis of a small population of cross-linked structures of the type indicated in Figure 27e. It is interesting to remark that also in the study of Chatenay et al.[167] with MBP, a small high molecular weight component was observed. What remains to be explained is whether this feature is caused by the presence of the protein or whether a small part of the cross-linked structure (one could say percolating structure) is also present in the unfilled micelles per se.

The acceptance of the water-shell model for hydrophilic biopolymers should not be taken to signify that the behavior of proteins or nucleic acids hosted in reverse micelles is completely the same as in aqueous solutions. There are in fact some differences which are not in contradiction with the water-shell model, but which require, in order to be explained, some changes in the nature of the water inside the reverse micelles.

The "anomalies" of the water constituting the water pool of the reverse micelles have been discussed in Section I.I for the case of unfilled micelles. When a biopolymer is hosted in the water pool, the situation may become even more complicated. At low levels of water, for example, the protein and the polar micellar wall may compete for water with the result that at any given w_o, the content of "free" water inside the micelle may be considerably lower in the filled micelles than in the unfilled ones. Furthermore, the protein may have an

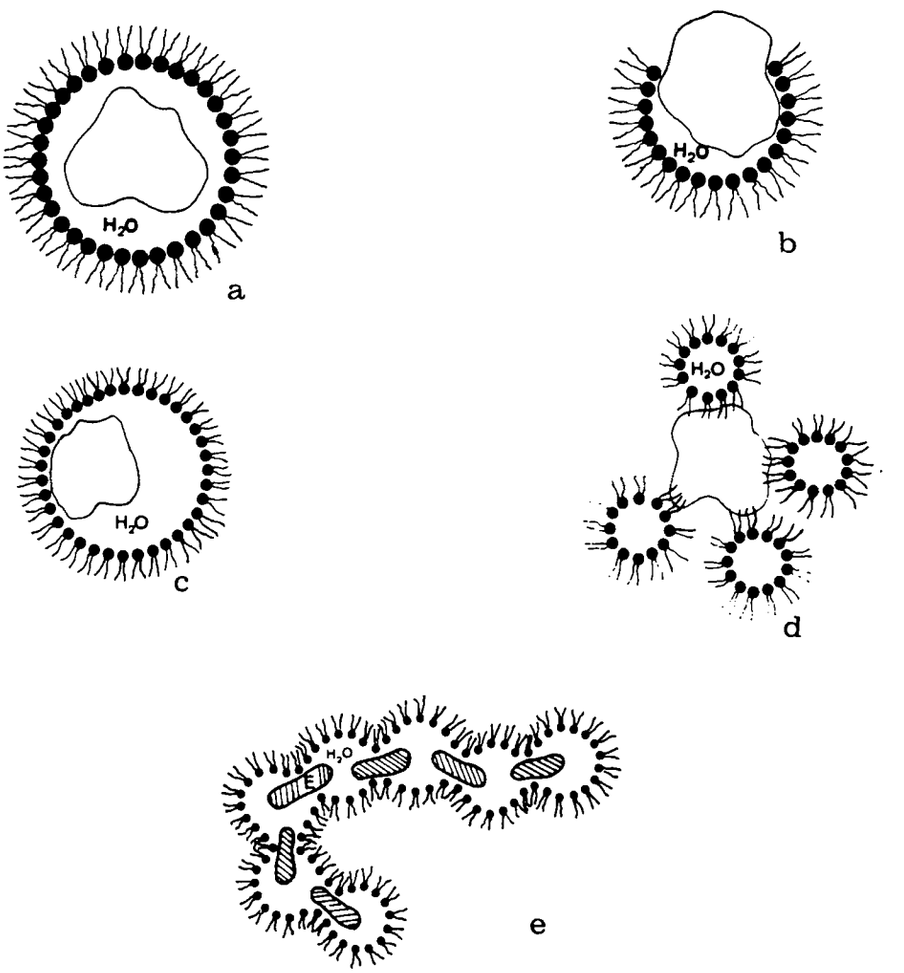


FIGURE 27. Possible models for a protein hosted in a reverse micelle. As explained in the text, Figure 27a is the water-shell model whereby the protein is located in the water pool and is protected from the micelle wall by layers of water. Figure 27b represents the case of a protein having a very lipophilic part, which tends to interact directly with the hydrocarbon bulk. Figure 27c represents the protein adsorbed to the micelle wall; Figure 27d is the case of a protein solubilized by the help of several small micelles which interact with the hydrocarbon tails with the hydrophobic portions of the protein. Figure 27e schematizes the formation of a network among several micelles, bridged by the protein molecules.

influence on the structure of water. In turn, the structure of water may influence certain dynamic processes and certain physical parameters, like the pKs or the pI of the guest protein.

Whereas the understanding of all these structural details of water may require some time, it is possible already now to accept the notions that (1) the water inside the water pool does not have the same physical properties as bulk water, and that (2) this difference may be one of the most important factors for the physical perturbations experienced by the biopolymers hosted in the reverse micelle. It is a point of delicate balance, because on the one hand we state that the protein is embedded in essentially an aqueous environment (water-shell model), while on the other we are stating that the guest biopolymers are experiencing a novel environment. With these observations in mind, let us consider some spectroscopic data which may relate directly to the structure/conformation of the guest biopolymer.

Let us consider for this Table 6, which collects UV-absorption and fluorescence data for proteins in reverse micelles with various surfactant systems. In UV-absorption spectra, there is a general tendency towards a red shift in reverse micelles with respect to water solution. This can be explained with the somewhat less polar character of the water pool environment. One may recall here that the dielectric constant inside AOT reverse micelles, for example, has been evaluated to be similar to that of methanol.[97] Changes in the extinction coefficients in micelles with respect to water are absent or very modest. As expected, perturbations are larger, the smaller the w_o value, and this is true also for all other kinds of spectroscopic analyses. In this regard, one should bear in mind the data of Table 5 according to which the protein actual w_o value is somewhat larger than the nominal w_o value. In other words, the spectra reported at a $w_o = 5$ correspond to a local environment of the protein with a w_o value of ca. 13.

Fluorescence spectra are also of some interest. Note first of all that as already mentioned at w_o values larger than ca. 10 the emission properties in water and in reverse micelles are very close to each other. Only at very small w_o values there is, at least in the AOT system, a sizable enhancement of the quantum yield and blue-shift of the emission maxima. This is also in general agreement with a more hydrophobic environment of the protein.

It is worthwhile perhaps to mention that still much information can be gathered from electronic spectroscopy about structure. For example, a systematic investigation of fluorescence polarization could be very useful to determine the relative mobility of the guest protein. In fact, if this kind of study would be accompanied by a parallel investigation of the fluorescence polarization of the micelle (containing, for example, a fluorescence cosurfactant probe), one could explore whether the mobility of the protein and of the micelle are the same or not. The mobility is also related to the dynamics and exchange processes of protein-containing reverse micelles. Here, too, systematic investigations are missing (the dynamic processes with macromolecules may well differ from those of small components). Fluorescence techniques can be very valuable experimental tools here also.

Let us consider now the CD data, again not dwelling on the properties of single systems, but indicating only general considerations. Table 6 shows that the general trend is similar to that observed with UV-absorption and fluorescence, inasmuch as perturbations with respect to water solution are larger, the smaller the w_o, and tend to disappear at larger water contents. In addition, there is an increase of ellipticity in the far UV-region of the spectrum, namely, in correspondence with the electronic transitions of the peptide chromophore. This can be taken to suggest an increase of secondary (in general helical) structure. This is in keeping with the notion of a more hydrophobic environment: hydrogen bonding tends obviously to increase in a solvent less polar than water.

What does this notion of a more hydrophobic environment really mean? At w_o around ten, for example, all water molecules inside the protein-containing micelle are in all likelihood bound (no free water) partly by the AOT sulfonate groups, partly by the protein. The spectroscopic perturbations may then simply reflect this absence of free water around the protein. The possibility of an interaction of the protein with the hydrocarbon tails of the surfactant, or with the solvent, might also be taken into account, but we should not forget that enzymes are generally active at w_o values around ten, and that, on the other hand, surfactants or apolar solvents act as powerful denaturing agents of proteins. An example of CD data in reverse micelles vis a vis water is given in Figure 28. This figure also shows the behavior in the near UV-region which reflects the aromatic chromophores of the protein, and therefore is an index of the conformational behavior of tryptophan and tyrosine residues (since the UV-absorption spectra in this region show practically no change with respect to water). In the cases reported in the figure, changes are small even at rather low w_o values, which is typical for most of the other proteins investigated until now.[174-177] Hemoglobin appears to give much larger changes, but it is quite likely that this is due to denaturation although the authors do not specifically say so.[178]

Table 6
SPECTROSCOPIC PROPERTIES OF SOME PROTEINS IN REVERSE MICELLAR SOLUTIONS[a]

Protein	System	UV	Fluorescence	CD	Ref.
α-Chymotrypsin (E.C. 3.4.21.1)	CTAB/CH/*n*-hexanol (10%) 2—5% water			Modified	165,194
	SDS/CH/*n*-hexanol (10%) 2—5% water			As in water	165
	AOT/IO w_o = 5—25	Diff. spectra taken, decr. at incr. w_o	Blue shift of 4 nm Q.Y. 113—90%	At small w_o higher int. at 230 nm	175
	Methyltrioctylamm. chloride/CH	Red shift of 2 nm	Red shift of 12 nm incr. of Q.Y.	Similar to water	150 186
Trypsin (E.C. 3.4.21.4)	Methyltrioctylamm. chloride/CH	Red shift of 4 nm	Blue shift of 3 nm Q.Y. much higher	Smaller int. especially in near UV	150
Pepsin (E.C. 3.4.23.1)	Methyltrioctylamm. chloride/CH	Red shift of 4 nm	Blue shift of 6 nm Q.Y. much higher		150
Lysozyme (E.C. 3.2.1.17)	AOT/IO w_o = 3—25	Identical to water	10 nm blue shift Q.Y. higher	Large changes[b]	153 174
	Tetraethylene-glycoldodecyl-ether/IO, w_o = 1—5	Identical to water	10 nm blue shift Q.Y. higher	Very close to water	174
	CTAB/CHCl, IO 1:1 or CTAB/IO/butanol 1:4	Identical to water	5 nm blue shift	Near UV similar to water	174
			10 nm blue shift	Higher int. at 220 nm, near UV very close to water	174
Folch-Pi proteolipid	AOT/IO w_o = 5.5	Identical to spectra in 2-chloroethanol	Blue shift with resp. to 2-chloroeth.-spectrum	High helix content	154
Horse liver alcohol dehydrogenase (E.C. 1.1.1.1)	AOT/IO w_o = 10—60	Very small changes	Blue shift decrease of Q.Y.	Small changes at 200—215 nm	174

[a] The red or blue shift and quantum yields are compared to a water solution at the same (or similar) conditions. Abbreviations used: CH = cyclohexane, IO = isooctane, O = octane, decr. = decreasing, incr. = increasing, Q.Y. = quantum yield, int. = intensity, CD = circular dichroism. For more data on the spectroscopy, see Reference 182.

[b] In the absence of bound substrate. With bound NAG_3 the changes are modest.

From Luisi, P. L. and Steinmann, B., *Methods in Enzymology*, Mosbach, K., Ed., in press. With permission.

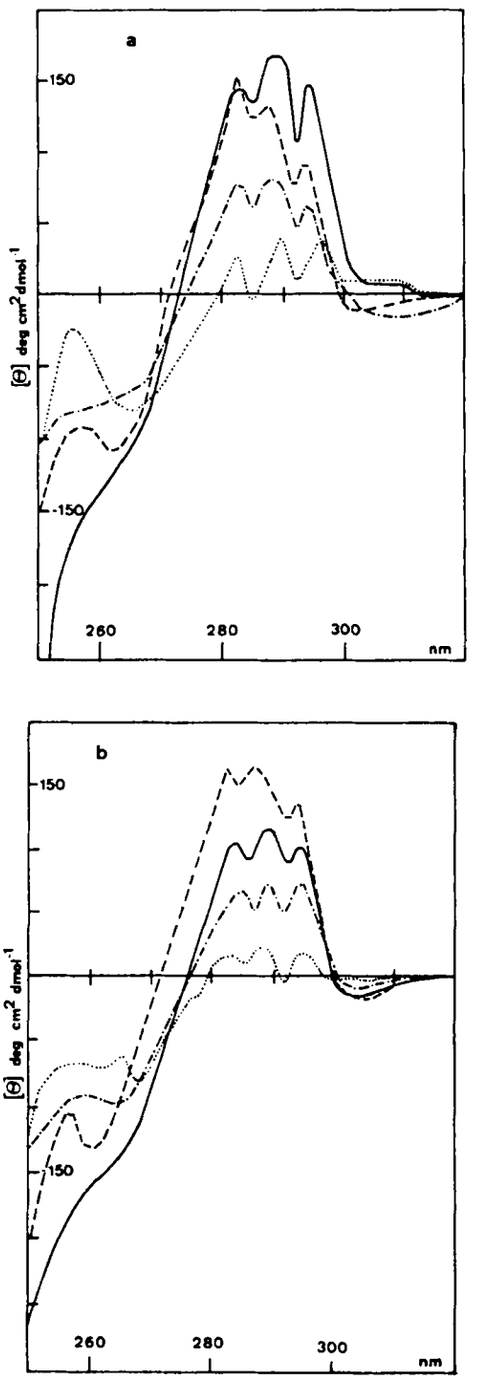


FIGURE 28. Circular dichroic spectra of lysozyme in water and in AOT/isooctane solutions. The ellipticity is expressed per average residue. The enzyme concentration in this experiment was 10 μM. (a) Lysozyme-NAG in water (——) and in micelles at w_o = 3, 6, and 9, in the order of decreasing intensity; (b) Lysozyme-NAG_3 complex in water (——) and in micellar solutions at w_o = 3, 16, 25, in the order of decreasing intensity. (From Steinmann, B., Jackle, H., and Luisi, P. L., *Biopolymers*, 1986, in press. With permission.)

Electronic spectroscopy is not the only way to gather information about the structure of the guest biopolymer. NMR is of course a method which in principle is very powerful. The problem here is on the one hand the sensitivity of the method in the concentration range obtained with proteins in reverse micelles, and on the other hand the problems of frequency assignment in a novel solvent system. Systematic studies with proteins are still lacking, although spectra of both lysozyme[174] and epidermial growth factor[179] in AOT/isooctane reverse micelles have been recorded. More advanced is the state of the art with small peptides in reverse micelles, thanks mostly to a series of papers from the group of Gierasch.[81,82,180]

There is another consideration which is apparent from Table 6. This concerns the comparison among different surfactant systems. Generally, the AOT system is the one in which spectroscopic (and by inference structural) perturbations are the greatest. Conversely, biopolymers in nonionic surfactants show the smallest changes with respect to water. The positively charged CTAB surfactant appears to be in an intermediate position. This probably can be compared to the water perturbations, which are larger in AOT than in the other systems.

Finally, a few observations on nucleic acids. As we have already mentioned, experimental studies on size, form, and structure of reverse micelles which contain DNA or RNA have not yet been performed. The case of high molecular weight double-stranded DNA should be particularly interesting, as one may wonder whether the micelles maintain their spherical form upon uptake of the biopolymer, or whether instead they assume a cigar-shaped form like the rigid guest molecule. The problem in this case is again a technical one: the fractional saturation of micelles is indeed very small in the case of high molecular weight DNA, and this may prevent clarification on the structure for a long while.

As far as the conformation of DNA and RNA inside the reverse micelles is concerned, very modest changes are observed for the low molecular weight components. In the molecular weight range 30,000 to 250,000, a hypsochromic effect is observed in AOT/hydrocarbon reverse micelles with respect to water. This is attended by an increase of the molar ellipticity.[158,159] An example of such behavior is represented in Figure 29. These data can be interpreted in a way which is consistent with that given in the case of proteins: a more hydrophobic environment tends to increase the extent and intensity of H-bonds, with a consequent increase of base pair stacking, which in turn causes a decrease of the absorptivity — a well-known effect in the biophysics of nucleic acids in aqueous solution. This also causes an increase of the conformational rigidity of the biopolymer, with a consequent increase of the CD intensity at 260 nm.

As already mentioned, a considerable surprise in this field was that even a plasmid with a molecular weight of 2.7×10^6 could be solubilized in reverse micelles.[159] Whereas the absorption properties are the same as in water (with no appreciable light scattering), the circular dichroic properties are remarkably changed (see Figure 29b and d). The spectral features correspond to those of a psi-spectrum, which is taken as an indication of a very condensed or superpackaged form of DNA. Details about this system as well as data on the interaction with proteins and polypeptides, are reported elsewhere.[159] Here, restricting ourselves to the question of structure, it should only be added that the features represented in Figure 29 are due to an intermolecular aggregation phenomenon, more than to the collapsing of a single plasmid molecule within one micelle. Even so, this experiment raises more questions about the compartmentalization of biopolymers in reverse micellar solutions, and about the relationship between geometrical constraints of the compartmentalization and the conformation of the guest biopolymer. One corollary question concerns the maximal dimensions of biopolymers which can be hosted in reverse micellar solutions. In this regard, one does not know yet where to set the limit. It has been possible to solubilize bacterial cells readily in a Tween/isopropyl palmitate system[181] and to maintain them viable. In this case, too, questions of structure of both the host and guest systems promise to be very challenging.

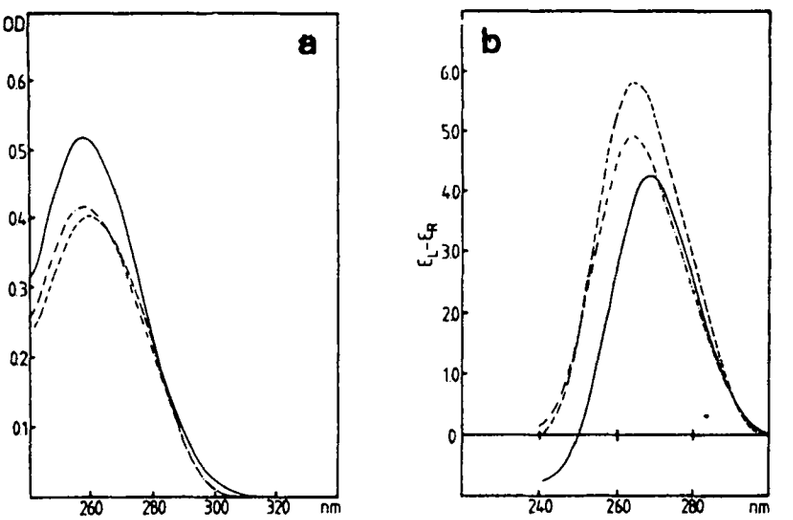


FIGURE 29. Absorption and circular dichroic spectra of nucleic acids in reverse micelles. Absorption (a) and ellipticity (b) properties (1 cm light path) of RNA (molecular weight 20,000 to 30,000 Dalton) in water and in hydrocarbon micellar solutions. (——) RNA in an aqueous solution of borate buffer pH = 9.0; (---) in isooctane/AOT solution at w_o = 14.8 (prepared from an aqueous stock solution of borate buffer pH = 9.0); (—) same as (---), but w_o = 22.2. These solutions have been prepared by the injection procedure, namely, adding the same number of microliters of an aqueous stock solution at 3 mℓ of water or of hydrocarbon micellar solution. [AOT] = 50 m*M*. The ellipticities in this figure are given in (degrees × l./nucleotide-mol × cm), under the assumption that the molar concentration in the micellar solutions and in water are the same. The extinction coefficient in water for RNA was taken as 7000 per nucleotide. (From Luisi, R. L., Imre, V. E., Jackle, H., and Pande, A., *Topics in Pharmaceutical Sciences 1983*, Breimer, D. D. and Speiser, P., Eds., Elsevier/North Holland, 1983, 243. With permission.) Absorption (c) and ellipticity (d) properties (1 cm light path) of DNA (molecular weight ca. 250,000 Daltons) in water (——) and in isooctane/AOT (50 m*M*)/water micellar solutions: (---) w_o = 18.5 (prepared from an aqueous stock solution of borate buffer pH = 9.0): (—·—·) w_o = 18.5 (prepared from an aqueous stock solution of tris buffer pH = 7.5); (·····) same as (----), but w_o = 25.9; (-·-·-) same as (----), but w_o = 14.8; (-·-·-·-) in a) same as (----), but w_o = 22.2. The extinction coefficient of DNA in water was taken as 6450 per nucleotide. The CD spectrum of the plasmid (pBR 322) is qualitatively the same as that in d). (From Luisi, P. L., Imre, V. E., Jackle, H., and Pande, A., *Topics in Pharmaceutical Sciences 1983*, Brimer, D. D. and Speiser, P., Eds., Elsevier/North Holland, 1983, 243. With permission.)

E. Activity of Enzymes in Hydrocarbon Reverse Micellar Solutions

Several enzymes have been by now solubilized in reverse micellar solutions using generally AOT, but also other surfactants. An extensive review of all these data has been presented recently;[182] however, for the sake of completeness we repeat here some of the main observations arising from such data.

Hydrocarbon micellar solutions can be handled (e.g., for activity assay, for the determination of kinetic parameters, and for mechanistic studies) just like normal aqueous solutions. When adding substrate to the enzyme, one can always utilize two micellar solutions of E and S at the same w_o; or add a few microliters of enzyme in bulk aqueous solution to the micellar solution of the substrate (thus, altering w_o); or use the opposite procedure, i.e., add substrate from the stock aqueous solution to the micellar solution of the enzyme. The preferred procedure depends on the particular enzyme in consideration. For example, under conditions where the enzyme is relatively unstable in reverse micelles, as is the case at large

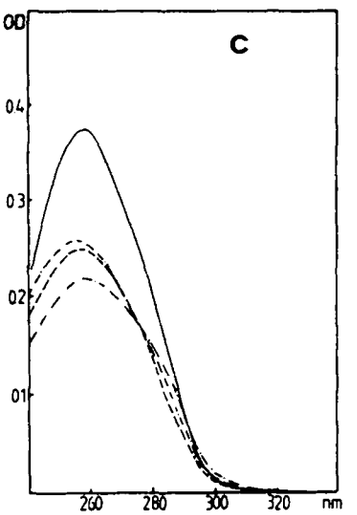


FIGURE 29c

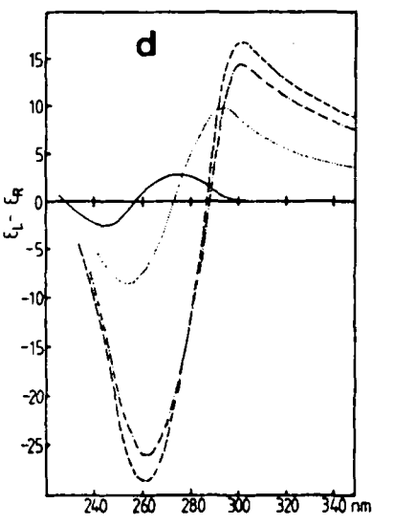


FIGURE 29d

w_o values, or in the particular case of lysozyme,[174] then it is better to add the enzyme last in the form of an aqueous stock solution.

The determination of the kinetic parameters, K_m and k_{cat}, can be made using classical Lineweaver-Burk plots, as in aqueous solutions. Attention should, however, be given to the question of concentration. Usually, substrate at increasing concentrations is added to the enzyme micellar solution, using the same amount of injected stock solution in order to keep w_o constant. In this case, both $[S]_{ov}$ and $[S]_{wp}$ increase simultaneously (for substrates which are only soluble in water, $[S]_{wp} \cdot F_w = [S]_{ov}$. Enzyme saturation, or half-enzyme saturation (at K_m) will occur when a certain number of molecules of substrate have been added to a given enzyme solution; notice that this given amount of substrate molecules will correspond, according to the protocol described above, to two different concentrations, $[S]_{ov}$ and $[S]_{wp}$. Accordingly, one can define two distinct K_ms, which differ from one another by the factor F_w. However, obviously it would be absurd to say that enzyme saturation is numerically different if considered as overall or as water-pool concentration. There is only one physical system, enzyme saturation occurs at a given ratio of enzyme to substrate molecules, and to argue that $K_{m,wp}$ is larger than $K_{m,ov}$ and to explain this difference in physical terms would be erroneous.

The K_m micellar value to be compared with the aqueous value is then $K_{m,ov}$ or $K_{m,wp} \cdot F_w$. Of course, the micellar value does not need to be equal to the aqueous value: the micellar environment can bring about all kinds of changes in the enzyme behavior (due, for example, to conformational changes, to the effect of the dielectric constant on the affinity constant, to diffusion effects). This is apparent from Table 7: K_m in micelles are often very close to the values in aqueous solutions, but occasionally significant deviations are apparent.

It is possible to construct Lineweaver-Burk plots maintaining either the overall concentration constant and varying the water pool concentration or vice versa. This is of course very tedious and not very easy to do.

From Table 7 it is apparent that mostly monomeric enzymes, preferentially proteases and other hydrolases, have been studied. The groups of Martinek, Robinson, Balasubramanian, Luisi, and Douzou have been the most active over the last 3 to 4 years. The notion of enzymes in reverse micelles can be traced back, however, to Wells and collaborators, who investigated phospholipases.[183] In this case the surfactant was the substrate itself (phospholipids) in aqueous organic solvents, and since the hydrophobic enzyme is soluble in certain

Table 7
KINETIC PARAMETERS FOR ENZYMES IN REVERSE MICELLES[a,b]

Enzyme	System	Aqueous Solution		Reverse Micelles		Ref.
		k_{cat}	K_m	k_{cat}	$(K_m)_{ov}$	
α-Chymotrypsin (E.C. 3.4.21.1)	AOT/IO[d]	0.83s^{-1} pH 7.9 (opt)	0.6 m*M*	5.06s^{-1} opt. conditions	0.4 m*M*	175
(E.C. 3.4.21.1)	AOT/O	$7 \times 10^{-3}s^{-1}$ pH 8	$1 \times 10^{-1}M$	$9 \times 10^{-4}s^{-1}$ pH 8	$1 \times 10^{-4}M$	186,187,194
(E.C. 3.4.21.1)	AOT/hept.	45.6s^{-1}	$3.9 \times 10^{-4}M$	98s^{-1} opt. conditions		187
Trypsin (E.C. 3.4.21.4)	CTAB/$CHCl_3$ O (1:1)	0.3s^{-1} pH 8	$1 \times 10^{-3}M$	$4 \times 10^{-2}s^{-1}$ pH 8	$3.6 \times 10^{-4}M$	186
(E.C. 3.4.21.4)	AOT/hept.	8.7s^{-1} opt. pH	$4 \times 10^{-6}M$	11.0s^{-1} opt. cond.	$3.5 \times 10^{-5}M$	78
Ribonuclease (E.C. 3.1.4.22)	AOT/O	0.165s^{-1}[c]	—	1.23s^{-1}[c]	—	176
Peroxidase (E.C. 1.11.1.7)	AOT/O	$1 \times 10^2s^{-1}$ pH 8	—	$19 \times 10^2s^{-1}$ pH 8	—	193
Lipoxygenase (E.C. 1.13.11.12)	AOT/IO	9s^{-1}[c] pH 10	$2.7 \times 10^{-5}M$	4.5s^{-1}[c] pH 10	$20 \times 10^{-5}M$	189
Lysozyme (E.C. 3.2.1.17)	AOT/IO	$3 \times 10^{-4}s^{-1}$	$4 \times 10^{-5}M$	$4 \times 10^{-4}s^{-1}$	$5 \times 10^{-5}M$[f]	174
		0.140s^{-1}	$10 \times 10^{-6}M$	0.130s^{-1}	$2 \times 10^{-6}M$[g]	153
Lysozyme (E.C. 3.2.1.17)	CTAB/$CHCl_3$ IO (1:1)	$2.5 \times 10^{-3}s^{-1}$ pH 5	3×10^5M	$8.2 \times 10^{-4}s^{-1}$ pH 5	$1.6 \times 10^{-5}M$	174
(E.C. 3.2.1.17)	$EO_4 - C_{12}$[e] IO	$3 \times 10^{-4}s^{-1}$ pH 7	$4 \times 10^{-5}M$	$4 \times 10^{-4}s^{-1}$ pH 7	$3 \times 10^{-6}M$	174
LADH (E.C. 1.1.1.1)	AOT/IO	114s^{-1}[c] pH 7.1 (opt)	$3.7 \times 10^{-4}M$	80s^{-1}[c] opt. cond.	$3 \times 10^{-4}M$	177

[a] All K_m values in reverse micelles are expressed as $(K_m)_{ov}$, i.e., referring to the overall concentration ($K_{m,ov} = K_{m,wp} \cdot F_w$, where F_w is the volume percent of water).

[b] The pH of the water pool of the reverse micellar solutions is generally taken as the pH of the stock aqueous solution used to prepare the micellar system with the injection procedure. See Reference 113—118 for a more detailed discussion about the pH problem in reverse micelles.

[c] Initial velocities v/e_o

[d] Abbreviations, see Table 6.

[e] Tetraethyleneglycoldodecylether

[f] Using 2,4 dinitrophenol – NAG_3 as substrate
[g] Using chitin as substrate
[h] For this enzyme, also see the extensive kinetic work from Martinek's group, reviewed in Reference 194.

From Luisi, P. L. and Steinmann, B., *Methods in Enzymology*, Mosbach, K., Ed., in press. With permission.

organic solvents, the report on the enzyme activity in such a system was not very surprising. A couple of years earlier, Montal and Gitler had reported about the solubilization of proteolipids in alkanes,[184] but the notion of micellization was not recognized at that time. Only in 1977 a report on the solubilization of α-chymotrypsin in cyclohexane with the help of methyltrioctyl ammonium chloride, which included the first UV-spectrum of a hydrophilic protein in such an apolar environment, was reported.[185] However, the enzyme was inactive.[150,185] A few years later, in the groups of Martinek[186] and Menger,[187] activity of α-chymotrypsin in hydrocarbon using AOT was reported, and independently by Wolf and Luisi[176] in the case of ribonuclease. In this case, circular dichroism spectra were also reported, which indicated that the enzyme overall conformation was not significantly changed with respect to water solution.

There followed a series of papers, with basically two main directions: (1) elucidation of the structure of the enzyme-containing micelles, and of basic questions regarding the enzymology (including the relationship between conformation and activity) of these novel catalytic systems; and (2) applications to biotechnology, or to organic chemistry at large.

The structure of these host/guest aggregates has been discussed already in the previous sections. Concerning the activity of enzymes in reverse micellar systems, the following generalizations are possible: (1) enzymes maintain activity comparable to that found in aqueous solutions; (2) there are not significant changes in the kinetic behavior, and a Michaelis-Menten behavior has been observed in reverse micellar solutions just as in water; (3) the maximal activity in reverse micelles is not found at the maximal water content (e.g., at w_o around 50 to 70), but at rather small w_o values (typically 10 to 13); (4) enzymes in reverse micelles are able to accept not only water-soluble substrates, but also water-insoluble ones, namely, those which are directly solubilized in the hydrocarbons; and (5) the stability of enzymes in reverse micelles is generally comparable to that in water, being greater at small w_o values and generally rather small at large w_o values (in other words, it follows the pattern of enzyme activity).

From Table 7, one can obtain some actual numerical values concerning these general principles. As already mentioned, more detailed tables about enzymes in reverse micelles can be found in other recent reviews (e.g., Reference 182). As a way of illustrating the activity of enzymes as a function of w_o, we report in Figure 30 the case of α-chymotrypsin. The maximal activity is around $w_o = 8$, and even allowing for the fact that the local w_o value is probably around 12 (considering the arguments presented in Table 5), one reaches the surprising conclusion that the enzyme shows its best catalytic power in micelles in which most of the water is not free. Notice that water is a cosubstrate of the enzyme. In addition, k_{cat} of α-chymotrypsin in reverse micelles is larger than the maximal value in water. This feature, first reported by Menger and Yamada,[187] has been confirmed by Luisi and Barbaric using a different substrate,[175] and in turn these last data have been confirmed by Robinson and co-workers,[188] though with some minor numerical differences. Although the enhancement is not dramatic (it is about a factor of three to six), it opens interesting questions about the activity and physical nature of water in reverse micelles.

Most of the data discussed until now concern the system AOT/hydrocarbon/water. In fact, this is the system which has been most widely investigated. However, this is not the only one which permits the solubilization of enzymes in reverse micelles, and actually other systems, e.g., those based on neutral surfactants, can occasionally be of great interest. For example, ethyleneoxide-based surfactants are generally much less intrusive than AOT, e.g., they do not give the large pK perturbations observed for guest dyes or substrate molecules as in the case of AOT. Also, spectroscopic perturbations of the guest biopolymers are much smaller in ethyleneoxide-based surfactants, or even in CTAB, than in AOT. The same is true for nucleic acids.[159] In the case of lysozyme, whereas the AOT system rapidly denatures the enzyme, in CTAB or $H(OCH_2CH_2)_4OC_{12}H_{25}$ reverse micelles the enzyme is stable and

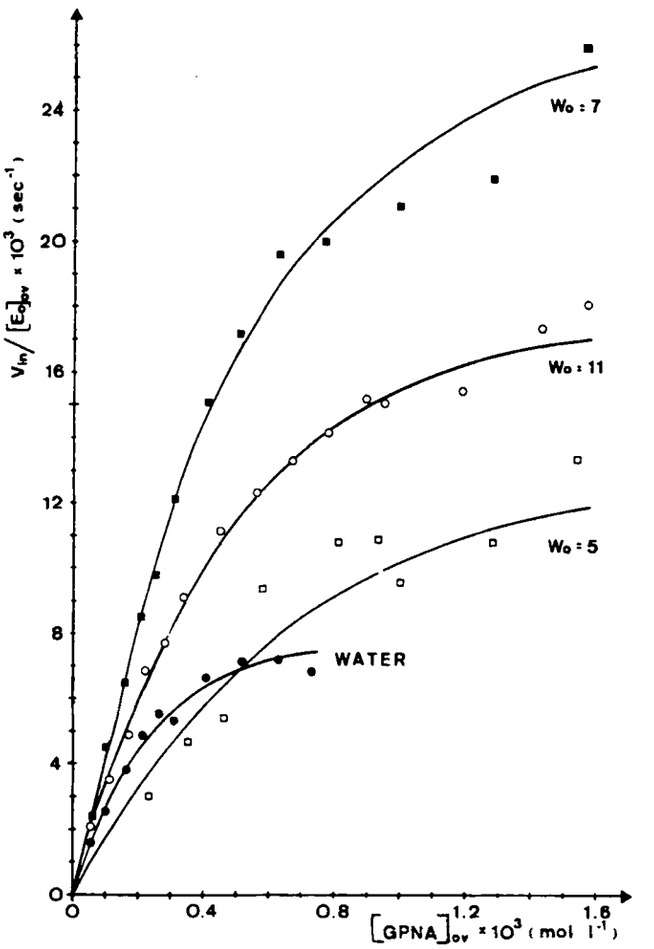


FIGURE 30. Activity of α-chymotrypsin in water and reverse micellar solutions at different w_0. Conditions are pH 8.3 in 0.1 *M* glycine/NaOH buffer, in the presence of 0.1 *M* NaCl (with this stock solution the micellar solutions at different w_0 were prepared). $[E_{0,ov}] = 1\ \mu M$. AOT was 50 m*M* for the curve at w_0 7 and 11, and 100 m*M* for the curve at w_0 5. Temperature: 22°C.

shows the same spectra as in water solution.[174] Also with respect to the solubilizing power, AOT is not always the best choice: for example, we have been unable to solubilize bacterial cells using this surfactant, whereas the solubilization of *Escherichia coli* and other cells was readily possible with Tween surfactants.[181]

F. Applications of Enzymes in Reverse Micelles

The possibility of the biotechnological application of enzymes in reverse micelles is based mostly on the possibility of catalytically converting substrates which are water-insoluble.[189] In this respect, enzymes in reverse micelles seem to offer indeed considerable advantages with respect to alternative systems (for example, enzymes used in biphasic systems). Also, due to the fact that one is dealing with homogeneous solutions, studies on the kinetics and mechanisms are more easily carried out.

Let us see first one example in which enzymes in reverse micelles are used to catalyze

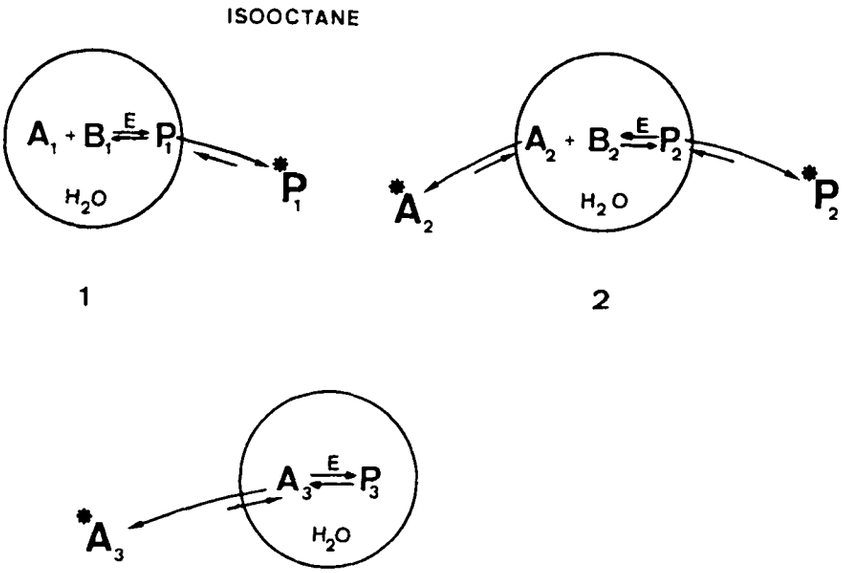


FIGURE 31. Three examples of compartmentalization of reactants in reverse micelles in enzymatic reactions. In the first case (1) the two reagents A and B are preferentially soluble in water and the product C in hydrocarbon. The second case (2) represents a different reaction, where one of the two reagents (A) is also soluble in hydrocarbon. Finally (3), the case of a quite different reaction is schematized, where an overwhelmingly hydrocarbon-soluble compound A yields, upon enzymatic cleavage, a water soluble product B which would remain entrapped in the water pools. (From Luthi, P. and Luisi, P. L., *J. Am. Chem. Soc.*, 106, 7285, 1984. With permission.)

advantageously an organic synthesis. The reaction is the synthesis of a water-insoluble peptide, catalyzed by α-chymotrypsin, taking advantage of the compartmentalization of the reverse micelles. The example is shown in Figure 31, Case 2: one of the two starting reagents is water-soluble, the other partitions between water and the hydrocarbon solution. The enzyme is confined in the water pool. The product of the synthesis is water-insoluble and, after being produced in the water pool, is expelled into the external phase.[190] One can see here clearly the advantages of the micellar solution: although the reaction takes place in a homogeneous solution, reagents and products partition differently in the two microphases, and this provides an efficient driving force for shifting the chemical equilibrium in a particular direction. One can think of various other ways of utilizing the micellar system. For example, if the reaction product from a water-insoluble compound is only water soluble (e.g., the hydrolysis product of a fatty acid ester), it will tend to concentrate in the water pools. There are indeed several cases in which synthesis occurs with a marked change of solubility.

The above principle can be combined with an appropriate enzyme reactor, which allows physical separation between enzyme and reagents.[190] A more ambitious application of enzymes in reverse micelles has been proposed by Hilhorst and co-workers.[191] They were able to solubilize three enzymes (hydrogenase, lipoamide dehydrogenase, and a steroid dehydrogenase) to specifically reduce a water-insoluble ketosteroid starting with gaseous hydrogen as the reducing species.

An interesting possibility for biotechnological applications is offered by the recent observation that also bacterial cells can be solubilized in the hydrocarbon micellar system.[181] There is here the possibility of utilizing certain bacterial strains which are able to metabolize hydrocarbons; in this way the bacterial cells would utilize the solvent as a carbon source. Studies in this direction are in progress.

One should mention also another recent finding, namely, the gelation of hydrocarbon micellar solutions. When gelatin is solubilized in AOT/isooctane/H_2O at rather high concentrations and at rather high w_o values at temperatures above 40°C, and then cooled down below 37°C (the gelation point of gelatin in water), the entire hydrocarbon system becomes a gel.[192] Gels can be obtained also with biocompatible solvent/surfactant systems. This offers the possibility of producing systems which are potentially very interesting for pharmaceutical and cosmetic applications (and, in fact, several chemical industries are now actively working in the area). One can obtain viscous oils or creams containing active biopolymers or other active hydrophilic biomolecules in the water droplets. An application which is more directly correlated to the use of enzymes, is the use of such gels as supports for enzymes (which can be cosolubilized with gelatin and remain active in the gel form). For example, when lipooxygenase is entrapped in such gels, in the form of a small column, the substrate lineolic acid can be added to the column as a hydrocarbon solution, and the product can be collected in the eluant.

As a further example of biotechnological applications, phase transfer methods are now under study as a chromatographic separation process for proteins. Work is in progress in the group of Hatton at M.I.T. and in the group of van Rijt in Wageningen, The Netherlands.

Finally, we mention again nucleic acids solubilized in reverse micelles. Although the biotechnological exploitation of these findings does not seem to be timely yet, the interest is growing in the possibility that due to the particular physical environment, certain DNA reactions can be advantageously modified.

G. Miscellaneous Applications of Reverse Micelles

Two aspects not previously discussed deserve note here. Although most successful surfactant-based drug delivery systems use vesicles or liposomes (often constructed from polymerizable surfactants), Speiser[195] has prepared hardened drug delivery systems starting from AOT reverse micelles. Several groups[196-199] have used the water pools of reverse micelles as microenvironments to prepare highly monodisperse, unusually small colloidal metal particles having (in some cases) high catalytic activities. Leong and co-workers[200] have made similarly monodisperse lattices by polymerizing acrylamide solubilized in water pools.

III. CONCLUDING REMARKS

The fascination of the field of biopolymers in reverse micelles is based mostly on the dual nature of the system: on the one hand we are dealing with homogeneous solutions, which allow for detailed molecular conformational and mechanistic studies; on the other hand, the biopolymer is compartmentalized in a niche having properties which differ largely from those of the bulk solvent. The biopolymer, although confined in the micelle and thereby stabilized, can freely exchange material with the environment, taken either from other micelles and/or directly from the hydrocarbon solvent. This quality expresses itself in other interesting forms: the hydrophilic enzyme is essentially in an aqueous environment, yet the water of the water pool has a character which markedly differs from "normal" bulk water. Thus, we have a situation in which the biopolymer, although being in a water environment and maintaining several properties which are typical of normal aqueous solutions, also acquires novel properties — at least in AOT reverse micelles.

Among these novel, or at least unexpected properties, one may enumerate the following: (1) the solubilization of proteins from the solid state at very low w_o values; (2) the highest enzyme activity being found at very low water content; (3) the activity enhancement with respect to water for chymotrypsin and other enzymes; (4) the compartmentalization effects

in the case of high molecular weight DNA, which results in the formation of the psi-spectrum; (5) the surprisingly efficient phase transfer of proteins from water to a supernatant micellar hydrocarbon solution; (6) the fact that an enzyme solubilized in reverse micelles can also accept a lipophilic substrate present in the bulk hydrocarbon solvent; (7) the gelation effect; and (8) the fact that very large biochemical systems, like plasmids or bacterial cells, can be solubilized in hydrocarbon solutions with the help of reverse micelles. All these observations give ample material for study both from the basic and from the applied sciences.

In fact, concerning basic research, most of the fundamental questions are still not yet satisfactorily answered. Concerning, for example, the process of solubilization, neither the nature of the driving forces (thermodynamics), nor the kinetic mechanism is clarified to any extent for guest biopolymers. The reason why, in a phase-transfer process from the water solution to the supernatant micellar solution, a protein can be transferred almost quantitatively, is not well understood even qualitatively. Also, the reason for the large reactivity of certain enzymes at low w_o values is still kind of a mystery.

One can say, however tentatively, that the nature of water in the water pools is perhaps the most important factor for all these properties. As already mentioned, several studies have been performed on this subject, but the precise determination of the physical parameters of water, like dielectric constant, microviscosity, or simply the structure, remain to be elucidated — and actually one does not see at the moment how they can be directly determined.

More and more scientists are, however, attracted by the field, and perhaps due to the collaboration of quite different techniques and mentalities, progress on the basic science of this difficult area will come.

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