

Enzyme reactions in reverse micelles

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Abstract

Enzyme reactions are important not only in life processes but also for the commercial production of various chemicals and foods. Many enzyme reactions of practical interest involve water-insoluble substrates and/or products. To improve the efficiencies of such reactions, one can exploit the system of reverse micelles to immobilize the enzymes and serve as a microheterogeneous reaction medium. Enzyme reactions in reverse micelles require lower reaction volumes, are not restricted by mass transfer considerations and are not subject to product-inhibition. Also, microbial contamination is minimized. The unique microenvironment of the enzyme allows for biocatalytic activity, stability and substrate specificity that is different and vastly improved as compared to that in the aqueous environment. However, to make reverse micellar enzymology a commercial reality, problems linked to product separation, enzyme recovery and re-use need to be addressed. This paper discusses various aspects of enzyme reactions in reverse micelles and other closely related media.

1. INTRODUCTION

Quality constraints on the production of biologically active components can be very stringent. Unwanted byproducts undermine the properties anticipated from desired products. Valuable feedstock is lost through its conversion into these byproducts. Moreover, the usually expensive separation processes have to handle a larger throughput of material, adding to the cost of the desired product. Conventional chemical processes permit synthesis of biologically active components, but often produce undesirable byproducts. Enzymatic biosynthesis provides an effective alternative because of its remarkable specificity and high catalytic activity (1). Enzymes show great potential for the manufacture of food products, pharmaceuticals and agricultural chemicals (2,3). They possess significant catalytic activity at mild conditions of pH, temperature and pressure and their activity can be easily regulated. Thus, they permit chemical synthesis at small energy costs and relatively low capital costs. In contrast, conventional catalytic processes necessitate the use of extreme temperature, pressure and pH conditions. As such, biotechnological growth areas stand to benefit from the use of enzymes.

Despite their apparent advantages, commercial exploitation of enzymatic synthesis remains rather limited (4). One of the principal reasons is the need for a water-rich environment to preserve enzymatic biocatalytic activity. This necessity for an aqueous phase constitutes a significant problem for a number of biochemical reactions of commercial interest. If the reactant is sparingly soluble in water, its

accessibility to the enzyme is limited by the low concentration in water. Similarly, if the product is sparingly soluble in water, its presence inhibits the continued catalytic activity of the enzyme even at early stages of the reaction. For example, enzymatic steroid conversion is limited by the poor aqueous solubility of steroids. Typically steroids are soluble only to the extent of 10-100 micromoles per liter in water. Transformation of these compounds by oxidation or reduction reactions into useful pharmaceutical products requires immense process volumes, and large amounts of enzymes (5,6). Further, more than one-third of the known enzymes need cofactors that are expensive and which are transformed during the reaction. For a potential commercial application, it is necessary to recover, regenerate and re-use the cofactor. This goal is also difficult to realize if the process volumes involved are too large as in the use of aqueous media. Another example is enzymatic peptide synthesis which can be carried out utilizing hydrolytic enzymes such as pepsin and α -chymotrypsin. However, in aqueous medium, the reaction equilibrium favors hydrolysis rather than synthesis (5). The functional stability of enzymes in the aqueous phase is also less than satisfactory. Last, but not the least, bacterial contamination of technological equipment is another problem that needs to be overcome in aqueous media. Thus, enzymatic biosynthesis does not compare favorably with classic organic synthesis which is conducted using organic solvents as a reaction medium. In order to overcome these limitations and to make an enzymatic process attractive, it is necessary to develop reaction media more suitable than the aqueous phase.

Researchers have suggested various kinds of media for enzymatic biosynthesis where the organic phase is a significant proportion of the total reaction volume. Several advantages can be realized through the use of low-water organic media for biocatalytic reactions. The immensely high concentrations possible for the substrates and the products in the organic media, compared to their negligible solubility in water, ensures low reaction volumes. Since the substrate is present primarily in the organic phase and the product is also in the organic phase upon reaction, problems of substrate and product-inhibition are avoided. The product can be conveniently concentrated by evaporation of low-boiling solvents. For reactions such as peptide synthesis and transesterification, where water is a product of the reaction, the low-water environment shifts the equilibrium toward synthesis rather than hydrolysis. Similarly, the use of organic media reduces undesirable water-dependent side reactions such as hydrolysis of acid anhydrides and polymerization of quinones. In several instances, it has been observed that the use of a low-water environment freezes the enzyme in a catalytically active conformation and improves its thermal stability compared to that in aqueous media. Moreover, certain enzymes such as α -chymotrypsin exhibit the phenomenon of "superactivity" (7). Here, the catalytic activity in organic media is increased several-fold compared to that in aqueous media. Cofactor-dependent reactions can be conducted more efficiently in organic media since the enhanced local concentration of the cofactor in the vicinity of the enzyme permits reduced usage of the expensive cofactor. The insolubility of the enzyme in organic media permits recovery and re-use of the enzyme by filtration procedures. Microbial contamination is another problem that can be overcome with the use of organic media. Obviously, not all of the above advantages can be achieved with every type of non-aqueous reaction media.

2. LOW-WATER SYSTEMS

The major categories of low-water organic systems are solid enzyme suspensions in organic solvents which constitute liquid-solid systems, supercritical fluids, single-phase aqueous-organic solvent systems and two-phase water-organic solvent systems. The choice of the organic system used is a judicious one that must be made after consideration of all the factors involved.

2.1. Solid enzyme suspensions in organic solvents

Certain enzymes have been found to retain their catalytic activity when suspended in solid state in almost anhydrous organic solvents. The first studies in this direction were conducted as early as in 1966 when Dastoli et al.(8) found that suspensions of α -chymotrypsin and xanthine oxidase retained their catalytic activity in organic media (9,10). Since then, Klibanov (11,12) has conducted extensive research in this area. It is believed that this retention of activity is due to a layer of denatured protein forming around the enzyme suspension, protecting the inner layers from inactivation.

The selection of the solvent is critical in the use of solid enzymes suspended in organic solvents. It has been observed that it is the most hydrophobic organic solvents which serve as effective biocatalytic reaction media. The reasoning behind this is that hydrophilic solvents strip the essential hydration shell from the enzyme molecule which is necessary for maintaining its catalytically active conformation. The amount of water bound to the enzyme decreases dramatically with the increasing hydrophilicity of the solvent. For example, the reactivity of α -chymotrypsin in octane is 10^4 -fold higher than that in pyridine (13). Similar results were obtained for lipase (14) where the enzyme was totally inactivated by solvents miscible with water such as dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF).

The percentage of water in the nearly anhydrous solvent is another factor that is critical in maintaining high catalytic activity of the enzyme and varies depending on the type of enzyme in use. A monolayer coverage of water on an enzyme suspended in organic solvents necessitates the presence of approximately 500 molecules of water per molecule of enzyme. Enzymes such as chymotrypsin were found to exhibit full catalytic activity in organic solvents with less than 50 molecules of water per molecule of enzyme (15). In contrast, oxidoreductases such as alcohol dehydrogenase, tyrosinase and alcohol oxidase required complete monolayer coverage before exhibiting any catalytic activity. An interesting feature observed by Zaks and Klibanov is that water added to an organic solvent partitions onto the suspended enzyme. The amount of water required by the enzyme to express catalytic activity in an organic solvent was found to be a function of the amount of bound water retained by the molecule to maintain its catalytically active conformation. Enzymes such as chymotrypsin and lipase suspended in organic solvents were found to exhibit catalytic activity comparable to that in water (13-15). Though oxidoreductases such as horse liver alcohol dehydrogenase (HLADH), mushroom tyrosinase and yeast alcohol oxidase were virtually inactive in completely anhydrous organic solvents, addition of a few percent of water increased their activity to values comparable to that in aqueous solution (13-15). All the enzymatic reactions obeyed conventional Michaelis-Menten

kinetics.

A very interesting feature of enzyme suspensions in organic solvents is the phenomenon of "pH memory". It has been observed that enzyme suspensions in organic solvents must be lyophilized from buffer solutions at the optimum pH for maximum expression of catalytic activity. This phenomenon is attributed to the enzyme acquiring the ionization state corresponding to the pH in aqueous solution which is retained even upon incorporation into the organic solvent. For example, when lipase was precipitated from buffers of pH ranging from 5-9, and then dispersed in organic solvents, the lipase precipitated from the buffer at pH 8.4 exhibited maximum catalytic activity (13-15). This is interesting in view of the fact that pH 8.4 is the optimum pH for the enzyme in aqueous solution.

Enzymes suspended in organic solvents have been observed to exhibit altered substrate specificity compared to that in aqueous media. Though in aqueous solution, lipase reacts with any alcoholic substrate, in organic solvents it is found to be unreactive to tertiary alcohols. This is attributed to the steric hindrance caused by the lack of conformational mobility of the enzyme in organic solvents preventing the bulky alcohol from occupying the active site of the enzyme molecule (14). Similarly, the stereoselectivity of proteases in organic media was dramatically reduced compared to that in aqueous solution. This was interpreted to be due to the substrate's inability to displace water from the hydrophobic binding pocket of the enzyme molecule in organic media (16). For example, use of subtilisin suspensions in organic solvents permits the synthesis of peptides containing D-amino acid residues which is not possible in water due to the L-stereoselectivity of the enzyme in water. Another reason for the changed substrate specificity in organic solvent is the absence of hydrophobic interactions that are present in water. For example, in water, the activity of chymotrypsin drops by a factor of 5×10^4 when phenylalanine is replaced by serine in esters of N-acetyl-L-amino acids. In contrast, the ester of serine is three times more reactive than that of phenylalanine in the chymotrypsin-catalyzed trans-esterification reaction in n-octane (17). Moreover, though the ester of histidine in water is 200-fold less reactive than the ester of phenylalanine in water, the opposite effect is observed in n-octane where the histidine ester becomes 20-fold more reactive than that of phenylalanine.

Since dehydration drastically reduces the conformational mobility of enzymes, it would be expected that the thermal denaturation process would be slowed down considerably for enzymes suspended in organic solvents. This is indeed true for several enzymes suspended in organic solvents. For example, though chymotrypsin is irreversibly inactivated in water at 60°C within minutes, it is found to be active for several hours in octane at 100°C. The increased thermal stability of the enzyme is dependent upon the hydrophilicity of the organic solvent and the pH of the buffer solution from which it was lyophilized (13,15). Storage stability of enzymes in organic solvents also improves dramatically compared to that in water. Though chymotrypsin is active for just a few days in water, it was found to be fully active for a period of 6 months in octane. Similar results have been obtained also for subtilisin and porcine pancreatic lipase (18). The thermal stability of the enzymes was strongly dependent on the water content of the organic solvent. Though porcine pancreatic lipase at

100°C was highly stable at 0.3% water in organic solvent, the half-life dramatically dropped to that in bulk aqueous media at 0.8% water. Similarly, Deetz and Rozzell (19) observed that though the half life of HLADH in dry butyl acetate was greater than 100 hours, as the concentration of water increased to 2%, the stability of the enzyme decreased.

Solid enzymes suspensions in organic solvents have been used for a number of biotechnological applications. An important application is the lipase-catalyzed transesterification reaction. Though in aqueous media, hydrolysis is usually the prevalent reaction, in the low-water environment provided by organic solvents, transesterification takes place in a very high yield (14,20). In fact, Unilever has been reported to be commercializing a lipase-catalyzed trans-esterification process for upgrading triglycerides (21). Another important biotechnological application is the production of 2-halopropionic acids which are intermediates in the manufacture of herbicides by lipase catalyzed stereoselective esterification in organic solvents (20,22). Though it was initially believed that enzymatic reactions involving cofactors could not be conducted using solid enzyme suspensions in organic solvents, this has since been disproved and multi-enzyme systems involving nicotinamide adenine dinucleotide (NAD) have also been successfully incorporated in organic solvents (19,23). Moreover, it has been found that cofactor usage can be reduced due to the high local concentrations of cofactor in the vicinity of the enzyme. Other important enzymatically catalyzed conversions in this medium include hydroxylation of phenols with polyphenol oxidase, polymerization of phenols catalyzed by peroxidase (24) and peptide synthesis (25).

In spite of the advantages of solid enzyme suspensions in organic solvents, these systems are plagued by severe mass transfer limitations common in heterogeneous catalysis. Vigorous agitation is necessary to overcome the external diffusion problems while covalent modification to polyethylene glycol (26) and immobilization to solid supports (27,28) have been suggested as methods to overcome the intra-particle diffusion limitations. Moreover, not all enzymes are obtainable in a lyophilized form, thus restricting the universal applicability of this mode of enzymatic biosynthesis..

2.2. Supercritical Fluids

Supercritical fluids formed by the compression of gases such as carbon dioxide have been suggested as interesting media for biocatalytic reactions. Such systems permit high mass transfer rates and easy separation of reaction products. Randolph and coworkers (29) were the first group of researchers to utilize supercritical fluids as media for biocatalytic reactions. They investigated the conversion of disodium p-nitrophenyl phosphate to p-nitrophenol catalyzed by alkaline phosphatase in supercritical carbon dioxide at 100 atm pressure with 70% yields. Later Hammond et al.(30) conducted the polyphenol-catalyzed oxidation of phenols to quinones in the same reaction medium. Recently Randolph and coworkers (31,32) carried out the oxidation of cholesterol to cholestenone by cholesterol oxidase in supercritical CO₂. Using electron spin resonance (ESR) spectroscopy they found that the enzyme-catalyzed oxidation of cholesterol in supercritical fluids was enhanced due to the formation of cholesterol aggregates. However, the solubility of cholesterol in this

medium was much lower than that possible in organic solvents. Apart from the lower solubility of extremely lipophilic substrates in this medium, the high energy cost necessitated by the use of high pressures is also an important disadvantage for the commercial use of this medium.

2.3. Single-phase water-organic solvent systems

The addition of a miscible organic solvent such as an alcohol to the aqueous enzymatic reaction medium results in improved substrate and product solubility (33). Solvents commonly used include ethanol, acetone, acetonitrile, dimethylformamide and dioxane.

The major criterion for the selection of the organic solvent is the ability to maintain the catalytically active conformation of the enzyme without stripping its essential hydration shell. This criterion is satisfied by organic solvents capable of forming a cage of protective hydrogen bonds around the protein. Based on this, mixtures of water and polyols such as glycerol and ethylene glycol are considered to be appropriate enzymatic reaction media (34-36). However, drawbacks of these solvents are high viscosity and inability to dissolve highly lipophilic substrates.

An important disadvantage of single-phase water-organic solvent systems in general is that organic solvent concentrations greater than 50-70% strip the hydration shell of the protein causing it to denature (37). Guigliardi et al.(38) have recently investigated the effect of water-miscible solvents on the stability of the oxidoreductase malic enzyme. They found that while the enzyme was completely active after 24 hour exposure to 50% dimethyl formamide at room temperature, it lost 15% of its activity in aqueous solutions of methanol and ethanol. Moreover, though malic enzyme is noted for its remarkable thermostability, the presence of miscible organic solvents caused rapid denaturation of the enzyme at higher temperatures. The residual activity of the enzyme was inversely correlated to the logarithm of the octanol-water partition coefficient of the solvent. This was completely contrary to the behavior of enzymes in biphasic aqueous-organic systems where the use of more hydrophobic solvents resulted in higher enzyme stability.

Water-miscible organic solvents have not been extensively used to improve the solubility of reactants and products in enzymatic reactions due to the less than desirable stability of the biocatalyst in these systems. One of the few studies of enzymatic reactions in this medium is the protease-mediated peptide synthesis in 50-60% aqueous solutions of DMF (39). It was found that though esterase activity of the protease was preserved, the inactivation of the undesirable amidase activity resulted in a 10,000-fold increase in the rate of peptide synthesis as compared to anhydrous DMF. However, this is not surprising in view of the fact that in enzymatic catalysis in anhydrous organic solvents, hydrophobic solvents are preferred over such hydrophilic solvents as DMF since they are better able to maintain the integrity of the hydration shell surrounding the protein (12).

2.4. Two-phase water-organic solvent systems

Two-phase water-organic solvent systems have demonstrated strong potential as enzymatic reaction media (5,40,41). The biocatalyst located in the aqueous phase is

spatially separated from the organic solvent phase which acts a reservoir for the substrate and a sink for the products. Such biphasic systems not only permit high substrate and product solubility, but equilibrium-controlled reactions are driven to completion. Since the enzyme and the product are located in different phases, product recovery is easily facilitated and the enzyme can be recovered for re-use.

Several factors govern the choice of the organic solvent in such systems. Firstly, the organic solvent must have high solubilizing capacity for the reactants and the products. Moreover, the partition coefficients of the substrates and the products between the aqueous and the organic phase influence both the rate of the reaction as well as the equilibrium yield of the reaction. Product-inhibition can be reduced by selecting solvents which facilitate enhanced product partitioning into the organic phase. It is also important that the biocatalyst should be stable in the presence of the organic solvent. It has been found that solvents with octanol-water partition coefficients that are greater than 4, result in the highest stability of the biocatalyst (42). This is due to the extremely low solubility of hydrophobic solvents in the aqueous phase containing the enzyme.

Several biocatalytic conversions have been successfully conducted in two-phase systems. Carrea and Cremonesi (43) have extensively investigated the activity and stability of steroid-converting enzymes in biphasic aqueous/organic systems. They conducted the dehydrogenation of testosterone using 3- β hydroxysteroid dehydrogenase as well as the 20- β hydroxysteroid dehydrogenase-catalyzed reduction of progesterone and cortisone in two-phase mixtures of water and several organic solvents. Ethyl acetate and butyl acetate were found to be the best organic solvents due to the high solubility of the steroids coupled with appreciable enzyme stability in these systems (5,41,44,45). Legoy et al.(46) have used biphasic systems for the alcohol dehydrogenase catalyzed production of long chain aromatic aldehydes from alcohols. They observed that the enzyme was most stable in water-hexane two-phase mixtures.

Klibanov et al.(47) proposed the use of biphasic systems to shift the equilibrium of synthetic reactions employing hydrolases as biocatalysts. The partitioning of the product into the organic phase made it possible to shift the equilibrium constant of chymotrypsin-catalyzed peptide synthesis by several orders of magnitude in biphasic systems of water and chloroform. Cambou and Klibanov (48) used esterase-catalyzed trans-esterifications to produce optically active esters from racemates in biphasic systems. Though it was initially believed that the shift in the equilibrium constant in favor of the synthetic product was due to the reduction of water activity in these systems, it has since been proved that the increase in the equilibrium yield is due to the extraction of product into the organic phase rather than a reduction of water activity (49). Halling and coworkers demonstrated that only a significant reduction of the water activity below unity would have an effect on the position of the reaction equilibrium (50-52). Such a reduction in water activity is not possible in biphasic aqueous-organic systems.

A very important factor that governs the rate and equilibrium yield of biocatalytic reactions in biphasic systems is the volume ratio of the organic phase to the aqueous phase (53). The effect of the phase ratio is closely tied in with the mass transfer limitations prevalent at high biocatalyst concentrations. As the concentration of the

biocatalyst in the aqueous phase increases, there is increased substrate limitation in the aqueous phase. However, increase in the volume of the organic phase in relation to that of the aqueous phase increases the interfacial area available in the aqueous phase and reduces the ensuing mass transfer limitations. Another effect of increasing the phase ratio is the increased partitioning of product into the organic phase reducing product-inhibition. This beneficial effect of increased phase ratio on the product yield of the reaction has been observed in the case of chymotrypsin-catalyzed peptide synthesis in biphasic systems of water and ethyl acetate (54).

Since two-phase systems cause denaturation of the biocatalyst due to adsorption at the liquid-liquid interface, immobilization of the biocatalyst on a suitable support has been suggested as a method to alleviate this problem (55). Moreover, the low interfacial area of contact between the two-phases in such systems causes the reaction to be mass transfer limited. Larger interfacial areas of contact require significant intensity of agitation that increase energy costs and may have a detrimental effect on the structural stability of the enzyme. Furthermore, the formation of emulsions at the interface causes contact denaturation of the enzyme (56,57). Therefore, methods are needed that could retain the advantages of the two-phase reaction system while reducing the problems accompanying it.

2.5. Microheterogeneous media

All the reaction media that have been described so far are of the "macroheterogeneous nature" in which the enzyme present in one phase is spatially separated from the organic solvent containing the reactants and products on a macroscopic level. Such systems afford ease of product separation and enzyme recovery and re-use. However, diffusional limitations severely limit the use of enzymes in biphasic systems.

Microheterogeneous media such as the system of reverse micelles offer a novel approach to two-phase aqueous-organic biocatalysis. Reverse micelles are molecular aggregates made up of surfactant molecules and are spontaneously generated in an oil-water system in the presence of a suitable surfactant. Since the separation between the aqueous and organic domains in these systems occurs at "molecular dimensions" it eliminates mass transfer limitations. Unlike macroheterogeneous media, the reverse micelles are optically transparent which makes them amenable to analysis by various spectroscopic and analytical techniques.

Structurally, a reverse micelle consists of a core region made up of the polar parts of the surfactants surrounded by a non-polar shell region made up of the hydrophobic tails of the surfactants. The micelles are present in a continuous medium of oil. Water can be solubilized in the interior of the reverse micelles and the resulting structures are also known as water-in-oil microemulsions. The enzyme is solubilized in the aqueous core of the micelle and allowed to react with the substrate present in the organic continuous phase. Since the enzyme is spatially separated from the oil phase at the "molecular level", it is protected from the detrimental effect of the organic solvent.

Microheterogeneous media have a number of advantages as enzymatic reaction media over conventional two-phase systems. The enzyme located in an essentially

aqueous microenvironment retains its activity and the problem of inactivation is reduced as compared to that in organic solvents. Surprisingly, in many cases, the functional stability of the enzyme appears to be even better than in bulk water (7,58). Because of their small dimensions (in the range 20-200 Å), they are optically transparent and amenable to physical and chemical studies by various techniques. The amount of water in the reverse micelles can be precisely controlled. One can modify the activity of the water without simultaneously modifying the amount of the water. The nature of the oil-water interface can be designed to specification through the selection of the surfactant molecules, a wide variety of which are commercially available. For example, in case of synthetic reactions catalyzed by hydrolases, a reduction of water activity below unity dramatically increases the equilibrium constant in favor of the synthetic product (59). Though such reduction of water activity is not possible in biphasic systems, it can be easily achieved in reverse micellar systems by reduction of micellar size or by changing the nature of the surfactant and cosurfactant (60).

Reverse micelles with reproducible structural properties can thus be designed by manipulating the constituent molecules, such that the size of the water core, the polarity of the micelle interface and the stereochemistry of the micelle interface can all be varied to specification. Such variations can be expected to influence the enzymatic activity, substrate accessibility and the selectivity of the enzymatic reactions. Another significant advantage afforded by the small size of reverse micelles is the immense interfacial area of contact (10-100 m²/ml) between the substrate containing oil phase and the enzyme containing aqueous phase. This large interfacial area is achieved without requiring any agitation of the system (61). Since intermicellar exchange processes are rapid compared to the rates of common enzymatic reactions, mass transfer limitations are eliminated (62,63). Lastly, the reverse micellar system can be scaled-up rather easily for any large-scale application of the biosynthetic process. Since they are thermodynamically stable systems with reproducible structural properties, they are expected to yield products of consistent quality (64). In short, reverse micelles provide a microheterogeneous analog of the two-phase system with remarkably improved characteristics.

3. OPTIMIZATION OF ORGANIC SOLVENT FOR BIOCATALYSIS IN NON-AQUEOUS MEDIA

It has been observed by several researchers that the use of different organic solvents as media for biocatalysis leads to extremely different biocatalytic behavior. It would therefore be extremely useful to relate the activity and stability of the biocatalysts to certain known physical properties of the solvents. In general solvents of low polarity have been found to provide more favorable biocatalytic behavior as compared to those of high polarity.

The first important contribution in this area is that of Playne and Smith (65) who tested different organic solvents for their toxicity to anaerobic bacteria. This was followed by the work of Brink and Tramper (55) who proposed the use of the Hildebrand solubility parameter in conjunction with the molecular weight of the

solvent as a semi-empirical measure of its use as medium for biocatalytic reactions. They studied the activity and operational stability of the biocatalyst in the epoxidation of propene and 1-butene by *Mycobacterium* species in various organic solvents and found a weak correlation between these properties and the stability of the biocatalyst in the organic solvent. The lowest activities were exhibited by the biocatalyst in the region of high solubility parameter and low molecular weight which corresponds to solvents of small size and high polarity. Conversely, low polarity and high molecular weight leads to high activity retention of the biocatalyst in the water/organic solvent medium.

A more effective approach is that of Laane and coworkers (42,66) who propose that the activity retention of biocatalysts in organic media is related to the logarithm of the partition coefficient of the organic solvent in the octanol/water system. The logarithm of the partition coefficient ($\log P$) for any organic solvent can be obtained from hydrophobic fragmental constants (hfc) using a group contribution procedure (66). Values of hfc's were assigned to each functional group in a compound. By adding the hfc values for all the functional groups in a particular compound, the value of $\log P$ can be obtained. It is thus possible to predict the behavior of biocatalysts in organic media from knowledge of the relevant physical properties of the organic solvents. From experimental data of activity retention in a number of organic solvents, Laane and coworkers concluded that activity retention was low in solvents of $\log P < 2$ and high in solvents of $\log P > 4$. Solvents of $\log P$ in the range 2-4 resulted in moderate activity and stability of the biocatalysts.

Reslow et al.(67) observed that immobilized α -chymotrypsin esterification proceeded at a higher rate in solvents of $\log P > 0.7$. In order to rationalize better the solvent behavior for $\log P$ values in the range 0.5 to 1.5, they suggested that $\log P$ values should be corrected for the water content of the solvents. Laane and coworkers (66,68,69) also postulated that optimization of biocatalysis in organic solvents necessitated an adjustment of the polarity of the microenvironment of the biocatalyst ($\log P_i$) and that of the continuous phase ($\log P_{cph}$) to the polarities of the substrate ($\log P_s$) and the product ($\log P_p$). According to them, [$\log P_{cph} - \log P_s$] and [$\log P_i - \log P_p$] should be maximized, while [$\log P_{cph} - \log P_p$] and [$\log P_i - \log P_s$] should be minimized. This would ensure that at the same overall substrate concentration, the substrate concentration in the immediate vicinity of the biocatalyst is maintained at a high value while product inhibition is reduced due to the preferential partitioning of the product in the organic continuous phase. In the case of substrate inhibition, they postulate that $\log P_i$ should be optimized with respect to $\log P_s$.

In the case of biocatalysis in reverse micelles, Laane and coworkers (42,66) suggest that the use of a cosurfactant such as an alcohol permits greater control of the polarity of the interface and the organic continuous phase. The ratio of the cosurfactant to the surfactant in the interface would determine $\log P_i$ and $\log P_{cph}$ and affect substrate concentration in the microenvironment of the biocatalyst. A semi-empirical formula for $\log P$ of mixtures is $\log P = X_1 \log P_1 + X_2 \log P_2$, For reverse micelles where the molar ratio of the cosurfactant to the surfactant in the interface is a_0 and c_0 is the mole fraction of the cosurfactant in the continuous phase, this relationship becomes $\log P_i = a_0/(a_0 + 1) \log P_{cosurf} + 1/(1 + a_0) \log P_{surf}$ and \log

$P_{\text{cph}} = c_o \log P_{\text{cosurf}} + (1 - c_o) \log P_{\text{orgsolv}}$. This procedure was tested by them to optimize the catalytic activity of 20- β hydroxysteroid dehydrogenase in reverse micelles of CTAB in octane using hexanol as a cosurfactant. The highest catalytic activity of the enzyme was obtained at those concentrations of the surfactant and cosurfactant at which $[\log P_i - \log P_s]$ was minimal and $[\log P_{\text{cph}} - \log P_s]$ was maximal. In this manner, it is possible to design reverse micelles for optimal bioconversion in microemulsions.

The log P model however, cannot account for the surprisingly high activities of porcine pancreatic lipase in pyridine or subtilisin in DMF (70). Following the earlier suggestion by Reslow et al. (67), Dordick (70) recommends that the log P value of the solvent should be corrected for the water solubilizing power of the solvent via $\log P_{\text{corr}} = (1-x) \log P_{\text{org solv}} + x \log P_{\text{water}}$ where x is the mole fraction of water in a water saturated solvent.

The choice of the organic solvent also affects the equilibrium yield of product in enzymatically-catalyzed reactions. This is particularly true for reverse hydrolytic or synthetic reactions catalyzed by hydrolases in organic media where it is possible to shift the equilibrium in favor of the synthetic product by proper choice of the organic solvent. In biphasic reaction systems, knowledge of log P values for the reactants and the products can be used to obtain the equilibrium constant for the reaction (52). Solvents that cause favorable extraction of the product into the organic phase give maximum equilibrium yield of the synthetic product.

4. ASPECTS OF ENZYME SOLUBILIZATION IN REVERSE MICELLES

4.1. Formation of reverse micelles

Figure 1 is a schematic representation of a reverse micelle. It consists of a spherical aqueous core of radius r_w surrounded by the hydrocarbon tails of the surfactant of length l making the overall radius of the micelle r_o .

$$l = r_o - r_w \quad (1)$$

The surface area of the water core and the volume of the hydrophobic tail region in a reverse micelle are both functions of the aggregation number N_{agg} (the number of surfactant molecules per micelle).

$$N_{\text{agg}} = \frac{4 \pi}{3} \frac{(r_o^3 - r_w^3)}{V} \quad (2)$$

$$N_{\text{agg}} = \frac{4 \pi r_w^2}{a} \quad (3)$$

N_{agg} is obtained explicitly by dividing the volume of the hydrophobic region in a single micelle by the molecular volume V of the hydrocarbon tail of the surfactant. The aggregation number is also the ratio of the total surface area of the aqueous core of

the micelle to the surface area per head group "a" of the surfactant. The surface area a per head group depends on various intra-micellar interactions and is obtained by minimizing the thermodynamic potential of the reverse micellar system. The area a is thus an equilibrium variable and not simply related to the geometrical size of the surfactant head group.

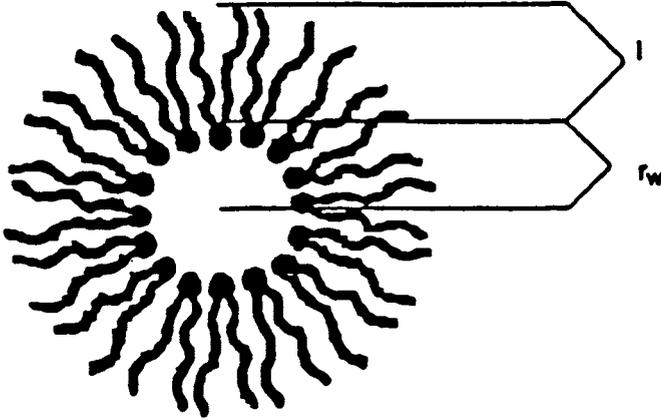


Figure 1. Schematic representation of a reverse micelle

Combining eq.(2) and (3), a relationship between the overall and the water core radii of the micelle is obtained. This relationship depends on the molecular volume of the surfactant tails and the surface area per surfactant head group.

$$\frac{3V}{a(r_o - r_w)} = \frac{(r_w^2 + r_o^2 + r_w r_o)}{r_w^2} \quad (4)$$

Substituting $l = r_o - r_w$ and $x = r_o / r_w$,

$$\frac{3V}{al} = 1 + x + x^2 \quad (5)$$

Since reverse micelles can only be present if $r_o > r_w$, the criterion for the formation of reverse micelles is that $(V/al) > 1$. For surfactants with a single tail, (V/l) is roughly 21 \AA^2 while for double tailed surfactants (V/l) is about 41 \AA^2 . Certain

surfactants require a cosurfactant for the formation of reverse micelles since the equilibrium area per head group is too large compared to (V/l) . Since long chain alcohols are uncharged and have a small head group, they are usually the best candidates as cosurfactants. They are capable of increasing the hydrophobic tail volume without a concomitant increase in the equilibrium area of the head group. However, alcohols with hydrocarbon chains longer than the hydrophobic tails of the detergent are not capable of functioning as cosurfactants (71-76).

The most commonly used surfactant for the formation of reverse micelles is sodium dioctyl sulfosuccinate (Aerosol OT or AOT). The structure of AOT is depicted in Figure 2. The minimum radius of the reverse micelle r_0 for AOT is 15 Å which corresponds to the sum of the head group length of 9 Å and the hydrocarbon tail length of 6 Å. A minimum of seven water molecules are required to hydrate each surfactant molecule of AOT in a hydrocarbon solvent while in the reverse micelles in equilibrium with an aqueous phase there are about 55 molecules of water per molecule of AOT (74).

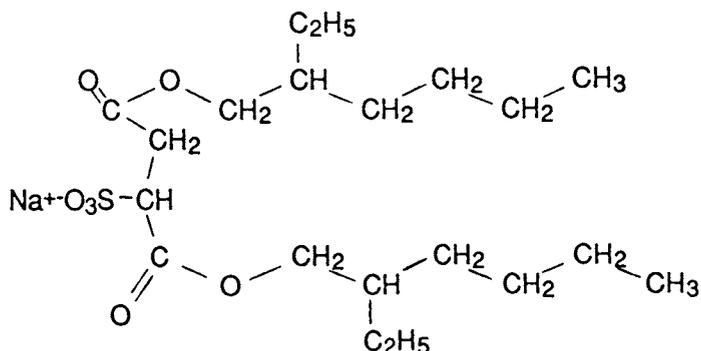


Figure 2. Structure of AOT molecule

Other common surfactants used in reverse micellar enzymology include the cationic surfactants Cetyl Trimethyl Ammonium Bromide (CTAB) and Trioctyl Methyl Ammonium Chloride (TOMAC), the anionic surfactant Sodium Dodecyl Sulfate (SDS), the non-ionic surfactants of the Tween, Triton and Brij series and naturally occurring phospholipids such as lecithin.

The phase behavior of the ternary system AOT/isooctane/buffer has been well documented in the literature (71-75). The ternary phase diagrams for the AOT/Isooctane/Water system is shown in Figure 3 where the L_2 region corresponds to the large compositional domain over which reverse micelles containing solubilized water are formed (75).

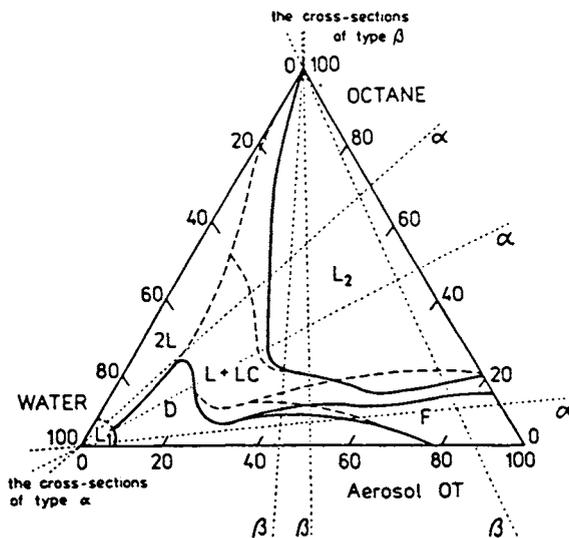


Figure 3. Phase diagram for the AOT/octane/water system (75)

4.2 Solubilization of enzymes in reverse micelles

A number of water-soluble enzymes such as ribonuclease, peroxidase, α -chymotrypsin, trypsin, lactate dehydrogenase, pyruvate kinase, pyrophosphatase, lysozyme and alcohol dehydrogenase have been solubilized in reverse micelles and their catalytic behavior has been studied. Other enzymes that have been solubilized include phospholipase, lipase, α -amylase, cytochrome-c and hydrogenase (77,78). Most of the research in this area has been conducted using reverse micelles formulated with AOT as the surfactant. These studies have shown that it is possible to solubilize the enzymes inside the reverse micelles at certain suitable conditions of pH and ionic strength as well as to release them from the micelles by modifying these conditions. The enzymes have been found to retain their activity to an appreciable extent within the reverse micelle medium.

4.3. Techniques for the incorporation of enzymes in reverse micelles

Three techniques have been suggested and used so far for the entrapment of proteins (enzymes) into reverse micelles forming an optically transparent solution. The first procedure called the injection technique (36,79) is the most extensively used. Here, an aqueous solution of the enzyme is injected into a solution of the surfactant in organic solvent. The incorporation of the enzyme into the aqueous core is almost instantaneous and only requires agitation for a few seconds. This procedure is extremely simple, quick and makes it possible to easily and precisely control the water

content of the resulting micellar solution.

The second technique was suggested and used by Luisi and coworkers (80, 81) in their earlier research. It involves the phase-transfer of enzyme from a bulk aqueous solution into a surfactant-solvent solution of approximately equal volume. Important drawbacks of this procedure are the long time duration for attainment of equilibrium and the inability to control the degree of hydration of the resultant micellar solution.

The third procedure was developed by Menger and Yamada (82) and is used for the preparation of a concentrated solution of the enzyme in reverse micelles. A lyophilized preparation of the protein is contacted with a solution of the surfactant in the organic solvent with the desired degree of hydration. The dry protein is incorporated into the micelles after a period of time which is dependent on the degree of hydration of the micelles. The encapsulation of the enzyme is quicker in reverse micelles with a higher degree of hydration. Repeated contacting of dry protein with the reverse micellar solution has shown that it is possible to achieve a 25 mg/ml solubility of α -chymotrypsin in the reverse micelles (83). However, this procedure is no longer in use due to the rapid denaturation of the protein that occurs during the encapsulation process. Another disadvantage of this method is that most proteins are not available in the lyophilized form preventing its universal applicability.

The mechanism of protein uptake into and release from reverse micelles is still poorly understood. A dynamic exchange process between the aqueous core and the organic phase is considered to be responsible for the protein solubilization. Many researchers subscribe to the theory that it is the reverse micelle that forms around the protein as opposed to the protein being transported across the organic solvent into the micelle (84).

4.4. Limit of protein solubilization and localization of protein in reverse micelles

A substantial amount of protein can be solubilized in reverse micelles by the use of suitable surfactants (83). The limit of solubility is usually a function of temperature, pH, degree of hydration and size of the micelle, nature and concentration of the surfactant and the isoelectric point of the protein. A solubilization curve (Figure 4) for the enzyme α -chymotrypsin as a function of the degree of hydration R in AOT/Isooctane reverse micelles showed that solubilization of the protein commences only after a certain critical value of R after which a limit of solubility (in this case 25 mg/ml) is reached (83).

Reverse micelles afford the unique possibility for the enzyme to localize in a microenvironment that is most compatible with its native or intracellular conformation and continued catalytic activity (Figure 5). Spectral characteristics of proteins such as peroxidase (85) and alcohol dehydrogenase (86) show little change from that in the aqueous media. This shows that hydrophilic or water-soluble proteins tend to localize within the aqueous core of the micelle and are thus protected from the detrimental effect of the organic solvent. Surface active proteins such as lipases can interact with the surfactant layer of the micelle while enzymes that are typically membrane-bound in nature can come into contact with the organic solvent. Spectral studies of cytochrome-c in AOT reverse micelles show significant changes suggesting that this protein interacts with the interface or even the bulk organic solvent in the micellar

medium (87,88).

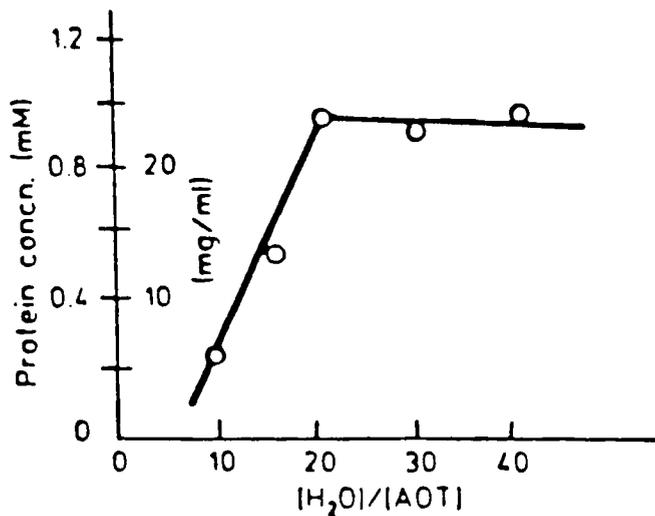


Figure 4. Solubilization curve for α -chymotrypsin in AOT/isooctane/water reverse micelles (83).

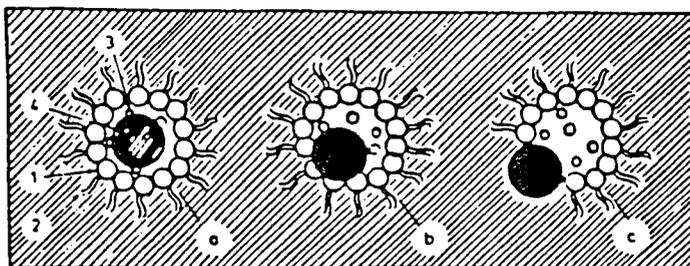


Figure 5. Localization of proteins within reverse micellar aggregates (84). (a) hydrophilic protein, (b) surface active protein, (c) membrane protein, (1) polar head, (2) surfactant tail, (3) counter-ion or water molecule, and (4) protein molecule.

By studying the reaction between hydrated electrons generated by pulse radiolysis and proteins in AOT reverse micelles, it was possible to find the location of proteins such as cytochrome-c, α -chymotrypsin and ribonuclease in reverse micelles. Since

hydrated electron quenching rate constants are smaller when proteins locate at the interface than for those in the water pool, it could be shown that ribonuclease and α -chymotrypsin localize in the water pool, while cytochrome-c is present near the interface (87,88). Moreover, it was found by X-ray scattering that the localization of the protein affects the micellar size which in turn could affect the catalytic activity of the enzyme (87).

4.5. Effect of protein solubilization on structure and size of reverse micelle

Besides the effect of solubilization on the structure and conformation of the enzyme, the structural organization and size of the enzyme-containing micellar aggregate has important implications from the point of view of micellar catalysis. Knowledge of the size of the microemulsion droplets as well as the mobility and the orientation of the solubilized enzyme and their effect on the enzyme activity would permit one to design reverse micellar systems of desirable structural properties. Such basic research facilitates controlling and predetermining the catalytic activity of the solubilized enzyme. Several studies have been conducted to determine the perturbation and change in size of the micellar structure as a result of protein incorporation. These studies reveal that it is possible to utilize the degree of hydration, surfactant concentration and the protein concentration as effective variables for the optimization of enzymatic activity in reverse micelles.

The first study that was conducted in this regard was that of Bonner and Luisi (89). They utilized the measurements of sedimentation diffusion coefficients of protein-containing and protein-free micelles to probe the effect of protein solubilization on the structural properties of the microemulsion droplets. These studies were carried out on the enzymes lysozyme, ribonuclease and HLADH solubilized in reverse micelles of AOT/isooctane. They observed that there was an increase in the sedimentation coefficient and the molecular weight of the protein-containing aggregate with the degree of hydration of the micelle. Based on these experimental observations as well as the assumption that there was no effect of protein incorporation on the degree of hydration of the micelle, they proposed a model known as the "water-shell" model for enzyme-containing reverse micelles. This model postulates that the micellar volume after protein incorporation is the sum of the volumes of the empty micelle and that of the protein.

Further experimental evidence for the water-shell model was provided by quasi-elastic laser light scattering studies conducted on the insertion of myelin basic protein (MBP) into reverse micelles of AOT (90). At low values of R, the hydrodynamic radii of protein-filled micellar aggregates were significantly larger than that of the unfilled micelles. The experimental evidence also revealed that 3 empty (water-filled but protein-free droplets) were necessary to build up a sufficiently large droplet capable of accommodating a protein molecule. The authors concluded that the competition between the surfactant and the protein for interfacial water layers caused a redistribution of the surfactant and water molecules. In subsequent work, Chatenay and coworkers (91) utilized a non-perturbative technique, fluorescence recovery after fringe pattern photobleaching (FRAPP), to determine the radii of filled and unfilled micelles for the system MBP in AOT/isooctane reverse micelles. They observed that

protein containing micelles were significantly larger than the empty micelles at low values of the degree of hydration. However at high R, though the hydrodynamic radii were virtually unaffected by protein incorporation, AOT concentration significantly affected the micellar size. The radii of the filled and unfilled micelles were identical at surfactant concentration extrapolated to zero. This effect was interpreted to be due to the modification of the inter-micellar interactions by MBP localized in the aqueous core. Though later studies revealed the incorrectness of some of the assumptions, the water-shell model has remained a sufficiently simple and often-used model for structural studies on protein-containing micelles.

Pileni and coworkers (88) have used small-angle X-ray scattering to investigate the effect of protein solubilization on the structural rearrangement of the protein-containing micellar aggregates and the localization of the protein in the microemulsion droplets. They observed that when the protein is solubilized in reverse micelles, the micellar size was dependent upon the localization of the protein within the reverse micelle. Proteins like α -chymotrypsin which were located primarily in the water-pool caused an increase in micellar size upon insertion into the micelles. However, pulse radiolysis studies revealed that if the size of the protein was much larger than the micellar size (eg. at low water content), smaller protein containing micelles existed in equilibrium with protein-free micelles. In contrast proteins such as cytochrome-c, which are present in the interface decrease the size of the micelle similar to the effect of surfactant addition to the micellar solution. Since the data from small-angle X-ray scattering (SAXS) agreed with pulse radiolysis studies, it was possible to confirm that cytochrome-c is indeed present at the interface. Recent studies conducted by Huruguen et al.(92,93) on the solubilization of cytochrome-c in reverse micelles composed of AOT in isoctane have revealed the interesting phenomenon of percolation upon protein incorporation in the reverse micelle. Though the percolation phenomenon, whereby micellar droplets are connected with each other as a result of van der Waals attractive interactions usually requires high temperatures, solubilization of cytochrome-c reduced the percolation threshold of the micellar droplets. By increasing the water content at a given protein concentration, or alternatively the protein concentration at high water content, the percolation process could be made to occur at room temperature. Interestingly, if the protein occupancy in each micelle exceeded two molecules, increase in water content caused phase splitting into two optically transparent phases-an upper isoctane phase and a lower phase containing AOT, water and protein. Similar phenomenon has also been reported for ribonuclease in AOT/isoctane reverse micelles (94). In contrast, the absence of the percolation phenomenon in the case of α -chymotrypsin confirmed that this protein does not perturb the micellar structure. Percolation could have important implications from the point of view of biocatalysis in reverse micelles. Addition of water after completion of the reaction would cause phase separation allowing product recovery and enzyme re-use.

Fletcher et al.(95) have investigated the size and polydispersity of micellar droplets by small angle neutron scattering (SANS) when solubilizing α -chymotrypsin in water-in-oil microemulsions. They observed virtually no change in the scattering profile as a result of protein incorporation into the micelles. Though no quantitative interpretation concerning localization of the enzyme was possible, the authors

concluded that the structure of reverse micelles was negligibly perturbed by the incorporation of α -chymotrypsin.

Zampieri and coworkers (96) utilized analytical ultracentrifugation with the double dye technique in a novel procedure to determine the degree of hydration of filled and unfilled reverse micelles. Solubilization of α -chymotrypsin, lysozyme and MBP in AOT/isooctane reverse micelles was studied. Unlike scattering studies, ultracentrifugation and fluorescence recovery after fringe pattern photobleaching (FRAPP) permit selective measurement of micellar populations of different sizes existing in equilibrium. Protein uptake was found to significantly increase the dimensions of the micelle. The redistribution of the water and surfactant molecules between the micelles caused the resulting solution to consist of smaller unfilled micelles in equilibrium with larger filled micelles. This study provided the first experimental confirmation that the assumption of equal R for protein-filled and unfilled micelles was incorrect.

Sheu and coworkers (97) have utilized small-angle neutron scattering (SANS) to determine the effect of cytochrome-c incorporation on size of micelles in the system AOT/isooctane. SANS is a particularly effective technique since it permits direct measurement of the hydrodynamic radii of the micelles. They found that protein solubilization caused a significant redistribution of the surfactant and water molecules thereby causing the radius of the filled micelle to greatly exceed that of the unfilled micelle. Their results are opposite of those of Pileni and coworkers (88) who observed a decrease in micellar size upon protein incorporation. This contradiction can be resolved when one notes that Sheu and coworkers (97) have used the water-shell model for cytochrome-c in reverse micelles which assumes that the protein occupies the micellar core. However, evidence is now available to prove that cytochrome-c occupies the micellar interface (89). This confirms that localization of the protein molecule is a very important factor to consider when determining size of protein-containing micellar aggregates.

Levashov and coworkers (98) have proposed a model (Figure 6) which is an alternative to the water-shell model. Based on sedimentation analysis of solubilization of several proteins in reverse micelles, the authors propose that if the size of the micelle exceeds that of the protein, there is no effect of protein solubilization on micellar radii (Fixed-size model). However, in the event the protein molecule is larger than the micelle, the model postulates an increase in micellar size to accommodate the larger protein (Induced-fit model). This model has been criticized since it violates the area and volume constraints imposed on the system by predetermined water, protein and surfactant concentrations. It has been suggested that the results of Levashov and coworkers could be due to artifacts in the analysis procedure (96,97,99). A rebuttal to this contention has been provided by a $^{13}\text{C}_{\text{nmr}}$ study conducted on the solubilization of α -chymotrypsin in reversed micelles of AOT in isooctane (100). Levashov's group interprets the 3-fold increase in the spin lattice relaxation times for the CH_2 segments of the AOT molecule to be due to an expulsion of the micellar water out of the aqueous core and its deeper penetration into the alkyl chains of the surfactant resulting in no change in micellar size upon protein solubilization. The issue of the effect of protein solubilization on the size of the reverse micelle remains unresolved and further

studies are necessary to predict the effect of protein solubilization on the micelle.

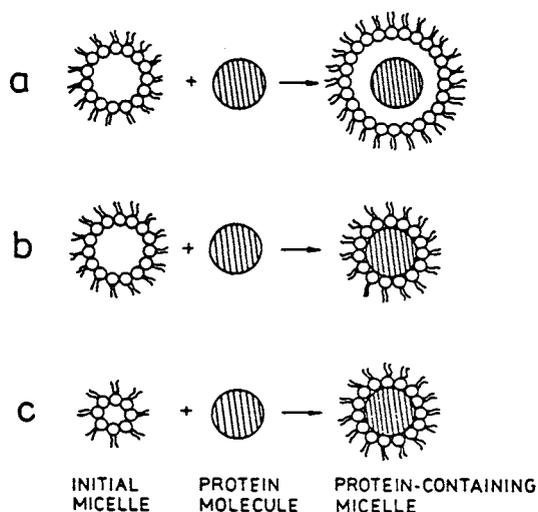


Figure 6. Effect of protein solubilization on the size of micelles. (a) water-shell model, (b) fixed-shell model, (c) induced-fit model (98).

4.6. Theoretical models for predicting micellar size upon protein solubilization

A number of theoretical studies to predict the effect of protein solubilization on micellar size based upon the thermodynamic treatment of protein solubilization have appeared in the literature. Bratko et al.(101,102) have developed a thermodynamic model for protein solubilization in reverse micelles based upon the electrostatic contribution to the free energy of transfer of the protein from an aqueous solution into the microemulsion. Their model correctly predicts that low ionic strength and pH values below the isoelectric point (pI) of the protein increases uptake of the protein. The model predictions of the effect of salt concentration on protein uptake are in agreement with experimental observations (103). However, it assumes the simple shell and core model for protein solubilization, where the size of filled and unfilled micelles do not differ from each other irrespective of the pH and ionic strength in the micelles while evidence suggests that changes in ionic strength affect protein solubilization (99).

Casselli and coworkers (104) have developed a simplified thermodynamic model

for protein solubilization in water-in-oil microemulsions. Based on the minimization of free energy upon protein uptake coupled with a microcapacitor model for electrostatic interactions, they have been able to predict the hydrodynamic radii of filled and empty micelles. Their model predicts a significant increase in micellar size upon protein incorporation. Hydrodynamic radii are found to be affected by the initial R as well as the protein concentration in the reverse micelles. Recently they have refined their model to account for the effect of bulk salt concentration and pH on protein uptake by the micelles (105).

A recent study by Rahaman and Hatton (99) has criticized the model proposed by Casselli et al. (104,105). According to the model of Casselli, the radius of the filled micelle is equal to that of the unfilled micelle before protein uptake at complete occupancy. They also assume constant surfactant head coverage implying that the number of filled micelles is the same as the number of empty micelles prior to protein solubilization. Since every micelle is considered to contain one molecule of protein, the constraint of constant water volume in the system is violated. Rahaman and Hatton (99) have developed a thermodynamic model for predicting the hydrodynamic radii of protein-filled and empty reverse micelles as a function of salt concentration, net charge, size and concentration of protein as well as degree of hydration in the micellar phase for the phase-transfer and the injection methods of protein solubilization. The model identifies the free energy of protein-reverse micellar charge interactions as the major driving force for solubilization in micellar systems. Their model predictions agree well with the SANS data for solubilization of α -chymotrypsin in AOT/isooctane reverse micelles where the radius of the filled-micelle exceeds that of the unfilled-micelle. This model predicts that for the injection technique, an increase in the degree of hydration only increases the radius of the empty micelles while that of the filled micelles remain unchanged. Similarly, an increase in AOT concentration at constant water concentration would affect the radii of the empty rather than the filled-micelles. These predictions have important implications from the point of view of biocatalysis in reverse micelles. The change in catalytic activity with the degree of hydration could no longer be due to a change in the free and bound water present in the protein-containing micelle. Nevertheless, the authors say that the nature of the microenvironment of the enzyme in the filled-micelle could be affected by the rapid dynamic interchange between the filled and empty reverse micelles.

5. CONFORMATIONAL CHANGES IN ENZYMES UPON SOLUBILIZATION

It has been speculated that the difference in the catalytic activity of enzymes upon solubilization in reverse micelles is a result of the perturbation in the structure and orientation of the enzyme in the micellar matrix. The change in the nature of micellar water as compared to bulk aqueous medium could be responsible for intra-molecular hydrogen bonding in the macromolecule changing its conformation. Moreover, interactions of the protein with the surfactant layer could also be responsible for changes in the localization and structure of the enzyme in the micelles. The questions to be addressed here are: What is the effect of solubilization upon the conformation of the enzyme? How does the degree of hydration and surfactant concentration affect

enzyme structure? Does the localization of the enzyme in the micelles have any effect on its conformation? Is there any correlation between structural changes and enzymatic activity? The optical transparency of reverse micelles has made it possible to answer these questions through their characterization by several techniques such as ESR, circular dichroism, fluorescence and absorption spectroscopy. These techniques have been used to probe the conformation and localization of solubilized enzymes and attempts have been made to correlate these with the enzymatic activity. Such correlations would make it possible to design reverse micellar systems that can precisely control enzymatic activity.

Absorption spectroscopy, circular dichroism and fluorescence were the first techniques used (106-111) to visualize the conformational changes as well as alterations in the exposure of aromatic chromophores in the enzyme upon protein solubilization. The reader is referred to an excellent review in this regard (107). UV absorbance spectra of the solubilized protein revealed a tendency towards a red shift in the wavelength of maximum absorbance with respect to aqueous media (106-109). This phenomenon is due to the increased hydrophobicity of the microenvironment of the enzyme in reverse micelles. Fluorescence spectra of solubilized enzymes on the other hand, demonstrated a blue shift with respect to aqueous media (107,110). All these changes were found to be more pronounced at lower values of R and are a result of the increased hydrophobicity of the enzyme microenvironment as opposed to the aqueous solution. A recent study on the fluorescent properties of indole derivatives, lysozyme and azurin in AOT/n-hexane reverse micelles revealed that the fluorescent decay of the proteins was complex and strongly dependent on the degree of hydration of the microemulsion (111). Steady-state polarization measurements confirmed that the amount of water influences the conformational flexibility of the protein so that at high hydration ratios the properties approach that in bulk media (111).

Circular dichroism studies have also been widely and effectively used to monitor conformational changes in solubilized proteins (107). Two cases of significance that will be discussed are that of α -chymotrypsin and lysozyme. α -chymotrypsin is the most widely studied enzyme in reverse micelles. At certain conditions of R and surfactant concentration in AOT/isooctane reverse micelles, it exhibits the phenomenon of "superactivity" where its catalytic activity is increased several-fold compared to that in the aqueous solution. Interestingly, the CD spectra of the solubilized enzyme have revealed that at certain values of R where superactivity has been observed, there is a significant conformational difference in the enzyme as compared to that in the aqueous solution. On the other hand, the CD spectrum of another protease trypsin, which does not exhibit superactivity in reverse micelles shows that the secondary structure of the enzyme is relatively unperturbed in reverse micellar solution (112). Another interesting case in point is that of lysozyme (109,113). The CD spectrum of lysozyme in AOT/isooctane reverse micelles revealed a significant difference in its conformation as compared to that in aqueous solution indicating a denaturation of the enzyme in the microemulsion. Paradoxically, the enzyme retained its complete catalytic activity in reverse micelles. This paradox was resolved when it was observed that in the presence of the substrate, the denaturation process was absent and the enzyme retained its native conformation. This was also

confirmed by fluorescence and nmr studies. Myelin basic protein (MBP) is another protein whose structure in reverse micelles has been extensively investigated (114). Absorption spectroscopy, CD and time-resolved fluorescence anisotropy of MBP in reverse micelles of AOT/isooctane revealed that while insertion into microemulsions increased conformational rigidity, the hydration ratio and the surfactant concentration had virtually no effect on the structural characteristics of the enzyme. However, the nature of the surfactant affected the binding of the protein to the interface and affected its conformation.

The effect of the microenvironment of reverse micelles on the conformational mobility of solubilized enzymes has been investigated widely using electron spin resonance (ESR) spectroscopy. Belonogova and coworkers (115) used ESR spectroscopy to study the conformational rigidity of α -chymotrypsin in reverse micelles of AOT in octane. They observed that the degree of hydration had a significant impact on the conformation of the enzyme thereby affecting its catalytic activity. At optimum hydration ratios, the enzyme was frozen into a catalytically active conformation accounting for the phenomenon of superactivity. Cazianis and Xenakis (116) used hydrophilic and hydrophobic spin labels localized in different areas of the enzyme to monitor its structural variation. In AOT/isooctane microemulsions at low R, only the hydrophilic portion was preferentially buried in the aqueous core, while at high R, the entire enzyme was present in the aqueous core. The mobility of the spin labelled moiety of the enzyme depended on the hydrophobicity of the site to which the probe was bound and on the size of the microemulsion droplets. Clark et al. (117) investigated the structure-activity relationship for HLADH and tryptophanase in Brij 35/cyclohexane/hexanol microemulsions by ESR spectroscopy. They observed that for tryptophanase the optimum activity at a certain value of R was attributable to a structural change in the enzyme. Marzola et al. (118) have recorded ESR spectra of spin-labelled human serum albumin in AOT/isooctane reverse micelles. They observed that while an isotropic reorientational model could be successfully used for proteins in aqueous solution, an anisotropic model was necessary for micelle-incorporated proteins due to enzyme interactions with the AOT molecule. These structural changes monitored by ESR spectra were in agreement with circular dichroism and fluorescence studies.

Vos and coworkers have investigated the effect of protein interactions with the micellar layer on the structure and mobility of the enzyme (119). They conducted triplet-state kinetics on Zn-porphyrin cytochrome-c in aqueous solution and in reverse micelles. The triple-state decays of the protein in reverse micelles of AOT in octane were strongly affected, which was indicative of the fact that protein-surfactant interactions were responsible for conformational variations in the protein. However, there was not much change in the triple-state kinetics of the protein in CTAB microemulsions showing that the nature of the surfactant plays a very important role in determining the structure and hence catalytic activity of the enzyme. Similarly, time-resolved fluorescence and CD spectroscopy studies on porphyrin cytochrome-c and Zn-porphyrin cytochrome-c incorporated in reverse micelles confirmed that electrostatic interactions between the protein and the charged interface perturbed the native conformation of the enzyme molecule. CD and fluorescence studies on

cytochrome-c in AOT/isooctane reverse micelles revealed that even at high hydration ratios, the structure of cytochrome-c does not approach that in bulk solution, showing that the protein is located at the surfactant interface and the electrostatic interactions between the polar head groups of the surfactant and the protein cause a change in the secondary structure of the protein (120-122). Eremin and Metelitsa (120) propose that the hydrophobic amino acid residues of the enzyme are buried in the non-polar region of the alkyl chains in the surfactant. Time-resolved fluorescence anisotropy on HLADH in reverse micelles showed that while the protein structure is not significantly altered, strong electrostatic interactions between the protein and the AOT interface are responsible for the pH-dependent spectra of the protein. However, in CTAB reverse micelles, these interactions were much weaker than in AOT reverse micelles.

Battistel et al.(123) have investigated the thermodynamic parameters of the unfolding of ribonuclease, cytochrome-c and lysozyme as a function of composition in AOT/isooctane reverse micelles by differential scanning calorimetry (DSC). The thermograms for ribonuclease and cytochrome-c showed a single sharp transition corresponding to protein unfolding. For lysozyme, no such transition was observed indicating that the protein was denatured upon insertion into the micelles. The thermal stability of the enzymes was dependent on the degree of hydration of the microemulsions. Since the predominant contributions to the free energy of unfolding were entropic in nature, it was indicative of subtle conformational changes accompanying protein solubilization.

Nuclear magnetic resonance (NMR) of proteins and peptides has also been utilized as a technique to probe the interaction of the protein with the micellar wall in AOT/isooctane reverse micelles (124). It was found that while Met-enkephalin in reverse micelles adopts a folded conformation, pancreatic secretory trypsin inhibitor (PSTI) is flexible suggesting non-specific interaction with micelles. This is consistent with the fact that PSTI is not a membrane receptor. On the other hand, epidermal growth factor which binds to specific cellular surface receptors rearranges the conformation of its terminal fragment when contacted with the AOT micellar surface.

Gonnelli and Strambini (125,126) used tryptophan phosphorescence lifetime studies to probe the dynamic structure of hydrolytic enzymes alkaline phosphatase and HLADH in AOT/isooctane reverse micelles and correlated it to enzymatic activity. They found that the rigidity of alkaline phosphatase conformation observed in a certain range of R values was also accompanied by an increase in enzymatic activity. However, as micellar size increased further, the decrease in catalytic activity was not accompanied by any detectable change in the structure of the protein. They suggested that besides the structural perturbation of the protein caused by protein surfactant interactions, the altered activity of micellar water could itself be responsible for the altered reactivity of the hydrolytic reaction.

Levashov and coworkers (60,127) have also proposed that conformational changes in proteins solubilized in reverse micelles are responsible for the alterations in enzymatic activity as compared to that in aqueous solution. They observed that the optimum micellar size at which the enzyme exhibits optimum activity bears a very strong correlation to the dimensions of the micelle-incorporated protein. Moreover, they found that replacing the water in the aqueous core of reverse micelles with a

water/glycerin mixture increased the catalytic activity of γ -chymotrypsin in proportion to the percentage of glycerin in the mixture and was indirectly dependent on the viscosity of the micellar fluid. The maximum activity was observed when a 94% glycerin solution was present in the micellar core. The maximum catalytic activity also coincided with a minimum in the rotational frequency of the enzyme as observed by ESR spectroscopy. They interpreted this increase in catalytic activity on replacement of the water by glycerin as a result of the freezing of the enzyme in a catalytically active conformation (128). Another study conducted by the same group demonstrated that the increased structural rigidity of the enzyme in AOT/isooctane reverse micelles resulted in almost similar activity for both specific as well as non-specific substrates of the enzyme (129). This confirmed that incorporation of the enzyme in micelles of optimum size froze the enzyme in a catalytically active conformation. Moreover in the presence of the water-miscible solvent DMSO in CTAB reverse micelles, the increased catalytic activity of the enzyme was accompanied by a concomitant increase in the conformational rigidity of the enzyme observed by ESR spectroscopy (129,130).

These studies demonstrate the effects of micellar size, and nature and concentration of the surfactant on the enzyme structure and activity. This information could permit the design of reverse micelles for most efficient catalytic conversion.

6. CATALYTIC PROPERTIES OF ENZYMES IN REVERSE MICELLES

Several studies have shown that enzymes solubilized in reverse micelles exhibit classical Michaelis-Menten kinetics. Nevertheless, the catalytic properties of enzymes in reverse micelles differ from that in bulk aqueous media. The effect of variables such as the degree of hydration (R) and micellar size, micellar pH, surfactant concentration and ionic strength of the buffer solution on the substrate specificity and catalytic properties of the enzymes will be discussed in the following section.

6.1. Effect of the degree of hydration (R)

One of the most striking effects of the incorporation of enzymes into reverse micelles on their catalytic activity is that of the degree of hydration (R). R is the number of water molecules per surfactant molecule in the micelle and is directly correlated to the size of the reverse micelle (76). For a number of enzymes, the catalytic activity varies in a "bell-shaped" manner with R and at a certain value of R, the enzyme displays maximum activity. This is not universal and a monotonic increasing or monotonic decreasing dependence on R has also been reported (130-140). For most enzymes this value of the maximum activity at optimum R is of the same order of magnitude as that in bulk aqueous solution. However, certain enzymes such as α -chymotrypsin (7), peroxidase (85), laccase (140) and acid phosphatase (141) exhibit the phenomenon of "superactivity", where the micellar activity is increased several-fold compared to that in the aqueous solution.

A very interesting effect of the degree of hydration on the enzymatic catalytic activity arises in the case of oligomeric enzymes such as γ -glutamyl transferase (142) and lactate dehydrogenase (143). Unlike the single maximum in catalytic activity that is observed as a function of R for monomeric enzymes, several maxima were observed

for lactate dehydrogenase each corresponding to the monomer, dimer, trimer/tetramer and octamer of the enzyme. Similarly for g-glutamyl transferase, two optima were observed corresponding to the two subunits of molecular weight 20,000 and 53,000 Daltons respectively. The presence of the different subunits in the reverse micelles was confirmed by ultracentrifugation which made it possible to separate the micellar solution into the light and heavy subunits of the enzymes.

Several theoretical models have been formulated to explain this dependence of enzymatic activity on R. The basis of the models includes intermicellar enzymatic exchange (143), enhanced local substrate concentrations near the enzyme at optimum R in charged ionic reverse micelles (145-147), enzyme partitioning between the pseudophases of the micellar system (134,135) and diffusion theory (148-151).

Kabanov et al.(144) have proposed a model to account for the variation of enzymatic activity with R by considering the following factors: a well-defined optimal micellar radius exists for every enzyme at which it displays maximal activity, the polydispersity of the micelles, the collisions between the enzyme-filled and empty micelles resulting in inter-micellar enzyme transfer and inactivation of enzyme molecules in micelles of low-water content. Though the bell-shaped dependence on R could be explained by their model, it involves the use of several parameters which need to be experimentally determined.

A simple theoretical model which attempts to explain the effect of R on the enzymatic catalytic activity in reverse micelles is that of Bru and coworkers (134,135). They propose that the enzyme is partitioned between the free-water, bound-water and surfactant tails of the micelle. The activity of the enzyme is determined by its immediate microenvironment and values of catalytic rate constants are assigned to the enzyme depending on its localization. Though this approach attempts to quantify effects of enzyme microenvironment on its activity, it involves arbitrarily assigned parameters that cannot be individually ascertained.

An interesting treatment has been proposed by Ruckenstein and Karpe (145-147) to explain the effect of hydration ratio on catalytic activity of enzymes and superactivity in ionic reverse micelles on the basis of the electrical double layer theory. They have developed this model for the specific case of α -chymotrypsin catalyzed hydrolysis of N-glutaryl-L-phenylalanine p-nitroanilide (GPNA) in reverse micelles of AOT/isooctane. The model involves a substrate mass balance governing the partitioning of the substrate GPNA between the water-pool and the interface in conjunction with the electrical potential set up by the charge on the solubilized enzyme and the polar head groups of the surfactant. The latter has been determined with the use of the Poisson-Boltzman equation. Based on this they found that at optimum R, the substrate concentration in the immediate vicinity of the enzyme is much greater than the overall substrate concentration. This increase in the substrate concentration results from the repulsive interactions between the charged substrate and the charged surfactant head groups which pushes the substrate toward the enzyme surface. The absolute value of the charge on the surfactant layer increases with R increasing the local concentration of the substrate near the enzyme. However, as R increases, the increased distance between the enzyme and the micellar layer and the increased charge of the surfactant layer act in opposite directions, and are responsible for the optimum

in catalytic activity with R . The authors propose that the consequent superactivity of the enzyme is due to the enhanced substrate concentrations in the vicinity of the enzyme at optimum R . The calculations have also been recently modified by accounting for the role of the dielectric constant of the water-pool on enzymatic activity and improving the agreement between experimental values and model predictions.

Maestro, Bianucci and Walde (148,151) have proposed a model based on diffusion theory to explain the bell-shaped dependence of enzymatic activity on R in the case of hydrolytic reactions. The cornerstone of the model is the inter-micellar exchange of the substrate caused by collisions between enzyme-filled and unfilled micelles. The model also takes into account the intra-micellar diffusion of the substrate from the micellar wall to the enzyme surface which is described by a second-order differential equation. The authors contend that the opposite dependencies of the inter-micellar and the intra-micellar diffusion processes cause the bell-shaped dependence on R .

Another model that aims to account for the dependence of enzymatic catalytic activity on R in the micelles based on inter-micellar exchange is that of Verhaert and coworkers (149,152). They propose that enzymatic biocatalysis in reverse micelles occurs in two steps: diffusion and collision of substrate and enzyme filled micelles resulting in exchange of micellar contents followed by enzyme-substrate reaction and conversion. They have utilized this approach to describe the kinetics of 20- β hydroxysteroid dehydrogenase and enoate reductase in reverse micelles (153,154). However, the model contains several adjustable parameters which represent the rate of inter-micellar exchange of contents that have been fitted to experimental data. A third paper which uses diffusion and dynamic inter-micellar exchange between substrate-filled and enzyme-filled micelles to account for dependence of catalytic activity on micellar size is that of Oldfield (150). Though these diffusion based theories of reversed micellar kinetics of enzymatic reactions appear attractive, their estimates of the time scales involved in the inter-micellar exchange processes indicate that these models are inadequate to explain the commonly studied slow, enzymatic reactions but are only suited for fast reactions such enzyme catalyzed photosynthetic reactions.

Another school of thought attributes this unique dependence of the biocatalytic activity on R to the conformational rigidity of the enzyme at the optimal R or the optimal size of the micelle. It is believed that this lack of conformational mobility of the enzyme forces the enzyme to acquire a catalytically favorable structure resulting in high catalytic activity. Evidence in support of this theory will be discussed in a later section.

6.2. Effect of pH

Similar to the pH-dependent behavior of enzymatic activity in aqueous solution, the catalytic behavior of enzymes and consequently the kinetic parameters of enzymatic reactions are profoundly affected by the pH of the buffer solution inside the core of the micelle. Generally, for charged surfactants the micellar pH is not the same as that of the buffer solution used to form the micelles. However, the concept of the

effective pH of the aqueous core experienced by enzymes in reverse micelles is still not clear because of the relatively few water molecules per reverse micelle.

As a result of the difference in the micellar pH compared to that in bulk aqueous solution, catalytic studies on enzymes solubilized in ionic reverse micelles have revealed marked shifts in the pH profile compared to that in aqueous solution depending on the type of surfactant used (7,35,82,106). Although in the case of non-ionic surfactants there was no change in the optimal pH, the use of charged surfactants such as AOT have caused a noticeable shift of about 1-2 units in the alkaline direction in the pH profile of the solubilized enzyme. This shift in pH was found to be more pronounced at low values of R. Several explanations have been proposed to account for this phenomenon. Firstly, the use of charged, ionogenic surfactants could cause the formation of an electrical double layer around the enzyme and changes the local concentration of the hydrogen ions in the aqueous core of the micelle from that in the stock buffer solution used for enzyme preparation. This is analogous to the pH effect observed for enzymes immobilized in charged polyelectrolyte matrices (155). Secondly, it is possible that the incorporation of the enzyme in the reverse micelle could cause a change in the acid-base properties of the ionogenic groups of the enzyme itself resulting in a changed pH optimum for the enzyme. Lastly, changes in the conformation of the solubilized enzyme could also alter the pKa of the ionogenic groups in the enzyme resulting in a change in its pH-dependent catalytic activity.

Smith and Luisi (156) used $^{31}\text{P}_{\text{nmr}}$ chemical shifts to determine the pH of the water-pool in AOT micellar solutions and found the pH of the micellar core to be within 0.4 pH units of the bulk aqueous solution used to prepare the reverse micelles. However, their procedure measured a pH that was an average of the free and bound micellar water. The alkaline shift in the pH profile of enzymes solubilized in anionic reverse micelles has been attributed to the acidic impurities in the surfactant AOT (157).

Ruckenstein and coworkers (145-147,158) invoked the electrical double layer theory with the Poisson-Boltzmann equation to estimate the electrostatic potential in ionic reverse micelles at different hydration ratios R. They theorized that the pH near the enzyme surface for enzymes solubilized in AOT reverse micelles was a function of R and the method of solubilization of the reverse micelles. For the injection method of solubilization of enzymes in AOT reverse micelles, the pH near the enzyme surface was found to be more alkaline than that in bulk aqueous solution. This is consistent with recent studies on enzymatic kinetics in AOT reverse micelles using purified surfactant samples where an acidic shift in the pH profile has been observed for the enzymatic activity (58,159).

6.3. Effect of surfactant concentration

A change in the concentration of the surfactant used for the stabilization of reverse micelles alters either the size and/or the micellar concentration. If the surfactant concentration is varied at a constant volume fraction of the water in the micelles, it results in a decrease in the micellar size. On the other hand, an increase in the surfactant concentration at a constant degree of hydration R, results in an

increase in the micellar concentration without any alteration in the structure of micelles. Figure 7 is a schematic representation of the interplay of water and surfactant concentration on the micellar dimensions and concentration (160).

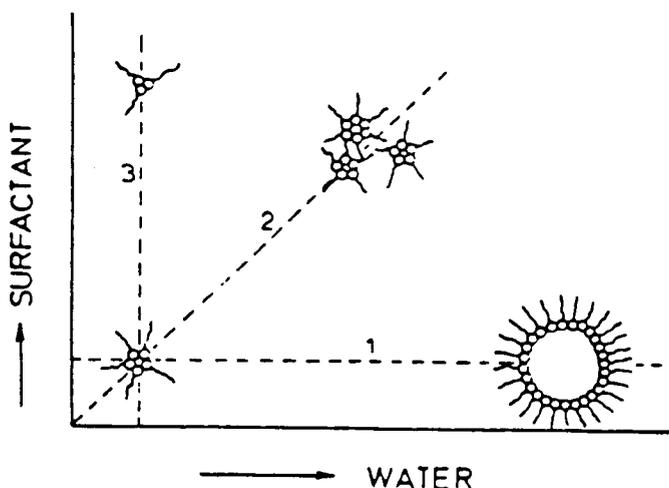


Figure 7. Effect of water and surfactant concentration on number and size of reverse micelles (160). (1) surfactant concentration constant, water increasing, (2) surfactant and water concentrations both increasing, (3) water constant, surfactant concentration increasing.

Since the micellar properties remain unaffected by a change in surfactant concentration at a constant degree of hydration, one expects that the catalytic properties of the enzyme remain unaltered in such a case. This is indeed true for a number of enzymes such as trypsin (35) and alkaline phosphatase (161) where the pseudo first-order rate constant k_{cat} is not affected by the surfactant concentration at constant R . However, for some enzymes such as peroxidase (85,137), laccase (139,140) and acid phosphatase (141), the catalytic activity is a very strong function of the surfactant concentration and the value of k_{cat} can be reduced by as much as two orders of magnitude by an increase in the surfactant concentration at constant R . It was observed that the highest value of k_{cat} obtained at surfactant concentration extrapolated to zero was independent of the nature of the surfactant and was an intrinsic property of the enzyme itself. Interestingly, this latter group of enzymes is also characterized by the presence of hydrophobic "anchoring groups" that are capable

of interaction with the micellar membrane. It is believed that this interaction is responsible for the observed effect of surfactant concentration on enzyme activity. A confirmation of this theory was obtained from the study of the effect of AOT concentration on the activity of native and stearylated α -chymotrypsin. While the activity of native α -chymotrypsin was not influenced by surfactant concentration, the stearylated enzyme (which has "anchoring groups" capable of membrane interaction) exhibited a marked reduction in its activity with surfactant concentration. Similar results were obtained for soluble and membrane forms of γ -glutamyltransferase and aminopeptidase demonstrating that catalytic dependence on surfactant concentration is a test for membrane activity of the enzyme (162).

The partitioning of the substrate into the surfactant layer as well as the inhibition of the enzyme by the surfactant have also been suggested as possible reasons for the effect of surfactant concentration on enzymatic activity. Schoemaeker et al. (163) have conducted a detailed study of the interaction of two enzymes lipase and α -chymotrypsin with surfactants in aqueous solution and correlated this behavior with that of the same enzymes in reverse micelles. They observed that in aqueous solution, α -chymotrypsin was competitively inhibited by AOT at or around the critical micelle concentration. Similar behavior was also observed in AOT-isooctane reverse micelles, where the pseudo first-order rate constant k_{cat} was unchanged on incorporation into reverse micelles but the Michaelis constant K_m was increased by a factor of 100. It was believed that AOT behaves as a non-competitive inhibitor of α -chymotrypsin. However, studies conducted on the partitioning of α -chymotrypsin between bulk aqueous solution and the microemulsion revealed that it was the partitioning of the substrate GPNA in the surfactant layer and not the inhibition of the enzyme that played the major role in causing the increased value of K_m in AOT reverse micelles (164). Though competitive inhibition was observed for lipase in aqueous solution containing SDS and CTAB and non-competitive inhibition in aqueous solutions containing AOT, there was no effect of surfactant on the enzyme activity in reverse micelles.

O'Connor and Walde (165) investigated the effect of AOT concentration on the catalytic rate constants of esterase activity of human milk lipase and 4-nitrophenyl propionate as substrate. They observed that with increasing surfactant concentration there was a decrease in k_{cat} and an increase in K_m of the enzymatic reaction in reverse micelles. The authors interpret this behavior as mixed inhibition by the surfactant.

The enzyme acid phosphatase, which exhibits superactivity in AOT-isooctane reverse micelles has also been reported to be non-competitively inhibited by the surfactant AOT (141). It is believed that the sulfo group, which is contained in the polar portion of the AOT molecule is capable of blocking the active site of the enzyme.

Recently, in our laboratory we have investigated (58,138) the effect of surfactant concentration on the catalytic activity of cholesterol oxidase in AOT/isooctane reverse micelles. We observed a decrease in k_{cat} and an increase in K_m with surfactant concentration in the micelles. Models which took into consideration the partitioning of the substrate cholesterol between the water core, interface and organic continuous phase of the reverse micellar system satisfactorily explained the increase in K_m with AOT concentration. However, the decrease in k_{cat} with AOT concentration in reverse

micelles was due to non-competitive inhibition of the enzyme by AOT. It was found that the maximum value of k_{cat} at surfactant concentrations extrapolated to zero was the same as the value for the enzyme in aqueous solution. These observations were consistent with the fact that cholesterol oxidase has an amphipathic character with a hydrophobic anchor region connected to a hydrophilic active site linked together by a proteinase sensitive segment. It is this hydrophobic region that is capable of binding to the surfactant layer of detergent micelles. These studies which demonstrate that the surfactant is not a passive component of the reaction mixture but may be actively involved in the enzymatic reaction are very significant in the enzymatic biocatalysis in reverse micelles.

6.4. Change in substrate specificity

In addition to the changes in magnitude of the catalytic constants of enzymes solubilized in reverse micelles, there could also be changes in the substrate specificity of the enzymes as compared to aqueous solution. This phenomenon is exemplified by the behavior of alcohol dehydrogenase in reverse micelles (166). Though in water, octanol is the best substrate for alcohol dehydrogenase, it has been observed that in reverse micelles, alcohol dehydrogenase catalyzes the dehydrogenation of butanol much more rapidly than octanol. This can be explained on the basis of the fact that more hydrophobic long chain alcohols such as octanol partition to a greater extent into the organic solvent than in the aqueous core. Therefore, the local concentration of octanol in the micelles is lower than that of butanol in the vicinity of the enzyme. This change in the Michaelis-Menten constant K_m is responsible for the change in the substrate specificity of the enzyme. Using a pseudophase substrate partitioning approach, Khmel'nitsky et al. (168) obtained the true value of K_m for the alcohol substrates in the micellar medium and showed that this was the same as that in aqueous medium. This confirmed that the change in substrate specificity for alcohol dehydrogenase in reverse micelles is indeed an artifact observed as a result of substrate partitioning between the various phases of the micellar system. Pancreatic lipase, in contrast, exhibits a genuine change in substrate specificity. Though in aqueous solution it catalyzes the hydrolysis of all triacylglycerols at the same rate (169), in AOT/isooctane reverse micelles it hydrolyses triolein 15 times more rapidly than tributyrin (170).

6.5. Retention of enzymatic activity in reverse micelles

Enzymes in reverse micelles are stable over appreciable lengths of time. At optimum values of R the enzyme is retained in its active conformation allowing the catalytic activity to be appreciably preserved, even more so than in aqueous medium (171,172). However, the stability of the enzyme in the micellar system is a strong function of the nature of the surfactant, the pH and R. For example, trypsin in reversed micelles retained its activity for periods ranging from a few minutes to a week depending upon the type of substrate, buffer and degree of hydration (173-175). Whereas some studies show that α -chymotrypsin degrades more rapidly in reverse micelles than in aqueous solution (95), Levashov and coworkers (129) have reported that the same enzyme remains stable for two years in micellar solutions.

7. KINETICS OF ENZYMATIC REACTIONS IN REVERSE MICELLES

7.1. Michaelis-Menten kinetics for enzymatic reactions

Initial rate kinetics of enzyme-catalyzed reactions follow a substrate-saturation mechanism, well-known in the literature as the Michaelis-Menten kinetics. For the enzymatic reaction



where E, S, ES and P represent the enzyme, the substrate, the enzyme/substrate complex and the product respectively, the various phenomenological rate constants are denoted by k_+ , k_- and k_{cat} . Using the quasi steady-state approximation, the initial rate kinetics is represented by

$$v = \frac{dS}{dt} = \frac{v_{max} S}{K_m + S} = \frac{k_{cat} E_o S}{K_m + S} \quad (6)$$

where $K_m = (k_- + k_{cat})/k_+$, is the steady-state dissociation constant of the enzyme-substrate complex, also known as the Michaelis constant, v is the reaction velocity and $v_{max} = k_{cat} E_o$ is the maximum velocity attained at saturating substrate concentrations. E_o refers to the total concentration of the enzyme within the system, i.e. the sum of the free enzyme, E and that bound to the substrate, ES. Linearizing the Michaelis-Menten equation gives

$$\frac{1}{v} = \frac{1}{k_{cat} E_o} + \frac{K_m}{k_{cat} E_o S} \quad (7)$$

A plot of $1/v$ versus $1/S$, called the Lineweaver-Burke plot, enables one to obtain the Michaelis-Menten constant K_m and the rate constant k_{cat} from the slope and the intercept.

7.2 Kinetic model for enzymatic reactions in reverse micelles

The encapsulation of enzymes in the reverse micelles causes a difference in their kinetic behavior compared to that in the aqueous medium (60). This is due to several reasons. Firstly, the interaction between the enzyme and the substrate takes place in a different environment than that existing in the aqueous solution. This will modify the values of k_{cat} and K_m , with the experimentally observed values being denoted as $k_{cat,app}$ and $K_{m,app}$. Secondly, the substrate can partition between the various microdomains of the reverse micelles so that the local concentration of the substrate in the vicinity of the enzyme differs from the overall experimentally measurable concentration of the substrate. Further, the reaction in the microheterogeneous medium could be controlled by diffusional or molecular exchange processes. Lastly, there could be conformational changes in the encapsulated enzyme resulting in modified values for k_{cat} and K_m .

Martinek and coworkers (173) developed a kinetic model, referred to hereafter as the "Biphasic Model", on the basis of the following simplifying assumptions: (i) The enzyme is present solely in the water-pool of the reverse micelles; (ii) The reaction takes place entirely within the water-pool; (iii) The substrate is partitioned between the organic continuous phase and the water-pool (hence, "Biphasic"). Using these assumptions, they developed quantitative relationships between the apparent and the true Michaelis-Menten kinetic constants. Since the enzyme is present entirely within the water-pool, an enzyme mass balance gives

$$E_{o,t} = E_{o,wp} \theta_{wp} \quad (8)$$

Since the substrate is partitioned between the organic continuous phase and the water-pool, a substrate material balance gives

$$S_{o,t} = S_{o,wp} \theta_{wp} + S_{o,org} \theta_{org} \quad (9)$$

In the above relations, the subscripts "t", "org" and "wp" refer to the total or overall system, the organic continuous phase and the water-pool of the reverse micelle, respectively. θ_{org} and θ_{wp} are the volume fractions of the organic continuous phase and the water-pool in the overall system, respectively. The overall or experimentally measurable reaction velocity v_t based on the total system concentrations, is written in analogy with eq.(6) as

$$v_t = \frac{k_{cat,app} E_{o,t} S_{o,t}}{K_{m,app} + S_{o,t}} \quad (10)$$

Introducing eq.(8) and (9) in eq.(10), we can write

$$v_t = \frac{k_{cat,app} [E_{o,wp} \theta_{wp}] [S_{o,wp} \theta_{wp} + S_{o,org} \theta_{org}]}{K_{m,app} + [S_{o,wp} \theta_{wp} + S_{o,org} \theta_{org}]} \quad (11)$$

Since the reaction is confined to the water-pool, the overall reaction velocity can be related to the reaction velocity in the water-pool. One gets,

$$v_t = v_{wp} \theta_{wp} = \frac{k_{cat,wp} E_{o,wp} S_{o,wp}}{K_{m,wp} + S_{o,wp}} \theta_{wp} \quad (12)$$

Here v_{wp} is the reaction velocity in the water-pool of the reverse micelle and is written analogous to eq.(6) but in terms of the concentrations prevailing in the water-pool. A comparison of eq.(11) and (12) allows the apparent kinetic constants to be related to the kinetic constants in the water-pool as follows:

$$k_{cat,app} = k_{cat,wp} \quad (13)$$

$$\frac{K_{m,app}}{\theta_{wp} + P_{org} \theta_{org}} = K_{m,wp} \quad (14)$$

where, P_{org} is the partition coefficient of the substrate between the organic continuous phase and the water-pool, given by

$$P_{org} = \frac{S_{o,org}}{S_{o,wp}} \quad (15)$$

When the substrate is much more soluble in the organic phase than in water, $P_{org} \gg 1$, and eq.(14) simplifies to

$$K_{m,app} = K_{m,wp} P_{org} \theta_{org} \quad (16)$$

At the other limit, when the substrate is much more soluble in water than in the organic phase, $P_{org} \ll 1$, hence

$$K_{m,app} = K_{m,wp} \theta_{wp} \quad (17)$$

The original Biphasic model proposed as above has some limitations. Several studies on the kinetics of enzymatic reactions in reverse micelles have shown that $k_{cat,app}$ is seldom equal to $k_{cat,wp}$. Moreover, this model is not able to account for the dependence of the apparent rate constants on the surfactant concentration, which is an important variable in the formation of reverse micelles.

Several suggestions have since been made to improve upon the predictions of the Biphasic model. A simple extension of the Biphasic model is based on the suggestion that the interface composed of the surfactant polar groups and hydrophobic tails plays a significant role in determining the enzymatic reaction kinetics in reverse micelles (134,135,168,176). The reverse micellar system is now considered to be multiphasic with the free-water present in the aqueous core, bound-water entrapped in the surfactant polar heads groups, surfactant tails and the rest of the system constituted of the organic continuous phase. Not only the substrate, but also the enzyme is believed to partition into the multiple phases of the micellar system. In contrast to these phenomenological kinetic approaches, a more fundamental treatment of the enzyme kinetics in ionic reverse micelles based on a detailed analysis of the electrical double layers has been developed by Ruckenstein and Karpe (145-147).

7.3 Modified kinetic model for enzymatic reactions in reverse micelles

The Biphasic model has recently been modified by us (138) based on the following three considerations. Firstly, the reverse micellar system is considered to be composed of three pseudophases: a lumped phase called the micellar core which includes both the free-water and the bound-water layers entrapped into the surfactant head groups, the interfacial phase which is constituted of surfactant tails and the organic solvent continuous phase. Secondly, it is assumed that the enzyme is not

completely present in the micellar core but is also partitioned into the interface as an enzyme-AOT complex. Thirdly, since the active site present in the hydrophilic domain of the enzyme is entirely in the water core, the reaction takes place only in the water-pool. Thus, although the enzyme and the substrate are both present in the interface, no reaction takes place in the interface since the active site is not conformationally accessible to the substrate in this region.

Based on these assumptions, the following relationships between the known Michaelis-Menten kinetic constants in the water pool and the experimentally measured (or apparent) kinetic constants in the reverse micelles can be arrived at. Below, the subscript "wp" refers to the micellar core, "I" refers to the micellar interface and "org" refers to the organic continuous phase while θ refers to the volume fractions of various domains in the reverse micellar system. Since the enzyme is present both in the micellar core and the interface,

$$E_{o,t} = E_{o,wp} \theta_{wp} + E_{o,I} \theta_I \quad (18)$$

From a substrate mass balance

$$S_{o,t} = S_{o,wp} \theta_{wp} + S_{o,I} \theta_I + S_{o,org} \theta_{org} \quad (19)$$

The overall reaction velocity can be written as before in terms of the apparent kinetic constants,

$$v_t = \frac{k_{cat,app} E_{o,t} S_{o,t}}{K_{m,app} + S_{o,t}} \quad (20)$$

Introducing eq.(18) and (19) in eq.(20), we get

$$v_t = \frac{k_{cat,app} [E_{o,wp} \theta_{wp} + E_{o,I} \theta_I] [S_{o,wp} \theta_{wp} + S_{o,I} \theta_I + S_{o,org} \theta_{org}]}{K_{m,app} + [S_{o,wp} \theta_{wp} + S_{o,I} \theta_I + S_{o,org} \theta_{org}]} \quad (21)$$

Since the reaction is assumed to be confined to the water-pool, we can also write

$$v_t = v_{wp} \theta_{wp} = \frac{k_{cat,wp} E_{o,wp} S_{o,wp}}{K_{m,wp} + S_{o,wp}} \theta_{wp} \quad (22)$$

where v_{wp} is the reaction velocity in the aqueous core of the micelle. Comparing eq.(21) and (22), the apparent and true reaction rate constants are related as follows:

$$k_{cat,app} = \frac{k_{cat,wp}}{1 + (E_{o,I} \theta_I) / (E_{o,wp} \theta_{wp})} = \frac{k_{cat,wp}}{1 + p [AOT]} \quad (23)$$

If the molar ratio R of water to surfactant is maintained constant at different surfactant concentrations, the ratio of the volume fraction of the interface to that of the water-pool is a constant. Since the partitioning of the enzyme into the interfacial region is

assumed to occur as a consequence of interactions with the surfactant, the concentration of the enzyme in the interfacial region can be taken proportional to the total concentration of AOT in the system. Thus, the second equality in eq.(23) is written with p as a constant of proportionality. The effect of surfactant concentration on $k_{cat,app}$ thus represents the active interaction of the surfactant AOT with the enzyme present in the interface.

The true and apparent values of the Michaelis constant K_m are related as

$$\frac{K_{m,app}}{\theta_{wp} + P_I \theta_I + P_{org} \theta_{org}} = K_{m,wp} \quad (24)$$

Here, P_I is the partition coefficient of the substrate between the interface and the water pool.

$$P_I = \frac{S_{o,I}}{S_{o,wp}} \quad (25)$$

while P_{org} has already been defined by eq.(15). Eq.(24) reveals how the value of $K_{m,app}$ depends on the surfactant concentration through the variable θ_I . This modified model has been shown to describe well the kinetics of cholesterol oxidation by cholesterol oxidase in AOT reverse micelles (138).

7.4 Enzyme recovery from reverse micelles

Enzymatic biocatalysis in reverse micelles cannot become an economically feasible proposition unless the enzyme can be recovered for reuse at the end of the catalytic cycle. Several strategies have been suggested to overcome this bottleneck in the commercialization of reverse micellar enzymology.

The first strategy involves the contacting of the enzyme-laden reverse micellar phase with bulk aqueous buffer. Depending on the pH of the buffer, the pI of the enzyme, the temperature and the ionic strength of the buffer, it is possible to back extract the protein from the reverse micellar phase into the buffer for reuse (164,177-179).

Another method that could be employed is the use of microporous membrane reactors which could retain the reverse micelles containing the enzyme within the reactor while allowing the reaction medium containing the product to flow through (49,180). However, the success of such a strategy would necessitate the reverse micelles to be resistant to the shear forces characteristic of membrane devices. Recently, Leser and coworkers (Personal communication) have proposed the utilization of silica particles to back extract the enzyme from reverse micelles. The methodology involves adsorption of the solubilized protein to silica powder added to the micelles. Subsequently, the protein is recovered from the silica into buffered aqueous solution. The procedure was used for AOT and lecithin micellar solutions and found to more effective than extraction of the protein with salt solution.

Still another procedure involves the use of microemulsion based solid phases containing the entrapped enzyme which exist in equilibrium with the organic solvent.

This permits easy separation of the gel entrapped enzyme for re-use and could easily facilitate continuous operation. Examples of this are organogels (181), polyacrylamide entrapped enzymes in microemulsion (182) and lyotropic liquid crystals (183). However, all these strategies are accompanied by mass transfer limitations.

Recently, a novel strategy for enzyme recovery has been suggested that involves temperature-induced phase changes in the microemulsions (184). Small changes in temperature make it possible to shift the microheterogeneous phase into two phases in equilibrium with each other: a microemulsion phase containing enzyme and surfactant and an oil phase containing the product. The oil phase containing the product can then be separated from the enzymatic catalyst. Fresh solvent containing substrate can be added to the microemulsion and the temperature readjusted so that a single microemulsion phase is obtained. Another effective strategy has been proposed by Carlson and Nagarajan (84) who demonstrated that the addition of a small amount of isopropyl alcohol to reverse micelles containing enzymes results in an almost quantitative expulsion of solubilized protein from the micelle, without destroying the integrity of the reverse micellar structure.

8. ENZYME CATALYSIS IN REVERSE MICELLES: APPLICATIONS TO BIOTECHNOLOGY

The use of enzyme-catalyzed reactions in reverse micelles is still in its infancy and much work needs to be done before commercialization of this process is possible. However, this section will focus on active research that has been and is being conducted toward that end. Enzyme catalyzed processes are chiefly used in the food and pharmaceutical industry and also in fine organic synthesis (2, 3). All these aspects will be individually considered in this section.

8.1. Applications of reverse micellar enzymology in the food industry

The commercial use of enzymes has been mainly in the food industry. Immobilization of enzymes on solid supports which was the first alternative to using soluble enzymes in bulk aqueous media was first commercialized in the food industry. The most interesting aspect of micellar enzymology in the food industry is the use of lipase-catalyzed reactions in reverse micelles. Lipases are enzymes that hydrolyze a variety of fats and oils as well as synthetic mono-, di- and triglycerides. Hydrolysis of glycerol esters yields free fatty acids and glycerol. Since by nature, lipases are enzymes that act upon water-insoluble substrates and require the presence of an oil-water interface for expression of their enzymatic activity, the use of reverse micelles greatly facilitates the use of lipases. The substrates which have to be emulsified prior to enzymatic hydrolysis, can be solubilized to a large extent in the oil-continuous phase while the enzyme is hosted in the reverse micellar phase. The enormous interfacial area that is possible in reverse micelles tremendously improves enzymatic activity. Since the substrate is mainly concentrated in the oil-continuous phase, substrate inhibition problems are mitigated.

The first example of lipase-catalyzed hydrolysis in reverse micelles was provided by the work of Malakhova et al.(170). Since then, Han and Rhee (185-189) have

conducted a detailed study of the kinetics of the hydrolysis of the substrate olive oil by lipase in reverse micelles composed of AOT in isooctane. No substrate inhibition was detected upto a concentration of 40% (v/v). Complete hydrolysis and substantial increase in volumetric productivity was achieved when compared to bulk aqueous phase as the reaction medium. The initial activity of the enzyme in reverse micelles was strongly dependent on the degree of hydration of the micelles and was optimum at $R = 10$. The temperature and pH profiles of the enzyme activity were similar to that in buffer solution. The stability of the enzyme was also a function of R , and at $R = 5$, the enzyme was almost as stable as that in aqueous solution. While hydrolytic reactions in water are usually considered to be of pseudo first-order, the low-water environment in reverse micelles necessitated the reaction to be modelled as a two-substrate reaction. Equilibrium fractional conversion was a function of the initial water concentration and was shifted to higher values at high water concentrations. Similar results were obtained by Kim and Kiomin (190) who investigated lipase-catalyzed hydrolysis of palm kernel olein in AOT/isooctane reverse micelles. No substrate inhibition was observed upto a concentration of 20% (w/v) of the substrate. This was in contrast to the behavior in macroemulsions where substrate inhibition could be detected at 3-5% (w/v) of the substrate.

Fletcher et al.(191) investigated the kinetics of lipase catalyzed hydrolysis of nitrophenyl alkanolate esters in AOT/heptane reverse micelles. Though the intrinsic activity of the enzyme was similar to that in water, substrate partitioning into the oil phase reduced the conversion. The enzyme was fairly stable in the microemulsion and retained 60% of its activity at 35°C for 6 days. However, the use of microemulsions based on CTAB/heptane/chloroform caused a drastic reduction in enzymatic activity due to inhibition of the enzyme by the surfactant. Similar behavior was observed for the esterase activity of human milk lipase in AOT/isooctane microemulsions where the surfactant AOT reversibly inhibited the enzyme activity. Holmberg and Osterberg (192-193) obtained monoglycerides in 80% yield by the lipase-catalyzed hydrolysis of triglycerides in AOT/hydrocarbon water-in-oil microemulsions. The monoglyceride was subsequently hydrolyzed to glycerol and fatty acids. Using a 1,3 specific lipase, both glycerolysis and hydrolysis of a triglyceride in AOT/isooctane could be achieved. The molar ratio R was critical in determining the amount of monoglycerides and fatty acids obtained as a result of the reaction. However, the type of lipase used did not influence the ratio between glycerolysis and hydrolysis (194).

Another useful application of lipases in the food industry involves transesterification of fatty acid esters and glyceride synthesis. Esters of fatty acids are important flavor and aroma components in the food industry. Lipase-catalyzed synthesis reactions necessitate the use of low-water environments so as to hinder the reverse hydrolytic reaction from taking place. Reverse micelles have been shown to be particularly useful in this regard.

Holmberg and Osterberg (192) demonstrated that microemulsions composed of the anionic surfactant AOT in hydrocarbon solvents as well as non-ionic surfactants such as triethylene glycol monodecyl ether in hydrocarbon could be used for the lipase-catalyzed inter-esterification of triglycerides and fatty acids. The reaction rate in the non-ionic surfactant microemulsion was higher than that in the AOT reverse

micelles. Moreover, addition of water to the reverse micelles composed of the non-ionic surfactant resulted in phase-splitting into an aqueous phase containing the enzyme while the surfactant was concentrated in the organic solvent phase permitting facile enzyme recovery. Similarly Bello et al.(195) studied the lipase-catalyzed inter-esterification in a quaternary system composed of triglyceride and fatty acids, water, surfactant Brij 35 and alcohol. As the water activity was reduced, inter-esterification was favored over hydrolysis. At optimum conditions, 40% conversion could be achieved for the inter-esterification reaction. Abraham et al.(196) observed that in reverse micelles composed of AOT in hexane, triacetin-tributyryn inter-esterification was favored over hydrolysis as the degree of hydration of the micelles was lowered. Hayes and Gulari (197) conducted a detailed kinetic study of lipase-catalyzed inter-esterification in AOT/isooctane reversed micelles. They observed that while the pH profile was unchanged, the ratio of water-to-surfactant had a profound effect on activity as well as stability of the enzyme.

Lipase-catalyzed synthesis of triglycerides has also been found to be favorable in reversed micelles. Morita et al.(59) conducted the synthesis of triacylglycerol by lipase in phosphatidylcholine reverse micelles in n-hexane. Though in other media, 1,2 diacylglycerol was hydrolyzed to 2-monoacyl glycerol, triacyl glycerol synthesis took place in reverse micelles. The initial activity of the synthesis was optimum at $R = 10$. Lauric, myristic, palmitic, stearic, oleic and arachidic acids were found to be effective substrates for the synthesis of triacylglycerol. Other studies conducted by Hayes and Gulari (197) as well as Fletcher et al.(191) showed that a 60% conversion of fatty acids to triglycerides could be achieved in reversed micelles composed of AOT/isooctane. Legoy et al.(198) obtained 95% synthesis of ester from heptanol and oleic acid using *Candida cylindracea* lipase in Brij 35 microemulsions after 14 days of reaction at optimum reaction conditions.

Another important food application of biocatalysis in reverse micelles is enzyme-catalyzed peptide synthesis. Oligopeptides are used widely as artificial sweeteners in the food industry. Luthi and Luisi (180) were the first to investigate this reaction in reverse micelles. They obtained a 40-60% yield for the synthesis of a hydrocarbon-soluble tripeptide in AOT/isooctane reverse micelles catalyzed by α -chymotrypsin. The separation of the enzyme-containing reversed micelles from the product-containing hydrocarbon phase was achieved by the use of a semi-permeable hollow fiber reactor that permitted the hydrocarbon phase to pass through while the enzyme was still retained within the membrane. Shield et al.(199) also successfully conducted the α -chymotrypsin-catalyzed synthesis of dipeptides in reverse micelles composed of CTAB in octane with hexanol as the cosurfactant. The dipeptide formation was enhanced due to increased local concentration of the substrate in the water-pool containing the enzyme. Similarly the oil-soluble product was thrown back into the oil phase reducing product-inhibition and shifting the equilibrium toward completion of the synthesis reaction.

Enzyme-catalyzed hydrolysis of proteins in reverse micelles also has potential applications in the food industry. Protein hydrolysates are very important ingredients in the food industry. They can be used for the alteration or inducement of desirable functional properties in proteins and for the production of small peptides and amino

acids useful in dietetic foods and flavoring agents (200). Protein hydrolysis catalyzed by α -chymotrypsin and trypsin are among the most extensively studied reactions in reverse micelles. α -chymotrypsin demonstrates a "superactivity" for protein hydrolysis in ionic reverse micelles. This enhanced activity is attributed to increased conformational rigidity of the enzyme in the surfactant shell of the micelle. Another explanation in this regard has been provided by Ruckenstein and Karpe (145-147) who believe the superactivity is due to charge induced effects by the substrate and surfactant.

Amylases are very important enzymes in the starch industry. Srivastava et al.(201) entrapped α -amylase in reverse micelles composed of Tween 80 in n-hexane. They observed a 3-fold increase in activity as compared to that in bulk aqueous medium when starch was used as a substrate. The same group also investigated the activity of cellulase in Tween 80/hexane reverse micelles. Cellulase is important in the shell-fish industry. The activity of this enzyme was also increased 3-fold in reverse micelles. Similarly Sinitsyn, et al.(202) used reverse micelle entrapped cellulase to catalyze the hydrolysis of cellulose to glucose.

Madamwar et al.(203) investigated the activity and stability of invertase in reverse micelles of the surfactants sodium tauroglycocholate and sodium lauryl sulfate. Under mild conditions, a more than four-fold increase in enzymatic activity was observed using sucrose as a substrate. Moreover, the reverse micelle-entrapped enzyme also displayed a lower K_m value compared to that in the aqueous phase.

Lipoxygenases are key enzymes in the production of off-flavors in foods. It is desirable to measure the activity of this enzyme in the processing plant so that loss of enzyme activity during heat treatment and blanching can be correlated with quality retention on frozen storage. Though lipoxygenase activity has been assayed for extensively in aqueous medium, Kurganov et al.(204) have used reverse micelle entrapped enzyme to assay for soybean lipoxygenase-catalyzed peroxidation of linoleic acid in organic solvents. The same group has also used AOT/isooctane reverse micelles to study the effect of water-insoluble inhibitors such as dihydroxyflavin esters on lipoxygenase activity. Though there is no effect of the inhibitor on activity when it is suspended in aqueous medium, enzyme activity was found to be affected by inhibitor in AOT/isooctane reverse micelles.

The interest in peroxidase and catalase in the food industry stems from their ability to destroy hydrogen peroxide after it has been utilized as a bactericidal agent to preserve food quality. Peroxidase has been extensively studied in reverse micelles and its activity is found to be increased a 100-fold upon incorporation in AOT/isooctane reverse micelles (167). Catalase has also been studied in reverse micelles of AOT in toluene and its activity is comparable to that in aqueous solution (205).

Finally, the enzyme glucose oxidase which is important in the fruit products and wine industry has also been solubilized in reverse micelles of CTAB in octane using hexanol as cosurfactant (206). Using a sensitive fluorescence detection assay, it was found that in CTAB reversed micelles the reaction rate was 30 times faster than in aqueous solution.

8.2. Use of reverse micellar enzymology in the pharmaceutical industry

The most important application of enzymes in the pharmaceutical industry is in steroid conversions to important products. Steroids are complex chemical molecules that are used as anti-inflammatory, diuretic, anabolic, contraceptive, progestational and anti-cancer agents (207). Due to the large number of potential reaction sites on the steroid molecule, the stereo and regio selectivity of enzyme catalysis is often utilized in preference to traditional chemical synthesis. The low solubility of steroids in water necessitates the use of an organic solvent medium for the reaction (5,6). Since enzymes are rapidly inactivated in the presence of organic solvents, reverse micelles could be very useful for enzyme-catalyzed synthesis of steroids.

The enzymatic conversion of apolar steroids in reverse micelles was first investigated by Hilhorst and coworkers (6,208). They developed a multi-enzyme system in reversed micelles composed of CTAB/octane/hexanol containing lipoamide dehydrogenase, 20 β -hydroxy steroid dehydrogenase and hydrogenase (Figure 8).

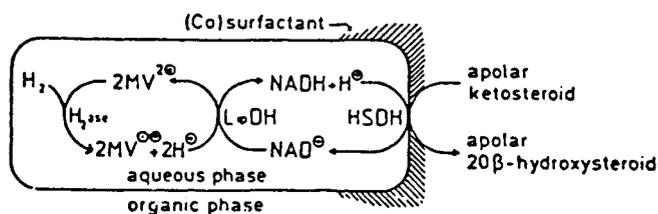


Figure 8. Multi-enzyme system in reverse micelles as proposed in ref.(208)

This system catalyzed the site-specific reduction of progesterone and utilized an *in situ* NADH regenerating system with hydrogen as the ultimate reductant. In contrast to the high rate of conversion observed in this system (90% conversion), no conversion of progesterone was observed in two-phase water/butyl acetate system. On further investigation of the reverse micellar composition on the enzymatic activity, it was found that the water content and the ratio of hexanol-to-CTAB strongly affect the enzymatic activity (6) The rate of steroid conversion was affected by the hydrophobicity of the steroid substrate relative to the hydrophobicity of the continuous phase and the interface. The system that gave the highest local concentration of the substrate steroid in the vicinity of the enzyme resulted in the highest rate of conversion.

Lee and Biellmann (209) also investigated the activity of the same enzyme in microemulsions made of the anionic surfactant SDS, cationic CTAB and non-ionic Triton X-100. They observed that the nature of the cosurfactant used in the formation of the microemulsions had a strong effect on the enzymatic activity. Ishikawa et al. (210) investigated the 3α - and 20β -hydroxy steroid catalyzed reduction of progesterone in AOT/isooctane reversed micelles. They observed that the rate of hydroxysteroid dehydrogenase catalyzed reduction of steroids in reverse micelles was enhanced 750-fold as compared to that in the aqueous buffer. Another enzyme that has been used for steroid conversion in reversed micelles is cholesterol oxidase (134-136,138,211). Cholesterol oxidase catalyzes the oxidation of cholesterol to cholestenone which is a precursor to pharmaceutically important products such as androstene-4,17-dione. Cholesterol oxidase also oxidizes 3β hydroxy sterols such as dehydroepiandrosterone yielding useful products. Since the solubility of cholesterol in aqueous medium is only to the extent of $4\ \mu\text{M}$, the advantage of using an almost completely organic medium are obvious.

Gupte et al.(138) conducted a detailed kinetic investigation of the enzymatic cholesterol oxidation in reverse micelles. The reaction was conducted in optically transparent reverse micelles composed of the anionic surfactant AOT in isooctane, the cationic surfactant CTAB in octane with hexanol as cosurfactant and the non-ionic surfactant Triton X-100 in cyclohexane with hexanol as cosurfactant. The enzyme was appreciably active in the microemulsions and catalyzed the conversion of the substrate cholesterol present in the organic continuous phase to product cholestenone. The rate of the enzymatic reaction was strongly influenced by the pH of the water pool, the surfactant concentration and the degree of hydration of the micelles.

The pH profile of the enzyme exhibited an acidic shift in anionic AOT reverse micelles while it displayed an alkaline shift in cationic CTAB reverse micelles. This was consistent with recent studies conducted by Ruckenstein and Karpe (145-147) who theorized that the pH of the micellar core in AOT/isooctane reverse micelles formed by the injection procedure was more alkaline than in aqueous media.

Cholesterol oxidase in AOT/isooctane reverse micelles showed the highest activity at the lowest values of R . This demonstrated that the enzyme was most active in the bound-water present in the polar head groups of the surfactant AOT. In contrast, the enzyme exhibited a bell-shaped dependence of its biocatalytic activity on R in cationic CTAB reverse micelles. In non-ionic Triton X-100 reverse micelles, the enzyme exhibited a monotonic increase in its activity with R . This can be readily explained when one considers the absence of electrostatic and ionic hydration effects with the use of non-ionic surfactants.

The enzymatic reaction in reverse micelles composed of all the three surfactants was seen to follow classical Michaelis-Menten kinetics. However, the values of the apparent rate constants k_{catapp} and K_{mapp} differed from the true, known reaction rate constants in aqueous medium. K_{mapp} was 100 to 1000-fold higher than the true constant in aqueous medium depending on the nature and concentration of the surfactant used. This phenomenon could be explained by accounting for the partitioning of the substrate between the various phases of the microemulsion including the micellar core, the bulk organic solvent, and the interface composed of the surfactant tails. k_{catapp} was reduced

2 to 4-fold as compared to k_{cat} in aqueous media depending on the concentration and type of surfactant used. The model of Gupte et al.(138) invoked non-competitive inhibition of the enzyme by the surfactant AOT to explain the discrepancy between the apparent rate constant in reverse micelles and that in aqueous media. The observation that the surfactant is not a passive component of the reaction mixture but may be actively involved in the reaction has important implications from the point of view of biocatalysis in reverse micelles.

Since product-inhibition is dramatically reduced in these systems due to the extraction of the product into the organic phase, complete conversion of high concentrations of cholesterol was possible under stationary conditions in relatively short periods of time. This demonstrated that biocatalysis in reverse micelles is not only superior to that in aqueous media, where substrate solubility limited the product yields, but is also a significant improvement over two-phase biocatalysis where high agitation requirements increase energy costs and cause shear denaturation of the sensitive biocatalyst. The enzyme in reverse micelles was even more stable than in aqueous media and retained more than 60% of its activity after a week in the micellar medium demonstrating the practical utility of such systems.

Lee and Biellmann (211) have also investigated the kinetics of the same reaction in cationic, anionic and nonionic microemulsions. They found that while k_{cat} was reduced 3 to 4-fold in the microemulsions, K_m was increased 100 to 1000-fold compared to that in aqueous media. Doddema et al.(212) successfully conducted the same reaction in reverse micellar media of CTAB using a continuous stirred tank reactor and a plug flow reactor with high yields.

Mevkh et al.(213) have studied the kinetic properties of prostaglandin H synthetase in reverse micelles composed of AOT/octane. The interest in prostaglandin H synthetase stems from its ability to catalyze the synthesis of pharmaceutically important prostaglandins from 20-member polyunsaturated acids. Since prostaglandin synthetase is insoluble in water, it is usually solubilized in the aqueous solution with the aid of detergents such as Triton X-100 and Tween. However, the enzyme is rapidly inactivated in aqueous micellar media. In contrast, the inactivation constant of the enzyme in reverse micelles was found to significantly decrease with increasing concentration of the electron-donor and resulted in increased product yield. In reverse micelles, the activity of the enzyme was found to be extremely sensitive to pH, degree of hydration and concentration of surfactant.

Another interesting application of reverse micelles in the pharmaceutical industry is in the synthesis of protein-protein conjugates. The creation of synthetic vaccines and drugs on a polymeric basis has necessitated effective synthesis of protein conjugates with linear polyelectrolytes such as poly(methacrylic acid) and quarternized poly(4-vinyl pyridine) (214). The synthesis of these conjugates is conventionally conducted in aqueous solution causing covalent bond formation between the functional groups of the macromolecules. In aqueous medium, it is not possible to control the direction of the reaction and the extent of conversion resulting in the formation of large insoluble aggregates. Kabanov et al.(214) have utilized reverse micelles as a medium for the tailoring of macromolecular protein-protein conjugates. By controlling the degree of hydration and hence the micellar size, they have shown that it is possible

to form macromolecule conjugates of desired size and composition.

Lastly, peptide synthesis catalyzed by α -chymotrypsin which has been discussed earlier in this section also has pharmaceutical applications (180,189). A wide variety of synthetic peptides are currently in use or are being developed for use in the pharmaceutical industry.

8.3. Use of reverse micellar biocatalysis in chemical and energy industry

Similar to peptide and triglyceride synthesis, enzyme catalysis can be used for other reactions in fine organic synthesis where water is a product of the reaction. For example, in the oxidation of alcohols to aldehydes catalyzed by alcohol dehydrogenase, the equilibrium constant could be altered by a factor of 10^6 in reverse micelles as compared to that in aqueous media (35,173-175). Larsson et al.(215) found that the specific activity of alcohol dehydrogenase in reversed micelles was appreciably retained upto two weeks.

Hilhorst et al.(171) have investigated the photosensitized production of hydrogen by hydrogenase in reversed micelles composed of CTAB/chloroform/octane. The biophotolysis of water catalyzed by hydrogenase was promoted in this medium and the stability of hydrogenase was enhanced as compared to aqueous media. This constitutes a potential methodology for the utilization of solar energy. The same reaction was studied by Castro and Cabral (216,217) in AOT/isooctane reverse micelles. The exchange of micellar contents was found to be extremely rapid. The activity and the stability of the enzyme was strongly affected by the degree of hydration and the pH of the water-pool in the micelles.

The use of enzyme catalyzed reactions in reverse micelles is still in the research stage and much work needs to be done before any of the processes are commercialized. Specifically, the problems of product separation and enzyme recovery and re-use need to be addressed. Several alternatives to reverse micelles that involve low-water, microheterogeneous media but involving simplified workup procedures have been proposed and these are discussed in the next section.

9. NEW TRENDS

Recent research on biocatalysis in organic media has focussed on the development of microheterogeneous systems that facilitate product separation as well as enzyme recovery and re-use. The enzyme hosts include: 1. Microemulsion based organogels, 2. Block copolymer microdomains, 3. Lyotropic liquid crystals, 4. Gel-entrapped enzymes in reverse micelles 5. Polyethylene glycol-modified enzymes, 6. Surface-modified polymeric nanogranules, 7. Clathrate hydrates in reverse micelles, 8. Detergentless microemulsions, 9. Uni- and bicontinuous microemulsions, 10. Winsor III systems.

9.1. Microemulsion-based organo gels

A new and interesting non-aqueous system that is being used for the biocatalysis of water-insoluble substrates is that of microemulsion-based organogels (MBG's). It has been observed that under certain conditions, it is possible to transform reversed

micellar solutions to rigid, optically transparent gel-like structures with extremely high viscosity. These gels serve to entrap proteins and enzymes with retention of catalytic activity. Microemulsion-based gels have a distinct advantage over conventional reverse micelles in that they facilitate enzyme re-use and easy product separation. The two distinct kinds of microemulsion-based gels that have been reported are gelatin gels and lecithin gels.

9.1.1. Gelatin gels

Gelatin gels are formed under certain conditions when fairly high concentrations of gelatin are solubilized in AOT/hydrocarbon/water micellar systems. The preparation of gelatin gels was first described by Luisi and coworkers (181). The procedure involves the addition of an AOT/hydrocarbon micellar solution to gelatin powder under vigorous agitation at which it is maintained for 30 minutes. The mixture is subsequently heated to 50-60°C and upon dissolution of the gelatin, the turbid solution is cooled to 25°C forming a transparent gel. A variation of this procedure has been described by Quellet and Eicke (218). It involves heating gelatin powder in water at 50°C-60°C till it has completely dissolved. This is followed by addition of a solution of AOT/alkane at the same temperature to the aqueous gelatin solution under vigorous agitation. The viscous, turbid solution is then cooled to 25°C forming a transparent gel.

A phase diagram of the AOT/isooctane/water/gelatin system (219) reveals that the formation of gel is very strongly influenced by the water content R of the system and that gel formation can occur at a maximum water content of 25. Onset of gelation takes place when the gelatin content in the system (depending on the water content of the system) is greater than 5%. The sol-gel transition is also a function of the surfactant concentration. At higher AOT concentration, the overall concentration of gelatin required for gel formation is lower than at low AOT concentration. The sol-gel transition boundary is independent of temperature and decreases with an increase in the chain length of the hydrocarbon. Gel formation is also affected by the pH, ionic strength and salt concentration of the system.

Luisi and coworkers report that gelation in microemulsions takes place only with AOT as surfactant and is not observed in non-ionic and cationic surfactants demonstrating that AOT-gelatin interactions are crucial in the gelation process. The only other microemulsion system found to be favorable for gel formation was a mixed surfactant microemulsion system Tween 85/AOT/hexadecane/water/gelatin system where Tween 85 acts as a cosurfactant to AOT.

The biotechnological potential of gel formation in microemulsions has sparked great interest and in-depth studies have been conducted to elucidate the microstructure of the system. Extensive conductivity measurements, DSC, optical rotation studies and pulsed NMR studies have been conducted to probe the microstructure of the gels (219-222). Quellet and coworkers (218-223) have used light scattering, X-ray scattering and circular dichroism data to propose the first structural model of this system (Figure 9). Their model proposes the generation of nanogel structures in the water-pool of the microemulsions as an initial nucleation step in the gelation process. As soon as the nucleation step is terminated, they propose the occurrence of percolation of the

nanophases forming "infinite fractal clusters" in the water droplets. Gelatin is intimately involved in the percolation process in which the polypeptide segments of gelatin form helices extending into the organic phase. Percolation is accompanied by a drastic change in the microstructure and a dramatic increase in the electrical conductivity. The final sol-gel transition step involves the formation of a 3-dimensional network of nanogels interconnected by crosslinking bridges made of gelatin helices.

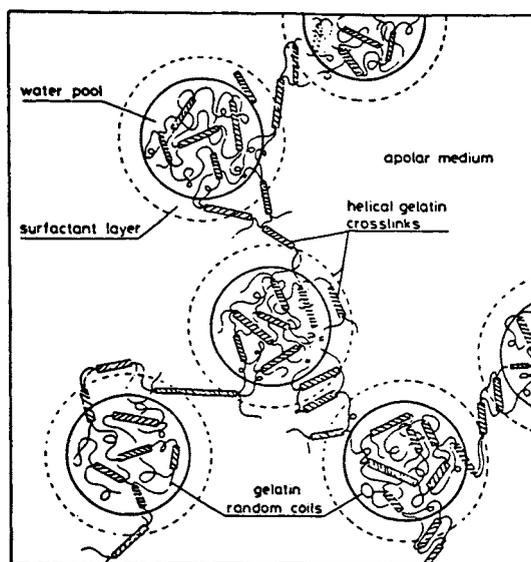


Figure 9. Proposed microstructure of organogels in microemulsions made of AOT/gelatin/oil/water (218).

A conflicting structural model has been proposed by Atkinson and coworkers based on SANS data in conjunction with tracer-diffusion studies, NMR and conductivity measurements of the organogels (Figure 10). Their model proposes the formation of an extensive network of rods containing gelatin and water which coexist with a surfactant shell of AOT in equilibrium with microemulsion water droplets (224,225).

Regardless of the structure of MBG's, their biotechnological relevance stems from their potential as hosts for guest biopolymer molecules. The field of organogel based enzymology is still in its infancy and only a few enzymes have been tested in this system. The first enzymatic reaction that has been investigated in this medium is

the hydrolysis of linoleic acid by lipoxygenase (219). It was observed that the activity of lipoxygenase decreases on being incorporated into MBG's of increasing gelatin content and the enzyme is completely inactivated at 6% gelatin. Even though the enzyme is rapidly inactivated, there was an increase in stability as compared to AOT/hydrocarbon reversed micelles. The activity of this enzyme could only be investigated below the sol-gel transition.

β -galactosidase or lactase, on the other hand, exhibited more promise in AOT reverse micelles containing gelatin (219). The first-order rate constant k_{cat} in AOT/isooctane reverse micelles was higher than that in water while K_m was lower by an order of magnitude. On addition of gelatin to the reverse micellar solution, in a concentration ranging from 0-4% by volume, K_m increased by a factor of 2 with only a marginal increase in k_{cat} . Though the Michaelis-Menten parameters at the gelatin concentration corresponding to the sol-gel transition point could not be investigated due to experimental error during preparation, the gel-entrapped enzyme retained its full activity.

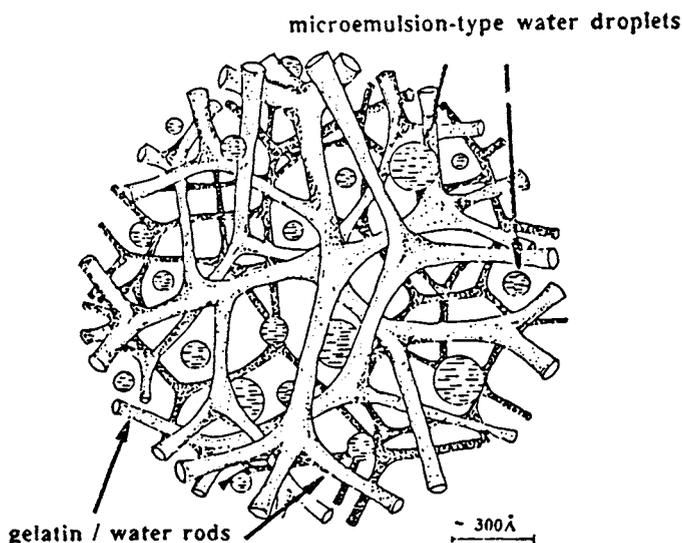


Figure 10. Proposed microstructure of AOT/gelatin/oil/water system (225).

Recently Jenta et al.(226) have conducted a detailed kinetic study of the enzymatic trans-esterification activity of *Chromobacterium viscosum* lipase in gelatin based organogels where octyldecanoate was synthesized from decanoic acid and octanol. Concentrations of the enzyme to the extent of 0.6 mg enzyme/ml of gel could be achieved. The catalytic activity of the enzyme was essentially invariant on changing gelatin concentration and water content of gel over a wide range. Though an attempt was made to increase the surface area by gel fragmentation, severe mass transfer

limitations of the substrate from the gel phase to the oil continuous phase still prevailed. This dramatically reduced the catalytic efficiency of the gel-entrapped lipase.

9.1.2. Lecithin gel

Scartazzini and Luisi (227) recently demonstrated that addition of small amounts of water to soybean/lecithin organic solvent reverse micelles causes a dramatic increase in viscosity and the formation of a rigid, optically transparent gel matrix. Solutions of lecithin in upto 50 different solvents are amenable to gelation on the addition of small amounts of water which causes an increase in viscosity by a factor of 10^6 . Since lecithin solutions do not contain any polymeric material, the formation of such highly viscoelastic solutions is intriguing. Recent studies have aimed at elucidating the microstructure of lecithin gels (219,228-230). A phase diagram for the ternary system soybean lecithin/isooctane/water revealed that only a very small amount of water is necessary for gel formation (Figure 11). Increasing the water content beyond a certain critical value induces phase separation and a sharp decrease in viscosity.

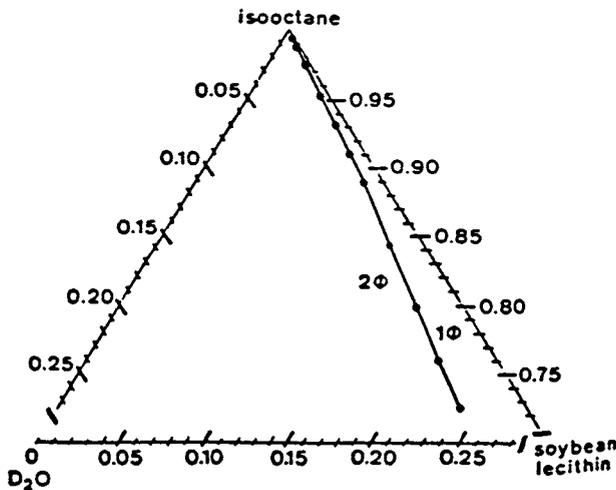


Figure 11. Phase diagram for the lecithin/isooctane/water system (219). Concentrations are expressed as weight fractions.

Extensive rheological and light scattering studies have revealed an interesting microstructure for lecithin-based organogels (219). Rheological studies have shown that the elastic modulus of the gels is related to the lecithin volume fraction by a

power-law relation where the exponent is very similar to that in semi-dilute polymer solutions leading to the nickname, "polymer-like reverse micelles". Viscosity and light scattering data suggest that the gelation process occurs in the system resulting from self-association of lecithin molecules into long cylindrical reverse micelles. Since the viscosity increases upon the addition of water, the aggregation of lecithin molecules is water-induced.

Lecithin has the advantage of biocompatibility compared to other surfactants and lecithin based gels have several potential medical and pharmaceutical applications. Lecithin-based organogels have been shown to be suitable hosts for the entrapment of biopolymers such as proteins and enzymes (219). UV spectra of two proteins (perylene and erythrosin) entrapped into lecithin MBG's have revealed that there is no significant structural change in the protein and it probably resides in the micellar interface (219).

Scartazzini and Luisi (231) have investigated the lipase-catalyzed hydrolysis of tricaprilyn using enzyme immobilized in microemulsion gels of soybean lecithin in cyclooctane. The reaction was slow and was carried out over a period of 8 days to obtain moderate yields. The enzyme was active in the gel for about a month. However, the free fatty acids produced as a result of the hydrolysis reaction acted as cosurfactant and destabilized the gel causing the viscosity to decrease appreciably. Eventually, the gel was converted to a viscous solution.

Since this is the only enzymatic study conducted in this system and the effect of operating variables on enzymatic activity remains to be explored, conclusions on the efficacy of this biocatalytic medium would be premature. However, some general conclusions and recommendations can be made regarding this medium. Though MBG's offer essentially the same advantages as immobilized enzymes in organic solvents, i.e. easy enzyme reuse and product recovery, the essential advantage of low diffusion path length in microemulsion systems is lost. Mass transfer limitations will have to be dealt with to realize the full potential of these systems. Nevertheless, organogels afford a novel immobilization protocol for enzymes catalyzing reactions with substrates poorly soluble in water.

9.2. Block copolymer microdomains

Block copolymers can be visualized as assemblies of homopolymers that are linked together by chemical bonds. For example, a diblock copolymer $[A_k B_m]$ consists of a block of k repeating units of kind A covalently bonded to a block of m repeating units of kind B. The two different types of blocks within the copolymer usually do not mix with one another. This behavior is a consequence of the high molecular weight of polymers which results in a very small entropy of mixing, that is insufficient in magnitude to offset the small but positive enthalpy of mixing. Consequently, at equilibrium, the dissimilar segments in block copolymers undergo separation into different phases. These segregated phases are referred to as microdomains (232). These microdomains can be spherical, cylindrical or lamellar in shape and they can be disordered or ordered. These structural features of the microdomains are determined by the composition and molecular weight of the block copolymer as well as by the system conditions at each temperature.

The microdomain formation occurs also in solutions of block copolymers (233). For example, when a AB diblock copolymer is present in a solvent selective for the B block, the copolymer molecules spontaneously self-assemble to form microdomain structures characterized by a core region made up of the A blocks and a shell region made up of the B blocks and the solvent. Such a microdomain structure generated in block copolymer solutions is analogous to reverse micelles formed from low molecular weight surfactant or lipid molecules (234).

A characteristic feature of microdomains in block copolymer assemblies, whether in pure states or in solution is the large interfacial area between the segregated domains. Another distinguishing feature of the microdomains is their ability to solubilize chemical species that are compatible with the domains (Figure 12). Both these features contribute to the potential use of block copolymer systems in various practical applications. We have exploited the microheterogeneity of block copolymer microdomains to immobilize enzymes and carry out enzymatic reactions (136).

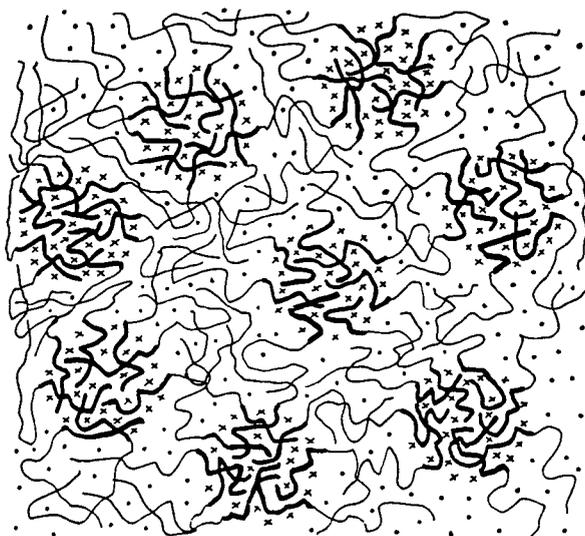


Figure 12. A schematic representation of block copolymer microdomains (136).

The use of this novel microheterogeneous system, namely the microdomain structure of hydrophilic-hydrophobic block copolymers as an effective enzymatic reaction medium was demonstrated by solubilizing enzymes within the hydrophilic domains of the block copolymer. The immobilized enzyme reacts with water-insoluble substrates that are solubilized within the hydrophobic domains acting as micro-

reservoirs of substrates. It is possible to use the block copolymer microdomains profitably also in case of water-soluble substrates, if the reaction product is water-insoluble. In this case, the product is continuously removed from the hydrophilic domains by its transfer into the hydrophobic domains acting as micro-sinks, thus the problem of product-inhibition is avoided.

Two enzymatic reactions were explored. In the first example, the enzyme cholesterol oxidase was used to oxidize cholesterol to cholestenone. The advantage derived from the increased solubility of cholesterol in the block copolymer system (approximately 22,000 μM in the pure block copolymer, and upto 50,000 μM in block copolymer solutions, depending on the concentration of copolymer in solution) compared to the aqueous phase solubility of 4.7 μM , is obvious. The second enzymatic reaction involves horse radish peroxidase which was used to catalyze the oxidation of pyrogallol to purpurogallin by hydrogen peroxide. In this case, the substrate has considerable water solubility while the reaction product has a lower water solubility. The dissolution of purpurogallin in the hydrophobic microdomains removes it from water as it forms, and helps overcome product-inhibition from affecting catalytic activity.

The block copolymer used was a triblock copolymer of ethyleneoxide and propyleneoxide. It is distributed under the commercial name of Pluronics by the BASF Corporation. The block copolymer has the general structure $\text{HO}-(\text{EO})_x-(\text{PO})_y-(\text{EO})_z-\text{H}$, where EO and PO refer to ethyleneoxide and propyleneoxide and x,y and z refer to numbers of monomeric units in the different blocks. Pluronics are available in a range of molecular weights and at various compositions of EO and PO. Block copolymers have low toxicity, are mild in terms of skin and eye irritation and are thus safer to use when compared against synthetic surfactants used in the formation of analogous microheterogeneous structures such as reverse micelles.

The enzymatic activity of cholesterol oxidase decreased with increasing water content in the microdomains. This behavior was similar to that in conventional reverse micelles formed by low molecular weight surfactants (138). A pH optimum was discerned which depends upon the value of the water content ratio R in the microdomains. This pH optimum is practically identical to that in the aqueous medium. Intuitively, this may be expected given the nonionic nature of the block copolymer studied.

An obvious feature of interest is the possibility of utilizing pure block copolymers (i.e without any solvent such as cyclohexane) as the enzymatic reaction medium. This would make the immobilization medium a very simple one since it would be based on a single component system. When the concentration of the copolymer in solution is modified, changes occur in the dimensions of the hydrophobic and hydrophilic microdomains as well as in the nature of the domain morphologies. These changes can modify the microenvironment of the enzyme and thus potentially give rise to altered intrinsic activities. It was observed that the activity of cholesterol oxidase increased with increasing copolymer concentration, the maximum activity being exhibited in pure block copolymer. The stability of the enzyme cholesterol oxidase in the microdomains was even better than that in the aqueous medium and the enzymatic activity in microdomains was retained over extended periods of time.

In contrast, the activity of horseradish peroxidase increased with increasing water content in the microdomains. Though cholesterol oxidase exhibited maximum activity in the pure block copolymer, peroxidase activity decreased with increasing copolymer concentration. The absorbance spectra of the enzymes in the microdomains revealed that incorporation of cholesterol oxidase in micelles of block copolymer microdomains caused alterations in the backbone conformation of the enzyme and hence its catalytic activity. The increase in catalytic activity of the enzyme upon incorporation into the microdomains was due to the favorable effect of the hydrophobicity of the enzyme microenvironment on enzymatic activity. Peroxidase activity was detrimentally affected on the other hand, due to the preference of peroxidase for a more hydrophilic environment.

We have shown that block copolymer microdomains can serve as an effective microheterogeneous medium for carrying out enzymatic biosynthetic reactions. The observed activities of cholesterol oxidase and horse radish peroxidase make the block copolymer microdomains interesting systems for further exploration involving various types of enzymes. The illustrative studies with the two types of enzymes suggest that if the hydrophobicity of the microenvironment is increased, the activity of cholesterol oxidase is enhanced while that of horse radish peroxidase is diminished. The nature of the enzyme's microenvironment can be conveniently manipulated because block copolymer microdomains are equilibrium entities, are easily reproduced and possess well defined geometrical characteristics depending upon the molecular weight, block composition, temperature and the type and amount of solvent present. Further, substantial amounts of water-insoluble substrates can be solubilized within the hydrophobic microdomains thus eliminating the substrate availability from limiting the rate of practical enzymatic reactions. The substrates and the reaction products can be easily removed from the block copolymer microdomains by manipulations of system temperature, which significantly affects polymer solubility behavior. The closest analog of the block copolymer microdomains are the reverse micelle systems formed using conventional low molecular weight surfactants. Here, one requires not only a surfactant and solvent, but often an alcohol cosurfactant as well to form reverse micelles. In contrast, one can potentially work with the single component system of pure block copolymers. Block copolymer microdomains permit extremely low water or highly hydrophobic microenvironments as compared to reverse micelles. This makes them very interesting systems to explore for such reactions as trans-esterification by lipases where water is a product of the biosynthetic process. By choosing the appropriate block copolymer, one can ensure minimal diffusional resistances. These resistances can be easily manipulated, if necessary, by the addition of solvents or by small changes in temperature.

9.3. Lyotropic liquid crystal mesophases

Lyotropic liquid crystal mesophases are aggregates formed by amphiphilic molecules such as surfactants upon dissolution in water/organic solvent mixtures. Most studies of enzymatic reactions in microheterogeneous media have concentrated on the region of the phase diagram corresponding to the formation of reverse micelles. However, it has been shown that it is also possible to entrap enzymes in surfactant

aggregates where the spherical micellar structures do not prevail. In these regions of the phase diagram, the entrapment of enzymes in liquid crystalline structures with lamellar, reversed hexagonal (cylindrical) and reversed cubic packing of surfactant molecules occurs with retention of catalytic activity.

Figure 13 is a phase diagram for the ternary system AOT/octane/water. It is apparent from the phase diagram that while reverse micelles form in the region L_2 , liquid crystalline structures exist in the other regions of the phase diagram. D, F and I_2' represent lamellar, reversed hexagonal and reversed cubic structures respectively (161).

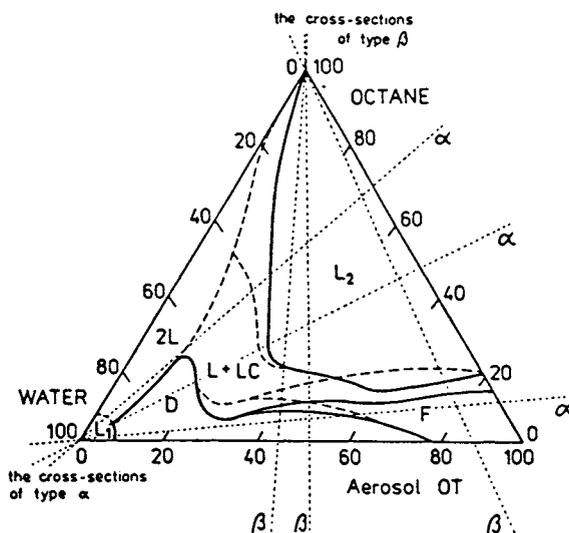


Figure 13. Phase diagram for AOT/octane/water system (75)

Klyachko et al.(161) were the first group to investigate the catalytic activity of enzymes in liquid crystalline structures formed in the system AOT/octane/water and Brij 96/cyclohexane/water. They examined the potential of these systems to serve as biocatalytic media, using acid and alkaline phosphatase as well as peroxidase as model enzymatic systems. Entrapment of enzymes into the reversed hexagonal and lamellar structures was achieved by the addition of increasing amounts of buffer to an enzyme-containing reverse micellar solution in organic solvent till a transparent, viscous gel was obtained. Hexagonal packing of reversed cylinders was obtained by preliminary heating to 30°C, followed by addition of aqueous enzyme solution and subsequent cooling to 20°C. Enzymatic activity was determined by addition of substrate just prior to formation of liquid crystalline structures. The catalytic activity

of the enzymes was found to be independent of the order of priority in which the reagents were added demonstrating that diffusional limitations could be neglected. The enzymatic reactions catalyzed by the solubilized enzymes followed substrate saturation Michaelis-Menten kinetics. The catalytic activity of the enzyme was strongly influenced by the water content of the system even within the same phase. The transition from one phase to the other was readily evident from the dramatic change in catalytic activity of the solubilized enzymes as well as the overall conductivity of the system.

The catalytic activity was also affected by the pH of the buffer forming the system similar to that in aqueous solution. The value of the first-order rate constant for p-nitrophenyl phosphate hydrolysis catalyzed by alkaline phosphate was highest in spherical reverse micellar phase and lowest in the reversed cylindrical structures formed in the ternary system AOT/isooctane/water. The change in activity caused by phase transition from one liquid crystalline structure to the other was completely reversible. In contrast to alkaline phosphatase, the specific activity of peroxidase was higher in the lamellar crystals as compared to that in reverse micelles and dramatically increased in the reversed cubic structures formed in Brij 96/cyclohexane/water system (161). Similar results were obtained for the activity of laccase in ternary systems of Brij 56/cyclohexane/water and Aerosol OT/octane/water (140). They observed that the activity of the enzyme was strongly influenced by the type of hydrated aggregate formed. Alteration in the packing of the micellar structures caused sharp changes in the enzyme activity. This was attributed to the change in the conformation of the enzyme upon incorporation into micellar aggregates of differing morphology. The pseudo first-order rate constant k_{cat} decreased with increasing surfactant concentration.

Recently, Miethe and coworkers (183) have used liquid crystals formed by the ternary system polyoxyethylene/hexane/water in the region of the phase diagram where the liquid crystals coexist with the organic solvent. H_2 -NMR signals revealed that the liquid crystals were reverse cubic. The potential applications of this system was demonstrated by investigating the kinetics of various dehydrogenase reactions. It was observed that the activity of the enzymes was comparable and in some cases even higher than that in the water-phase. The partitioning of the substrate between the liquid crystal and the solvent-phase was more rapid than the enzymatic reaction indicating the absence of diffusion limitations. Moreover, alcohol dehydrogenase was substantially stabilized in this system as compared to that in water. Using the dehydrogenation system of *Pimellobacter simplex* as an example, the same group has demonstrated that microbial biocatalysts are also appreciably active and stable in this medium (235).

Another enzyme that has exhibited substantial biocatalytic activity in this system is R-oxynitrilase which catalyzes the synthesis of optically active cyanohydrins (235). The liquid crystal/organic solvent system has been utilized in continuous reactors for the reduction of cinnamaldehyde by alcohol dehydrogenase as well as for the continuous production of R-oxynitrilase catalyzed production of R-mandelonitrile. Yields were appreciable and separation of the enzyme-containing LC phase from solvent hexane was simply achieved by utilizing a large macroporous filter. If mass transfer limitations can be completely eliminated, liquid crystal systems demonstrate

potential as a reaction medium for the biocatalysis of water-insoluble substrates. However, since the water content of the systems in regions where the LC phase coexists with organic solvents is substantial, this system may not be as effective as low water systems such as reverse micelles or block copolymer microdomains in reverse hydrolytic reactions catalyzed by hydrolases where water is a product of the enzymatic reaction.

9.4. Gel-entrapped enzymes in reverse micelles

Gel-entrapped enzymes in reverse micelles are yet another alternative to reverse micelles from the point of view of simplification of enzyme re-use and product recovery. This concept aims at combining the advantages of continuous operation afforded by immobilized enzymes with those of reverse micellar enzymology. The procedure involves the immobilization of the enzymes in acrylamide copolymer gels as a preliminary step, followed by equilibration of the gel-entrapped enzyme with the reverse micellar solution. The system can be visualized as a suspension facilitating transport of substrates and products across the gel matrix into the organic solvent. The potential of this concept was demonstrated by investigating the kinetics of trypsin-catalyzed hydrolysis of N α -benzoyl-L-arginine ethyl ester (BAEE) and the α -chymotrypsin catalyzed hydrolysis of GPNA (182). The activity of the gel-entrapped trypsin in aqueous and reverse micellar solutions was 50% of the activity of the soluble enzyme. The value of K_m for the gel-entrapped enzyme was however increased 10-fold in aqueous medium and by a factor of 1000 in reverse micellar solution. This leads to the belief that the affinity of the substrate toward BAEE is substantially lowered upon entrapment in gel.

In case of α -chymotrypsin, the activity of the gel-entrapped enzyme was of the same order as that of the soluble enzyme in aqueous and reverse micellar solution. However, the superactivity of chymotrypsin in reverse micelles was not observed in the case of gel-entrapped enzyme. Moreover, the gel-entrapped enzyme did not exhibit any variation in its activity with R of the micelles.

The advantages of using reverse micellar solutions as compared to organic solvent mixtures was demonstrated for peptide synthesis catalyzed by gel entrapped chymotrypsin. Reaction yields in AOT/isooctane reverse micellar solutions were substantially higher than that in organic solvent mixtures. Though similar yields were obtained as for soluble enzymes in both aqueous and reverse micellar solutions, the stability of the enzymes improved dramatically upon gel-entrapment as compared to that in free solution. Moreover, the separation of products could be easily achieved by simple filtration.

It appears that gel-entrapped enzymes in reverse micelles do not offer the interesting structurally-induced changes in the catalytic behavior of enzymes in reverse micelles. Unlike reverse micelles, it is not possible to utilize variables such as R, pH and surfactant concentration to obtain optimal enzymatic activity. However, since they are not plagued by mass transfer limitations that normally exist in heterogenous catalysis and afford easy enzyme recovery and reuse, they demonstrate promise. Their potential as enzymatic reaction media should be explored by investigating the kinetics of other enzymes in reverse micelles, particularly those catalyzing reactions involving

water-insoluble substrates or products.

9.5. Polyethylene glycol-modified enzymes

The amphipathic properties of polyethylene glycol have been exploited by Inada and coworkers (26) in a novel method for the use of enzymes in non-aqueous media. The procedure for enzyme modification with PEG first involves the activation of PEG with cyanuric chloride to form activated polyethylene glycol (PEG₂). The residual chlorine atom in the activated PEG₂ then reacts with the available amino acid groups on the surface of the enzyme molecule. Due to the hydrophobic chains of PEG, the resultant modified-enzyme is soluble in organic solvents forming a transparent solution. However, the hydrophilic character of the molecule allows the preservation of enzymatic activity by preventing exposure to the organic solvent. The degree of modification can be precisely controlled by varying the ratio of PEG to protein.

Four different enzymes: lipase, catalase, chymotrypsin and peroxidase have been modified by this procedure and their catalytic activity in organic solvents has been investigated. The modified enzymes retained appreciable activity in organic solvents which was sometimes even greater than that of the free enzyme in aqueous solution. Lipase catalyzed several enzymatic reactions of biotechnological interest such as transesterification and synthesis of esters, ester hydrolysis as well as polymerization of hydroxydecanoic acid and fatty acid exchange in fats and oils. PEG-modified lipase displayed appreciable stability in organic solvents. The thermostability of the enzyme was markedly improved and the optimum temperature for the enzyme was increased to 70°C as compared to 45°C for the unmodified enzyme. Similarly, the PEG-modified chymotrypsin was catalytically active and catalyzed the synthesis of peptide and amide bonds. A comparison of PEG-modified trypsin and chymotrypsin for the synthesis of peptides in organic solvents revealed that there was no change in enzyme specificity upon replacement of water by hydrophobic solvents (236). This was an indication that hydrophobic interactions between enzyme and substrate are not important during catalysis in the organic phase. Catalase modified by PEG displayed a UV spectrum in benzene which was almost identical to that of unmodified catalase in aqueous solution, demonstrating that the structural characteristics of the enzyme were almost unaltered by the modification. The maximum activity of modified-catalase was higher than that of free-catalase in aqueous solution. Similarly, horse radish peroxide modified by PEG was catalytically active and its absorption spectrum was unaltered by PEG-modification.

The retention of catalytic activity in PEG-modified enzymes is believed to be due to the existence of an essential hydration shell around the enzyme necessary for preservation of catalytic activity. This belief is confirmed by the observation that the PEG-modified lipase is catalytically inactive in water-miscible solvents capable of destroying the essential hydration shell. Though PEG-modified enzymes demonstrate great promise, they do not have universal applicability due to the need for amino groups present on the surface of the enzyme for modification. Moreover, the enzyme modification procedure does not have the ease and simplicity of preparation afforded by reverse micellar media.

9.6. Enzymes entrapped into polymeric nanogranules

Enzymes entrapped into polymeric nanogranules constitute yet another novel procedure for the immobilization of enzymes used in organic media with the retention of catalytic activity. Entrapment of enzymes in polymeric nanogranules involves a complicated three-step procedure. Firstly, the enzyme is entrapped in mixed surfactant reverse micelles composed of the surfactant AOT and the triblock copolymer Pluronic F 108 (Figure 14). The end hydroxy groups are esterified with methacrylic acid prior to use so as to obtain polymerizable double bonds at both ends. Polymerization is then achieved by UV-irradiation of the solution using α - α' -azodiisobutyronitrile as an initiator. Finally AOT is chromatographically removed and the solvent is removed from the nanogranules containing enzyme by vacuum evaporation of the solvent.

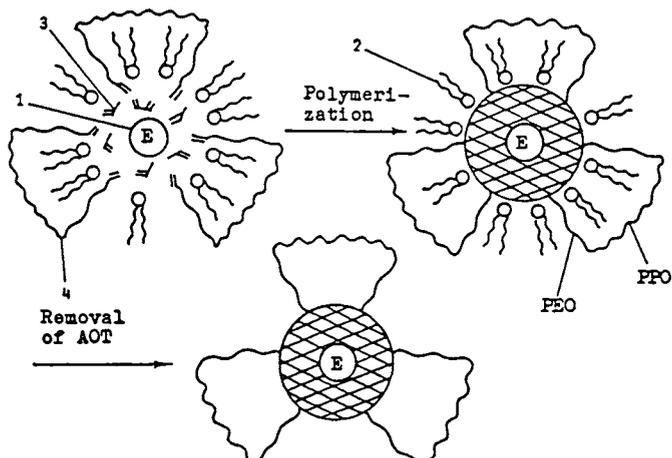


Figure 14. Entrapment of enzymes into surface-modified polymeric nanoglobules (130). 1 = enzyme, 2 = AOT, 3 = monomers, 4 = Pluronic F-108.

The presence of the hydrophobic poly (propyleneoxide) chains on the surface of the polymeric nanogranules facilitate their solubilization in organic solvents forming an optically transparent solution. The enzyme is however protected from the detrimental effect of the organic solvent by being sequestered in the core of the nanogranules.

The potential of this system for biocatalysis was demonstrated by investigating

N-acetyl-L-tyrosine ethyl ester (ATEE) synthesis catalyzed by α -chymotrypsin entrapped in polymeric nanogranules (130). The catalytic rate of the reaction was an order of magnitude higher than in other non-aqueous systems such as solid enzyme suspensions in organic solvents, biphasic water-chloroform systems and immobilized enzymes in organic solvents. Use of membrane reactors permitted enzyme retention within the reactor while easy product recovery could be achieved by evaporation of the organic solvent. Polymeric nanogranule entrapped α -chymotrypsin also displayed enhanced thermostability as compared to the free enzyme in aqueous medium or AOT/octane reverse micelles (237).

These initial studies demonstrate the potential for this system as a novel biocatalytic medium. This system affords the advantage of simplified product recovery compared to reverse micelles. Mass transfer limitations in polymeric nanogranules are much less compared to conventional immobilized enzymes or organogel entrapped enzymes. They also have more universal applicability compared to PEG-modified enzymes which require the presence of amino groups on the enzyme surface for PEG modification. However, the complicated immobilization procedure may prevent this technique from being widely used for biocatalysis in non-aqueous media.

9.7. Clathrate hydrates in reverse micelles

Clathrate hydrates are crystalline inclusions of gas and water, formed when an aqueous phase is contacted with a light gas phase at enhanced pressures and reduced temperatures. It has been shown that by admission of gas at elevated pressures it is possible to convert the microdroplets in the water-pool of reverse micelles to hydrates which nucleate and drop out of solution (238-241). Formation of clathrate hydrates in reverse micelles is a novel methodology for the modification of the catalytic activity of enzymes entrapped in reverse micelles. Hydrate formation reduces the water content of the micelles and consequently the micellar size. This process is reversible and permits *in situ* control of micellar size by changing the pressure. Since enzymatic activity is strongly influenced by the water content of the micelles, pressure can be used as a reversible control of enzymatic activity.

The application of clathrate hydrate formation in reverse micelles containing enzymes has been investigated for two model enzymes: lipase which locates in the micellar interface and α -chymotrypsin which is solubilized in the aqueous core of the micelles (240). It was observed that pressurization with methane induces the formation of gas hydrates in the reverse micelles and substantially reduces the micellar size of both enzyme-filled and empty micelles. The enzymes were found to retain their full enzymatic activity after hydrate modification of the micelles and enzyme deactivation was found to be minimal. The catalytic activity of the enzymes increased to the values obtained from preparation of reverse micelles of optimal water content. However, the optimal R for the enzymes was shifted to a lower value due to some modification of micellar water upon gas hydrate formation. In contrast, other procedures capable of reducing micellar size such as surfactant addition and mixing of micelles of lower water content were not able to restore enzymatic activity to the optimum. This procedure of micellar size modification has been suggested as a technique for improving the activity of enzymes recovered from fermentation broths by phase-

transfer into a reversed micellar phase (99). Since enzyme recovery from fermentation broths is usually achieved by phase-transfer into reverse micelles of higher than optimal water content, hydrate formation is a possible procedure for tuning the size of the micelles for optimal expression of enzymatic activity.

Recently, the same group has also used clathrate hydrate formation in reverse micelles as a technique for the recovery of proteins from reverse micelles (241). Solubilization of ethylene into reverse micelles containing cytochrome-c caused a decrease in the solvent density and at a certain critical value of this density, micellar instability and phase-splitting was observed leading to a precipitation of the encapsulated protein. Clathrate hydrates formed into the microaqueous phase containing protein forming a solid phase that can be separated out by filtration. This permitted recovery of protein in a concentrated form without need for pH or ionic strength modification. Contamination of the recovered protein by surfactant could be avoided by partial depressurization after precipitation of the protein in the presence of added electrolyte. This allowed reincorporation of the surfactant into the micellar phase without simultaneous uptake of protein.

However, in spite of their potential applications, clathrate hydrate formation in reverse micelles necessitates the use of high pressure conditions which may be uneconomical. The use of ambient temperature and pressure conditions which is an attractive advantage of enzymatic biosynthesis over conventional chemical synthesis is lost with the operation at elevated pressure conditions.

9.8. Detergentless microemulsions

Detergentless microemulsions are thermodynamically stable and optically transparent microdroplets of water in a continuous medium of oil that are formed in the presence of an alcohol, an example being certain regions of the phase diagram of the ternary system hexane/2-propanol/water. It has been shown that it is possible to solubilize enzymes in these microemulsion droplets forming transparent solutions with retention of catalytic activity (133,242-244). Since these systems do not require the presence of surfactant for their formation but are stabilized by the presence of alcohol at the micellar interface, they offer the advantage of simple product separation and enzyme re-use.

Figure 15 shows a phase diagram of the ternary system hexane/2-propanol/water (243). Region A corresponds to unstable macroemulsions, C corresponds to hydrogen-bonded aggregates of water and 2-propanol dispersed in the organic continuous phase while D comprises true solutions of the three components. It is in Region B that there is the existence of detergentless microemulsions where droplets of water in hexane are stabilized by the presence of alcohol at the interface. Five enzymes have been solubilized in detergentless microemulsions and their catalytic activity has been investigated.

Khmelnitsky and coworkers (242) were the first group of researchers to advocate the use of detergentless microemulsions as media for enzymatic reactions. They investigated the kinetics of the trypsin-catalyzed hydrolysis of N-benzoyl-L-arginine ethyl ester (BAEE) in the system hexane/isopropanol/water. It was found that the catalytic activity of the enzyme in this ternary system exhibited a maximum in the

region of the phase diagram corresponding to the formation of detergentless microemulsions. The maximum activity was of the same order of magnitude but lower than that in aqueous solution. Similarly the stability of the enzyme was also greatest in detergentless microemulsions and was even higher than that in aqueous medium.

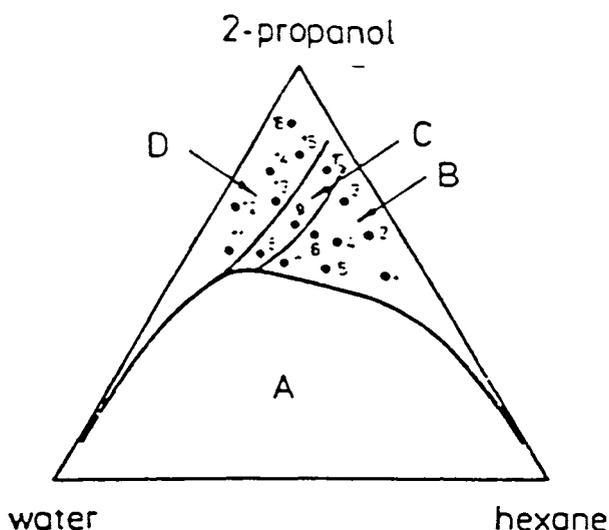


Figure 15. Phase diagram for the hexane/2-propanol/water system (243).
A = separated phases, B = microemulsions, C = hydrogen-bonding of water and 2-propanol, and D = ternary molecular solution.

For the α -chymotrypsin catalyzed synthesis of N-carbobenzoxy-L-tryptophan isopropyl ester in detergentless microemulsions, the catalytic activity increased by an order of magnitude compared to that in aqueous buffer. This was similar to the behavior of α -chymotrypsin in reverse micelles.

Similarly, in the case of the laccase-catalyzed oxidation of pyrocatechol to benzoquinone, there was a maximum in the activity of the enzyme in the region of the phase diagram permitting the formation of detergentless microemulsions (244). However, this maximal activity was only 20% of that in the aqueous solution. More importantly, though conventional reverse micelles permit improved stability of most enzymes, the inactivation constant of laccase in reverse micelles was 10-fold higher than that in water. Time-resolved laser fluorescence and analytical ultracentrifugation of the microenvironment of detergentless microemulsions with nitrate anion revealed

that the polarity of the droplets in detergentless microemulsions was of the same magnitude as a 75% (v/v) solution of 2-propanol in water (133). Since hydrophilic organic solvents are known to detrimentally affect enzyme stability, the inactivation of laccase in detergentless microemulsions may be caused by the increased polarity in the hydrophilic microdomains of detergentless microemulsions.

In the case of cholesterol oxidase (243), the catalytic activity of the enzyme exhibited two maxima in the ternary system hexane/2-propanol/water. Surprisingly, the catalytic activity of this enzyme was highest in the region of the phase diagram corresponding to the formation of a true ternary solution and was comparable to the activity in water. In the region corresponding to the formation of detergentless microemulsions, the maximum was 50% lower than that in aqueous solution. Moreover, the enzyme stability was dramatically reduced and was lowest in the region of formation of detergentless microemulsions. This is in complete contrast to the behavior of the enzyme in AOT/isooctane reverse micelles and block copolymer microdomains where the enzyme exhibits enhanced stability as compared to that in water (58,136). Sub-zero temperatures were required for the complete conversion of cholesterol to cholestenone in detergentless microemulsions due to the rapid inactivation of the enzyme in this medium. In contrast, it has been found that complete conversion could be achieved at room temperature in reverse micelles (58,136).

Similarly, though the catalytic activity of *Candida cylindracea* lipase was highest in the region of the phase diagram corresponding to the formation of detergentless microemulsions, only 9% of the substrate methyl palmitate ester could be hydrolyzed in this system (245). It appears that the high solubility of 2-propanol in water detrimentally affects enzyme stability. Another possible explanation is that the presence of interfacial substrates such as cholesterol and methyl palmitate ester dramatically alter the structure of the microemulsion.

Finally, a significant disadvantage of detergentless microemulsions is that they are able to solubilize typically, only 1 μ M of enzyme in the system, leading to poor reaction yields for enzymes having low intrinsic catalytic activity. In conclusion, though enzyme recovery and product separation are easily facilitated in this medium, the poor intrinsic catalytic activity and stability of enzymes catalyzing reactions involving weakly surface active substrates, makes the system less than desirable.

9.9. Use of bicontinuous microemulsions as media for enzymatic reactions

Most studies of enzymatic reactions in microemulsion media have concentrated on the oil-rich region of the ternary system AOT/isooctane/water. However, recently Larsson and coworkers (246) have extended this to the entire range of oil-to-water ratios from oil-in-water to water-in-oil microstructures via a bicontinuous microemulsion at comparable amounts of buffer and oil. Bicontinuous microemulsions involve a three-dimensional continuous dividing surface of surfactant film of highly connected topology.

The effect of varying the oil/water ratio on the enzymatic activity was investigated using HLADH as a model enzyme. Keeping the AOT concentration constant, the effect of the change in the microstructure on the enzymatic activity from O/W to W/O microemulsions was investigated by varying the oil/water ratio in the

system. It was found that HLADH exhibits a decrease in the enzymatic activity with an increase in the oil content of the system. This change in the catalytic activity as a function of the oil-to-water ratio could be successfully modelled by accounting for substrate partitioning in the oil and aqueous microdomains. HLADH demonstrated improved stability with an increase in the percentage of oil in the system. This observation is in agreement with previous studies where the presence of oil was seen to reduce the inactivation of HLADH by the surfactant AOT.

Self-diffusion coefficients of water and oil using the Fourier transform pulsed gradient spin echo technique provided insight into the structure of the microemulsion system. The diffusion constants of water and oil were found to be strong functions of the oil-to-water ratio and directly provided information on the radius of the microdroplets in the oil-rich and water-rich ends of the spectrum through the Stokes-Einstein relation.

In a novel approach to product recovery from microemulsions, Larsson et al. (184) demonstrated that temperature-induced phase separation could be used to recover enzyme. Small changes in temperature caused the bicontinuous microemulsion to separate into an oil-rich phase containing the product and a water-rich phase containing the surfactant and enzyme. Two surfactant systems were explored, the AOT/isooctane/water system undergoes phase separation by an increase in temperature while the Epps/isooctane/water system necessitates a decrease in temperature to cause phase splitting.

This study has demonstrated that microemulsions can be utilized for enzymatic reactions over the entire range of oil-to-water ratios. Temperature-induced phase separation has provided a novel methodology to recover the enzyme for reuse. Further studies with other enzymes could examine the universal applicability of this medium for biocatalytic reactions.

9.10. Winsor III systems

Winsor III systems represent a microemulsion in equilibrium with excess oil and water phase. The three-phase system is also referred to as middle phase microemulsion. This middle phase coexisting with excess oil and water phases can exhibit bicontinuity of the kind mentioned above. Winsor III systems have also been explored as a potential medium for biocatalytic reactions affording easy separation of products.

Use of Winsor III systems was investigated by the kinetics of the lipase catalyzed hydrolysis of triglycerides (247). Preparation of the systems was achieved by the addition of a polyoxyethylene ether phosphonate surfactant to a 1:1 volume mixture of buffer and isooctane. Since lipase is a membrane protein, 95% of the enzyme was found to be concentrated in the middle phase microemulsion. The rate of enzymatic hydrolysis of trimyristin to 2-myristoyl glycerol and fatty acid was comparable though slightly lower than that in AOT/isooctane reverse micelles. Though the fatty acid was mainly retained in the bottom aqueous phase and could be separated, the surface active monoglyceride was distributed between the microemulsion and the top oil phase.

Though middle phase microemulsions are a potentially interesting system for biocatalytic reactions, the effective use of these systems for product recovery and

enzyme reuse is greatly dependent on the partition coefficients of not only the reactants and products but also the enzyme between the three phases of the system.

10. USE OF WHOLE MICROBIAL CELLS FOR BIOCATALYSIS IN REVERSE MICELLES

A recent and very interesting development in the field of biocatalysis in reverse micelles is the use of whole microbial cells. There are several advantages to the use of whole cells instead of purified enzymes for biocatalysis. The use of whole microbial cells makes it possible to utilize entire metabolic pathways in the cell involving several enzyme activities and permits the conversion of relatively simple starting materials to useful end products (248). It eliminates the expensive, long and tedious process of isolation and purification of the enzyme. Further, it eliminates the need to supply external cofactors required for conducting many enzymatic reactions of commercial importance.

In the past decade, microbial biocatalysis has been conducted in non-aqueous media where the organic phase is a significant proportion of the total volume. Buckland et al. (249) conducted the oxidation of cholesterol to cholestenone using *Nocardia* in various pure organic solvents. They demonstrated that highly hydrophobic solvents were more conducive to enzymatic conversion. The use of single-phase water/organic solvent mixtures has been less than successful due to the poor stability of microbial biocatalysts (250) as well as the poor solubility of extremely lipophilic substrates in such systems. On the other hand, immiscible two-phase microbial biocatalysis has proved to be a promising alternative to the use of aqueous media. Schwartz and McCoy (251) achieved a 5-fold increase in the yield of derivatives of octadiene by adding 20% cyclohexane to cultures of *Pseudomonas oleovorans* due to diminished product inhibition in two-phase media. Boeren and Laane (252) found that the rate of oxidation of dehydroepiandrosterone acetate (DHEA) to 4, androsten-3,17-dione (A-D) by *Flavobacterium dehydrogenans* in two-phase water/aliphatic alkane systems to be much higher than in aqueous media as a result of the beneficial effect of dissolved organic solvent on cell viability. However, a very important drawback of two-phase biocatalysis is the high diffusion path length necessitating vigorous agitation of the reaction mixture. This leads to problems such as biomass clotting and aggregation of the cells at the liquid-liquid interface. Immobilization of the cells on solid supports has been utilized as a solution to this problem (253-255).

A novel procedure used to improve the stability of microbial biocatalysts in non-aqueous media is the immobilization of the whole cells in colloidal aggregates. Very little work has been done in this field so far, the notable exception being that of Luisi and coworkers who have immobilized bacterial cells of *Escherichia coli*, *Acinetobacter calcoaceticus* and *Corynebacterium equi* in reverse micelles composed of the surfactant Tween 85, solvent isopropylpalmitate and water (256). In the absence of cells, the radii of reverse micelles ranges from 4-10 nm depending on the water content. Though the size of the bacterial cells exceeds this radius, they showed that it is possible to immobilize the whole cells in the hydrophilic microdomains of the

reverse micelles. It appears that the micellar system is capable of restructuring in such a way that it can accommodate particles of a much larger size than the original micelle. This novel methodology permits the cells to be shielded from the detrimental effect of the organic solvent and concomitantly allows high substrate and product solubility. Microemulsions containing whole microbial cells upto 10^8 cells/ml gave optically transparent solutions while the same concentrations of cells in aqueous media were turbid. Optical micrographs revealed that this phenomenon was due to a reduction in the size of the bacterial cells upon incorporation in reverse micelles. Interestingly, the *E. coli* cells retained their activity of β -galactosidase for over 24 hours in the microemulsions.

The same group of researchers has also immobilized yeast cells of *Saccharomyces cerevisiae* in reverse micelles stabilized by the surfactants Tween 85 and asolectins in the solvents isopropylpalmitate and hexadecane. They found the cells to be viable in this media for periods upto 10 days (257). Viability was significantly higher for second-generation cells that had been cultivated from a culture previously solubilized in microemulsions.

It has also been demonstrated that it is possible to solubilize organelles such as mitochondria in reverse micelles composed of the surfactant AOT in isooctane. It was observed that the respiratory activity of the mitochondria was the same as that in aqueous solution and enzymatic activity was retained. The possibility of using mitochondrial microreactors in reverse micelles is very interesting from the biotechnological point of view. Recently it has been shown that these systems are useful for microbial transformation in organic solvents. Haag et al. (258) reported that preparative β -ketoester reduction and ester hydrolyses are possible by using yeast cells in colloidal suspensions of asolectins and isopropylpalmitate. Similarly Fadnavis et al. (259) used reverse micellar media for the hydrolysis of amino acid methylesters by yeast cells cross-linked with glutaraldehyde. Lee and Yen (260) have utilized the microorganism *Thiobacillus ferrooxidans* hosted in reverse micelles in a novel approach to remove sulfur from coal. They observed that the microorganism in optically transparent reverse micelles was considerably more effective in sulfur reduction as compared to cells entrapped in water-in-oil emulsion media. Moreover, the cell-free extracts of the biocatalyst in reverse micelles exhibited higher rates of sulfur reduction than the whole microbial cells. A total sulfur removal of 50% was possible using the reverse micellar process with reaction times considerably lower than the conventional microbial process demonstrating the biotechnological potential of this novel system.

Recently in our laboratory, we have investigated the kinetics of the steroid conversion of dehydroepiandrosterone (DHE) to 4 androsten-3,17-dione (A-D) utilizing the microorganism *Flavobacterium dehydrogenans* in colloidal media (58). Several colloidal systems such as AOT/isooctane/water, CTAB/octane/hexanol/water, block copolymer/cyclohexane/water and Triton X-100/cyclohexane/water were investigated. However, the system that best supported the solubilization of whole microbial biocatalysts and the steroid bioconversion under consideration was Tween-85/isopropylpalmitate/water system proposed by Haering and coworkers (256).

The reaction kinetics and the stability of the biocatalyst was compared to that in

water saturated organic solvent and two-phase aqueous/organic mixtures. Though the rate of the reaction was appreciable in optically transparent microemulsions, the reaction rate increased with water content and was highest in the region of the phase diagram corresponding to the formation of cloudy emulsions. Unlike two-phase systems where mass transfer severely limited the rate of the reaction, complete conversion could be achieved in colloidal systems under stationary conditions demonstrating that energy requirements for the biocatalysis in colloidal media can be substantially reduced as compared to the two-phase system. Furthermore, the stability of the biocatalyst under stationary conditions was also substantially higher than under conditions of agitation demonstrating the biotechnological potential of the system.

11. CONCLUSION

For the past decade, the field of biocatalysis in reverse micelles has fascinated researchers in biotechnology because of the microheterogeneous characteristics of these systems. The protein is confined in an essentially aqueous microenvironment allowing hydrophobic substrates and products to be solubilized in the hydrophobic microdomains. The dual characteristics of the medium offer several advantages for biocatalytic reactions involving water-insoluble substrates and products such as lowered reaction volumes, no mass transfer limitations, absence of product-inhibition and lowered microbial contamination. Simultaneously the biocatalyst is protected from the detrimental effect of the organic solvent. The unique microenvironment of the enzyme results in biocatalytic activity, stability and substrate specificity that is different and often vastly improved as compared to that in the aqueous media. Research efforts have demonstrated that the variables water-to-surfactant ratio R , pH and surfactant concentration can be effectively used to optimize enzymatic activity in this medium. The optical transparency of microheterogeneous media has also made possible the utilization of absorption spectroscopy, CD, fluorescence and ESR spectroscopy to characterize the structure and localization of the solubilized biopolymer. Moreover, light scattering, SAXS and SANS as well as novel techniques such as FRAPP have also been utilized to determine the effect of solubilization on the size of the protein-containing aggregate. Such fundamental experimental research in conjunction with theoretical modelling studies on the effect of protein solubilization in reverse micelles on biocatalytic activity would permit the design of microemulsion systems that precisely control enzymatic activity. However, the problems of product separation and enzyme recovery and reuse need to be addressed before the commercialization of reverse micellar enzymology is possible. Recently proposed methodologies for biocatalyst recovery and reuse such as temperature induced phase separation (184) and isopropyl alcohol addition (84) may prove useful in this regard. Moreover, several new microemulsion-based systems that are specifically geared toward overcoming the bottleneck of product separation demonstrate promise. The concerted effort of several researchers worldwide leading to novel ideas such as whole microbial biocatalysis in colloidal media has ensured growth in this field by leaps and bounds. It is anticipated that the day is not far when the commercialization of this novel technology will become a reality.

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